Daily rhythms in muscle mitochondria

Effects of time-restricted feeding and exercise

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Chapter 10

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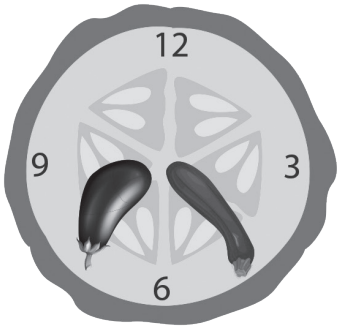
A technical note



Time-restricted running alters clock gene expression in *Soleus*, but not *Gastrocnemius* muscle

A technical note

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Introduction

The circadian timing system and activity are closely intertwined. Not only has wheel running activity long been used as the prime read-out of circadian behavior, disturbing the circadian clock can strongly affect activity patterns and vice versa. Lesioning of the master pacemaker in the suprachiasmatic nucleus (SCN) as well as a global knockout of the core clock gene Bmal1 results in arrhythmic (running wheel) activity, even in regular L/D conditions. Conversely, running wheel activity is one of the best-known non-photic Zeitgebers able to phase-shift the central clock. Moreover, activity has been long suspected to be a potent Zeitgeber for peripheral clocks, especially skeletal muscle. Several lines of evidence indicate that exercise can synchronize the muscle clock. Denervation of the muscle for example leads to decreases in the expression of several clock genes as well as to phase-advances in the daily rhythms of Bmal1 and Dbp expression (Dyar et al., 2015; Nakao et al., 2015). However, the exact contributions of muscle activity are not fully understood, as in the above mentioned denervation experiments the complete loss of muscle activity did not results in a complete loss of rhythmicity in the muscle clocks, which clearly indicates that other factors can also entrain the muscle clock.

Many studies have been performed to investigate the phase-shifting effects of running wheel activity on the central clock, but also several studies have been performed on the effects of the timing of exercise on peripheral clocks. Two hours of either voluntary or forced activity for 4 weeks during the light period shifted the peripheral clocks in skeletal muscle and lung, but not SCN (Wolff & Esser, 2012). However, animals either were forced to run on a treadmill or were moved on a daily basis between the home cage and the running wheel cage for voluntary activity, both methods likely inducing stress in the animals. Stress can activate the hypothalamus-pituitary-adrenal (HPA) axis and thereby lead to alterations in glucocorticoid signaling. In the previous chapter, we showed that disturbing glucocorticoid signaling as a result of adrenalectomy can affect both clock and metabolic gene expression in *Soleus*. Forced exercise therefore seems an unfavorable option to study the effects of (timing of) exercise as a Zeitgeber. One study thus far has directly compared voluntary with forced running wheel activity (Sasaki et al., 2016). They found that when mice were subject to forced exercise in a running wheel during the light period the phase-shifts in peripheral clocks (kidney, liver and submandibular gland) were larger compared to those of animals on a voluntary exercise schedule (Sasaki et al., 2016). Interestingly, for the *Gastrocnemius* clock no difference between forced and voluntary exercise was found. As mentioned before, the stronger effects on the peripheral clocks during the forced exercise protocols can likely be attributed to stress and increased HPA-axis activity. Indeed, compared to voluntary exercise, the forced exercise protocol was accompanied by increased levels of noradrenaline in the peripheral tissues and strongly increased systemic levels of corticosterone, both indicative of a physical stress responses in the animals. Removing the adrenal gland combined with adrenergic receptor blockers negated the effects of forced exercise on the peripheral clocks (kidney, liver and submandibular gland) (Sasaki et al., 2016). It thus seems necessary to study the effects of exercise as a Zeitgeber under stress-free conditions such as voluntary exercise, preferably without handling the animals. One such study on voluntary exercise found that four weeks of *ad libitum* access to a running wheel phase-advanced the rhythms of Per1, Per2, Reverbα and Dbp in liver and white adipose tissues, but not in brown adipose tissue and skeletal muscle of mice (Yasumoto, Nakao, & Oishi, 2015). Although timing of the acrophases were not found to be different in muscle, peak expression levels or Per1, Per2 and Reverbα were increased in the group that had access to a running wheel.

Experiments that employ a model of time-restricted running (TRR), i.e., studies that limit the duration of access to a running wheel without forcing the animals to run, are rather scarce. One study allowed animals to run either *ad libitum*, for 4h in the beginning of the dark period or for 4h at the end of the dark period (Schroeder et al., 2012). Compared to sedentary animals *ad libitum* access to a running wheel delayed the peaks of PER2 in the liver and kidneys, but not in heart and the SCN. TRR during the early dark period and not during the late dark period also phase delayed PER2 expression in the kidney, whilst both TRR groups displayed a phase delay in liver. This indicates that the timing of voluntary exercise differentially affects peripheral clocks. However, these results also underline the importance of more research on the effects of voluntary exercise (both unrestricted as well as time-restricted) on the molecular clock since the study of Yasumoto *et al*. found phase-advances in liver Per2 mRNA, whilst Schroeder *et al.* found phase-delays of liver PER2 protein levels in their *ad libitum* running groups.

Finally, also several experiments performed in this thesis indicate that activity can contribute to entrainment of the muscle clock. In various of our time-restricted feeding (TRF) studies (chapters 3.1, 3.2, 4, 6 and 8) we found changes in the muscle clock concurrently with altered activity levels, especially reduced light/dark differences in locomotor activity in animals fed during the light period together with reduced clock gene expression rhythms in muscle. It thus seems likely that altered patterns in activity can at least partly, entrain the muscle clocks.

Taking everything together, we hypothesized that manipulation of the timing of activity can alter the peripheral clocks in muscle tissues. More specifically, we hypothesized that the timing of voluntary running wheel activity during the light period will reduce the amplitude of the muscle clock.

Methods

Forty-four male Wistar rats were randomly assigned to one of 4 groups: sedentary (no access to running wheel, n=8), *ad libitum* running (ALR, unrestricted access to a running wheel, n=12), dark period running (DR, restricted access to the running wheel between ZT13-23, n=12), and light period running (LR, restricted access to the running wheel between ZT1-11, n=12). Animals were individually housed in the same room under similar environmental conditions as described in other chapters in this thesis. Pelleted chow and drink tap water were available *ad libitum* throughout the entire experiment. Body weight and food intake were measured weekly after which animals were scanned in an EchoMRI device to additionally measure fat and lean mass. Food intake during the period that the LR group could run was measured once a week, but on different days. During the first two weeks, all animals that had access to a running wheel could run *ad libitum* in order to get accustomed to running in the wheel. After this two-week baseline phase, the running wheels of the DR and LR groups were blocked except for 10 hours in the middle of the dark and light period, respectively. This TRR phase lasted 4 weeks after which all animals were sacrificed at ZT0 or ZT12 and plasma and *Soleus* and *Gastrocnemius* muscle were collected. In a subgroup of 16 animals (n=2-6 for each experimental group) also perirenal (bilateral), epididymal (bilateral) and subcutaneous (unilateral) fat depots were carefully excised and weighed.

Glucose measurements, RNA isolation, cDNA synthesis and qPCR were performed as described earlier in this thesis, with the exception that as for now only 1 housekeeping gene was used to standardize gene expression levels.

Results

During the baseline phase animals in the different experimental groups ran similar distances per day (Figure 1a) and also the daily pattern of running activity was highly similar with nearly all running activity taking place during the dark period (Figure 1b). Although during the baseline phase animals almost did not run at all during the light period, starting from the third day of the TRR phase LR animals ran during the light phase a distance comparable to roughly 40% of the total daily distance they ran during the baseline phase. Intriguingly, both the ALR and DR gradually started running less 2 weeks after the start of the TRR phase, but such a decline in activity was less prominent in the LR group. In the final week of the TRR phase the LR group ran approximately 50% of the distance of either the ALR or DR group. Unlike the DR group, the LR animals mainly ran during the first 4 hours of their 10h running period (Figure 1c). The DR animals ran more constantly during the full 10h of running wheel access. This likely caused the LR animals to run less per day compared to both DR and ALR animals. Lastly, ALR animals showed some running activity during the light period and DR animals were slightly more active during the dark period as compared to ALR animals.

Compared to all three TRR groups the sedentary animals showed significant increases in body weight and relative fat mass together with reduced lean mass starting immediately during the first week of baseline running (Figures 1d-f). However, the difference in body weight between the LR and sedentary animals diminished and even became non-significant in the third week of TRR (Figure 1d). At the end of the experiment LR animals had a reduced lean mass as compared to both the ALR and DR group. After 2 weeks of TRR relative fat mass of the LR group was higher as compared to the ALR group and at the end of the experiment LR animals also had significant more fat mass compared to the DR animals. Body weight and relative fat and lean mass never significantly differed between ALR and DR animals.

Although during the first two experimental weeks of *ad libitum* running daily food intake was higher in the sedentary group compared to the three TRR groups, this difference diminished and became non-significant from the first week of the TRR phase onwards (Figure 1g). Total daily food intake did not differ between the three TRR groups, but LR animals ate significantly more during the daytime as compared to the other three experimental groups (Figure 1h). Furthermore, DR animals ate significantly more compared to LR animals during the dark period and trends were observed for increased food intake of ALR compared to LR and decreased food intake for ALR as compared to DR animals in the dark period.

Overall daily plasma glucose levels only differed significantly between ALR and DR animals, this can likely be attributed to the lowered glucose levels for the DR group at ZT12 as none of the groups significantly differed from each other at ZT0 (Figure 1i; two-way ANOVA with Tukey's post-hoc tests).

Furthermore, in the sedentary animals we observed the largest epididymal, subcutaneous and perirenal fat depots (Figures 1j-l). LR animals were found to have significantly larger perirenal fat depots compared to both ALR and DR animals, as well as larger epididymal fat depots compared to the ALR group.

Finally, clock gene expression was also affected (Figures 2a-d) in both a tissue- and clock-gene dependent manner. In the *Soleus* muscle, Bmal1 expression at ZT0 was lower for the LR group as compared to the ALR and DR groups. For Per2 the diurnal difference in expression levels between ZT0 and ZT12 as present in ALR and DR animals was lost in LR animals. Strangely, also in the sedentary animals no difference between ZT0 and ZT12 was found for Per2 expression in the *Soleus*. In *Gastrocnemius* no group differences in gene expression of Bmal1 or Per2 were found, but all groups had different expression levels between ZT0 and ZT12 for both these genes.

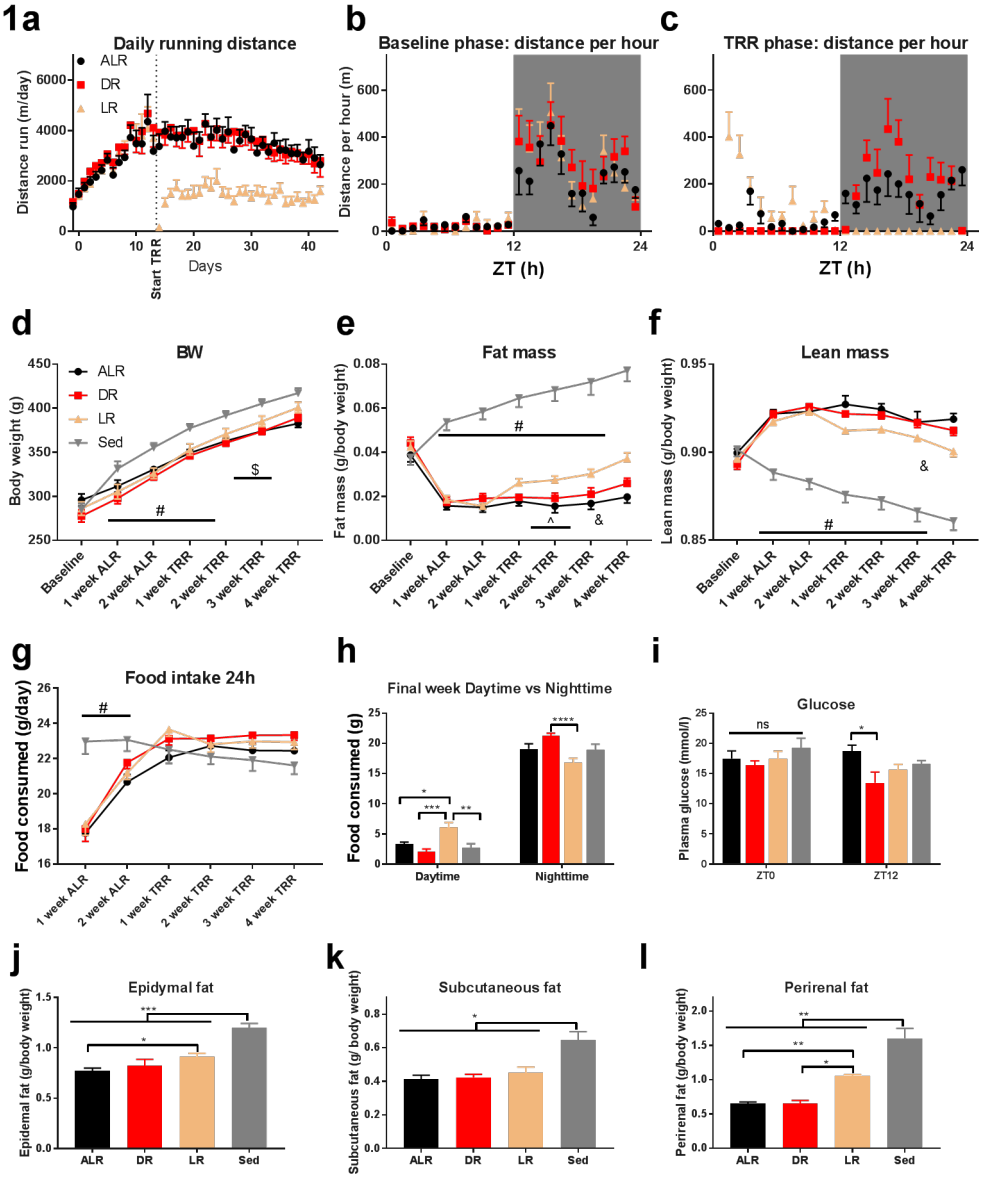

Figure 1 Physiological measurements. 1a-c Running wheel activity of the ALR, DR and LR group summarized per day (1a) and per hour during the last day of the Baseline phase (1b) and the last day of the TRR phase (1c). 1d-f Body weight (1d), relative fat mass (1e) and relative lean mass (1f) of the animals throughout the experiment. 1g-h Daily food intake throughout the experiment (1g) and in the last week of the TRR phase separated for daytime and nighttime (i.e., ZT1-11 (the period that the LR could run) versus ZT11-1 (the remainder of the 24h cycle)) (1h). 1i-l Plasma glucose values (1i) and fat depot weights (j-l) after sacrifice of the animals. # = sedentary group significantly differs from all other groups, $ = sedentary group significantly differs from ALR and DR groups, & = LR group significantly differs from ALR and DR groups, ^ = LR group significantly differs from ALR group. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Tukey's multiple comparisons test was used as post-hoc test for all comparisons. For figures 1a-I n = 8 for the sedentary group and n = 12 for the groups with (time-restricted) access to a running wheel. For figures 1j-l n = 4 for the sedentary and LR groups, n = 2 for the DR group and n = 6 for the ALR group.

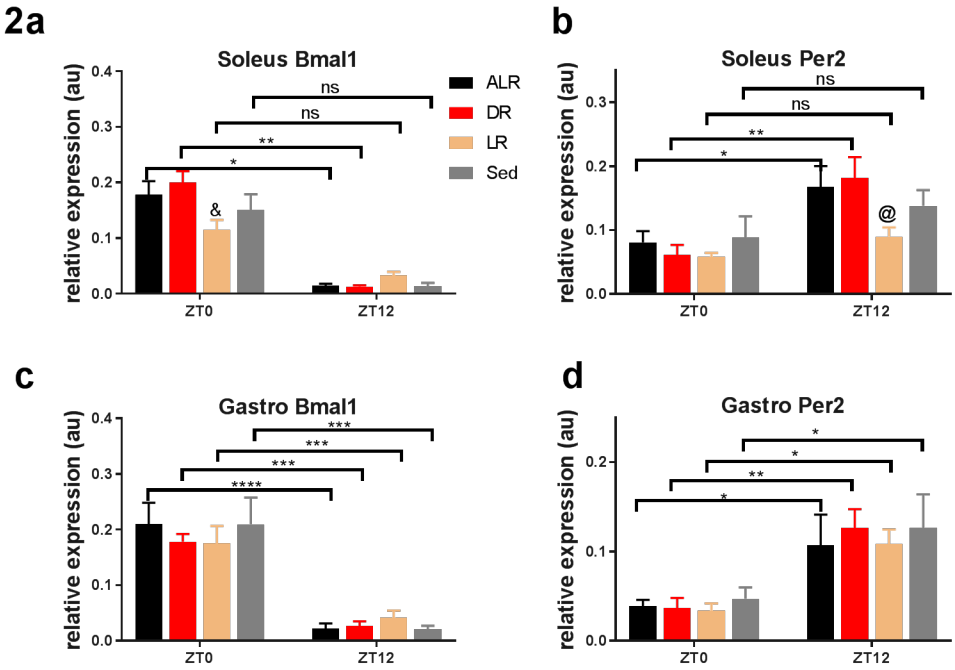

Figure 2 Clock gene expression of Bmal1 and Per2 at ZT0 and ZT12 in Soleus (a-b) and Gastrocnemius (c-d) muscle in the 4 experimental groups. & = LR group significantly differs from ALR and DR groups, @ = LR group significantly differs from DR group. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Tukey's multiple comparisons test was used as post-hoc test for between group comparisons. Holms-Sidak's multiple comparisons test was used for within group comparisons (i.e. ZT0 versus ZT12). For each of the ZT's n = 4 for the sedentary group and n = 6 for the groups with (time-restricted) access to a running wheel.

Discussion and conclusion

Exercise, regardless of the time of day clearly is beneficial for health as all three groups with access to a running wheel showed reduced body weight gain, reduced fat mass as well as increased lean mass compared to the sedentary animals. In line with the EchoMRI results, sedentary animals also had the largest fat depots of all groups. However, TRR during the light period was found to have less of these beneficial effects as compared to the DR and ALR group, as indicated by a higher fat mass, lower lean mass and larger fat depots. It is not clear whether these less pronounced beneficial effects are due to the abnormal timing of the running wheel activity in the LR animals or to their lower amount of activity in the running wheel, as they only displayed about half the total daily running activity compared to ALR and DR. On the other hand, ALR and DR showed similar amounts of running wheel activity, nevertheless the fat mass and lean mass of the DR animals were slightly unhealthier than those of ALR animals were. Interestingly, LR animals also ate more during the light phase, and less during the dark phase. Other than reduced plasma glucose levels, running only during the dark period did not seem to affect most measures as compared to ALR. This can likely be attributed to the fact that both total daily activity and daily activity patterns were highly similar between DR and ALR animals (Figures 1a&c).

Timing of voluntary exercise also had significant effects on clock gene expression in the *Soleus*, but not the *Gastrocnemius* muscle. Similar to our previous findings on feeding

behavior as a Zeitgeber, the effects of running as a Zeitgeber were both tissue and clock-gene dependent with Per2, but not Bmal1 losing the day/night difference in expression levels (i.e., ZT0 versus ZT12 expression). However, as at present we only have 2 time points it is impossible to determine whether the rhythm in Per2 expression in *Soleus* is shifted or dampened. The difference between the two muscle types can be explained by the differences in structure and function of the muscles. The *Soleus* is a slow-twitching muscle that mainly consists of type I (oxidative) fibers and is generally associated with endurance, whilst the *Gastrocnemius* mainly consist of type II (glycolytic) fibers and is associated with high power output such as during resistance training. As the rats in the three TRR groups spread their daily running wheel activity over a period of several hours it is not likely that they mainly used glycolytic muscle activity. Instead, the prolonged running periods more closely resemble endurance training (oxidative muscle usage) which would explain why we do find changes in clock gene expression in the *Soleus*¸ but not the *Gastrocnemius* muscle.

Unexpectedly, also the feeding behavior in the LR group differed from the other two groups with a running wheel, with increased food consumption in the light period, i.e., the period during which they also performed their voluntary wheel running activity. Therefore, at present we are unable to fully disentangle the entrainment properties of feeding behavior from those of running wheel activity. Future experiments that combine TRF with TRR should provide more insight into the separate contributions of food and activity, for example by allowing the animals to eat during daytime but exercise during nighttime or vice versa. Furthermore, in order to eliminate possible effects of differences in total running distance the daily window of wheel access could be shortened to 3-4 hours as the major part of LR activity took place during the first few hours. This way also the DR animals have less running time, which will probably reduce their running activity to the levels of the LR group. In addition, reducing the TRR window will also result in less variation in the timing of exercise within the groups, as all running wheel activity has to be performed in a shorter time span.

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