Title: Repeated phase shifts in the lighting regimen change the blood pressure response to norepinephrine stimulation in rats

Running title: Response of BP to norepinephrine in shifted rats

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Summary

Disturbed circadian activity of the sympathetic system may be involved in negative consequences of chronodisruption on the cardiovascular system. We studied daily changes in pressure response to adrenergic stimulation in rats exposed to repeated phase advance shifts (PAS) of light/dark (LD) regimen. Blood pressure (BP), heart rate (HR) and locomotor activity was measured by radiotelemetry in normotensive Wistar rats exposed to repeated PAS (three 8-h shifts per week) lasting for 12 weeks. Norepinephrine was administered subcutaneously in the middle of L and D during week 12 of PAS exposure. In the control LD cycle, cardiovascular parameters exhibited significant daily rhythms with expected higher values during D than L phase. Rats exposed to PAS showed disturbed rhythms without a BP and HR increase. Administration of norepinephrine to control rats revealed daily variability in the cardiovascular response with higher stimulation of BP during L than D. This daily pattern of BP response to norepinephrine was diminished in the PAS group. The damped daily variability in pressure response to norepinephrine and augmented response during the light phase of the day suggest that the increased and desynchronised activity of the sympathetic system may worsen responses of the cardiovascular system to load in individuals exposed to irregular LD conditions.

Key words: radiotelemetry, norepinephrine, circadian, blood pressure, phase advance shift

Introduction

Circadian oscillations developed as adaptation to rhythmic changes of environmental conditions and there is a strong presumption that the synchronized timing system can improve performance of animals and humans in predictable conditions (Menaker *et al*. 2013). Circadian rhythms in the cardiovascular system are well known (Millar-Craig *et al.* 1978). Blood pressure (BP) and heart rate (HR) exhibit distinct circadian rhythms with higher values during the active phase than the passive phase (Smolensky *et al.* 2007). The activity of the cardiovascular system starts to rise at the beginning of the active phase when a higher frequency of myocardial ischemia (Mulcahy *et al.* 1988), myocardial infarction (Muller *et al.* 1985), sudden cardiac death (Muller *et al*. 1987) and stroke (Marler *et al*. 1989) occurs. The circadian system helps to predict an occurrence of stress stimuli from the environment and prepare the cardiovascular system for increased demands. Stress activates the hypothalamic–pituitary–adrenal axis as well as the sympathetic branch of the autonomic nervous system with norepinephrine release (Levy and Tasker 2012). Norepinephrine stimulates the heart and vessels via adrenergic receptors to cope with challenges. The circadian pattern of a vessel sensitivity to α1-adrenoreceptor agonists was demonstrated under in vitro conditions in the rat thoracic aorta rings with the higher sensitivity in the middle of the passive than the active phase (Keskil *et al.* 1996).

Shift work and night-time work may disrupt the innate circadian organisation. Disturbed circadian rhythms may have negative consequences on cardiovascular functions. First, by inducing a non-dipping BP pattern that is connected with a target-organ damage (Cuspidi *et al*. 2001, 2012). Second, chronodisruption can change the response of the cardiovascular system to stressors and contribute to the augmented sympathetic response with a higher

3

incidence of myocardial infarction and ischemic stroke (Manfredini *et al*. 2004; Vyas *et al*. 2012).

Therefore, in the present study we investigated if repeated phase advance shifts (PAS) of the lighting regime can: 1) increase absolute values of BP and HR; 2) disturb the circadian rhythmicity; and 3) change the response of BP and HR to administration of norepinephrine, the dominant neurotransmitter of the sympathetic system.

Material and methods

Experimental animals

Normotensive mature male Wistar rats (13 weeks old, 356 ± 8 g at the beginning of the experiment) were included in the study. They were housed individually in cages with food and water *ad libitum* under 12L:12D conditions (lights on 06:00) with controlled room temperature 21 ± 2 °C, humidity 55 ± 10 % and light intensity 150 Lux (Datalogger KIMO KH100, Chevrier Instruments Inc., Canada) during the experiment. The experiment was approved by the Ethical Committee for the Care and Use of Laboratory Animals at the Comenius University in Bratislava, Slovak Republic and the State Veterinary authority of Slovak Republic.

Implantation of transmitters and data collection

The surgical procedure was performed under ketamine hydrochloride (75 mg/kg; i.m.) and xylazine hydrochloride (10 mg/kg; i.m.) anaesthesia. The pressure radiotelemetric transmitter TA11PA-C40 (Data Science Int., St. Paul, Minnesota, USA) was surgically implanted rostrally into the abdominal part of the aorta just above its bifurcation (Brockway *et al*. 1991). The catheter was then stabilised to the aorta with tissue glue (3M Vetbond; DSI, USA) and a cellulose patch (Cellulose Patch Kit – Small Animals; DSI, USA). The body of the transmitter was secured to the muscular wall and the whole procedure has been validated at our department previously (Molcan *et al*. 2009). Animals were included in the experiment two weeks after the surgical procedure.

Experimental design

After the 2-weeks recovery period, experimental rats (n = 8) were kept for one week in control LD. Afterwards, they were exposed to 8-h phase advance shifts (PAS) three times per week and these conditions lasted 12 weeks (Fig. 1). Due to catheter clothing, two animals

were removed from the experiment. At the end of the experiment, norepinephrine (arterenol bitartrate hydrate; Calbiochem, Germany) was administered subcutaneously (200 μgkg^{-1}; set on the basis of preliminary trials) to experimental rats (PAS, n = 5) and rats entrained to LD (control, n = 9) in the middle of the subjective light (ZT06) and the dark (ZT18) phase of the day. In the entrained rats, effects of NE (n = 5) in comparison to saline (n = 4) administration were tested during the subjective light (ZT06) phase.

Data collection and statistical analysis

Pressure and locomotor data were acquired and calculated to systolic (SBP) and diastolic (DBP) blood pressure, HR and locomotor activity by Dataquest A.R.T. 4.1 Gold system (DSI, USA) by scheduled sampling intervals every 5 minutes with segment duration 30 seconds. Radiotelemetry data were obtained during the first control LD week and PAS weeks 1, 5, 10 and 11. On week 12, BP and HR were measured only during the norepinephrine administration.

Data were averaged to 1h ± standard error of the mean (SEM) for visualization of daily changes and to 5 minutes ± standard error of the mean (SEM) intervals after NE administration. An area under the curve (AUC) was calculated using a trapezoidal rule and expressed as square relative units (SRU). Acquired data were analysed with a repeated analysis of variance with Fisher LSD post-hoc test used for evaluation of differences between groups. Time is expressed in relative units – Zeitgeber time (ZT), when ZT00 is defined as the beginning of the light phase of the day. Circadian rhythm evaluation (mesor, amplitude, acrophase) of the individual measured data was performed with Lomb-Scargle Periodogram by Chronos-Fit software (Zuther *et al.* 2009).

Results

Day and night differences

Rats exposed to control LD conditions showed significant circadian rhythms of measured parameters with higher values during the dark phase in comparison to the light phase of the day (Fig. 2). Both pressure parameters (SBP: ZT 17:35 ± 1:31 h; DBP: ZT: 17:22 ± 1:12 h) and HR (ZT 17:22 ± 0:32 h) oscillated with the acrophase calculated to the middle of D. Locomotor activity of rats followed the same pattern as HR and BP with acrophase at ZT 18:21 ± 0:29 h (Table 1).

After 11 weeks of PAS exposure, we did not find any increase in absolute values of SBP, DBP, HR and locomotor activity (Fig. 2). However, significant disturbances of circadian rhythms in HR, BP and locomotor activity were observed (Table 1).

Effects of norepinephrine administration in comparison with saline

At first, we tested effects of subcutaneous administration of NE in comparison with saline in control rats at ZT06. NE administration resulted in a significant increase in SBP ($p < 0.001$; $164 ± 5$ vs. $118 ± 2$ mm Hg; $+39\%$) and DBP ($p < 0.001$; $117 ± 3$ vs. $83 ± 1$ mm Hg; $+41\%$) and this increase persisted for 140 minutes in comparison with basal values at ZT06. Saline increased both SBP and DBP ($128 ± 1$ mm Hg and $84 ± 2$ mm Hg respectively) only temporarily and BP values returned to basal levels 30 minutes after the treatment. Similarly AUC increased substantially in both SBP ($p < 0.001$) and DBP ($p < 0.001$) after the norepinephrine treatment (SBP: $402.5 ± 34.8$ SRU; DBP: $280.6 ± 49.7$ SRU) as compared to the saline administration (SBP: $54.5 ± 7.4$ SRU; DBP: $40.8 ± 5.1$). In control rats, norepinephrine failed to affect HR as compared to saline administration.

Effects of norepinephrine on control and shifted rats

Blood pressure

In control rats, norepinephrine administration increased BP more during the light (ZT06; SBP: 161 ± 2 mm Hg; +36%; DBP: 113 ± 2 mm Hg +43%) than the dark phase (ZT18; SBP: 154 ± 3 mm Hg; +23%; DBP: 107 ± 2 mm Hg +25%; Fig. 3). Similarly, the area under the curve was increased more after the norepinephrine treatment in the middle of the light compared to the dark phase in entrained rats (SBP: $p < 0.05$; DBP: $p < 0.05$; Fig. 4).

In rats exposed to PAS, no differences were found in the BP response to norepinephrine between ZT06 (SBP: 163 ± 5 mm Hg; +34%; DBP: 113 ± 3 mm Hg; +36%) and ZT18 (SBP: 169 ± 8 mm Hg; +35%; DBP: 115 ± 5 mm Hg; +39%). A comparison of AUC after norepinephrine administration in ZT06 and ZT18 revealed a significant increase in SBP ($p < 0.05$; ZT06: 558.11 ± 81.82 SRU; ZT18: 385.87 ± 67.46 SRU) but not in DBP ($p = 0.35$; ZT06: 397.23 ± 72.85 SRU; ZT18: 328.67 ± 73.61 SRU) in the PAS group (Fig. 4). The increase in BP values after norepinephrine administration in the PAS group was observed in comparison to rats entrained to LD during the subjective dark phase (ZT18) of the day (control SBP: 154 ± 3 mm Hg; PAS SBP: 169 ± 8 mm Hg; $p < 0.01$). During the subjective light phase (ZT06), the return to control BP values was not observed over 4 h in the PAS group while entrained rats returned to basal values after 170 minutes.

Heart rate

Administration of norepinephrine to entrained rats resulted in a significant ($p < 0.05$) changes of HR at ZT06 (310 ± 9 bpm; -5%) and ZT18 (347 ± 10 bpm; -11%) (Fig. 3). On the other hand, a comparison of AUC between ZT06 (336.65 ± 78.34 SRU) and ZT18 (195.57 ± 88.67 SRU) did not reveal significant differences (Fig. 4).

A similar HR response was observed after norepinephrine administration to the PAS group at ZT06 (260 ± 16 bpm; -13%) and ZT18 (248 ± 13 bpm; -25%) with the more pronounced decrease of HR after norepinephrine administration in comparison with the control group, mainly at ZT06. Comparison of AUC between ZT06 and ZT18 revealed significant ($p < 0.05$) differences in the PAS group (Fig. 4).

Discussion

Animals exposed to control LD conditions showed distinct daily rhythms in HR, BP and locomotor activity with higher values during the dark than the light phase of the day and these results are in line with previous studies (Witte and Lemmer 1995). Rats exposed to PAS for 11 weeks exhibited a decrease in the circadian rhythm power during the last week of the exposure and a diminished circadian/ultradian rhythm power ratio (Molcan *et al*. 2013) in comparison with control group. Moreover, long-term PAS did not elevate absolute values of BP and HR.

Norepinephrine administration enhanced both SBP and DBP in comparison with the saline injection. The increase of BP after the saline injection was transitory and values returned to pretreatment levels after 30 minutes. The increased levels of BP after norepinephrine administration persisted much longer (140 minutes) in comparison with saline. A similar massive increase of BP after norepinephrine was described in humans (Jain and Sing 2010), mice (Masuki *et al*. 2005) and rats (Massett *et al*. 1998). A lower and short-lasting increase of BP values, corresponding to saline administration, was observed in rats after behaviour-stress-like responses (Dielenberg *et al*. 2001; Sharp *et al*. 2003). Due to a lack of HR differences between NE and saline administration and low capacity of NE to cross the blood-brain barrier in adults (Lasbennes *et al.* 1983), we expect predominant effects of NE on adrenergic receptors in vessels.

We found a different response of BP and HR to norepinephrine administration at ZT06 (the light phase) in comparison to ZT18 (the dark phase). Both systolic and diastolic BP increased more in entrained rats after norepinephrine administration at ZT06 than ZT18. The return to the basal levels was observed 170 and 140 minutes after norepinephrine administration at ZT06 and ZT18, respectively. Moreover, the more prominent decrease of HR was observed

after norepinephrine administration at ZT06 than the ZT18. A time of the day dependent pressure response of arteries was observed in humans (Tham *et al*. 1996), mice (Masuki *et al*. 2005) as well as with in vitro studies using aorta rings (Görgün *et al*. 1998). Thus, our results in line with published data, demonstrated the distinct daily rhythm in the response of the cardiovascular system to the sympathetic activation in freely moving animals.

In rats exposed to repeated PAS, the circadian variability in BP response to norepinephrine administration was eliminated. The return of BP to basal values lasted longer and did not reach the control values during analysed 240 minutes for systolic and diastolic BP after norepinephrine administration at ZT06. After norepinephrine administration at ZT18, the return to basal values took 180 minutes for both systolic and diastolic BP. Moreover, the decrease of HR was different between the ZT06 (-13%) and ZT18 (-25%) phases of the day in shifted rats in comparison with the HR response in the control group. Thus, our results show that PAS obviates the temporal BP control and even more importantly, lost daily rhythms of BP and HR increase the sensitivity of the cardiovascular system to the adrenergic stimulation. Similarly, the temporal variation in the pressor response was abolished in mice with clock gene (*Bmal-/-*) deletion (Curtis *et al*. 2007). Moreover, lost circadian variability of baroreflex sensitivity with no difference between light and dark values was also shown in *Cry*-deficient mice (Masuki *et al*. 2005).

In our present study we did not expose rats to constant lighting conditions to prove that the rhythms persist in constant environment and are truly circadian. However, studies performed in humans under constant routine conditions, i.e. during constant wakefulness, activity, ambient temperature, dim light and with evenly distributed isocaloric meals (Duffy *et al*. 2002) proved the circadian control of HR (Kraeuchi *et al*. 1994; Van Dongen *et al*. 2001) and cardiac autonomic regulation (Hu *et al*. 2004). These human data are in line with studies

performed in rats with the lesioned central circadian oscillator (Scheer *et al*. 2001) and our previous findings (Molcan *et al*. 2013) that the circadian component is present but suppressed in rats exposed for 11 weeks to the same lighting regimen as used in the present study. Moreover, measurement of clock gene (*Bmal1* and *Clock*) expression in the heart of rats exposed to both phase delay (Szantoova *et al*. 2011) and phase advance (Herichova 2013) shifts of lighting regimen showed that circadian oscillations were present but desynchronized in relation to actual LD cycles.

Therefore it seems that the intact circadian system is a prerequisite for the synchronised activity of the cardiovascular system and the target deletion of clock genes or irregular environmental LD conditions can suppress the circadian variability of cardiovascular traits. Indeed, a circadian phase-dependency was observed in tail-cuff-induced effects on HR and BP that were more pronounced during the rest than the activity period in Wistar rats (Grundt *et al*. 2009). Conversely, in spontaneously hypertensive rats, no circadian phase-dependency of the tail-cuff stress response was observed in SBP and DBP and only the response of HR was more pronounced during the rest phase of the cycle (Grundt *et al*. 2009). In our study we found comparable results after PAS exposure since the BP response was diminished after NE administration and HR responded more sensitively to the BP increase than in the control group exposed to LD. Our study suggests, that under irregular LD conditions, the sympathetic activation of the cardiovascular system is increased also during the actual rest period, similarly as in animals with the lesioned central circadian oscillator (Scheer *et al.* 2001). In humans, the increased α-adrenergic vasoconstriction activity was observed in hypertension (Amann *et al*. 1981), coronary stenosis (Brown *et al*. 1984) and angina pectoris (Collins and Sheridan 1985) and a higher sensitivity to stress hormones was observed in hypertensive patients (Panza *et al*. 1990; al'Absi and Arnett 2000). Therefore, we assume that the disrupted

daily variability in adrenergic stimulation may be involved in the worsened response of the cardiovascular system to environmental stressors after long-term exposure to irregular lighting cycles.

Conclusion

Our results demonstrated that the long-term PAS of the lighting regimen did not induce hypertension in rats and probably the combination of disrupted circadian rhythms with other risk factors is necessary for changes in a set point for BP control. Norepinephrine administration increased BP more in rats exposed to PAS than in rats exposed to control LD and the loss of the daily variability in the pressure response was observed. Higher maximal values of BP in the middle of the actual darktime (ZT18) and long-time lasting BP response during the lighttime (ZT06) were observed in rats exposed to PAS than in controls. Therefore, our results suggest that the lost daily variability in BP response to sympathetic stimulation and the augmented BP response to sympathetic stimulation during the light phase of the day may worsen the response of the cardiovascular system to a load in individuals exposed to irregular LD conditions.

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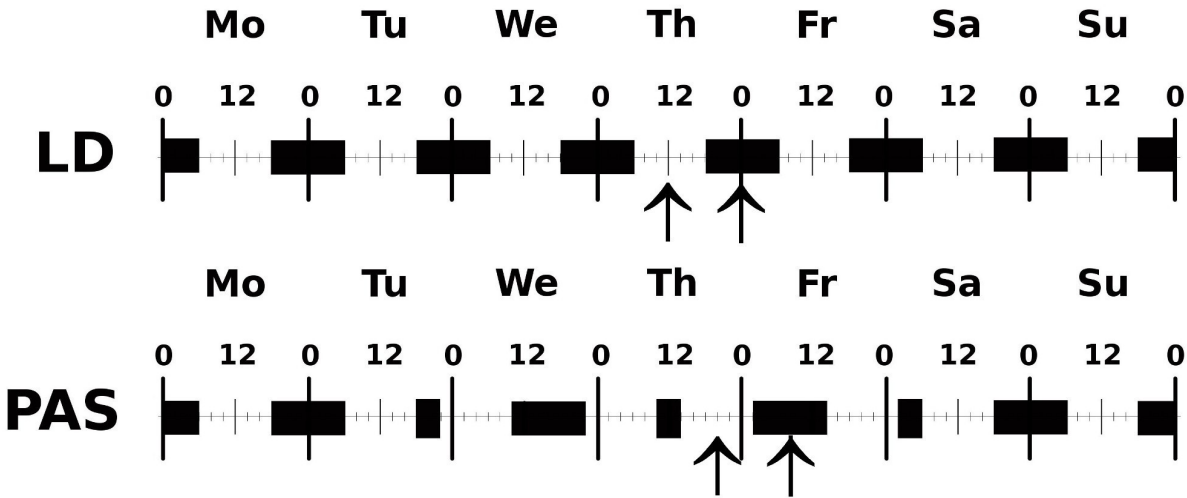
Fig. 1. Experimental design consisted of one week in control 12L:12D conditions followed by consecutive 12 weeks of phase advance shifts (PAS). Norepinephrine was administered during the last week of the PAS exposure in the middle of the subjective light and dark phase (arrows).
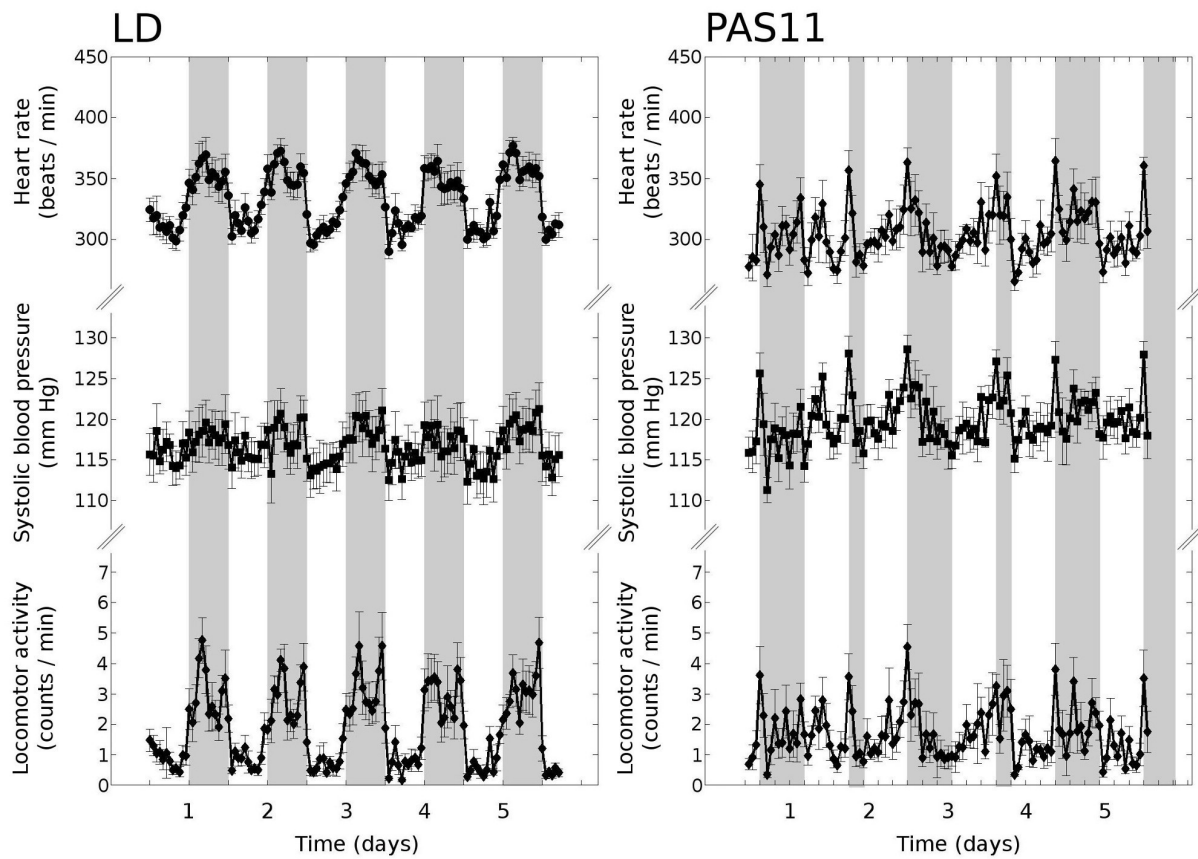
Fig. 2. Rhythmic pattern of heart rate, systolic blood pressure and locomotor activity in rats during the control light/dark (LD 12:12) regimen (n = 8) and during week 11 of exposure to 8-h phase advance shifts of lighting regimen (PAS, n = 6). Values are presented as means ± SEM of 1-hour intervals. Grey and white bars represent the darktime and lighttime, respectively.
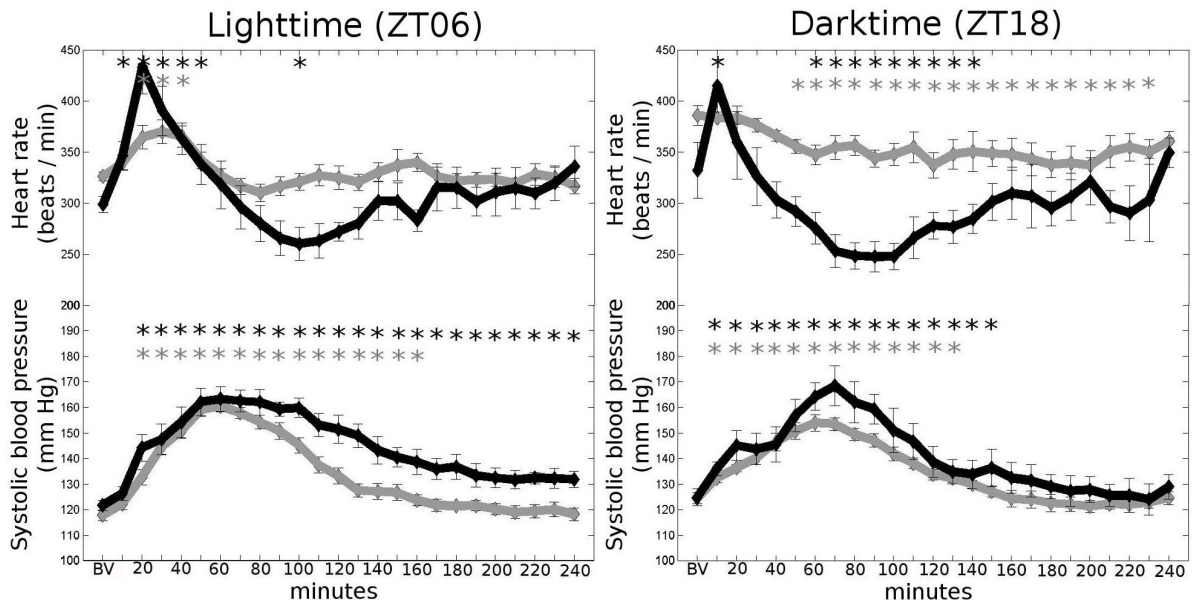
Fig. 3. Response of heart rate and systolic blood pressure to norepinephrine administration in control rats (grey line, n = 9) and in rats exposed to 12 weeks of 8-h phase advance shifts of lighting regimen (PAS, black line, n = 5) in the middle of the light (ZT06) and dark (ZT18) phase. Time is given as Zeitgeber time (ZT) with ZT00 at the dark-to-light transition. Asterisks represent significant (p < 0.05) differences compared to basal values (BV).
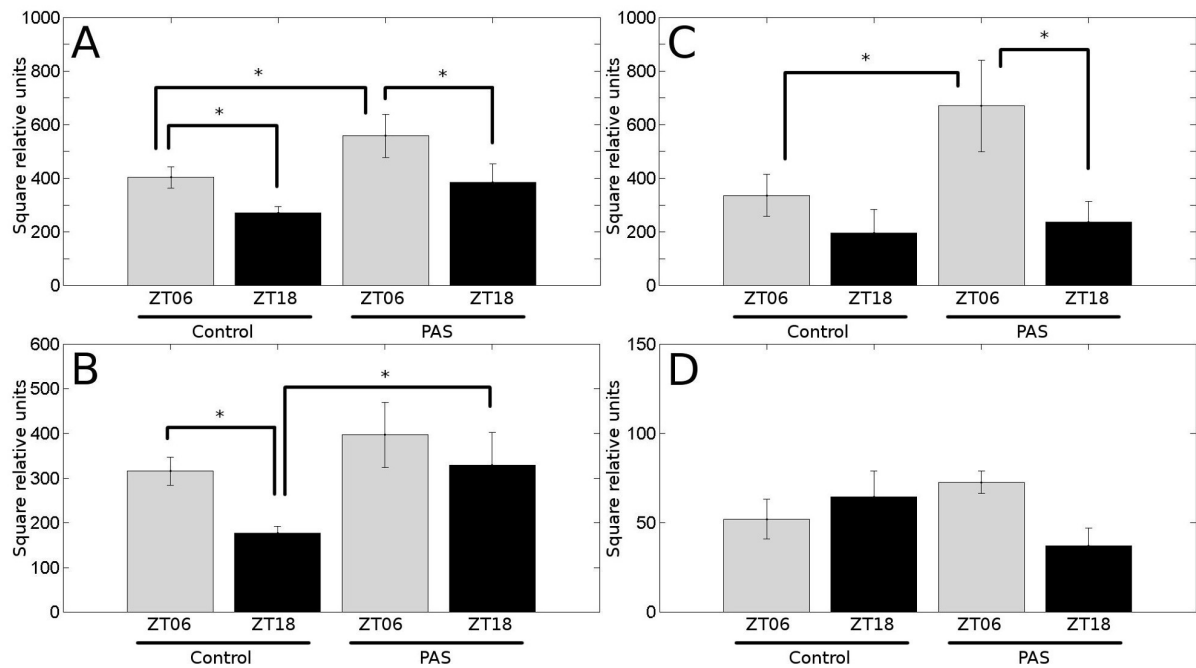
Fig. 4. Comparison of the area under the curve (AUC) in systolic (A) and diastolic (B) blood pressure, heart rate (C) and locomotor activity (D) after norepinephrine administration in control rats (n = 9) and rats exposed to 12 weeks of 8-h phase advance shifts of lighting regimen (PAS; n = 5) in the middle of the light (ZT06) and dark (ZT18) period. Time is given as Zeitgeber time (ZT) with ZT00 at the dark-to-light transition. Asterisks represent significant (p < 0.05) differences between columns.

Table 1. Effect of 11 weeks exposure to 8-h phase advance shifts of lighting regimen (PAS11) on rats expressed by mesor, amplitude, acrophase and % rhythm values in comparison to the control light/dark (LD) regimen. Time is given as Zeitgeber time (ZT) with ZT00 at the dark-to-light transition. Statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) is expressed in comparison to LD week. HR – heart rate; SBP – systolic blood pressure; DBP – diastolic blood pressure; LA – locomotor activity; bpm – beats per minute; cpm – counts per minute.

		Mesor		Amplitude		Acrophase	% rhythm	
HR	LD	334 ± 7		32 ± 3		17:22 ± 0:32	30.1 ± 3	
(bpm)	PAS11	305 ± 10	***	16 ± 5	*	17:28 ± 1:16	12.7 ± 3	**
SBP	LD	117 ± 2		3 ± 1		17:35 ± 1:31	12.5 ± 3	
(mm Hg)	PAS11	120 ± 2		2 ± 1		21:15 ± 1:59	8.3 ± 2	
DBP	LD	82 ± 2		2 ± 0.5		17:22 ± 1:12	13.6 ± 3	
(mm Hg)	PAS11	82 ± 1		2 ± 0.5		0:15 ± 2:30	7.3 ± 2	
LA	LD	1.9 ± 0.3		1.5 ± 0.2		18:21 ± 0:29	6.2 ± 1	
(cpm)	PAS11	1.7 ± 0.3		0.7 ± 0.2	**	20:04 ± 2:05	2.6 ± 1	**