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The 24-h pattern of *dgcr8*, *drosha*, and *dicer* expression in the rat suprachiasmatic nuclei and peripheral tissues and its modulation by angiotensin II

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Abstract. Study was focused on regulatory interactions between the circadian system and the renin–angiotensin system in control of microRNA (miRNA) biosynthesis. Responsiveness of the miRNA biosynthetic pathway, selected pre-miRNAs and clock genes to angiotensin II (AngII) infusion was analysed in the suprachiasmatic nuclei (SCN), liver, kidney and heart during a 24-h cycle. *per2* exerted a rhythmic expression profile in all analysed tissues. *clock* expression showed a rhythmic pattern in the peripheral tissues with tissue-specific response to AngII. *dgcr8* expression showed a tissue-specific rhythm only in peripheral tissues, which diminished in the heart and kidney after AngII delivery. Expression of pre-miR-30c was rhythmic in all studied peripheral tissues, pre-miR-34a expression exerted significant rhythm only in the liver. AngII delivery increased expression of pre-miR-30c and pre-miR-34a in the kidney. To conclude, peripheral oscillators are more likely to exhibit rhythmic miRNA biosynthesis responsive to AngII in a tissue-specific manner compared to SCN.

Key words: *clock* — miR-34a — miR-30c — Liver — Kidney

Introduction

Numerous physiological and behavioural processes in mammals are under the control of the circadian system, which generates endogenous rhythms over a period lasting approximately 24 h and synchronises the organism to cyclic changes in the environment. The circadian system is organised hierarchically. A main oscillator is localised in the suprachiasmatic nuclei (SCN) in the anterior hypothalamus while peripheral oscillators are located in all other tissues and are coordinated and synchronised by the main oscillator. Although a dominant synchronising factor for the circadian system is a light-dark (LD) cycle, there are other environmental cues that can be utilised to synchronise an organism (Stratmann and Schibler 2006; Dibner et al. 2009; Koop and Oster 2021). The regulatory influence of the SCN is transmitted to peripheral oscillators by both neuronal and humoral signals that influence peripheral oscillators with different strength depending on their innervation and available receptors (Guo et al. 2005; Albrecht 2012; Pilorz et al. 2020).

The molecular basis of the circadian oscillator is created by the transcription–translation feedback loop of clock genes and several transcription factors (Fig. 1). The positive limb of the transcription–translation feedback core consists of the transcription factors BMAL1 and CLOCK. The CLOCK–BMAL1 heterodimer induces the expression of the clock genes *per* and *cry*, through binding to a regulatory element E-box in the clock gene promoter. After accumulation of the

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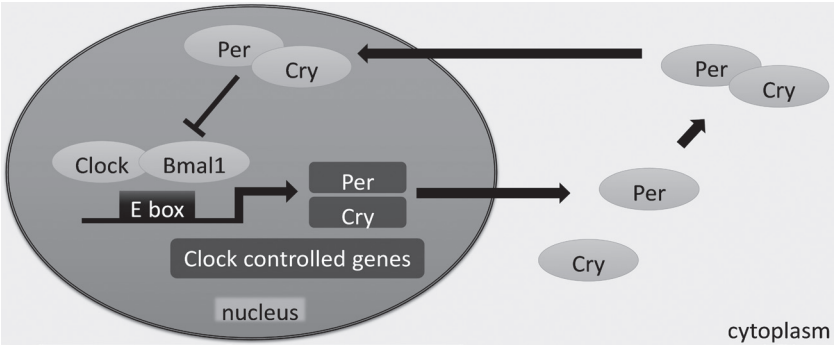

Figure 1 Transcriptional-translational loop of clock genes *per*, *cry* and transcription factors BMAL1, CLOCK. The CLOCK–BMAL1 heterodimer induces the expression of the clock genes *per* and *cry*, *via* a regulatory element E-box in the clock gene promoter. After accumulation of the *per* and *cry* protein products in the cytoplasm, heterodimer consisting of PER and CRY is translocated in the nucleus and inhibits the action of CLOCK–BMAL1 (modified according Finger et al. 2020).

per and *cry* protein products in the cytoplasm, PER and CRY heterodimerise and inhibit the action of CLOCK–BMAL1 and thus their own transcription (Finger et al. 2020; Patke et al. 2020; Pilorz et al. 2020). There are also additional regulatory loops that are involved in fine tuning the phase, amplitude and/or period of circadian oscillations (Patke et al. 2020; Kim and Lazar 2021).

Transcription factors involved in the circadian transcription–translation feedback loop also regulate the expression of so-called clock-controlled genes (Finger et al. 2020; Harder and Oster 2020; Kim and Lazar 2021). This regulation exhibits pronounced tissue-specific features (Zhang et al. 2014; Harder and Oster 2020). In addition to E-box-driven regulation, the circadian system controls gene expression *via* histone, post-transcriptional and post-translational modifications (de Assis and Oster 2021; Kim and Lazar 2021; Parnell et al. 2021). The regulatory influence of the circadian system on gene expression may also be mediated by non-coding RNAs such as microRNAs (miRNAs) (Kanaan et al. 2013; Du et al. 2014; Anna and Kannan 2021).

miRNAs belong to a large family of small non-coding RNAs, which regulate gene expression predominantly at the post-transcriptional level through RNA interference (Pu et al. 2019). miRNA biogenesis comprises two processing steps. After transcription by RNA polymerase II the primary transcript of miRNA (pri-miRNA) acquires a stem loop shape. pri-miRNA is processed in the nucleus by the protein complex Microprocessor composed from the enzyme Drosha and the cofactor DGCR8 (DiGeorge critical region 8) that cleaves pri-miRNA near the junction and thus creates precursor miRNA (pre-miRNA) (Nguyen et al. 2015; Kwon et al. 2016). In the cytoplasm, pre-miRNA is digested by the enzyme Dicer to create a miRNA duplex with a length of approximately 20–22 nucleotides (Taylor et al. 2013; Thunders and Delahunt 2021). One strand of the miRNA duplex – the so-called guide strand – is incorporated into the RNA-induced silencing complex (RISC) that executes gene silencing (Meijer et al. 2014).

Rhythmic expression profiles of miRNAs have been confirmed in various tissues, such as the SCN (Cheng et al. 2007; Gao et al. 2016; Kiessling et al. 2017), the mesencephalon (Kinoshita et al. 2014), the liver (Du et al. 2014; Wang et al. 2016), serum (Shende et al. 2011), the small intestine (Balakrishnan et al. 2010) and several cell lines (Gao et al. 2016; Han et al. 2016). Knockout of the gene *dicer*, a key miRNA biogenesis pathway component, was associated with a shorter free running period in mice (Chen et al. 2013), and knockout of the *dicer* or *dgcr8* gene led to loss of rhythmic expression of *per2* and inhibition of *clock* gene expression in embryonic stem cells of mice (Umemura et al. 2017). Several miRNAs are regulated directly by components of the transcription–translation feedback loop (Mehta and Cheng 2013; Kinoshita et al. 2020). Moreover, several studies indicate that miRNA processing enzymes or cofactors display a rhythmic expression profile in mammals (Lonsdale et al. 2013; Yan et al. 2013; Zhang et al. 2014).

The circadian system is also involved in regulation of the renin–angiotensin system (RAS) (Zhang et al. 2021) that controls blood pressure and electrolyte homeostasis (Forrester et al. 2018). Levels of AngII and AngI display a notable 24-h rhythm in the plasma of rats (Schiffer et al. 2001). Similarly, aldosterone, a mineralocorticoid hormone regulated by AngII, exerts a robust 24-h rhythmic pattern in the circulation of humans (Cugini et al. 1992) and rats (Lemmer et al. 2000). Moreover, a significant daily rhythm of renin and ACE activity in circulation has been observed in humans (Veglio et al. 1987; Cugini et al. 1992) and rats (Lemmer et al. 2000). The regulatory influence of AngII is mediated by two G-protein coupled receptors – AT1 (AngII receptor type 1) and AT2 (AngII receptor type 2) (Karnik et al. 2015; Forrester et al. 2018). AT1 exhibits a significant daily rhythm in the heart of rats (Herichová et al. 2013). The regulatory relationship between the circadian system and the RAS is reciprocal as AngII has been shown to regulate the central oscillator *via* AT1 (Brown et al. 2008). The regulatory influence of AngII in peripheral oscillators is executed mainly *via* AT1, which is highly abundant in the liver, heart and kidney (Kitami et al. 1992; Karnik et al. 2015).

AngII also influences the expression of miRNAs (Jiang et al. 2013; Huang et al. 2014; Pacurari and Tchounwou 2015;

Herichová 2016) and enzymes involved in their biogenesis (Adam et al. 2012) *via* several AngII-responsive transcription factors, including c-Myc (MYC proto-oncogene) (Shen et al. 2017), c-Fos (Fos proto-oncogene), c-Jun (Jun proto-oncogene) (Fang et al. 2011), ETS1 (ETS proto-oncogene 1) (Hao et al. 2015) and NF-κB (nuclear factor kappa B) (Rouet-Benzineb et al. 2000).

Although it is assumed that both the circadian system and AngII have the capacity to modify miRNA expression, nothing is known about their regulatory interactions and, even more importantly, effect of AngII on miRNA expression has never been studied during the whole 24-h cycle. Therefore, we designed a study to elucidate whether 24-h time course of miRNA biosynthesis is regulated by the circadian system and/or AngII and to what extent changes in miRNA biosynthesis pathway are reflected by miRNA levels. Daily pattern of enzymes involved in miRNA biogenesis and clock genes were measured in the central and peripheral oscillators in control and AngII treated rats along with miRNAs. Based on our previous screening (Hasakova et al. 2019) we focused on miR-34a and miR-30c showing bidirectional functional association with the circadian system (Agarwal et al. 2015; Hasakova et al. 2019).

Materials and Methods

Experimental animals

The experimental protocol was approved by the Ethical Committee for the Care and Use of Laboratory Animals at the Comenius University, Bratislava. Experimental animals (54 male Wistar rats) were obtained from the Institute of Experimental Pharmacology and Toxicology of the Slovak Academy of Sciences (Slovak Republic) at the age of 10–11 weeks. Animals were housed in the room with controlled temperature (21 ± 2°C) with food and water available *at libitum*. Animals were synchronised to an LD cycle with 12 h of light and 12 h of dark. Information about time in the animal facility is provided in relativised time units, Zeitgeber time (ZT), where ZT0 is defined as the beginning of the light phase of a 24-h cycle and ZT12 corresponds to the beginning of the dark phase of a 24-h cycle.

Implantation of Alzet minipumps

After acclimatisation, two groups were created from randomly selected animals. The first group was used to test the effect of AngII administered *via* implanted osmotic minipumps (n = 28). Osmotic minipumps for 28-day infusion of AngII (Alzet 2004, Calbiochem, USA) were implanted subcutaneously in rats under total anaesthesia (xylazine-ketamine combination applied intramuscularly) into the intrascapular area. This treatment ensured that AngII was released continuously at a dose of 100 ng/kg/min for 28 days. Control animals (n = 26) were sham-operated.

Tissue sampling

Tissue sampling was performed at the end of experiment (after 28 days). Samples from the AngII-treated and control groups were taken at 4-h intervals over a 24-h period (ZT2, ZT6, ZT10, ZT14, ZT18 and ZT22). Tissue samples from the heart, liver and kidney were snap frozen in liquid nitrogen and stored at −80°C until RNA extraction. Brain samples were frozen in dry ice and stored at −80°C; they were subsequently cut into 300-μm serial coronal sections in a cryostat (Reichert-Jung, Leica, Germany) at −10°C. The SCN were dissected bilaterally by using a micropuncture technique under a dissecting microscope with a metal punching needle (inner diameter of 400 μm) according to the punching guide atlas (Palkovits 1973; Palkovits and Brownstein 1988). Dissected tissues were stored at −80°C until RNA extraction.

RNA isolation, reverse transcription and quantification of gene expression by real-time polymerase chain reaction (PCR)

Total RNA from the SCN, heart, kidney and liver samples was isolated with the Tri Reagent (MRC, USA) or RNAzol (MRC, USA) according to the manufacturer's instructions. GlycoBlue Coprecipitant (Ambion, USA) was used to isolate RNA from the SCN samples.

Complementary DNA (cDNA) was synthetised with the ImProm-IITM Kit (Promega, USA) according to the manufacturer's instructions. The total reaction volume (20 μl) contained 1 μg of RNA. For the reaction, RNA was incubated with 2 μl of random hexamer primers at 70°C for 5 min and then immediately incubated on ice for 5 min. Reverse transcription was conducted with the following steps: annealing at 25°C for 5 min, extension at 42°C for 60 min and enzyme inactivation at 70°C for 15 min.

cDNA was then subjected to real-time PCR with use of the QuantiTect SYBR Green PCR Kit (Qiagen, Germany) and StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). The primer sequences used for the amplification are listed in Supplementary Material, Table S1. The PCR included hot-start polymerase activation at 95°C; 40–45 cycles of denaturation at 94°C for 15 s, annealing at 49–52°C for 30 s and extension at 72°C for 30 s; and a final hold at 72°C for 2 min. Melting curve analysis was used to ensure PCR specificity. Gene expression was normalised to the housekeeping genes *rplp1*, *mrpl19*, *gapdh* or *U6*.

Statistical analysis

Daily profiles of gene expression were analysed with the use of cosinor analysis. The time series data of gene expression in the control and AngII-treated groups were fitted to a cosinor curve with a 24-h period. When the fitted cosinor curve significantly matched the experimental data, the parameters of the cosinor curve were calculated: mesor (average value of fitted curve), amplitude (value of curve peak relative to the mesor) and acrophase (time of curve peak from ZT0 – dark-to-light transition) (Refinetti et al. 2007). Differences between the two groups at a specific time point were compared with an unpaired *t*-test. The data in the graphs are presented as the mean ± standard error of the mean (SEM).

Results

In the liver, we observed a significant daily rhythm in *per2* mRNA expression in the control and AngII-treated animals. In both groups, a peak in *per2* mRNA expression was detected in the first half of the dark phase of the LD cycle, however, AngII caused a significant phase advance in rhythmic *per2* mRNA expression (Fig. 2A, Table 1, cosinor, $p < 0.0001$). This phase advance was accompanied by an increase in *per2* expression in AngII-treated rats compared with control rats at ZT10 (Table 1, *t*-test, $p < 0.05$).

Expression of *clock* mRNA in the liver showed a pronounced daily rhythm with maximum expression in the light phase of the LD cycle in both groups (Fig. 2B, Table 1, cosinor, $p < 0.01$). AngII treatment was associated with an increase in *clock* mRNA expression at ZT2 (Table 1, *t*-test, $p < 0.05$).

Expression of pre-miR-30c in the liver exhibited a pronounced daily rhythm in the control and AngII-treated groups, showing a peak level in the middle and second half of the light phase of the LD cycle, respectively (Fig. 2C, Table 1, cosinor, $p < 0.001$).

pre-miR-34a expression showed a trend for a daily rhythmic profile in the control group (Fig. 2D, Table 1, cosinor, $p =$

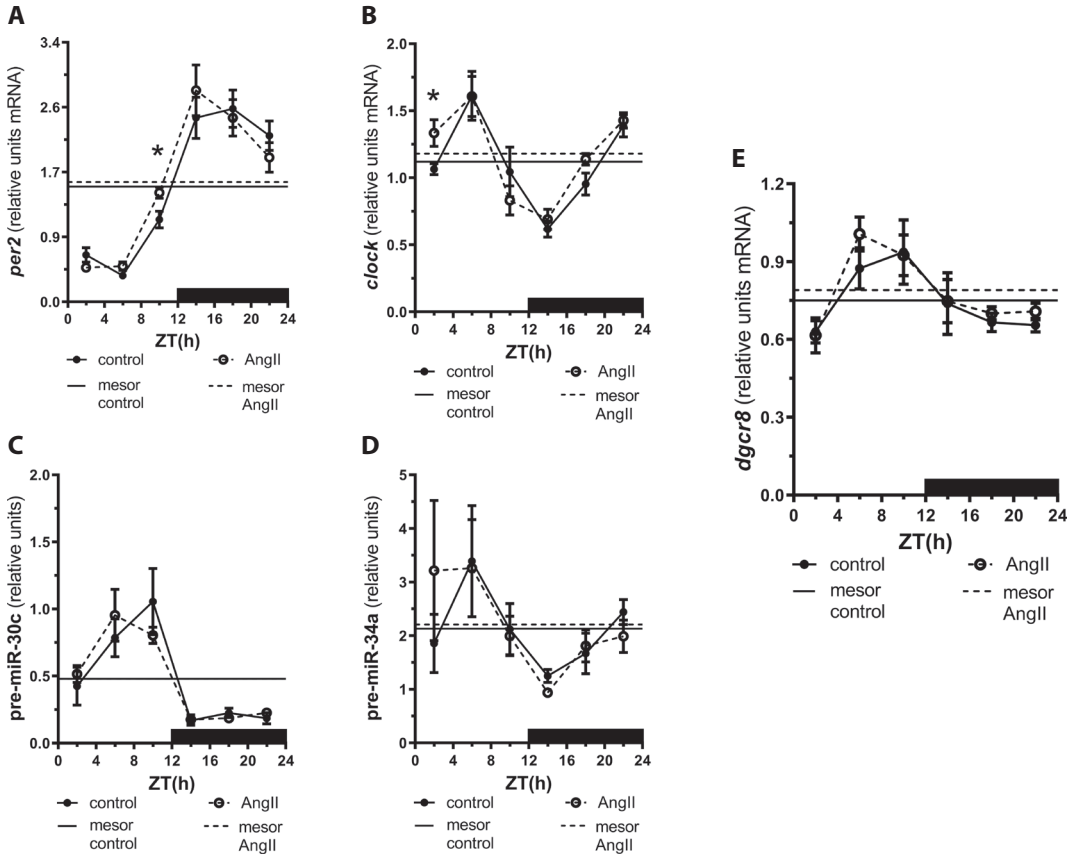

Figure 2. Effect of AngII infusion on daily profile of *per2* (**A**), *clock* (**B**), pre-miR-30c (**C**), pre-miR-34a (**D**) and *dgcr8* (**E**) expression in the liver. Time is expressed in relative time units ZT. The black bar at the x axis of graphs represents the dark phase of the LD cycle. The data are presented as the mean ± SEM ($n = 4–6$). The horizontal lines represent value of mesor calculated by cosinor analysis. An asterisk indicates a significant difference between the control and AngII-treated rats at the indicated time point; * $p < 0.05$, *t*-test.

Table 1. Cosinor analysis of clock genes, pre-miR-30c, pre-miR-34a and *dgcr8* expression in the control and AngII-treated groups

	Gene	Group	Mesor	Acrophase (h:min)	Amplitude	*p*	R	Significant difference between groups
Liver	*per2*	Control	1.51 ± 0.08	17:21 ± 0:22	1.20 ± 0.11	<0.0001	0.914	Phase advance ($p < 0.001$) ↑ ZT10 ($p < 0.05$)
		AngII	1.57 ± 0.09	16:20 ± 0:23	1.20 ± 0.12	<0.0001	0.889	
	clock	Control	1.12 ± 0.06	2:58 ± 1:02	0.32 ± 0.09	0.0050	0.607	↑ ZT2 ($p < 0.05$)
		AngII	1.18 ± 0.05	1:28 ± 0:37	0.39 ± 0.07	<0.0001	0.759	
	pre-miR-30c	Control	0.48 ± 0.06	7:45 ± 0:47	0.40 ± 0.08	0.0002	0.724	
		AngII	0.48 ± 0.04	6:39 ± 0:29	0.40 ± 0.05	<0.0001	0.855	
	pre-miR-34a	Control	2.13 ± 0.22	4:23 ± 1:53	0.66 ± 0.31	0.1294	0.404	
		AngII	2.21 ± 0.24	3:34 ± 1:14	1.02 ± 0.35	0.0242	0.507	
	dgcr8	Control	0.75 ± 0.03	9:18 ± 1:06	0.15 ± 0.04	0.0091	0.579	
		AngII	0.79 ± 0.03	8:42 ± 1:01	0.14 ± 0.04	0.0026	0.616	
Kidney	*per2*	Control	4.24 ± 0.22	15:16 ± 0:33	2.19 ± 0.32	<0.0001	0.823	
		AngII	4.47 ± 0.17	15:24 ± 0:21	2.41 ± 0.24	<0.0001	0.895	
	clock	Control	1.33 ± 0.04	1:04 ± 0:38	0.30 ± 0.05	<0.0001	0.760	Disappearance of the rhythm ↑ ZT18 ($p < 0.05$)
		AngII	1.40 ± 0.05	ns	ns	ns	0.366	
	pre-miR-30c	Control	1.24 ± 0.08	7:26 ± 1:17	0.35 ± 0.11	0.0171	0.546	↑ mesor ($p < 0.001$) ↑ ZT14 ($p < 0.01$)
		AngII	1.44 ± 0.08	6:04 ± 1:24	0.33 ± 0.12	0.0341	0.487	
	pre-miR-34a	Control	0.71 ± 0.05	ns	ns	ns	0.208	↑ whole group ($p < 0.05$) ↑ ZT14 ($p < 0.05$)
		AngII	0.94 ± 0.08	ns	ns	ns	0.109	
	dgcr8	Control	1.18 ± 0.04	5:08 ± 1:24	0.14 ± 0.05	0.0291	0.515	Disappearance of the rhythm
		AngII	1.24 ± 0.03	ns	ns	ns	0.393	
Heart	*per2*	Control	0.80 ± 0.05	15:42 ± 0:21	0.72 ± 0.07	<0.0001	0.913	Phase advance ↓ mesor ($p < 0.001$) ↓ ZT18; ZT22 ($p < 0.05$)
		AngII	0.69 ± 0.05	14:41 ± 0:24	0.60 ± 0.07	<0.0001	0.875	
	clock	Control	1.35 ± 0.04	0:40 ± 0:34	0.36 ± 0.06	<0.0001	0.790	
		AngII	1.28 ± 0.04	1:10 ± 0:36	0.38 ± 0.06	<0.0001	0.766	
	pre-miR-30c	Control	1.81 ± 0.13	9:44 ± 1:32	0.47 ± 0.19	0.0700	0.463	
		AngII	1.52 ± 0.08	8:06 ± 1:18	0.34 ± 0.11	0.0174	0.526	
	pre-miR-34a	Control	0.92 ± 0.06	7:53 ± 1:37	0.19 ± 0.08	0.0783	0.455	↑ ZT18 ($p < 0.05$) ↑ in D ($p < 0.05$)
		AngII	1.07 ± 0.05	7:56 ± 1:43	0.17 ± 0.07	0.0825	0.425	
	dgcr8	Control	1.10 ± 0.04	17:28 ± 1:01	0.20 ± 0.05	0.0022	0.642	Disappearance of the rhythm
		AngII	1.10 ± 0.05	ns	ns	ns	0.174	
SCN	*per2*	Control	0.82 ± 0.06	8:48 ± 0:45	0.43 ± 0.08	0.0001	0.750	
		AngII	0.89 ± 0.06	7:42 ± 0:38	0.51 ± 0.08	<0.0001	0.786	
	clock	Control	0.90 ± 0.05	ns	ns	ns	0.098	
		AngII	0.84 ± 0.05	21:41 ± 1:39	0.16 ± 0.07	0.0940	0.423	
	pre-miR-30c	Control	0.96 ± 0.07	ns	ns	ns	0.112	
		AngII	0.97 ± 0.07	ns	ns	ns	0.342	
	pre-miR-34a	Control	1.03 ± 0.12	ns	ns	ns	0.265	
		AngII	0.93 ± 0.07	ns	ns	ns	0.387	
	dgcr8	Control	0.83 ± 0.04	ns	ns	ns	0.130	
		AngII	0.81 ± 0.06	ns	ns	ns	0.177	

A cosine curve with a 24-h period was approximated to the time series data obtained from the control or AngII-treated group. The mesor and amplitude are given in relative units and the acrophase is given in relativised time units (ZT, where ZT0 is defined as the beginning of light phase of a 24-h cycle and ZT12 corresponds to beginning of the dark phase of a 24-h cycle). Parameters of rhythms were compared when the fitted curve for both groups showed a significant correlation with the experimental data. Significant differences are highlighted with grey colour. ns, not significant; *p*, level of significance of the fitted cosine curve; R, regression coefficient. Differences between the AngII-treated and control groups at a specific time point were compared by unpaired *t*-test. ↓, decreased expression in the AngII-treated group compared with the control group; ↑, increased expression in the AngII-treated group compared with the control group. The data are presented as the mean ± SEM ($n = 4–6$).

0.129). A significant daily rhythm in pre-miR-34a expression in the liver of AngII-treated rats showed maximum in the first half of the light phase of the LD cycle (Fig. 2D, Table 1, cosinor, $p < 0.05$).

Expression of *dgcr8* mRNA showed a rhythmic 24-h profile in the liver in both groups. The maximum level of *dgcr8* mRNA expression was detected in the second half of the light phase of the LD cycle in both the control and AngII-treated groups (Fig. 2E, Table 1, cosinor, $p < 0.01$). While expression of *dicer* was tonic during 24-h profile there was a pronounced trend to rhythmic expression in *drosha* mRNA which was more prominent in rats exposed to AngII treatment compared to control (Table S2, cosinor).

Expression of *per2* mRNA showed a daily rhythm with peak levels in the first half of the dark phase of the LD cycle in the kidney of control rats. Similarly, in AngII-treated rats *per2* mRNA expression showed a pronounced daily rhythm with a maximum level observed at ZT15 (Fig. 3A, Table 1, cosinor, $p < 0.0001$).

We observed a daily rhythm in *clock* mRNA expression in the kidney of control animals, with a peak level at the transition from the dark to light phase of the LD cycle (Fig. 3B, Table 1, cosinor, $p < 0.0001$). On the other hand, there was no rhythm in *clock* mRNA expression detected in AngII-treated rats (Fig. 3B, Table 1, cosinor). We observed an increase in *clock* mRNA expression in the kidney of the AngII-treated rats compared with the control rats at ZT18 (Table 1, *t*-test, $p < 0.05$).

pre-miR-30c expression exhibited a pronounced daily rhythm in the kidney in both groups, with a maximum level in the light phase of the LD cycle (Fig. 3C, Table 1, cosinor, $p < 0.05$). AngII treatment was associated with an elevated mesor of pre-miR-30c rhythmic expression (Table 1, cosinor, $p < 0.001$), which is in line with increased pre-miR-30c expression in the AngII-treated rats compared with the control rats at ZT14 (Table 1, *t*-test, $p < 0.001$).

pre-miR-34a expression in the kidney was arrhythmic in both groups (Fig. 3D, Table 1, cosinor). However, AngII treatment led to increased expression of pre-miR-34a compared with the control group (Table 1, *t*-test, $p < 0.02$). This effect was most pronounced at ZT14 (Table 1, *t*-test, $p < 0.05$).

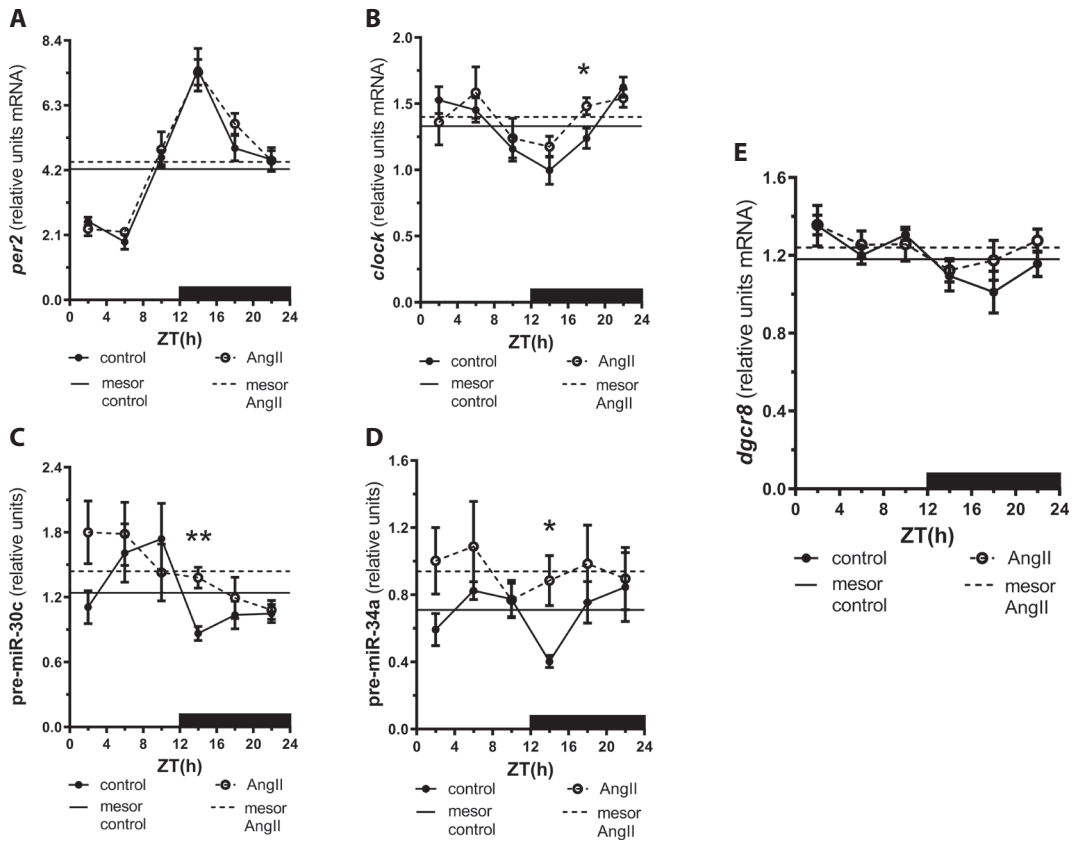

Figure 3. Effect of AngII infusion on the daily profile of *per2* (**A**), *clock* (**B**), pre-miR-30c (**C**), pre-miR-34a (**D**) and *dgcr8* (**E**) expression in the kidney. Time is expressed in relative time units ZT. The black bar at the x axis of graphs represents the dark phase of the LD cycle. The data are presented as the mean ± SEM ($n = 4–6$). The horizontal lines represent value of mesor calculated by cosinor analysis. An asterisk indicates a significant difference between control and AngII-treated rats at the indicated time point; * $p < 0.05$ and ** $p < 0.01$, *t*-test.

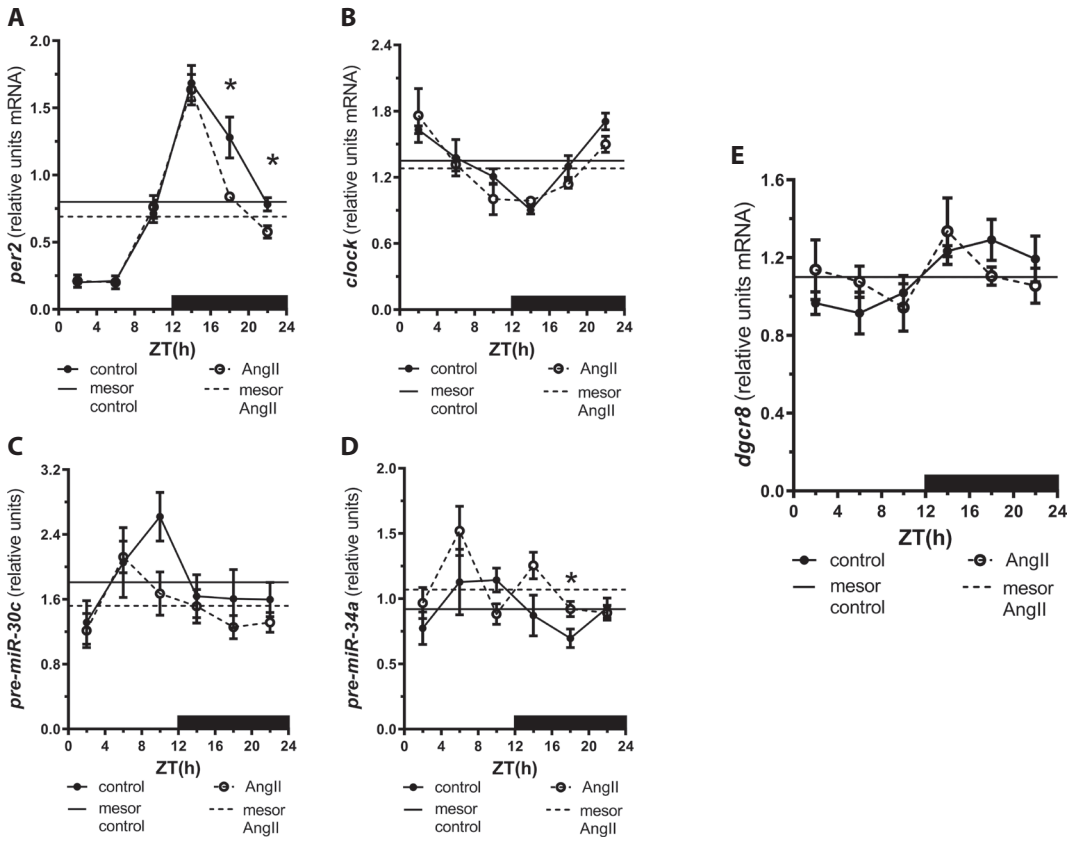

Figure 4. Effect of AngII infusion on the daily profile of *per2* (**A**, reproduced with permission from Herichova et al. 2013), *clock* (**B**), pre-miR-30c (**C**), pre-miR-34a (**D**) and *dgcr8* (**E**) expression in the heart. Time is expressed in relative time units ZT. The black bar at the x axis of graphs represents the dark phase of the LD cycle. The data are presented as the mean ± SEM (n = 4–6). The horizontal lines represent value of mesor calculated by cosinor analysis. An asterisk indicates a significant difference between control and AngII-treated rats at the indicated time point; * $p < 0.05$, t-test.

Among the miRNA biogenesis components analysed in the kidney, only *dgcr8* mRNA expression showed a rhythmic pattern, with a peak level in the first half of the light phase of the LD cycle (Fig. 3E, Table 1, cosinor, $p < 0.05$). AngII treatment was associated with the disappearance of the rhythmic pattern in *dgcr8* mRNA expression (Fig. 3E, Table 1, cosinor). AngII infusion was associated with significantly increased expression of *dicer* mRNA compared with the control group during the dark phase of the LD cycle; this effect was also significant during the entire 24-h profile (Table S2, t-test, $p < 0.05$).

In the heart, we observed a pronounced daily rhythm in *per2* mRNA expression in both groups. The maximum level of *per2* mRNA expression was detected at the beginning of the dark phase of the LD cycle in control as well as AngII-treated rats (Fig. 4A, Table 1, cosinor, $p < 0.0001$). However, AngII treatment was associated with phase advance in *per2* mRNA expression in the heart. Accordingly, treatment with AngII led to decreased expression of *per2* mRNA in the dark phase of the LD cycle at ZT18 and ZT22 compared with the control (Table 1, t-test, $p < 0.05$).

Expression of *clock* mRNA showed a significant daily rhythm in the heart of the control and AngII-treated animals, with peak levels at the transition from the dark to light phase of the LD cycle (Fig. 4B, Table 1, cosinor, $p < 0.0001$).

In the heart we observed a trend for rhythmic expression of pre-miR-30c in control rats (Fig. 4C, Table 3, cosinor, $p = 0.07$) and a significant daily rhythm in pre-miR-30c expression in animals infused with AngII (Fig. 4C, Table 3, cosinor, $p < 0.05$). The peak level of pre-miR-30c expression in both groups was observed during the light phase of the LD cycle (Table 3, cosinor).

pre-mi-34a showed a trend for rhythmic expression in the heart in control as well as AngII-treated rats (Fig. 4D, Table 1, cosinor, $p = 0.078$ and $p = 0.083$, respectively). AngII infusion increased pre-miR-34a expression in the dark phase of the LD cycle (Table 1, t-test, $p < 0.05$). This effect was most pronounced at ZT18 (Table 1, t-test, $p < 0.05$).

Expression of *dgcr8* mRNA in the heart showed a significant rhythmic profile in the control group (Fig. 4D, Table 1, cosinor, $p < 0.01$); however, this rhythm diminished after AngII treatment (Fig. 4D, cosinor). The peak level of *dgcr8* mRNA expression in the control group was observed in the middle of the dark phase of the LD cycle (Table 1, cosinor). Expression of the miRNA biogenesis components *drosha* and *dicer* mRNA was arhythmic in both groups (Table S2, cosinor). However, AngII treatment was associated with decreased expression of *drosha* mRNA in the heart (Table S2, *t*-test, $p < 0.05$).

In the SCN, expression of *per2* mRNA showed a significant daily rhythm in the control group, with a peak in the second half of the light phase of the LD cycle. This rhythm was not significantly influenced by AngII infusion (Fig. 5A, cosinor, $p < 0.001$), although in the AngII-treated rats we observed a trend for a more pronounced rhythmic pattern compared with the control rats. The daily profile of *clock* mRNA expression did not show a rhythmic pattern in the control group (Fig. 5B, cosinor). By contrast, in AngII-treated rats we observed a trend to rhythmicity in mRNA *clock* expression (Fig. 5B, cosinor, $p = 0.09$), with a peak at the end of the dark phase of the LD cycle.

In the SCN, the precursor-miRNAs pre-miR-30a and pre-miR-34a and the components of miRNA biogenesis *dicer*, *drosha*, *dgcr8* mRNA did not show rhythmic expression in the control or AngII-treated groups (Fig. 4, Table 1, Table S2, cosinor).

To analyse putative mechanisms of AngII-mediated regulation of miRNA biogenesis components and clock gene expression, we performed *in silico* promoter analysis by using the Eukaryotic Promoter Database (EPD). The search was limited to the −1 kb upstream promoter region relative to the transcription start site.

Transcription factors regulated by AngII were selected according to a literature search in PubMed. The genes *dicer* and *clock* as well as the genes coding miR-30c and miR-34a were excluded from *in silico* promoter analysis because information about their promoter regions in the rat is not available in EPD. *In silico* analysis revealed putative binding sites for the transcription factors ETS1, c-MYC, c-JUN, c-FOS and

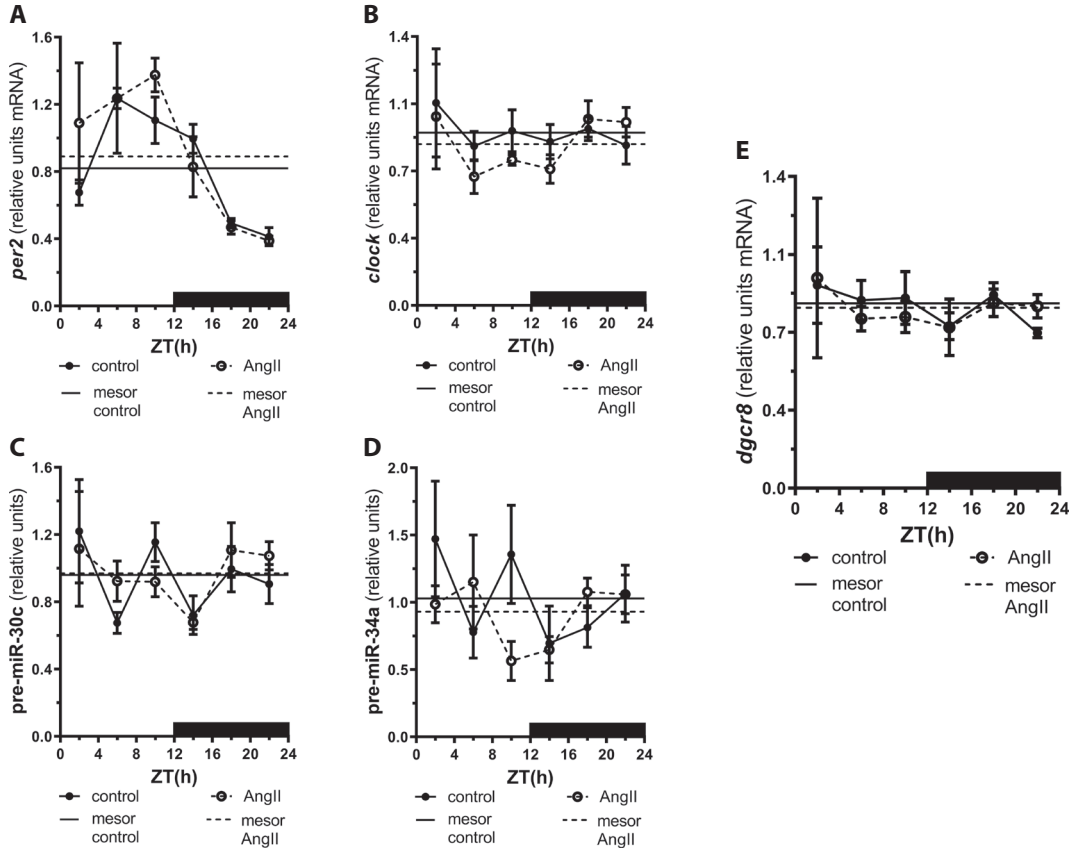

Figure 5. Effect of AngII infusion on the daily profile of *per2* (**A**), *clock* (**B**), pre-miR-30c (**C**), pre-miR-34a (**D**) and *dgcr8* (**E**) expression in the SCN. Time is expressed in relative units time ZT. The black bar at the x axis of graphs represents the dark phase of the LD cycle. The data are shown as the mean ± SEM ($n = 4–6$). The horizontal lines represent value of mesor calculated by cosinor analysis. SCN, suprachiasmatic nuclei.

Table 2. *In silico* analysis of binding sites for transcription factors regulated by AngII predicted in the promoter areas of the *drosha*, *dgcr8* and *per2* genes

	c-JUN	c-FOS	c-MYC	ETS1	NF-κB
drosha	✓(0.01)	✓(0.001)	✓(0.001)	✓(0.0001)	✓(0.001)
dgcr8	✓(0.01)	✓(0.01)	✓(0.00001)	✓(0.001)	✓(0.01)
per2	✓(0.001)	✓(0.001)	✓(0.001)	✓(0.0001)	✓(0.00001)

The symbol ✓ indicates the presence of a predicted binding site for the transcription factor in the rat genes. The number in parentheses represents the probability of the presence of a binding site within the −1 kb upstream promoter region relative to the transcription start site in the rat genes. Data were extracted from the Eukaryotic Promoter Database (EPD) (Dreos et al. 2015, 2017). c-MYC, MYC proto-oncogene; c-Fos, Fos proto-oncogene; c-Jun, Jun proto-oncogene; ETS1, ETS proto-oncogene 1; NF-κB, nuclear factor kappa B.

NF-κB in the upstream promoter region of the genes *drosha* and *dgcr8*; the ETS1 and c-MYC binding sites, respectively, had the highest binding probability for these genes (Table 2). Similarly, putative binding sites for all the above-mentioned transcription factors were found in the upstream promoter region of *per2* where NF-κB showed the highest binding probability (Table 2).

Discussion

Our study revealed prominent differences in the daily expression pattern of key components involved in miRNA biosynthesis, especially *dgcr8*, which exhibited a remarkable tissue-specific 24-h pattern. While *dgcr8* expression showed a pronounced daily rhythm in the liver, heart and kidney, surprisingly *dgcr8* expression in the SCN did not vary during a 24-h cycle. Similarly, *dgcr8* expression in the prefrontal cortex was arrhythmic (Herichová et al. 2021), indicating that the pattern of *dgcr8* expression can be regulated by some humoral factor that does not cross the blood–brain barrier. Interestingly, the acrophase of *dgcr8* in peripheral oscillators showed tissue-specific characteristics. The appearance of a rhythmic pattern in miRNA expression seems to be dependent on *dgcr8* in a tissue-dependent manner. The expression of *dicer* and *drosha* mRNA did not show a significant 24-h rhythm in the present study.

To elucidate whether the rhythm in *dgcr8* expression can be related to the cellular circadian oscillator, expression of *per2* and *clock* was analysed in the SCN, liver, heart and kidney. As expected, *per2* expression exhibited a clear-cut rhythm in all tissues with a peak at the second half of the light phase in the SCN and at the beginning of the dark phase in peripheral tissues. Expression of *clock* was arrhythmic in the SCN, consistently with previous findings (Shearman et al. 2000; Shieh et al. 2005; Herichová et al. 2007); while a clear-cut rhythm was observed in the heart, liver and kidney. A presence of rhythmic pattern in *clock* mRNA expression corresponds well with appearance of rhythm in *dgcr8* expression.

The transcription factor CLOCK can influence *dgcr8* expression at least in two ways. First, the EPD indicated the presence of a binding site for the transcription factor CLOCK that regulates gene expression *via* an E-box (Dreos et al. 2015, 2017) in the *dgcr8* sequence. Second, it has been also shown that *dgcr8* expression is regulated by deacetylation (Wada et al. 2012). This process can be influenced by CLOCK, as it was demonstrated that CLOCK is a histone acetyltransferase participating in regulation of gene expression (Doi et al. 2006).

Previously, microarray screening indicated rhythmic *dgcr8* expression in the white adipose tissue of mice, with a peak at the transition from the subjective dark to the subjective light phase under constant conditions (Zhang et al. 2014), and RNA sequencing implicated rhythm in *dgcr8* mRNA in the liver of mice, with maximum expression at the beginning of the dark phase of the LD cycle (Koronowski et al. 2019). We recently observed maximum *dgcr8* expression in the rat liver at the transition from the light to dark phase of the LD cycle, which is generally in agreement with previous measurement in the liver of mice.

However, a daily pattern in *dgcr8* expression in the kidney peaked in the middle of the light phase and in the heart, *dgcr8* expression peaked during the dark phase. A reason for the observed differences in the acrophase of *dgcr8* rhythmic expression in peripheral oscillators is currently not known. However, it has been shown that rhythmic expression of *dgcr8* in the liver is dependent on the presence of a central oscillator (Koronowski et al. 2019). Therefore, we hypothesise that SCN-driven humoral signals from the peripheral tissue synchronise *dgcr8* expression. The observed tissue-specific differences in the acrophase of *dgcr8* rhythmic expression could be related to the responsiveness of peripheral oscillators to this signalling.

The final pattern of *dgcr8* could be influenced by transcription factors under the control of basic feedback loops

including c-Myc, SP1 (Sp1 transcription factor) and HNF4A (hepatocyte nuclear factor 4 alpha) (Ripperger et al. 2010; Hirao et al. 2011), all of which have predicted binding sites in the *dgcr8* promoter in rats (Dreos et al. 2015, 2017). Unfortunately, the effects of these transcription factors on *dgcr8* expression have not yet been proved experimentally. There are currently only two experimentally validated transcription factors involved in the regulation of *dgcr8* expression (Gómez-Cabello et al. 2010; Shan et al. 2015), but neither of those seems to be involved in the generation of a tissue-specific 24-h pattern of *dgcr8* expression. Taken together, the generation of tissue-specific *dgcr8* expression seems to be a complex process involving several regulatory elements.

We did not observe a rhythmic pattern in *drosha* and *dicer* expression in this study. Previously, a daily rhythm in *dicer* expression has been implicated in the mouse liver, SCN, retina and bone marrow mononuclear cells of 2-month-old male mice (Yan et al. 2013). However, this observation was not confirmed later in the liver of mice (Du et al. 2014). Similarly, *drosha* and *dicer* do not exhibit a significant daily rhythm in expression in the liver of mice (Koronowski et al. 2019). Therefore, we suppose that *dicer* and *drosha* mRNA expression do not contribute significantly to induction of rhythmic expression of miRNA.

To further investigate the association between *dgcr8* and circadian oscillators and to determine whether the pattern of miRNA expression depends on *dgcr8*, we induced changes in *dgcr8* and *clock* gene expression by administration of AngII. This treatment allowed us to evaluate whether changes observed in *clock* gene expression are paralleled by changes in *dgcr8* and miRNA expression. Based on our previous screening (Hasakova et al. 2019), we analysed the regulatory influence of *dgcr8* on pre-miR-34a and pre-miR-30c expression as these miRNAs show a functional relationship with the circadian system. The effect of AngII on *per2*, *cry1*, *cry2*, *rev-erbα* and/or *dbp* mRNA expression in the aorta (Herichova et al. 2014), cells isolated from vascular smooth muscle of rat (Nonaka et al. 2001) and a cell line derived from human adrenal gland cells (Tetti et al. 2018) have been reported previously.

In the present study, AngII caused the disappearance of rhythmic *clock* gene expression in the kidney that was accompanied by the disappearance of the rhythm in *dgcr8* expression. AngII did not influence the daily rhythm in *clock* expression in the heart but decreased the mesor of the *per2* daily rhythm that was also accompanied by the disappearance of rhythm in *dgcr8* expression. *clock* gene expression in the liver of AngII-treated rats was more robust compared with the control group, a phenomenon that was also paralleled by increased robustness of *dgcr8* rhythmic expression. In spite of presence of AT1 receptors in the SCN (Thomas et al., 2004) we did not observe a significant effect of peripheral AngII administration on *clock* and *dgcr8* expression in the SCN under conditions of recent study. Although these parallels implicate a functional relationship between *dgcr8* and *clock* gene expression, *dgcr8* expression probably can also be regulated by AngII directly. A search in the EPD revealed that the *dgcr8* promoter region contains several regulatory regions responsive to AngII (Table 2). While domains for c-Myc and ETS1 binding to *dgcr8* show the highest probability, transcription sites for c-Jun, c-Fos and NF-κB show a lower level of significance (Table 2) (Dreos et al. 2015, 2017).

Pronounced differences between central and peripheral oscillators have also been observed in pre-miRNA expression. pre-miR-34a and pre-miR-30c, similarly to *dgcr8*, did not exhibit a rhythmic pattern in the SCN, and AngII did not influence their expression. On the other hand, both miRNAs showed a daily rhythm in expression in the liver. AngII treatment in this tissue increased the significance of rhythmic expression of both measured miRNAs as well as *dgcr8*.

Interestingly, only pre-miR-30c exhibited a rhythmic pattern in the heart and kidney while the expression of pre-miR-34a did not reach the level of significance, although there was a trend implicating a daily rhythm in the heart. In the rat, miR-34a is an independent transcription unit because it is not located within a host gene while, according to miRbase, miR-30c is expressed in a cluster (Kim et al. 2004; Landgraf et al. 2007; Linsen et al. 2010). Unfortunately, in rat there are insufficient data to perform *in silico* research focussed on transcription factors regulating miRNAs. In both the heart and kidney, the expression of pre-miR-30c and pre-miR-34a was responsive to AngII treatment probably *via* some AngII-responsive transcription factor(s). In both cases, AngII induced pre-miRNA expression without a parallel effect on *dgcr8* expression.

Conclusions

To conclude, under control conditions expression of *dgcr8* corresponded to the expression of *clock* in all studied tissues in terms of presence of rhythmic pattern. *dgcr8* exhibited a daily rhythm in expression in the liver, heart and kidney while expression of *dgcr8* was tonic in the SCN. Expression of pre-miR-30c and pre-miR-34a showed a greater parallel with *dgcr8* expression in the liver and SCN compared with the heart and kidney. AngII induced the expression of pre-miR-30c and pre-miR-34a in the kidney and pre-miR-34a in the heart, which was not observed in the liver and the SCN. It seems that the rhythm in expression of pre-miR-30c and pre-miR-34a is dependent on *dgcr8* expression mainly in the liver while other tissue-specific regulatory factors are more important in the SCN, heart and kidney.

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Supplementary Material

The 24-h pattern of *dgcr8*, *drosha*, and *dicer* expression in the rat suprachiasmatic nuclei and peripheral tissues and its modulation by angiotensin II

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Table S1. Sequences of primers used in real-time polymerase chain reaction reactions

Gene	Strand	Sequence (5′ → 3′)	Accession number	AT
clock	sense	CCA ACT CCT TCT GCC TCC TC	NM_021856	49°C
	antisense	ACC TCC GCT GTG TCA TCT TC		
dgcr8	sense	AAG AAG AGG CGA ATG GAG GA	NM_001105865.1	52°C
	antisense	CCC CAA GAA GTA GGG TCT GG		
dicer	sense	ATG AGA AGC AAA AAG GTC AGC A	XM_039113358.1	52°C
	antisense	ACA TAA CCA GGA GGA AGC CAA		
drosha	sense	GCA GGA GAC CCA CAA TAC CAA	NM_001107655.2	52°C
	antisense	AGA ACG AAT GCC AGT TTT CCA		
gapdh	sense	CTG AGA ATG GGA AGC TGG TC	NM_017008.4	52°C
	antisense	GTG GTT CAC ACC CAT CAC CAA		
mrpl19	sense	CCT GTG AAC AAG CTG AAA G	NM_001029898	49°C
	antisense	ATG GCT TGC TCC ACT TCT G		
per2	sense	GAG GTT CAG GGA AGT GAG CA	NM_031678.2	49°C
	antisense	TTG ACA CGC TTG GAC TTC AG		
pre-miR-30c	sense	TGT GTA AAC ATC CTA CAC TCT CAG C	NR_029833.1	52°C
	antisense	TGG CAG AAG GAG TAA ACA ACC		
pre-miR-34a	sense	TTG GCA GTG TCT TAG CTG GTT	NR_029610.1	52°C
	antisense	CAA CGT GCA GCA CTT CTA GG		
rplp1	sense	TCC ACA ACA TGG CTT CTG TC	NM_001007604.2	49°C
	antisense	ATT GCA GAT GAG GCT TCC AA		
U6	sense	GCT TCG GCA GCA CAT ATA CTA A	NR_004394.1	52°C
	antisense	AAA ATA TGG AAC GCT TCA CGA		

AT, annealing temperature; *clock*, circadian locomotor output cycles protein kaput; *dgcr8*, DiGeorge syndrome critical region gene 8; *gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *mrpl19*, mitochondrial ribosomal protein L19; *per2*, period circadian regulator 2; *rplp1*, ribosomal protein lateral stalk subunit P1; U6, U6 small nuclear RNA.

Table S2. Cosinor analysis of dicer and drosha expression in the control and angiotensin II (AngII)-treated rats

	Gene	Group	Mesor	Acrophase [h:min]	Amplitude	*p*	R	Significant difference between groups
Liver	*dicer*	Control	0.86 ± 0.02	ns	ns	0.105	0.422	
		AngII	0.88 ± 0.02	ns	ns	0.397	0.267	
	drosha	Control	0.58 ± 0.03	ns	ns	0.091	0.434	
		AngII	0.59 ± 0.02	ns	ns	0.050	0.462	
Kidney	*dicer*	Control	0.83 ± 0.02	ns	ns	0.144	0.394	↑ in dark; ↑ whole group ($p < 0.05$)
		AngII	0.89 ± 0.02	ns	ns	0.370	0.277	
	drosha	Control	0.86 ± 0.03	ns	ns	0.150	0.390	
		AngII	0.91 ± 0.03	ns	ns	0.937	0.072	
Heart	*dicer*	Control	1.10 ± 0.04	ns	ns	0.308	0.312	
		AngII	1.11 ± 0.03	ns	ns	0.322	0.295	
	drosha	Control	1.14 ± 0.05	ns	ns	0.117	0.413	↓ whole group ($p < 0.001$) ZT18 ($p < 0.05$)
		AngII	1.01 ± 0.05	ns	ns	0.642	0.187	
SCN	*dicer*	Control	0.99 ± 0.06	ns	ns	0.573	0.222	
		AngII	0.91 ± 0.07	ns	ns	0.558	0.214	
	drosha	Control	0.71 ± 0.03	ns	ns	0.710	0.175	
		AngII	0.71 ± 0.04	ns	ns	0.445	0.251	

A cosine curve with a 24-h period was approximated to the time series data of rats from the control or AngII-treated rats. The mesor and amplitude are given in relative units and the acrophase is given in relative time units ZT. ns, not significant; *p*, level of significance of the fitted cosine curve; R, regression coefficient. Differences between the control and AngII-treated groups at a specific time point were evaluated by unpaired *t*-test. ↓, decreased expression in AngII-treated group compared with the control group; ↑, increased expression in the AngII-treated group compared with the control group.