Rhythmic expression of cytochrome P450 epoxygenases CYP4x1 and CYP2c11 in the rat brain and vasculature

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Carver KA, Lourim D, Tryba AK, Harder DR. Rhythmic expression of cytochrome P450 epoxygenases CYP4x1 and CYP2c11 in the rat brain and vasculature. Am J Physiol Cell Physiol 307: C989–C998, 2014. First published July 23, 2014; doi:10.1152/ajpcell.00401.2013.—Mammals have circadian variation in blood pressure, heart rate, vascular tone, thrombotic tendency, and cerebral blood flow (CBF). These changes may be in part orchestrated by circadian variation in clock gene expression within cells comprising the vasculature that modulate blood flow (e.g., fibroblasts, cerebral vascular smooth muscle cells, astrocytes, and endothelial cells). However, the downstream mechanisms that underlie circadian changes in blood flow are unknown. Cytochrome P450 epoxygenases (CYP4x1 and CYP2c11) are expressed in the brain and vasculature and metabolize arachidonic acid (AA) to form epoxyeicosatrienoic acids (EETs). EETs are released from astrocytes, neurons, and vascular endothelial cells and act as potent vasodilators, increasing blood flow. EETs released in response to increases in neural activity evoke a corresponding increase in blood flow known as the functional hyperemic response. We examine the hypothesis that CYP2c11 and CYP4x1 expression and EETs production vary in a circadian manner in the rat brain and cerebral vasculature. RT-PCR revealed circadian/diurnal expression of clock and clock-controlled genes as well as CYP4x1 and CYP2c11, within the rat hippocampus, middle cerebral artery, inferior vena cava, hippocampal astrocytes and rat brain microvascular endothelial cells. Astrocyte and endothelial cell culture experiments revealed rhythmic variation in CYP4x1 and CYP2c11 gene and protein expression with a 12-h period and parallel rhythmic production of EETs. Our data suggest there is circadian regulation of CYP4x1 and CYP2c11 gene expression. Such rhythmic EETs production may contribute to circadian changes in blood flow and alter risk of adverse cardiovascular events throughout the day.

In rats, neurons, astrocytes, and endothelial cells express cytochrome P450 (Cyp450) epoxygenases (Cyp4x1 and/or Cyp2c11) that convert arachidonic acid (AA) to epoxyeicosatrienoic acids (EETs). EETs are involved in the functional hyperemic response, increasing nutritive blood flow to active neurons (5, 12, 13). Astrocytes play a major intermediary role in coupling CBF to neural activity. For example, astrocytes express Cyp450 epoxygenases and synthesize and release EETs in response to membrane receptor stimulation by glutamate and adenosine that are released from active neurons (3, 25). The released EETs induce cerebral vasodilation, an effect that is independent of nitric oxide (NO) induced vasodilation (19). EETs are released from astrocyte endfeet and diffuse to cerebral arterial muscle cells wherein EETs-signal activates Ca2+-activated K+ channel currents (6, 10, 11, 16), causing hyperpolarization of VSMC and subsequent vasodilation with increased CBF (9, 37). EETs released from neurons, astrocytes, and cerebral vascular endothelial cells onto VSMC can activate the phosphatidylinositol 3-kinase (PI3K)/phosphorylated Akt (P-Akt/protein kinase B) pathway (8), also resulting in VSMC hyperpolarization (23) and increased blood flow. In addition to the contribution of astrocyte-released EETs, it is also possible that cerebral VSMC have circadian changes in epoxygenase activity and synthesize EETs that are released onto blood vessels to function in an autocrine or paracrine fashion to regulate blood flow (27).

We investigate the hypothesis that circadian expression of CYP2c11 and CYP4x1 genes results in circadian variation in EETs production, whose rhythmic signaling could contribute to diurnal changes in CBF regulatory mechanisms such as the magnitude of the functional hyperemic response or the autoregulatory response capacity. Our study aims to help define circadian mechanisms that may play a role in modulating and coordinating blood flow, revealing potentially new therapeutic targets to regulate cerebral blood flow, as well as give new insight into the best time-of-day to administer treatments.1

Glossary

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CT</td>
<td>Circadian time</td>
</tr>
<tr>
<td>Cyp450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>Dbp</td>
<td>D-site albumin promoter binding protein</td>
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</tbody>
</table>

1 This article is the topic of an Editorial Focus by William J. Pearce (24a).
DBPE  D-site albumin promoter binding protein response element
E4bp4  E4 promoter binding protein 4
EC  Endothelial cells
EETs  Epoxycosatrienoic acids
eNOS  Endothelial nitric oxide synthase
NO  Nitric oxide
P-Akt  Phosphorylated Akt (protein kinase B)
Per1/Per2  Period 1/2
PI3K  Phosphatidylinositol 3-kinase
Reverbα  Reverse erythroblast leukemia viral oncogene homolog-α
RORE  Retinoic acid-related orphan receptor response element
VSMC  Vascular smooth muscle cells

MATERIALS AND METHODS

Bioinformatics analysis. The genomic sequences of human, rat, and mouse Cyp4x1 were compared from 4,000 bases upstream from the transcription start site to 2,000 bases downstream from the start site. Using the BLAST tool (http://blast.ncbi.nlm.nih.gov), we identified regions that were conserved among species. We then used a pattern-finding tool, fuzznuc (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=fuzznuc), as described previously (33), to search for E-box, retinoic acid-related orphan receptor response element (RORE), and D-site albumin promoter binding protein response element (DBPE) binding elements using the following consensus sequences: E-box, CA[TC-][AG][T][GT]; RORE, [AT][A][AT][NT][AG][GGTCA]; and DBPE, [GA][T][GT][AT][TC][GT][A][TC]. A one base mismatch was allowed for RORE and DBPE sites. The extent of conservation between the species was then determined. The same protocol was repeated to compare rat Cyp2c11 to its human homolog, CYP2C9.

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Medical College of Wisconsin. Male Wistar rats were obtained from Harlan (Indianapolis, IN). Animals were kept on a 12:12-h light-dark schedule for 14 days and then transferred to darkness for at least 24 h before being killed every 4 h beginning at 6:00 AM, circadian time 0 (CT 0) and then transferred to darkness for at least 24 h before being killed. Animals were kept on a 12:12-h light-dark schedule for 14 days.

Table 1. RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>E4bp4</td>
<td>ggtgcagcgaagcagacttc</td>
<td>agtttgcgcccgccccgttct</td>
<td>56°C</td>
</tr>
<tr>
<td>Reverbα</td>
<td>gagggtgtagaatttgccaa</td>
<td>cctccatgcgtgaacactgsgg</td>
<td>56°C</td>
</tr>
<tr>
<td>Dbp</td>
<td>agccgaaagagagcggcagtg</td>
<td>ctctggtgctctgacctcctt</td>
<td>56°C</td>
</tr>
<tr>
<td>Per1</td>
<td>acacccagaaaaagagagcacc</td>
<td>gctgagaagccttggtagttct</td>
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</tr>
<tr>
<td>Per2</td>
<td>gaggagagagagcagccgcccttccc</td>
<td>ttgacagcttgacctgctctc</td>
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</tr>
<tr>
<td>Bmal1</td>
<td>ttgcagctgtgacagttaaccgacc</td>
<td>gctgacctcagttacgagc</td>
<td>56°C</td>
</tr>
<tr>
<td>Cyp4x1 (tissue)</td>
<td>ggcgtgaaatgctctactggg</td>
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</tr>
<tr>
<td>Cyp4x1b (cells)</td>
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<td>ttggtagaggccagaggttct</td>
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</tr>
<tr>
<td>Cyp2c11</td>
<td>gaggacattgagagcgtttcttc</td>
<td>agccacaccggcagattgcttc</td>
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</tr>
<tr>
<td>Pol2a</td>
<td>cctggctgggtctgtgactaggg</td>
<td>ggtggacacgacgaggttct</td>
<td>56°C</td>
</tr>
</tbody>
</table>

All primer sequences are given 5’–3’.

Rat brain microvascular endothelial cell cultures. Rat brain microvascular endothelial cell cultures (RBMEC, Cell Applications, San Diego, CA) were plated on dishes coated with Attachment Factor (Cell Applications). Prior to plating, culture dishes were coated with Attachment Factor and incubated for at least 1 h at 37°C; the residual/nonadherent Attachment Factor was removed, and the rat brain vascular endothelial cell cultures (RBVEC) were plated and grown in RBMVEC Growth Media (Cell Applications). Cells from passages 5-15 were used for experiments. RBMVEC were synchronized using Growth Media with an additional 50% FBS and subsequently starved with RBMVEC Basal Media (Cell Applications) containing 1% horse serum and 25 U/ml penicillin and 25 μg/ml streptomycin (Invitrogen). Cells were harvested and processed as described for astrocytes (3) except that for protein samples, 600 μl of homogenization buffer with protease inhibitors were added to each 10-cm dish.

RNA isolation, cDNA synthesis, and RT-PCR. Tissue was first homogenized on ice using a mortar and pestle followed by passage through a QIAshredder column (Qiagen). Cells were scraped and sonicated on ice for 15–20 s at 25°C power before RNA purification was carried out using RNeasy Mini or Micro Kits followed by DNase I treatment (Qiagen) to remove any genomic DNA contaminants. RNA (1 μg) was used to synthesize cDNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) using the protocol recommended by the manufacturer. Primers were obtained from Operon Biotechnologies (Huntsville, AL), and sequences and annealing temperatures are given in Table 1. Samples of cDNA (25–50 ng per reaction) were analyzed by RT-PCR in triplicate using the following thermal cycling protocol: 3 min at 95°C, followed by 40 cycles of 30
s at 95°C, 30 s at annealing temperature (indicated in Table 1) and 30 s at 72°C.

Linearity of amplification for each primer set was verified by analysis of serially diluted cDNA samples. Product singularity and specificity were confirmed by melt curve analysis and agarose gel electrophoresis, respectively. Relative quantification of expression was determined by measuring the threshold (Ct) values of each sample using the $2^{-\Delta\Delta Ct}$ method (20). Relative mRNA abundance for each gene was normalized to Polr2 mRNA expression. We quantified expression of core clock and clock-controlled genes Per1, Per2, Rev-Erbα, E4bp4, Dbp, and Bmal1, as well as for the Cyp450 epoxygenases Cyp4x1 and Cyp2c11. The maximum expression value for each gene was set to 100 and the other quantities were normalized to that value.

**Microsome isolation.** Dishes were scraped and samples were transferred to 1.5-ml tubes and sonicated on ice, two times for 15–20 s each time using a Cole Parmer Ultrasonic Processor at 25% power (Model GEX130PB, Cole Parmer, Vernon Hills, IL). Cell lysates were stored at −80°C until they were used for Western blotting or processed for microsomes. For microsome preparations, homogenates were thawed on ice, then centrifuged at 10,000 g for 30 min at 4°C. The supernatant was transferred to an ultracentrifuge tube and centrifuged at 100,000 g for 90 min at 4°C. The microsomal pellet was resuspended in buffer (pH 7.25) containing (in mM) 27.5 KH$_2$PO$_4$, 73.5 K$_2$HPO$_4$, 1.0 disodium EDTA, 1.0 DTT, 60% (vol/vol) glycerol, with 10 μl of each of the phosphatase/protease inhibitors (described above) per 1 ml buffer. Samples were sonicated on ice in 15- to 20-s intervals until pellets were completely resuspended. Total protein in each sample was determined by measuring absorbance following treatment with a colorimetric Protein Assay Reagent (Bio-Rad) and referencing absorbance of each protein sample to an absorbance standard curve defined by colorimetric protein assay of a serial dilution of known BSA protein concentrations.

**Western blotting.** Protein from cell lysates (Bmal1) or microsomal preparations (Cyp450) (10–30 μg) was separated on 10% TGX Tris-glycine SDS PAGE gels (Bio-Rad) and transferred to an Immobilon-FL PVDF membrane (Millipore, Billerica, MA). Membranes were blocked in LI-COR blocking buffer and probed with mouse anti-Cyp2c11 (Genway Biotech, San Diego, CA) 1:500, mouse anti-Cyp4x1 (Abnova) 1:500, rabbit anti-Bmal1 (Abcam) 1:500, mouse anti-β-actin (Sigma) 1:20,000 or rabbit anti-γ-tubulin (Abcam) 1:800 diluted 1:1 in LI-COR blocking buffer: PBS pH 7.4 with 0.1% (vol/vol) Tween-20 (Bio-Rad). Blots were then probed with LI-COR donkey anti-mouse 680 or anti-rabbit 800 IR secondary antibodies at 1:5,000 to 1:10,000 in the same dilution solution used for primary antibodies. Blots were visualized using the Odyssey Infrared Imaging System and IR intensity was quantified using Odyssey software. Protein expression was normalized to β-actin or γ-tubulin and peak

![Fig. 1. Multiple species sequence alignments. Circadian regulatory elements conserved in rat (R), mouse (M), and human (H) Cyp4x1 (A) and in rat Cyp2c11 and human CYP2C9 (B) are shown. Regions where the sequence is conserved between species are enclosed in boxes, while lines with hashmarks indicate nonconserved regions larger than 100 bases. Nonconserved regions smaller than 100 bases are not depicted. Numbers represent nucleotide position relative to the transcription start site. Specific sequences are given for conserved and partially conserved regulatory sites with conserved sequences underlined. Only conserved E-box elements are depicted because we used specific search patterns instead of allowing mismatches for E-boxes in the analysis. For Cyp4x1 (A), the E-box at position −1,645 in rat and −1,729 in mouse is conserved in that each sequence perfectly matches an E-box search pattern that was used in the analysis. RORE, retinoic acid-related orphan receptor response element; DBPE, D-site albumin promoter binding protein response element; UTR, untranslated region.](http://ajpcell.physiology.org/)

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sample expression for each experiment was set to 100 with the remaining samples normalized accordingly.

Cyp450 activity assay. For activity assay experiments, astrocytes and RBMVEC were plated in 3.5-cm dishes and synchronized every 4 h for 24 h so that the assay could be performed on all seven time points simultaneously. All cells were starved for at least 12 h before the first well (time 24 h) was serum shocked. Following 2 h of serum shock, cells were returned to starvation media. The assay began 12 h after the last well (time 0 h) returned to serum starvation media. Starvation media were replaced (2 ml/well), and AA (Cayman Chemical, Ann Arbor, MI), diluted to 5 mM in denatured EtOH (Sigma), was added to a final concentration of 50 µM. Cells were incubated at 37°C for 60 min, placed on ice, scraped into 15-ml glass centrifuge tubes, and vortexed. Two 50-µl aliquots from each sample were removed to 1.5-ml tubes for protein quantification. The reaction was stopped by the addition of 1 M formic acid, and 5 ng of the internal standard deuterated fatty acid (20-HETE-d6, Cayman Chemical) were added. The lipid component of the reaction was extracted twice with 2 ml of diethyl ether (Sigma), back-extracted with 1 ml of distilled water, and the organic layer was then dried under nitrogen. Production of total EETs was measured by liquid chromatography-mass spectrometry (LC/MS). The samples were reconstituted with ethanol, and metabolites of AA were separated by HPLC on a Zorbax Eclipse Plus C18 Column (100 × 2.1 mm, 3.5 µm; Agilent Technologies, New Castle, DE) at a flow rate of 0.3 ml/min. The metabolites were eluted using a mixture of acetonitrile:methanol:water:acetic acid over a step gradient from 17:3:80:0.01 to 68:12:20:0.01 for 15 min. The effluent was ionized using negative ion electrospray, and peaks eluting with a mass-to-charge ratio (m/z) of 319>301 (HETEs and EETs), 337>301 (HETEs and DiHETEs), or 323>270 (internal standard, 20-HETE-d6) were monitored in multiple reactions using a triple quadrupole mass spectrometer (API 3000, Applied Biosystems, Foster City, CA). The ratio of ion abundance in the peaks of interest versus the internal standard was compared with standard curves generated with known concentrations of EETs and DiHETEs (from 0.2 to 10 ng). Individual EETs regiosomers were identified on the basis of their m/z ratio and specific retention time (DiHETEs: 12–14 min; EETs: 18–20 min). Product formation was normalized to the amount of total protein, peak formation was set to 100%, and other time points were normalized accordingly.

Statistics. Data are presented as means ± SE. Box-Cox analysis was used to find the optimal power transformation to stabilize variance; the log transformation was found to be optimal. Cosinor analysis performed on the log-scale was used to capture cyclic multiplicative changes about the original scale. Cosinor analysis was used to determine the presence of a significant rhythm in relative mRNA expression. In this analysis, expression data are fit by a nonlinear least-squares regression with the following equation: \( y = A + B \times \cos(\pi \times (t - C)/24) \), where \( A \) is the rhythm-adjusted mean, \( B \) is the amplitude of the rhythm, \( C \) is the phase given in circadian time representing the time of peak expression, and \( t \) is circadian time. The fit of a single cosine curve with a 24-h period is compared with that of a zero amplitude hypothesis (horizontal line) by nonlinear regression. The 24-h cosine model is preferred when the fit returns a \( P \) value of <0.05 indicating a significant circadian rhythm. For synchronized cell experiments, cosine curves with both 24- and 12-h periods were tested. One-way ANOVA followed by a Dunnett’s multiple comparison test was used to determine differences between time points by comparing

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**Fig. 2.** Cyclic clock and clock-controlled gene expression in rat brain and vasculature. Graphs show relative mRNA expression over circadian time (CT 0 = 6:00am = normal lights on time) for clock genes E4bp4 (A), Reverberα (B), Dbp (C), Per1 (D), and Per2 (E). Expression of each gene is relative to RNA polymerase II gene expression for the same samples. The specific tissue samples used are indicated above each graph. Data points represent mean ± SE; \( n = 5 \) for each time point. Cosinor statistical analysis was used, and \( P < 0.05 \) indicates the presence of a significant circadian component to the expression pattern. The phase \( \Phi \) or time of peak expression and \( P \) values are indicated on each graph. NS, no significant rhythm; Reverberα, reverse erythroblastic leukemia viral oncogene homolog-α; Per1/Per2, period 1/2; E4bp4, E4 promoter binding protein 4; Dbp, D-site albumin promoter binding protein. One-way ANOVA followed by Dunnett’s multiple comparison test was used to detect differences in expression between time points, using the peak expression mean as a reference. Significant differences from the peak (open circle ◦): * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \).
involved in circadian regulation of gene expression (33).

regulatory elements that have previously been shown to be bases, contains the promoter is a partially conserved RORE (7/11 bases, 63%) in mice and rats. The same bioinformatics approach is not conserved in rat and mouse, and a partially conserved

The 1,500 base region of the human sequence is an E-box that is over 60% homologous in rat or mice sequences. That said, the human sequence has an E-boxes, a RORE and a DBPE element, that are not conserved in rat and mice sequences. The coding region of exon 1 in the human gene is 83% homologous to the rodent sequences, while the 1,500 base region that is over 60% homologous among the two species; these regions contain two conserved E-boxes, two partially conserved (5/6 bases, 83%) E-boxes, one conserved RORE, and one partially conserved (8/11 bases, ~73%) RORE. These regions also include several nonconserved elements, such as a RORE that is present only in rats and E-boxes present only in mice. The human sequence also contains E-boxes, a RORE and a DBPE element, that are not conserved in rat or mice sequences. That said, the human sequence has an approximately 1,500 base region that is over 60% homologous to rat and mice sequences. The coding region of exon 1 in the human gene is 83% homologous to the rodent sequences, while rat and mouse exon 1 sequences are 96% homologous. Within the 1,500 base region of the human sequence is an E-box that is not conserved in rat and mouse, and a partially conserved (7/11 bases, 63%) RORE. The same bioinformatics approach was used to compare rat Cyp2c11 gene and its human homolog CYP2C9 (Fig. 1B).

The studied sequences contain a region with over 70% homology including the promoter region and exon 1, exon 2, and exon 3. Within the homologous region that contains the promoter is a partially conserved RORE (7/11 bases, ~64%) and a partially conserved DBPE (9/12 bases, 75%). We also found multiple potential sites for circadian regulation that are not conserved between human and rat sequences including E-boxes, ROREs, and DBPEs. That said, taken together, our data suggest that the promoter regions of Cyp4x1 and Cyp2c11 genes contain conserved transcriptional regulatory elements that have previously been shown to be involved in circadian regulation of gene expression (33).

Clock and clock-controlled genes in the brain and vasculature. To begin to examine circadian regulation of gene expression in the brain and vasculature, we used RT-PCR to profile circadian clock gene (Per1 and Per2) and clock-controlled gene (E4bp4, Reverb, Dhp) expression in the rat hippocampus, middle cerebral artery, and inferior vena cava. Although there are some phase differences between tissues, the sequential order of gene expression is the same in each tissue (Fig. 2) and is consistent with previous reports of core clock gene expression in peripheral tissues including heart, brain, and vasculature (1, 22, 36). Specifically, Dhp and E4bp4 are positive and negative transcriptional regulators of the clock genes Per1 and Per2.

RESULTS

Cyp4x1 and Cyp2c11 promoters are conserved across several mammalian species. Expression of Cyp4x1 and Cyp2c11 enzymes can regulate EET production, which we hypothesize may play an important role in circadian changes in blood flow. Here, we begin to examine whether Cyp4x1 and Cyp2c11 gene expression can undergo circadian regulation. To do this, we used bioinformatic tools to determine whether promoter regions of these Cyp450 epoxidase genes in rat, mice, and humans contain conserved transcriptional regulatory elements that are known to be involved in circadian regulation of gene expression. Specifically, we examined whether there is conservation of transcriptional regulatory sequences in the following: E-box, RORE, and DBPE using similar approaches as studies conducted by T. Yamamoto and colleagues (33). The sequence alignment of homologous regions is depicted in Fig. 1A. Greater than 50% of the studied regions (~4,000 to 2,000 bases) in mice and rats Cyp4x1 are ~80% homologous among the two species; these regions contain two conserved E-boxes, two partially conserved (5/6 bases, 83%) E-boxes, one conserved RORE, and one partially conserved (8/11 bases, ~73%) RORE. These regions also include several nonconserved elements, such as a RORE that is present only in rats and E-boxes present only in mice. The human sequence also contains E-boxes, a RORE and a DBPE element, that are not conserved in rat or mice sequences. That said, the human sequence has an approximately 1,500 base region that is over 60% homologous to rat and mice sequences. The coding region of exon 1 in the human gene is 83% homologous to the rodent sequences, while rat and mouse exon 1 sequences are 96% homologous. Within the 1,500 base region of the human sequence is an E-box that is not conserved in rat and mouse, and a partially conserved (7/11 bases, 63%) RORE. The same bioinformatics approach was used to compare rat Cyp2c11 gene and its human homolog CYP2C9 (Fig. 1B). The studied sequences contain a region with over 70% homology including the promoter region and exon 1, exon 2, and exon 3. Within the homologous region that contains the promoter is a partially conserved RORE (7/11 bases, ~64%) and a partially conserved DBPE (9/12 bases, 75%). We also found multiple potential sites for circadian regulation that are not conserved between human and rat sequences including E-boxes, ROREs, and DBPEs. That said, taken together, our data suggest that the promoter regions of Cyp4x1 and Cyp2c11 genes contain conserved transcriptional regulatory elements that have previously been shown to be involved in circadian regulation of gene expression (33).

Clock and clock-controlled genes in the brain and vasculature. To begin to examine circadian regulation of gene expression in the

Table 2. Phase values for gene expression in rat tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>E4bp4</th>
<th>Reverb α</th>
<th>Dhp</th>
<th>Per1</th>
<th>Per2</th>
<th>Cyp4x1</th>
<th>Cyp2c11</th>
</tr>
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<tbody>
<tr>
<td>Hippocampus</td>
<td>7:13am</td>
<td>2:05pm</td>
<td>3:41pm</td>
<td>NS</td>
<td>12:25am</td>
<td>2:14pm</td>
<td>8:35am</td>
</tr>
<tr>
<td>Middle cerebral artery</td>
<td>6:11am</td>
<td>1:47pm</td>
<td>3:45pm</td>
<td>NS</td>
<td>9:04pm</td>
<td>NS</td>
<td>10:12am</td>
</tr>
<tr>
<td>Inferior vena cava</td>
<td>2:29am</td>
<td>3:16pm</td>
<td>6:05pm</td>
<td>7:20pm</td>
<td>9:35pm</td>
<td>12:35pm</td>
<td>11:07am</td>
</tr>
</tbody>
</table>

Phase represents time of peak expression with (SE) in minutes. NS indicates that no significant rhythm was detected.

The mean of peak expression to all other means. GraphPad Prism Software (version 5.01) was used to carry out Cosinor analysis, ANOVA, and to generate Figs. 2–9.

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Fig. 3. Circadian/biphasic circadian expression of Cyp4x1 and Cyp2c11. Graphs show relative mRNA expression over circadian time (CT 0 = 6:00am) for Cyp4x1 (A) in hippocampus and inferior vena cava and Cyp2c11 (B) in hippocampus, middle cerebral artery, and inferior vena cava. Data points represent mean ± SE; n = 5 for each time point. Cosinor analysis was used to test for 12- and 24-h period rhythms. The phase (open circle ○): *P < 0.05, **P < 0.01.
to both tissues. Although we did not detect a circadian component with a midafternoon peak, while in inferior vena cava, peak Cyp4x1 expression occurred about 10 h later (Fig. 3). We did not detect rhythmic variation in Cyp4x1 expression in the hippocampus with a midafternoon peak, while in inferior vena cava, peak Cyp4x1 expression occurred about 10 h later (Fig. 3A). We did not detect rhythmic variation in Cyp4x1 expression in the middle cerebral artery; however, ANOVA showed that Cyp4x1 expression at circadian clock time 12 was significantly ($P < 0.05$) greater than expression at circadian clock time 16 (not shown).

Thus, peak expression of Dbp is followed by peak expression of Per1 and Per2 in the early/middle circadian night.

Further, after there is an early morning expression peak of their repressor, E4bp4, there is a trough in Per1 and Per2 expression in the late morning. Thus, Dbp and E4bp4 are antagonistic at the Per1/Per2 promoter DPBE site, and their peak expression is antiphasic with respect to each other.

Cyp450 epoxygenase gene expression in the brain and vasculature. To determine whether enzymes that generate vasoactive EETs are expressed in a circadian manner in the brain and vasculature, we used RT-PCR to measure mRNA expression of Cyp2c11 and Cyp4c11 genes in the hippocampus, inferior vena cava, and middle cerebral artery. Times of peak expression are shown in Table 2. We found that peak expression in the hippocampus and middle cerebral artery are much different than the phases observed in inferior vena cava (Fig. 3). We detected rhythmic variation in Cyp4x1 expression in the hippocampus with a midafternoon peak, while in inferior vena cava, peak Cyp4x1 expression occurred about 10 h later (Fig. 3A). We did not detect rhythmic variation in Cyp4x1 expression in the middle cerebral artery; however, ANOVA showed that Cyp4x1 expression at circadian clock time 12 was significantly ($P < 0.05$) greater than expression at circadian clock time 16 (not shown).

Cyp2c11 was rhythmically expressed in the hippocampus and middle cerebral artery (Fig. 3B), peaking in the morning in both tissues. Although we did not detect a circadian component to Cyp2c11 expression in inferior vena cava, we did detect a significant rhythm with a 12-h period, having peaks in the late morning and late evening (Fig. 3B).

Cyp450 gene and protein expression and Cyp450 enzyme activity in primary astrocytes and rat brain microvascular endothelial cells. Having shown that Cyp2c11 is expressed in a circadian manner in the middle cerebral artery and that both epoxygenase genes are rhythmic in the hippocampus, we decided to take a closer look at circadian gene expression in cells that produce vasoactive EETs, namely, astrocytes and endothelial cells. To investigate the possibility of sustained rhythmic expression having a 12-h period, we examined gene expression over 36 h in cultured neonatal hippocampal astrocytes and rat brain microvascular endothelial cells. Specifically, we measured mRNA expression of the core clock gene Bmal1, clock-controlled gene Reverba, and the epoxygenases Cyp2c11 and Cyp4x1 in cultured astrocytes (Fig. 4) and endothelial cells (Fig. 5) over 36 h. Bmal1 expression is negatively regulated by Reverba, and (as expected) we found that peak Bmal1 and Reverba mRNA expression is about 6 and 7.5 h out of phase from one another in both astrocytes (Fig. 4, A and B) and endothelial cells (Fig. 5, A and B). That said, in both cell types, we found that Cyp2c11 (Figs. 4C and 5C) and Cyp4x1 expression (Figs. 4D and 5D) has a significant rhythmic component with a 12-h period and that both genes are nearly in phase with one another. Although we detected a significant 24-h period rhythm in astrocytic Bmal1 expression (Fig. 4A) and a significant 12-h period rhythm in endothelial cell Cyp4x1 expression (Fig. 5D), significant differences in expression between time points was not detected by ANOVA. This is likely due to the fact that the Cosinor analysis takes into account the variance that is
due to the presence of a rhythm, while the ANOVA does not. In other words, differences in expression between time points are implicit when a significant rhythm is detected.

We used Western blotting techniques to measure Bmal1, Cyp2c11, and Cyp4x1 protein expression in cultured astrocytes (Fig. 6) and endothelial cells (Fig. 7) over a 24-h time period. Bmal1 mRNA expression (Figs. 4A and 5A) peaks about 12 h before the peak of Bmal1 protein (Figs. 6A and 7A); this delay between peak mRNA and protein expression likely reflects time needed for protein translation, as has been reported previously (31). Similarly, Cyp2c11 and Cyp4x1 mRNA (Figs. 4, C and D, and 5, C and D) and protein expression (Figs. 6, B and C, and 7, B and C) is rhythmic having a period of 12 h with peak mRNA and protein expression occurring at nearly the same time (Figs. 4–7). Cyp2c11 and Cyp4x1 protein expression (Figs. 7, B and C) and endothelial cell Cyp4x1 protein expression (Fig. 7C) showed the presence of a significant 12-h period rhythm, while the ANOVA did not detect significant differences in protein expression between time points. As explained above, the presence of a rhythm does indicate that the means are different.

Lastly, we investigated whether Cyp450 enzyme activity in astrocytes and rat brain vascular endothelial cells is rhythmic by measuring the amount of vasoactive EETs that these cells produce over 24 h (Figs. 8 and 9). In astrocytes, 8,9-EET formation is rhythmic with a 12-h period (Fig. 8A), while rhythmic 14,15- (Fig. 8B) and total EETs formation (Fig. 8C) has a significant rhythm with a 24-h period. In rat brain vascular endothelial cells, 8,9-EET (Fig. 9A), 11,12-EET (Fig. 9B), 14,15-EET (Fig. 9C), and total EETs formation (Fig. 9D) had a significant 12 h period rhythm.

DISCUSSION

In mammals, there are circadian changes in cerebral blood flow (CBF) and arterial pressure that may underlie circadian...
changes in the risk of having a cerebrovascular event (e.g., stroke) throughout the day and/or night. In rats, circadian regulation of CBF has been observed (32); however, the mechanisms underlying circadian regulation of CBF are unknown.

Cyp450 epoxygenases metabolize arachidonic acid into EETs, which are very potent vasodilators known to regulate basal and activity-dependent increases in CBF. Our study investigates the novel hypothesis that rhythmic expression of Cyp4x1 and Cyp2c11 genes results in rhythmic variation in EETs production in rat brain vascular endothelial cells (RBVEC) and astrocytes, potentially contributing to diurnal changes in CBF regulatory mechanisms.

In agreement with this hypothesis, our cell culture data from RBVEC and hippocampal astrocytes revealed rhythmic mRNA and protein expression of Cyp4x1 and Cyp2c11 (Figs. 4-7). However, expression of Cyp4x1 and Cyp2c11 was found to be rhythmically up- and downregulated within a period of 12 h, rather than in a diurnal fashion (once every 24 h) (Figs. 4-7). While Cyp4x1 and Cyp2c11 expression increases and decreases within 12 h, EETs production in RBVEC and hippocampal astrocytes is either rhythmically up- and downregulated within a 12 h period, or has a diurnal 24-h period. In addition, in astrocytes, Cyp4x1 and Cyp2c11 have a 12-h cycle period of expression (Fig. 6, B and C), yet produce 14,15-EET and total EETs in a circadian manner (Fig. 8, B and C). We speculate that this apparent temporal disparity between the timing of Cyp4x1 and Cyp2c11 expression and the generation of 14,15-EET and total EETs production in astrocytes may be due to the fact that EETs can be produced by additional Cyp450 enzymes, other than those monitored in this study and whose expression may also vary diurnally. Other regulatory scenarios are also possible.

Our data suggest the possibility that rhythmic EETs production from RBVEC and astrocytes have the potential to underlie and/or contribute to circadian changes in cerebral blood flow. However, we still do not know whether rhythmic EETs production, with either a 12-h or 24-h cycle period, directly leads to circadian changes in CBF. Owing to limitations of our in vitro studies, we also cannot rule out the possibility that in vivo, EETs production and release from RBVEC and/or astrocytes is circadian, rather than (in some cases) being rhythmically up- and downregulated every 12 h, as some of our in vitro studies suggest.

Conserved promoter sites may regulate rhythmic epoxygenase expression. It has been previously shown that the time of peak expression of clock-controlled genes is determined by the specific regulatory elements present in their respective promoters (33). We used bioinformatic tools to compare the promoter regions of Cyp4x1 and Cyp2c11 genes. We found that these genes contain relatively conserved transcriptional regulatory elements that have previously been shown to be involved in circadian regulation of gene expression (Fig. 1). Thus, our studies help define multiple potential regulatory sites in the epoxygenase gene promoters that may regulate rhythmic EETs expression; some of these regions are conserved between human, mouse and rat, including E-box elements, RORE and DBPE sites and are known to regulate circadian transcription of many clock-controlled genes (33). One DBPE site, for example, located −34 bases upstream of the transcription start

![Fig. 9. Rhythmic Cyp450 enzyme activity over 24 h in endothelial cells. Formation of EETs [8,9 EET (A), 11,12 EET (B), 14,15 EET (C), and total EETs (D)] from AA metabolism in cultured endothelial cells was measured by LC/MS. Cosinor analysis was used to test for 12 and 24 h period rhythms. Phase $\Phi$ and $P$ values are indicated on each graph. One-way ANOVA followed by Dunnett’s multiple comparison test was used to detect differences in expression between time points, using the peak expression mean as a reference. Significant differences from the peak (open circle $\circ$): *$P < 0.05$, **$P < 0.01$.](http://ajpcell.physiology.org/ by 10.22033.3 on October 4, 2017)
site in the Cyp2c11 promoter, has an identical sequence and position (~34 bases upstream of the transcription start site) to the DBPE site that regulates circadian expression of another Cyp450 enzyme, Cyp3a4 (29). Further studies will be needed to determine whether this position is a common motif in circadian regulation of Cyp450 genes that likely diverged following a series of gene duplication events.

Together, our data suggest there may be conservation of gene promoter sites that could play a role in circadian regulation of Cyp2c11 and Cyp4x1, whose activity produces EETs, which are potent cerebral vasodilators (11, 12, 13). These studies suggest the possibility of a relatively novel potential mechanism for circadian regulation of CBF, whereby circadian regulation of Cyp2c11 and Cyp4x1 expression may underlie circadian/rhythmic EETs production which in turn may regulate circadian changes in CBF. However, further studies are needed to determine whether rhythmic production of EETs contributes to circadian regulation of CBF.

Differences in phase of Per1 expression between different regions of the brain and different vascular tissues. Small differences in the phase of rhythmic gene expression between tissues are typically thought to be due to differences in hormonal regulation, neuromodulation, metabolic signals, and/or tissue-specific transcriptional/translational regulatory mechanisms. Differences in the temporal relationship of Per1 expression within the same organ have also been observed. For instance, in the brain, Per1 mRNA expression is rhythmically expressed in the rat superior chiasmatic nucleus but not rhythmically expressed within the hippocampal CA1/dentate gyrus or the cerebellum (26, 34). Still, we did not expect to find that Per1 mRNA expression is not rhythmic in the samples of the vasculature we tested, including the middle cerebral artery. A potential reason that we did not detect rhythmic expression of Per1 could be due to homogenization of tissue that may contain more abundant antiphasic or arhythmic Per1 expression in adjacent cell types. For example, phase differences in clock-controlled gene expression between peripheral oscillators, and between arteries and veins, has previously been demonstrated (7). Future studies will be needed to determine whether there is rhythmic Per1 expression in other arteries.

Our studies suggest a novel hypothesis that circadian regulation of EETs production in astrocytes, neurons, and/or endothelial cells potentially underlies and/or contributes to circadian variation in blood flow. While overall increases seen in global CBF during the active phase in rodents are largely due to increases in locomotor as well as neural activity, we are interested specifically at the phenomena of functional hyperemia as well as autoregulatory capacity, both of which likely vary diurnally. While EET concentration is one factor to be taken into consideration as far as these physiological events are concerned, other factors such as the ratio of EET regioisomers, other vasoactive factors such as other eicosanoids, other AA-derived factors including 20-HETE, anandamide-derived vasoactive compounds as well as the enzymes responsible for the breakdown of all of these factors need to be considered. The specific concentration of all of these and other factors is likely brain region specific and possibly synapse specific, so considering these factors requires investigating these phenomena at a much smaller scale than simply measuring global changes in cerebral blood flow.

While we use the fact that stroke occurrence and global CBF vary diurnally as a justification for our line of experimentation, we are actually studying, at a molecular level, factors that affect local CBF in terms of autoregulatory capacity and functional hyperemia, variations in which may contribute to stroke risk. These findings are particularly interesting in the context of global increases in metabolic demand in the brain, such as during transitions from sleep to wakefulness and during rhythmic neocortical activity in non-rapid eye movement (NREM) sleep. Finally, since Cyp450 enzymes are largely responsible for metabolizing pharmaceutical drugs, understanding circadian changes in Cyp450 expression and blood flow may give rise to new and more effective approaches to pharmacotherapy dosing and scheduling dose delivery.
10. Keskimäki Z, Gorgun CZ, Hodge E, Zengil H.


