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Research Report

Developmental expression of clock genes in the Syrian hamster

Xiaodong Li[a], Fred C. Davis[b,*]

[a]College of Life Sciences, WuHan University, WuHan City, Hubei Province, 430072, People's Republic of China
[b]Department of Biology, 134 Mugar Life Science Building, Northeastern University, Boston, MA 02115, USA

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Abstract

Transcription/translation feedback loops consisting of multiple clock genes are thought to be essential for circadian oscillations at cellular, tissue and organismal levels. We examined the developmental expressions of three clock genes (Bmal1, Cry1 and Per1) in the Syrian hamster to probe the oscillatory properties of the suprachiasmatic nucleus (SCN) over the first 4 days after the completion of SCN neurogenesis. Samples were taken at the dam's circadian times 6, 12, and 18 daily over 4 days in constant dim light and processed for in situ hybridization using ^{35}S-labeled RNA probes. Collection times were based on the phases of Bmal1 and Per1 rhythms in adult SCN and on an observed difference in Per1 mRNA at CT6 and 18 on postnatal day 2. For the developmental study, sections from each brain were processed in parallel for the three genes. Bmal1 was prominently expressed in the fetal SCN while Per1 and Cry1 were only weakly expressed. Transcripts of all three genes showed higher abundance just after birth. At subsequent ages, Bmal1 showed a significant decrease, while Per1 continued to be greater than prenatal levels. Significant variation was detected across circadian times for Cry1, but no circadian variation was detected for Per1 and Bmal1. Molecular oscillations equivalent to those observed in adults were not present in the fetal SCN despite evidence for an entrainable pacemaker at that time. An absence of robust oscillations during early SCN development may in part explain the strong phase-setting effects of pharmacological agents on the fetal/neonatal clock.

Theme: Neural basis of behavior
Topic: Biological rhythms and sleep

Keywords: Circadian; Suprachiasmatic; Bmal1; Cry1; Per1

1. Introduction

The suprachiasmatic nucleus (SCN) in the anterior ventral hypothalamus contains a circadian pacemaker essential for the expression of circadian rhythms in physiology and behavior. SCN neurons exhibit endogenous circadian oscillations in neural activity both as a population of coupled oscillators in vivo and in vitro and as individual cells in dissociated cell culture [14,19,44]. Through synaptic and/or paracrine signaling to downstream targets, the SCN drives circadian rhythms in endocrine, autonomic and behavioral events [22,36]. Physiological and behavioral rhythms are internally coordinated and have characteristic phase relationships to environmental cycles controlled through the entrainment of the pacemaker [45,46].

The emergence and maintenance of overt circadian rhythms during mammalian development requires the maturation of the SCN, the downstream effector systems, and their interactions [4]. Circadian oscillations in the SCN are likely to begin at least as early as the first developmental appearance of overt rhythms. Overt rhythms generally appear after birth in eutherian mammals, and a variety of evidence supports an earlier onset of pacemaker function (see [4,8,10]). For example, cross-fostering experiments in rodents have shown that pups obtain time of day information at or before birth. In these experiments, the phases of the pups' overt rhythms measured postnatally are correlated with the phases of maternal rhythms before birth. The rhythms used in these studies include pineal NAT activity,

* Corresponding author. Fax: +1 617 373 3724.
E-mail address: f.davis@neu.edu (F.C. Davis).

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plasma corticosterone level, and locomotor activity [5,12,18,31]. Although taken together, these experiments suggest that a circadian pacemaker, most likely the SCN, is oscillating before birth and is entrained by maternal rhythms, the approach of using postnatal rhythms provides limited information about the identity or properties of the pacemaker during early development.

An alternative approach is to directly measure physiological events in the SCN, the probable site of the circadian oscillations that are entrained before birth. Three rhythms have been measured in the fetal rat SCN, metabolic rate using the ^{14}C-labelled 2-deoxy-D-glucose (2DG) technique [27,28], abundance of mRNA for vasopressin [29], and spontaneous neural activity recorded in slice preparations [35]. Although these measurements were made in the SCN, the rhythms are probably downstream of the core oscillatory mechanism and, like overt rhythms, might develop sometime after the pacemaking oscillations. For example, in rats, the earliest known ages for rhythms in neural activity and vasopressin mRNA are E22 [29] and E21 [35], while a 2DG rhythm was measured at E19 [28]. Even the earliest rhythm (2DG uptake) might be an imperfect measure of pacemaker function since a robust 2DG rhythm is not required for pacemaker function in adult hamsters and a 2DG rhythm could not be detected in the fetal mouse SCN [3,32,33].

Several genes that are essential for the generation and maintenance of mammalian circadian oscillations at the cellular, tissue and organism levels have been identified [13,23,30]. Current knowledge indicates that these genes form a core transcription/translation feedback loop that controls the functional state of cellular machinery to produce circadian oscillations at the cellular level. Several of the 'clock' genes show circadian rhythms in their expression and/or in the intracellular distribution of their protein products in SCN cells [2,11,21,34].

The activity of genes essential for the generation of circadian oscillations in adults would be expected to also be essential for the initial onset of oscillations during development. Thus, the measurement of clock gene expression should provide direct assessment of pacemaker function within the SCN during development. To probe the functional development of the SCN, we characterized the developmental expression of 3 clock genes: Bmal1, Cry1, and Per1 in Syrian hamsters (*Mesocricetus auratus*). These genes all show circadian rhythms in mRNA abundance in the adult mouse and hamster SCN [2,11,21,24,34,39]. The Syrian hamster has a gestation length of about 15.5 days [40] and neurogenesis of the SCN is completed by 2 days before birth [9]. We collected brains at times corresponding to the mothers' circadian times 6, 12, and 18 on each day from embryonic day 13.5 (about 0.5 day after the end of SCN neurogenesis) to postnatal day 2. Those circadian times approximated the peak or trough of the Bmal1, Cry1 and Per1 expressions in the adult SCN [11,39]. We then performed in situ hybridization histochemistry for the mRNAs of those genes and performed densitometry analysis of the hybridization signals over the SCN region. Developmental and time of day related changes in mRNA abundance were assessed.

2. Material and methods

2.1. Animals

Adult male and female Syrian hamsters (*M. auratus*) from Charles River Laboratories (Kingston, NY) were entrained to a 14:10 LD cycle, with food and water continuously available. Matings were done during the last hour of the light phase of the LD cycle. Fertilization time was taken to be 6 h after lights off, which corresponded to about circadian time 18 of the females. This time was also designated as embryonic day 0. Hamsters have a gestation period of about 15.5 days, and embryonic day 15.5 was also designated postnatal day 0. During most of gestation, the dams were entrained to the same LD cycle. Before the day of fetal tissue collection, hamsters were transferred to constant dim light (<10 lx) at the normal time of lights off. The elapsed time of 6, 18, and 24 h in dim light was designated as the animals' CT18, 6, and 12, respectively. To collect tissue from pups, the dams were transferred to the dim-light room on the last day of gestation.

2.2. Tissue collection

To collect fetal brains, pregnant dams were anesthetized by an overdose of sodium pentobarbital (200 mg/kg body weight). Their heads were covered with hoods when they were taken out of the dim-light room. Fetuses were removed and the heads quickly cut into ice-cold PBS (0.01 M PBS, pH 7.4) for about 30 s before being immersed into the solution of 4% paraformaldehyde in 0.01 M PBS, pH 7.4 (PFA). For tissue collection at postnatal time points, pups were decapitated and the heads dropped into ice-cold PBS for about 30 s. The heads were then immersed into PFA with head skin removed to facilitate fixative penetration. Adult male hamsters were anesthetized by overdose of sodium pentobarbital (200 mg/kg body weight). Their heads were covered with hoods when they were taken out of the dim-light room and they were perfused transcardially with PBS followed by PFA. Brains were then removed and immersed in PFA.

Perinatal hamster heads and adult hamster brains were fixed in PFA for 24 h at 4 °C followed by PBS with 20% sucrose until the brains sank. Brains were then positioned in a 3% gelatin solution (w/v in PBS) in a plastic mold and cooled on ice until the solution solidified. A block was cut around the brain with about 1 mm of gelatin layer left around it. The whole block was then submersed in 2-methylbutane (Sigma) cooled to −30 to −40 °C for 3 minutes. 20-μm-thick coronal sections were cut on a cryostat at −20 °C and thaw-mounted onto gelatin and poly-L-lysine coated slides. For each brain, we collected at least 25 consecutive sections into five evenly

spaced series to cover the entire SCN region. Of the five series, three were used for in situ hybridization histochemistry for Bmal1, Cry1, and Per1, and one was used for Nissl staining. Sections were stored at −70 °C before in situ hybridization. All solutions for in situ hybridization were prepared in DEPC-treated water.

2.3. RNA probe synthesis and in situ hybridization

The Bmal1, Cry1, and Per1 cDNA templates used for RNA probe synthesis were PCR-amplified from a Syrian hamster SCN cDNA library using primers with T3/T7 promoter sequence extensions (for primer sequences used, see Table 1). The cDNA templates were provided by CJ Weitz of the Department of Neurobiology, Harvard Medical School. The ^{35}S-labeled RNA probes were synthesized using an in vitro transcription kit (Promega, Madison, WI) according to the protocol by Simmons et al. [37]. Final RNA probe hybridization solutions used for hybridization contain 50% formamide (v/v), 10% dextran sulfate (w/v), 0.3 M NaCl, 2% Denhart's solution (v/v), 0.01 M Tris, 0.001 M EDTA, 0.5 mg/ml tRNA and 0.01 M DTT (dithiothreitol) in DEPC-treated H_2O. The radioactivity of the hybridization solutions ranged from 10 to 15 × 10^6 cpm/ml. The hybridization protocol used was modified from that of Simmons et al. [37] with the 0.5% Triton X-100 (v/v) pretreatment instead of Proteinase K digestion. 60 μl of the RNA probe solution was applied to each slide, which was then coverslipped and incubated at 57 ± 1 °C for 16–24 h (depending on the probe). Slides were treated with RNase A (20 μg/ml) at 37 °C for 30 min and washed in saline sodium citrate (SSC) to final stringency of 0.1× SSC at 59 °C for 30 min. A ^{14}C radioactivity micro-scale (RPA504, Amersham Biosciences) was included on each film to calibrate exposures, which ranged from 2 to 3 weeks (depending on the probe). Hybridization with sense probes in either adult or fetuses never produced a distinct signal in any structure.

2.4. Densitometry analysis

Measurements of hybridization signal over the SCN region was done with a Northern Light Desktop Illuminator (Imaging Research, Ontario, Canada) using a Sony CCD video camera coupled to a computer running the AIS (6.0) software (Imaging Research). For each film, signals over the ^{14}C radioactivity micro-scales were measured in 8 steps (30–862 nCi/g of plastic substrate) to establish a standard curve. All densitometry measurements from sections fell within this curve.

The film image of each section analyzed was captured and digitized. A 190 × 190 μm circle (which corresponded to 10 × 10 pixels for the imaging setup used) was placed over the SCN region. The sampling circle abutted the wall of the 3rd ventricle medially and optic chiasm/optic tract ventrally (Fig. 1). For each individual animal and probe, 3 sections containing the SCN (rostral, middle and caudal)

Table 1
Primer sequences used for cDNA amplification

Genes	Sense[a] (5′ to 3′)	Antisense[b] (5′ to 3′)	Final size[c] (base)
Bmal1	AATTAACCCTCACTAAAGGGAAAGGCAGTGCCACCGACTACCAAG	TAATACGACTCACTATAGGGACGAAGAGGATCTTCCCTCTGTCAC	399
Cry1	AATTAACCCTCACTAAAGGGAACGCCGTGCACTGGTTCCGAAAGG	TAATACGACTCACTATAGGGCATTGAGTTCTATGATCTTGTCNAGGTC	419
Per1	AATTAACCCTCACTAAAGGGAGTCGGACATCATCATGATGGAAGAC	TAATACGACTCACTATAGGGATAGTTAGGGAGCACGGCAGGGGGA	564

[a] T3 promoter sequence (AATTAACCCTCACTAAAGGG) was attached.
[b] T7 promoter sequence (TAATACGACTCACTATAGGG) was attached.
[c] The size does not include the T3 or T7 promoter sequence.

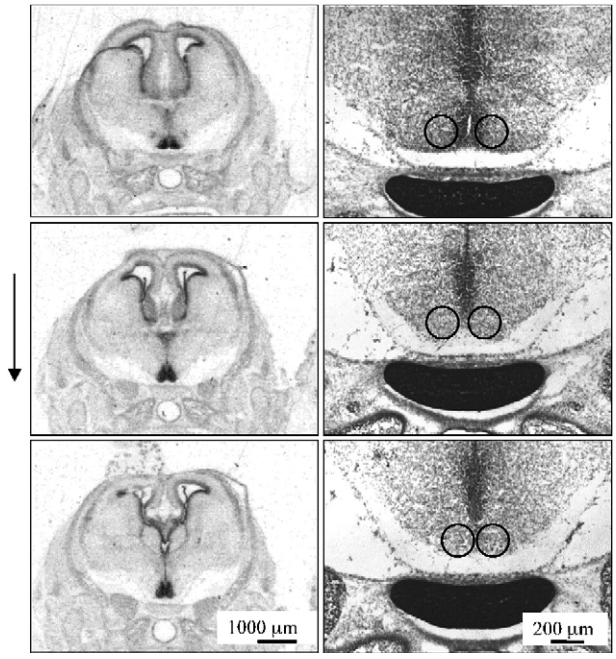


Fig. 1. Sampling area for the densitometry analysis of in situ signals over the SCN region. The autoradiographic images of brain sections hybridized with the Bmal1 probe are shown in the left panels. Examples of the sampling circles (approximately 190 μm in diameter) used for the densitometry are shown on the autoradiographs and at higher magnification on adjacent Nissl-stained sections (right panels). The sections are from a day 13.5 fetus. To reduce variation due to sampling position, a densitometry reading over each SCN was repeated 3 times. For each brain, 3 sections representing the rostral, middle, and caudal SCN were analyzed. The arrow to the left indicates a rostral to caudal direction.

were analyzed, and the left and right SCN on each section were measured 3 times. Eighteen values per animal were then averaged and taken as the SCN in situ signal strength for that particular brain.

Data were analyzed by one-way ANOVAs, and differences between pairs of groups were analyzed by Tukey's multiple comparisons if the overall ANOVA was significant. Unpaired *t* tests were used when only two groups were analyzed. Although 18 densitometric measurements were made on each brain to produce a value for each brain, the sample sizes used for statistical calculations and analyses were based on the number of brains in an experimental group.

3. Results

3.1. Developmental expression of Bmal1, Cry1, and Per1 in the SCN

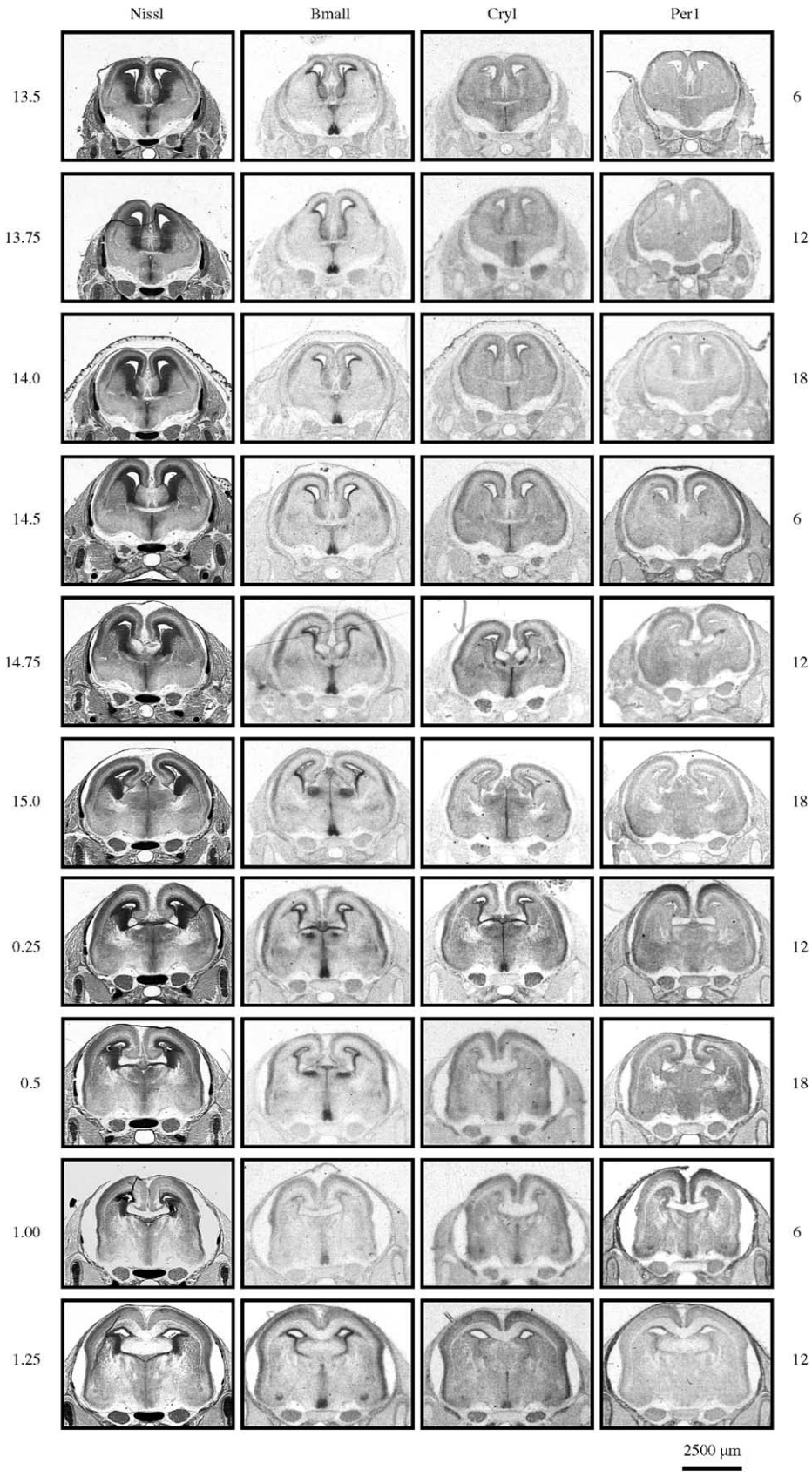
Fig. 2 shows representative in situ hybridization autoradiographs for three clock genes, Bmal1, Cry1, and Per1, during development. A Bmal1 signal was prominent beginning at the earliest age examined, 13.5 days after fertilization. The signal defined a structure corresponding to the SCN as determined by other anatomical criteria (vasoactive intestinal peptide mRNA expression [20] and surrounding landmarks). The signals for Cry1 and Per1 in the SCN region of prenatal brains were weaker than those for Bmal1 and in most cases were similar to those of the surrounding hypothalamic regions as judged visually (Fig. 2). At postnatal ages, however, stronger Cry1 and Per1 signals were observed within the SCN. Fig. 3 summarizes densitometry measurements over the SCN region for the three genes at different ages (for detailed information on the number of litters and fetuses/pups used for each developmental time, see Table 2).

The signal for Bmal1 was consistently high at all prenatal ages. The signal was also strong at the time point immediately after birth (postnatal 0.25, with birth at about E15.5 or PN 0). The signal at PN 0.25 was similar to those of prenatal ages ($P > 0.05$) but significantly higher than those of other postnatal ages ($P < 0.001$). Relative to prenatal signals, Cry1 and Per1 also showed strong signals immediately after birth. For Cry1, the signal at PN 0.25 was significantly higher than those of any other age ($P < 0.001$). For Per1, the signal was significantly higher than that of PN 1.25 and any prenatal age ($P < 0.001$) but similar to those at PN 0.5 and 1.0. When values were pooled into prenatal and postnatal groups (excluding PN 0.25 from both groups), Bmal1 expression significantly decreased while Per1 expression significantly increased after birth (Fig. 4). The expression levels for Cry1 did not differ significantly between the prenatal and postnatal ages.

3.2. Daily variation in levels of Bmal1, Cry1, and Per1 during development

The time points used in this study (3 time points per day corresponding to approximately the dam's circadian times 6, 12 and 18) were chosen on the basis of Bmal1 and Per1 rhythms in the adult hamster SCN (Fig. 5). Per1 mRNA abundance peaked at around CT6, while Bmal1 mRNA was high at CT14 and CT18. Per1 was low at CT18 and Bmal1 was low between CT6 and 10. These results agree with published results on the circadian expression profiles of Per1 and Bmal1 and their approximate anti-phase relationship [2,11,34,39]. Thus, the times chosen should have provided high, low, and intermediate (CT12) values for both genes. In preliminary experiments, low and high values for Per1 at CT6 and 18 were observed in brains collected between postnatal days 1 and 2 (Fig. 6). Although we did not characterize the circadian expression profile for Cry1 in

Fig. 2. Examples of autoradiographic images used for the analysis of Bmal1, Cry1, and Per1 during development. Each row shows a Nissl section and three autoradiographic images from a single brain. The age of the brain is shown down the left side of the figure, with 13.5 to 15.0 corresponding to days since fertilization (embryonic) and 0.25 to 1.25 corresponding to days after birth (postnatal). On the right side of the figure, the corresponding maternal circadian times (CT) are indicated. CT12 is the time when lights off of the prior light/dark cycle would have occurred. This corresponds approximately to the dam's time of activity onset.


Nissl
Bmall
Cryl
Per1
13.5
6
13.75
12
14.0
18
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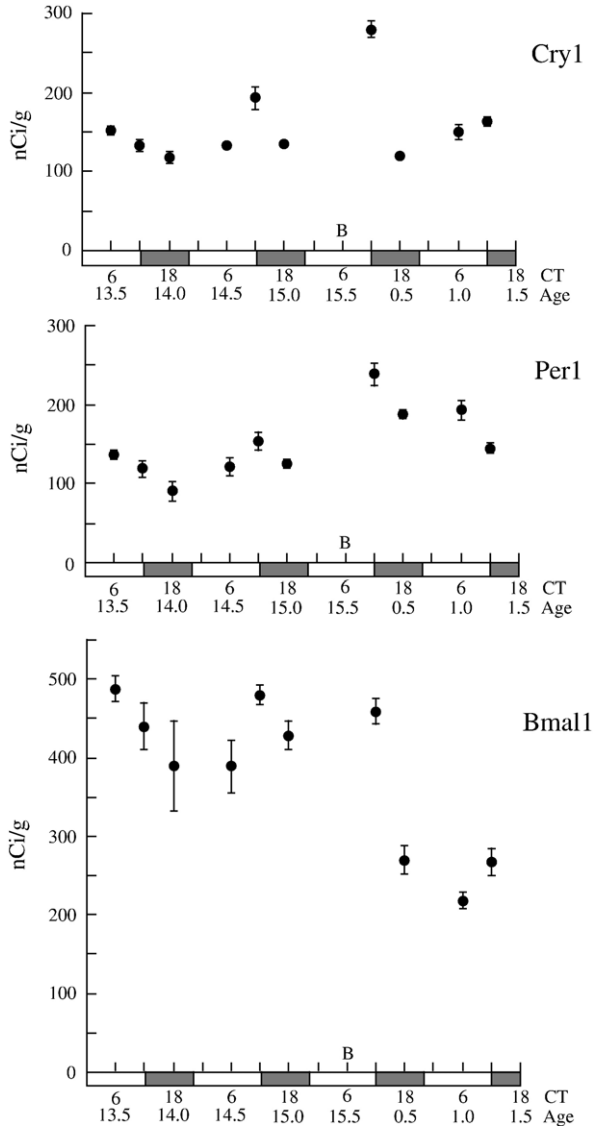

Fig. 3. Expression levels of Cry1, Per1, and Bmal1 in the SCN region during development. Each point shows the average expression level (±SEM) at a different age and circadian time (CT). Where error bars are not visible, they are contained within the symbol. Densitometry readings were converted to nCi/g using a standard curve (see Materials and methods) and nCi/g is proportional to the amount of probe hybridized to native mRNA. The light/dark cycle on which the dams were kept is shown below each graph. Dams were in dim constant light at the times of tissue collection. B indicates the approximate time of birth.

the adult hamster, results with mice and hamsters show that its expression in the SCN also has a circadian rhythm that peaks around CT12 [21,39].

Circadian variation during development for each gene was analyzed by combining all CT6 values, all CT12 values, and all CT18 values excluding the first time point after birth, which was especially high for all three genes. Each circadian time was therefore represented by two sets of prenatal values and one set of postnatal values. The average values for each circadian time are shown for all three genes in Fig. 7. Neither Per1 nor Bmal1 showed significant variation across circadian times ($P > 0.2$). Cry1, however, showed significant variation ($P < 0.001$), with the highest value at CT12. In contrast to Per1 and Bmal1, Cry1 did not show a significant change in mRNA levels between prenatal and postnatal ages (except for the first postnatal time point). Thus, less developmental variation in Cry1 may have allowed weak circadian variation to be detected. If prenatal data are analyzed separately, the results are the same for all three genes: no significant variation for Per1 and Bmal1 and a significant ($P < 0.05$) variation for Cry1, with the highest average value at CT12. When postnatal data are analyzed separately, both Per1 and Cry1 show significant variation ($P < 0.001$) and the variation in Bmal1 is close to significant ($P = 0.064$). As can be seen in Fig. 3, the highest average values postnatally (excluding the first time

Table 2
Summary information for tissue collection

Age (days postfertilization or postnatal)	Dam's circadian time	Number of fetuses (pups)/number of litters		
		Bmal1	Cry-1	Per-1
13.5	CT6	12/2	12/2	11/2
13.75	CT12	11/2	11/2	13/2
14.0	CT18	4/1	4/1	4/1
14.5	CT6	12/2	12/2	12/2
14.75	CT12	10/2	10/2	6/2
15.0	CT18	10/2	10/2	8/2
0.25	CT12	5/1	4/1	5/1
0.5	CT18	10/2	10/2	10/2
1.0	CT6	12/1	12/1	14/1
1.25	CT12	17/3	16/3	17/3

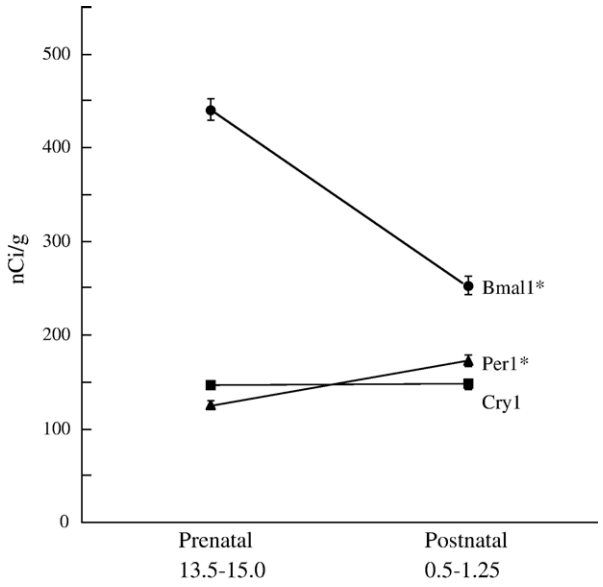

Fig. 4. Expression levels of Cry1, Per1, and Bmal1 in the SCN region before and after birth. Symbols indicate the average expression values (nCi/g, ±SEM) after combining the prenatal and postnatal ages (excluding PN 0.25). Where error bars are not visible, they are contained within the symbol. Bmal1 showed a significant decrease in hybridization signal and Per1 showed a significant increase (*$P < 0.001$, t test). There was no difference in the average Cry1 signal between pre- and postnatal ages ($P > 0.8$).

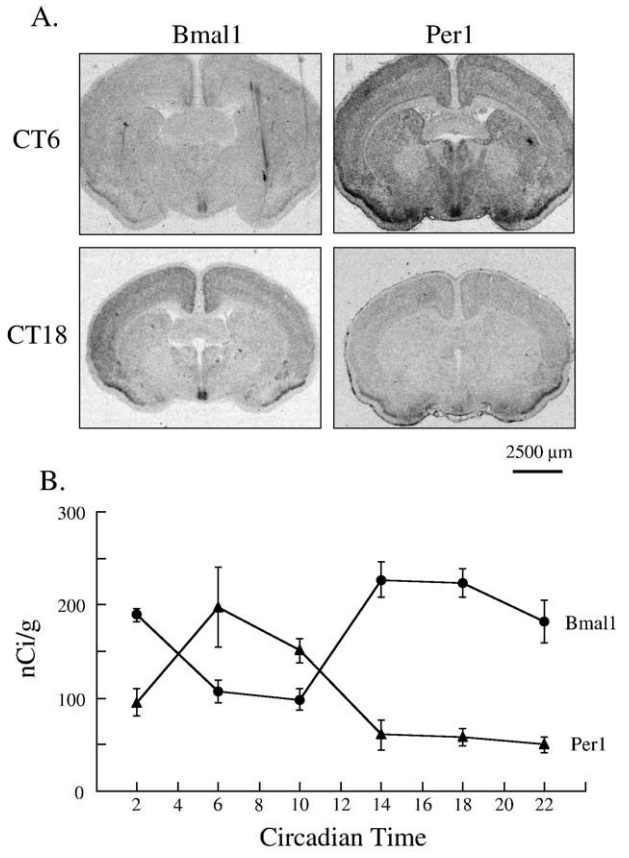

Fig. 5. Circadian expression of Bmal1 and Per1 in the adult SCN. (A) Representative autoradiographic images of adult brain sections collected at CT6 or CT18 and hybridized with Bmal1 or Per1 probes. (B) Circadian profile of Bmal1 and Per1 mRNA levels in the adult hamster SCN. Each point represents the average of measurements from three brains. Error bars represent standard errors. For both genes, variation over time was significant ($P < 0.01$, one-way ANOVA).

point after birth) were CT12, CT6, and CT18 for Cry1, Per1, and Bmal1, respectively.

4. Discussion

The phenotypic characteristics of SCN cells begin to appear soon after the cells are produced. Neurogenesis of the hamster SCN occurs between 10.5 and 13.0 days postfertilization [9] and the present results show that by 13.5 days after fertilization, SCN cells strongly express Bmal1 and are distinct from surrounding cells. The differentiation of SCN cells is likely to begin even earlier as indicated by the expression of vasoactive intestinal peptide (VIP) mRNA at 13.0 days after fertilization [20].

Our expectation at the start of this work was that "clock genes" would be expressed in the fetal SCN and would show 24 h rhythms in mRNA abundance. This expectation was based on the evidence for rhythmicity in the fetal rat SCN [27–29,35] and on evidence that fetal hamsters have an entrainable circadian pacemaker [7,42,43]. The rhythmic expressions of Per1, Cry1, and Bmal1 is thought to directly track molecular oscillations that are required for the generation of circadian rhythms [13,23,30]. Therefore, if circadian oscillations are generated and entrained in the fetal SCN, then rhythms in the expression of theses genes would be expected.

Despite a strong signal for Bmal1 in the fetal SCN, there was no evidence for prenatal rhythmicity in Bmal1 mRNA levels. Likewise, there was no evidence of a prenatal rhythm in Per1 mRNA even though a clear rhythm could be detected just 2 days after birth. Cry1, however, showed slight but significant variation across circadian times even when only prenatal data were analyzed. In addition, the highest average value for Cry1 before birth was at CT12 on the last of gestation, a time when Cry1 is expected to be high if molecular oscillations similar to those in the adult SCN are being expressed and are approximately in phase with maternal rhythms. The

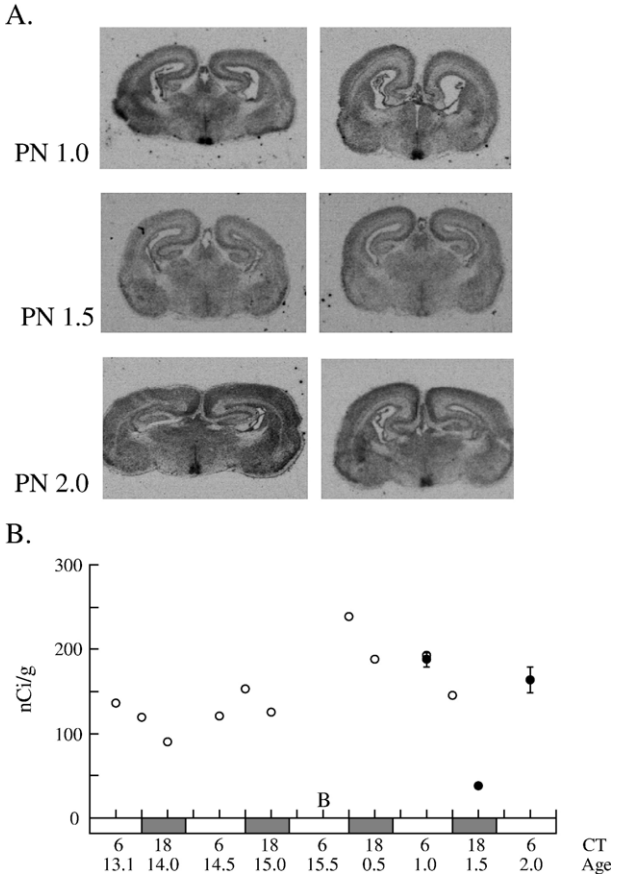

Fig. 6. Variation in Per1 expression on the second day after birth. (A) Autoradiographic images of sections from the brains of two hamsters at each of three ages. Postnatal (PN) 1.0 corresponds to 1 day after the approximate time of birth and corresponds to the dam's CT6. PN 1.5 corresponds to the dam's CT18. (B) Average expression levels of Per1 at three postnatal ages (filled symbols) plotted with the developmental data (open symbols) from Fig. 3. Standard errors are indicated by the vertical lines and, if not visible, are contained within the symbol (filled symbols) or were not plotted (open symbols). Per1 expression showed significant variation across the three postnatal ages ($P < 0.01$, one-way ANOVA, $n = 4$ per time point).

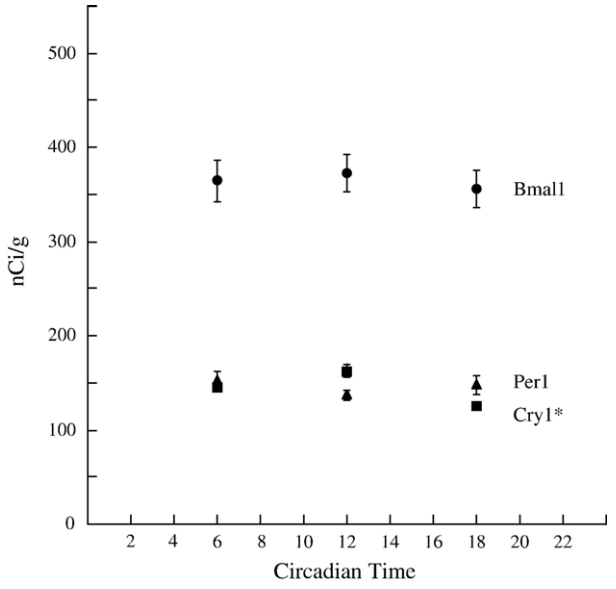

Fig. 7. Expression levels of Cry1, Per1, and Bmal1 in the SCN region at different circadian times during development. Symbols indicate the average expression values (nCi/g, ±SEM) after combining values from brains collected at the same circadian times (CT6, CT12, or CT18). Each CT includes values from two prenatal ages and one postnatal age. Where error bars are not visible, they are contained within the symbol. There was no variation in Bmal1 or Per1 ($P > 0.2$, one-way ANOVA). Cry1 showed significant variation, with the highest average value at CT12 (*$P < 0.001$, t test).

highest postnatal value (except for the first time point) was also at CT12. Thus, it is possible that circadian oscillations in Cry1 mRNA were expressed beginning on the last day of gestation even though oscillations in Per1 and Bmal1 could not be detected. Any such rhythmicity is not necessarily generated within the fetal SCN since the fetuses were exposed to maternal rhythms that could, hypothetically, acutely affect fetal physiology.

Rhythms in all three genes may begin to emerge around the time of birth. Except for the first time point after birth (PN 0.25), each gene over the next 24 h (3 time points) showed the highest average value at the circadian time predicted based on rhythms in adults (Bmal1: CT18; Per1: CT6; Cry1: CT12). Although levels of Per1 mRNA increased after birth in the main developmental study, a clear rhythm in Per1 expression on postnatal day 2 (from a separate study) appeared to emerge as a result of a return to low levels at CT18 (Fig. 6). It is possible that feedback inhibition of Per1 expression required an initial elevation in Per1.

It is unlikely that prenatal rhythms in Per1 and Bmal1 were expressed within individual fetuses but were not detected because the fetuses were out of phase with each other. Rhythms in 2DG uptake [27,28] and in VP mRNA in rat fetuses [29] indicate that fetuses are synchronized as long as the dam's rhythms are intact. In hamsters, prenatal synchrony is indicated by experiments in which the dam's SCN is lesioned. When such lesions are performed early in gestation, the behavioral rhythms of the pups within a litter are not synchronized, but when the lesions are performed late in gestation, synchrony is observed [6]. Thus, prior to the lesions late in gestation, the pups had been synchronized. Although we obtained tissues at times when each gene should be present in high and low amounts based on the rhythms in adults, it is possible that with three time points a day, the lowest and/or highest values for each gene were missed. This seems unlikely, and in rats (see below), even with 12 samples a day, molecular rhythms were not detected in the fetus.

The absence of robust rhythms in clock gene expression in the fetal hamster SCN is consistent with a recent report in rats. Sladek et al. [38] measured the expression of Per1, per2, Cry1, Bmal1 and clock on embryonic day 19 in rats. This is approximately 3 days before birth and 2 days after the completion of neurogenesis [1]. Similar to our results with Per1 and Bmal1, none of the genes showed significant variation in mRNA levels before birth. However, Per1, as well as Per2, Cry1, and Bmal1, was rhythmic by postnatal day 3. Also similar to our results, Per1 and Cry1 were consistently low in the fetus and Bma11 was consistently high. Per2, which we did not measure, showed levels in the fetal rat that were intermediate to the minimum and maximum levels observed postnatally.

It may be that under certain circumstances, the fetal rat SCN can exhibit daily rhythmicity. This is seen in 2DG uptake on E19 [27,28] and in VP mRNA levels on E21 [29]. In addition, a rhythm in Per1 mRNA has been observed on E20 [25]. Why the present results and those of Sladek et al. [38] did not reveal rhythms in the fetus (with the possible exception of Cry1 in the present study) is unclear. Until this apparent inconsistency between clock gene rhythms and other measures of SCN physiology is observed within the same study, it is premature to propose explanations.

In hamsters, except for the variation in Cry1 reported here, there is no direct evidence that the fetal SCN is rhythmic. There is strong evidence, however, that an entrainable pacemaker exists prenatally. Single injections of either melatonin or a dopamine agonist before birth can set the phase of behavioral rhythms expressed by the pups [42]. It is possible that oscillations would not normally be expressed in fetal hamster SCN but that exogenous melatonin or a dopamine agonist initiate the oscillations, thereby setting the phases.

The phase-setting effects of single prenatal injections in hamsters suggests that clock genes in the fetus, even if not yet oscillating, are sensitive to external stimuli. An analysis of how a single perturbation could, in theory, induce synchrony among fetuses revealed that a single perturbation would need to induce large phase shifts of ongoing oscillations in order to cause synchrony [42]. Equally effective would be the simultaneous initiation of oscillations in different fetuses. In either case, the fetal pacemaker appears to show unusual sensitivity to pharma-

cological agents. This might be expected if the underlying oscillations are on the verge of beginning to oscillate or are oscillating with low amplitude as suggested by the present results.

Our results raise the possibility that birth influences the expression of clock genes in the SCN. Per1 and Cry1 showed highest expression immediately after birth, and the expression of Bmal1 immediately after birth was the highest postnatal value for that gene (Fig. 3). Although previous evidence suggests that fetal oscillations that have been entrained by prenatal melatonin injections cannot be reset by the birth process [41], it is possible that under normal circumstances (without prenatal injections or surgery), physiological events at birth contribute to the initiation of oscillations. Birth in hamsters, measured as the first appearance of pups, occurs at restricted times of day and the timing of birth can be changed by shifting the mother's circadian rhythms [40]. These observations suggest that a phase-setting effect of birth could coordinate fetal oscillations with the rhythms of the mother. Some event associated with birth may be even more precisely timed than is the appearance of pups.

Any attempt to measure daily variation in gene expression or other endpoint during development is complicated by the possible masking of daily variation by developmental changes in the same endpoint. We observed a significant postnatal decrease in Bmal1 expression and a significant postnatal increase in Per1 expression in the SCN region compared to prenatal levels. If daily variations are just beginning around the time of birth, they may be difficult to observe because of changes related to birth or other aspects of development. Cry1, which showed no difference in prenatal and postnatal levels, showed small but significant circadian variation both before and after birth. We cannot exclude the possibility of low-amplitude oscillations in Per1 and Bmal1 or high-amplitude oscillations in only a small number of cells. It is clear, however, that SCN oscillations, if present, are not as robust as in adults [11] or even as robust as the Per1 rhythm in pups 2 days after birth (Fig. 7).

The present results raise new questions about the effects of prenatal phase-setting stimuli such as melatonin and dopamine agonists [7,15–17,42,43]. Whether these treatments initiate oscillations or set the phase of surreptitious oscillations, the affected oscillations come to dominate oscillations of the mature SCN. That is, the phase that is set prenatally is seen in the phases of behavioral rhythms at weaning. Thus, the phase of the mature SCN, which controls the behavioral rhythms [26], can be set by prenatal events, most likely within the fetal SCN. If only a small number of cells are oscillating in the fetal SCN, then those cells come to dominate the phase of the entire SCN. Alternatively, if phase is set as a result of oscillation being initiated by a treatment, then the oscillations must be initiated at approximately the same phase across cells and across fetuses, otherwise synchrony of phases among pups would not occur. An evaluation of these and other scenarios will require closer examination of the cellular and molecular events in the SCN around the time that oscillations begin.

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References

[1] J. Altman, S.A. Bayer, Development of the diencephalon in the rat: I. Autoradiographic study of the time of origin and settling patterns of neurons of the hypothalamus, J. Comp. Neurol. 182 (1978) 945–972.
[2] K. Bae, X. Jin, E.S. Maywood, M.H. Hastings, S.M. Reppert, D.R. Weaver, Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock, Neuron 30 (2001) 525–536.
[3] M. Bender, J. Drago, S.A. Rivkees, D1 receptors mediate dopamine action in the fetal suprachiasmatic nuclei: studies of mice with targeted deletion of the D1 dopamine receptor gene, Mol. Brain Res. 49 (1997) 271–277.
[4] F.C. Davis, Ontogeny of circadian rhythms, in: J. Aschoff (Ed.), Handb. Behav. Neurobiol., vol. 4, Plenum Publishing Corp, New York, 1981, pp. 257–274.
[5] F.C. Davis, R.A. Gorski, Development of hamster circadian rhythms: prenatal entrainment of the pacemaker, J. Biol. Rhythms 1 (1985) 77–89.
[6] F.C. Davis, R.A. Gorski, Development of hamster circadian rhythms: role of the maternal suprachiasmatic nucleus, J. Comp. Physiol., A 601 (1988) 610.
[7] F.C. Davis, J. Mannion, Entrainment of hamster pup circadian rhythms by prenatal melatonin injections to the mother, Am. J. Physiol. 255 (1988) R439–R448.
[8] F.C. Davis, S.M. Reppert, Development of mammalian rhythms, in: J.T. Takahashi, F.W. Turek, R.Y. Moore (Eds.), Handbook of Behavioral Neurobiology: Circadian Clocks, Plenum Publishing Co, Inc., New York, 2001, pp. 247–290.
[9] F.C. Davis, R. Boada, J. LeDeaux, Neurogenesis of the hamster suprachiasmatic nucleus, Brain Res. 519 (1990) 192–199.
[10] F.C. Davis, M.G. Frank, H.C. Heller, Ontogeny of sleep and circadian rhythms, in: F.W. Turek, P.C. Zee (Eds.), Regulation of Sleep and Circadian Rhythms, Marcel Dekker, Inc., New York, 1999, pp. 19–79.
[11] T. Deguchi, Ontogenesis of a biological clock for serotonin: acetyl coenzyme A *N*-acetyltransferase in pineal gland of rat, Proc. Natl. Acad. Sci. U. S. A. 72 (1975) 2814–2818.
[12] H.O. De la Iglesia, J. Meyer, A. Carpino Jr., W.J. Schwartz, Antiphase oscillation of the left and right suprachiasmatic nuclei, Science 290 (2000) 799–801.
[13] J.C. Dunlap, Molecular bases for circadian clocks, Cell 96 (1999) 271–290.
[14] M.U. Gillette, The suprachiasmatic nuclei: circadian phase-shifts induced at the time of hypothalamic slice preparation are preserved in vitro, Brain Res. 379 (1986) 176–181.
[15] J. Grosse, F.C. Davis, Melatonin entrains the restored circadian activity rhythms of Syrian hamsters bearing fetal suprachiasmatic nucleus grafts, J. Neurosci. 18 (1998) 8032–8037.
[16] J. Grosse, F.C. Davis, Transient entrainment of a circadian pacemaker during development by dopaminergic activation in Syrian hamsters, Brain Res. Bull. 48 (1999) 185–194.
[17] J. Grosse, A. Velickovic, F.C. Davis, Entrainment of Syrian hamster circadian activity rhythms by neonatal melatonin injections, Am. J. Physiol.: Regul., Integr. Comp. Physiol. 270 (1996) R533–R540.
[18] T. Hiroshige, K. Honma, K. Watanabe, Possible zeitgebers for external

entrainment of the circadian rhythm of plasma corticosterone in blind infantile rats, J. Physiol. 325 (1982) 507–519.
[19] S.-I.T. Inouye, H. Kawamura, Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 5962–5966.
[20] S.J. Kuhlman, In situ hybridization of vasoactive intestinal polypeptide mRNA: the time course, distribution, and rhythmicity of gene transcription in the fetal suprachiasmatic nucleus, Biology, Northeastern University, Boston, 1996.
[21] K. Kume, M.J. Zylka, S. Sriram, L.P. Shearman, D.R. Weaver, X. Jin, E.S. Maywood, M.H. Hastings, S.M. Reppert, mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop, Cell 98 (1999) 193–205.
[22] R.K. Leak, R.Y. Moore, Topographic organization of suprachiasmatic nucleus projection neurons, J. Comp. Neurol. 433 (2001) 312–334.
[23] P.L. Lowrey, J.S. Takahashi, Mammalian circadian biology: elucidating genome-wide levels of temporal organization, Annu. Rev. Genomics Hum. Genet. 5 (2004) 407–441.
[24] E.S. Maywood, N. Mrosovsky, M.D. Field, M.H. Hastings, Rapid down-regulation of mammalian period genes during behavioral resetting of the circadian clock, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 15211–15216.
[25] H. Ohta, S. Honma, H. Abe, K. Honma, Effects of nursing mothers on rPer1 and rPer2 circadian expressions in the neonatal rat suprachiasmatic nuclei vary with developmental stage, Eur. J. Neurosci. 15 (2002) 1953–1960.
[26] M.R. Ralph, R.G. Foster, F.C. Davis, M. Menaker, Transplanted suprachiasmatic nucleus determines circadian period, Science 247 (1990) 975–978.
[27] S.M. Reppert, W.J. Schwartz, Maternal coordination of the fetal biological clock in utero, Science 220 (1983) 969–971.
[28] S.M. Reppert, W.J. Schwartz, The suprachiasmatic nuclei of the fetal rat: characterization of a functional circadian clock using 14C-labeled deoxyglucose, J. Neurosci. 4 (1984) 1677–1682.
[29] S.M. Reppert, G.R. Uhl, Vasopressin messenger ribonucleic acid in supraoptic and suprachiasmatic nuclei: appearance and circadian regulation during development, Endocrinology 120 (1987) 2483–2487.
[30] S.M. Reppert, D.R. Weaver, Coordination of circadian timing in mammals, Nature 418 (2002) 935–941.
[31] S.M. Reppert, R.J. Coleman, H.W. Heath, J.R. Swedlow, Pineal *N*-acetyltransferase activity in 10-day-old rats: a paradigm for studying the developing circadian system, Endocrinology 115 (1984) 918–925.
[32] W.J. Schwartz, In vivo metabolic activity of hamster suprachiasmatic nuclei: use of anesthesia, Am. J. Physiol. 252 (1987) 419–422.
[33] W.J. Schwartz, Different in vivo metabolic activities of suprachiasmatic nuclei of Turkish and golden hamsters, Am. J. Physiol. 259 (1990) R1083–R1085.
[34] L.P. Shearman, S. Sriram, D.R. Weaver, E.S. Maywood, I. Chaves, B. Zheng, K. Kume, C.C. Lee, G.T. van der Horst, M.H. Hastings, S.M. Reppert, Interacting molecular loops in the mammalian circadian clock, Science 288 (2000) 1013–1019.
[35] S. Shibata, R.Y. Moore, Development of neuronal activity in the rat suprachiasmatic nucleus, Dev. Brain Res. 34 (1987) 311–315.
[36] R. Silver, J. LeSauter, P.A. Tresco, M.N. Lehman, A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms, Nature 382 (1996) 810–813.
[37] D.M. Simmons, J.L. Arriza, L.W. Swanson, A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radio-labeled single-stranded RNA probes, J. Histotechnol. 12 (1989) 169–181.
[38] M. Sladek, A. Sumova, Z. Kovacikova, Z. Bendova, K. Laurinova, H. Illnerova, Insight into molecular core clock mechanism of embryonic and early postnatal rat suprachiasmatic nucleus, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 6231–6236.
[39] B.B. Tournier, J.S. Menet, H. Dardente, V.J. Poirel, A. Malan, M. Masson-Pevet, P. Pevet, P. Vuillez, Photoperiod differentially regulates clock genes' expression in the suprachiasmatic nucleus of Syrian hamster, Neuroscience 118 (2003) 317–322.
[40] N. Viswanathan, F.C. Davis, Timing of birth in Syrian hamsters, Biol. Reprod. 47 (1992) 6–10.
[41] N. Viswanathan, F.C. Davis, The fetal circadian pacemaker is not involved in the timing of birth in hamsters, Biol. Reprod. 48 (1993) 530–537.
[42] N. Viswanathan, F.C. Davis, Single prenatal injections of melatonin or the D1-dopamine receptor agonist SKF 38393 to pregnant hamsters sets the offsprings' circadian rhythms to phases 180∞ apart, J. Comp. Physiol., A 180 (1997) 339–346.
[43] N. Viswanathan, D.R. Weaver, S.M. Reppert, F.C. Davis, Entrainment of the fetal hamster circadian pacemaker by prenatal injections of the dopamine agonist, SKF 38393, J. Neurosci. 14 (1994) 5393–5398.
[44] D.K. Welsh, D.E. Logothetis, M. Meister, S.M. Reppert, Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms, Neuron 14 (1995) 697–706.
[45] S. Yamazaki, R. Numano, M. Abe, A. Hida, R. Takahashi, M. Ueda, G.D. Block, Y. Sakaki, M. Menaker, H. Tei, Resetting central and peripheral circadian oscillators in transgenic rats, Science 288 (2000) 682–685.
[46] S.H. Yoo, S. Yamazaki, P.L. Lowrey, K. Shimomura, C.H. Ko, E.D. Buhr, S.M. Siepka, H.K. Hong, W.J. Oh, O.J. Yoo, M. Menaker, J.S. Takahashi, PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 5339–5346.