

Plasticity of circadian rhythmicity during simulated food scarcity and the role of the SCN.

L. de Wit, S2167743

Biology Master

Chronobiology, University of Groningen

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Supervisors: J.Riede & R.A.Hut

Abstract

Many aspects of behavior and physiology are subject to circadian rhythmicity, showing predictable patterns on a daily basis that continue even in constant environments. The biological clock has evolved in a way that certain environmental cues can have an impact on the behavioral output of the clock. It was shown that the hypothalamic suprachiasmatic nucleus (SCN) is the main circadian oscillator as in its absence circadian rhythmicity cease to persist while changes in the circadian period of the SCN also change the circadian period of the intact animal. The phase and period of SCN are mainly synchronized to the environment by light input. In addition, other environmental cues called Zeitgebers can also influence the rhythmic output of the circadian system without the involvement of the SCN.¹ Examples of these other Zeitgebers are temperature cycles, methamphetamine and food availability. The mechanisms and brain areas involved in these processes are largely unknown. In this paper we aim to find out how the SCN is involved in altering the circadian rhythmicity under simulated food scarcity. Previous research has shown that under the Work-For-Food protocol (WFF) nocturnal mice become day active if they have to work hard to obtain food. This phenotypic plasticity is the focus of our interest and is the underlying motivation for the lesion study. From our data can it can be concluded that SCNx animals do not shift their activity towards the day under a high workload, the SHAM animals do shift their activity towards the day. This means that the SCN is indeed necessary for the phenotypic flexibility shown during simulated food scarcity. The data also shows evidence for a slave oscillator that is expressed in the absence of the SCN under a high workload in complete light.

Keywords: circadian rhythmicity, food scarcity, metabolism, suprachiasmatic nucleus, phenotypic flexibility.

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Introduction

Circadian system

The circadian system is generally considered to serve as an internal biological clock network which oscillates with a period of around 24 hours. The oscillation of the daily rhythm is determined by the oscillation of different clock genes in the brain, these genes are cyclically expressed in the body ². The mammalian circadian clock is daily aligned by the light dark cycle of the sun by the rotation of the earth. The rhythm also persists when no information about time of day is given by the light dark cycle and other environmental parameters are kept constant³. They are driven by self-sustaining circadian oscillators that have a period of around 24 hours. This system is shaped over hundreds of thousands of years under evolutionary pressure for energy conservation. Evolution has fine-tuned the clock to not just respond to environmental cues but also anticipate them to maintain homeostatic balance and thereby promote survival ⁴. The output of this system is coordinated by the main circadian oscillator, the suprachiasmatic nucleus (SCN), which controls sleep-wake rhythms and timing of all sorts of other behaviors. It becomes more and more clear that the SCN is not solely responsible for this. Each living cell can sustain its own rhythmicity, therefore, it is not unlikely that there are other clocks in the body that drive certain pathways and thereby behaviors. The internal environment can send information to the SCN, in this way information about metabolic and physiological process can have a feedback on the main clock. The way this information is organized is by peripheral clocks that can synchronize activity of multiple cells in a tissue to send information about the body towards the SCN. Next to having different clocks, there are also different Zeitgebers, (German, meaning "time givers"), which can entrain these clocks. The light dark cycle is the most important Zeitgeber but also; food availability, temperature, reward, anxiety and metabolic information can influence the timing of the circadian system. The influence of metabolic

balance on the circadian system will be the main topic of my report. The circadian system consist of the SCN but also a possible slave oscillator. This slave oscillator is downstream of the SCN and can control behavior like the SCN⁵.

Temporal niche switching

Previous research has shown that animals can be flexible in their circadian timing when food is restricted or methamphetamine is administrated. However, there are also other instances when animals show flexibility in the timing of their behavior and switch from for instance nocturnal activity toward diurnal activity. The daily temporal niche of a species is usually defined as patterns of activity during daytime and night time ⁵. Flexibility within species has been found in multiple studies⁵. The golden hamster for instance is flexible in its behavior and whether it is diurnal or nocturnal depends on environmental factors. When these hamsters are in the lab they are nocturnal but in the wild they are crepuscular/diurnal ⁶.

Work for food

The work-for-food protocol (WFF) is based on the idea to simulate natural food shortage in a controlled setting. Mice in this paradigm obtain their food by working for it, completing revolutions in their running wheel. By determining the workload (amount of revolutions per pellet of food obtained) we can simulate environments with high or low food availability. In this protocol the animals can be food deprived by increasing the workload ⁷. Using the WFF-paradigm allows us to look at the timing of an animal's behavior when it is metabolically challenged without giving it a timing cue. The animals are allowed to run in their running wheel 24 hours a day and thereby they can obtain food 24 hours a day. The natural rhythmicity is not altered in a forced way and we can investigate the more natural behavior of the animals. By gradually increasing the workload (amount of

revolutions per food pellet obtained) we can simulate natural food scarcity ⁷. Previous research has shown, that mice that are usually nocturnal can shift their activity towards the day when exposed to a high workload. A possible explanation is that the mice in their natural habitat (subject to daily rhythms in environmental temperature) would save energy by shifting their behavior towards what is usually the warmest part of the day. By being active during the light-phase they reduce energetic costs of maintaining body temperature (Van der Vinne et al, 2014).

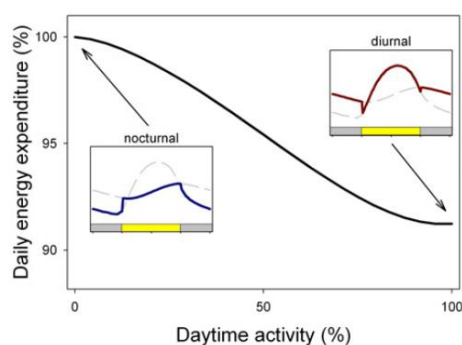


Figure 2. Daily energy expenditure decreases when mice increase daytime activity when subjected to natural daily temperature cycles⁸. *The mechanisms underlying this phenomenon are largely unknown. However previous research has shown that the SCN does not shift its activity at the level of gene expression of clock genes ⁸. It seems that the behavior and physiology are the parameters that shift their activity towards a diurnal pattern when faced with food scarcity.*

Food entrainable oscillator

The SCN is very important for determining the phase of activity, because after lesioning the SCN animals become arrhythmic in constant conditions. However rhythmicity can be restored in SCN lesioned animals by the induction of another oscillator - known as the

food entrainable oscillator (FEO) by timed feeding. The animals in these protocols are usually food deprived, receiving around 70% of their ad libitum food intake to increase their motivation. The animals are given an restricted time during the day when food is available. The animals will start becoming active around 2 to 3 hours before scheduled meal time; this is called food anticipatory activity (FAA)⁹. As mentioned before the circadian system can entrain to food availability. Rats and mice are nocturnal animals; this means that they show most of their spontaneous activity during the dark phase of the day. Restricting food access of nocturnal rodents to a limited amount of time during the light-phase, the animals will shift their activity towards the day. It is known that this behavior is driven by an oscillator outside of the SCN. In different lesion studies it has been proven that mice can still show FAA without a functional SCN ¹⁰, animals being able to anticipate daily meals by activity and body-temperature. When fasted this cycle persist for the next days. The big question remains which brain area(s) is/are involved in predicting and anticipating daily patterns in food availability. The system responsible is called the food-entrainable oscillator (FEO) and is believed to be an autonomous pacemaker outside of the SCN that can entrain on food availability ¹¹. There have been numerous studies in elucidating the FEO's anatomical location and mechanism, however the exact location in the brain remains a mystery. This raises the question if we can speak of a FEO as to be an oscillator at a specific location, or perhaps multiple brain areas are involved to drive FAA. In the last couple of years the idea of a multiple oscillator system gained more and more interest ¹².

Methamphetamine

Next to the FEO there is also another oscillator that can control behavior in the absence of the

SCN, namely the methamphetamine-sensitive circadian oscillator (MASCO). When rats and mice are administered a low dose of methamphetamine in their drinking water the MASCO wheel-running activity rhythm is observed. The methamphetamine increases period length and thereby the animals do not stay entrained to the light-dark cycle.¹³. During long-term treatment of methamphetamine the MASCO-controlled activity dissociates from the 24 hours rhythm of the SCN and under a LD cycle splitting of activity occurs¹⁴. Hereby the activity splits in two rhythmic components and is driven by two different oscillators namely the SCN and the MASCO^{14,15}. The rhythm of the MASCO persist when the SCN is lesioned, which proves that the MASCO is an autonomous oscillator outside of the SCN.

Ultradian rhythms

In addition to circadian rhythms, there is also a rhythm that is shorter than 24 hours namely, the ultradian rhythms. Ultradian rhythms are recurrent periods of around 2-6 hours and are usually superimposed on the 24 hours light-dark cycle of a day. Some examples of processes that can be arranged in a ultradian rhythm are: locomotion, sleep, hormonal release, heart rate, bowel activity and appetite. The generations of such ultradian rhythms are not dependent on the light-dark cycle nor on the SCN, as ultradian locomotor-activity in voles persists when the animals are kept in constant darkness and upon ablation of the SCN^{16,17}. These rhythms may not simply be driven by metabolic demand alone, since the ultradian rhythm in foraging activity observed in the common voles persists in the absence of food¹⁸.

Dopaminergic pathways; common ground in MASCO, FEO & ultradian rhythms?

It has been suggested that the MASCO and FEO originate from the same brain structure, since

there are so many similarities between the two oscillators. The MASCO and FEO both show dopaminergic characteristics. Methamphetamine is a strong stimulant of the dopaminergic and noradrenergic system in the brain¹⁹. Dopamine is also involved in restricted feeding protocols, as higher levels of dopamine are found in the striatum and midbrain in mice on restricted feeding²⁰. Furthermore food anticipation activity is increased when animals are treated with dopamine receptor antagonists²⁰. Together, this research suggests that the dopamine pathway is involved in both the oscillators.

Recently the link between the dopaminergic pathways and ultradian rhythms has also been suggested²¹. Disruption of the dopamine transporter gene lengthens the period of ultradian locomotor rhythms in mice. The authors concluded that there is strong evidence for a dopaminergic ultradian oscillator (DUO) driving behavioural rhythms alongside the SCN²¹. The SCN and DUO are synchronized and together can form the output of locomotor-activity²¹. Perhaps the same pathway is involved in the phenotypic flexibility observed in the WFF protocol, the first step towards the answer is to investigate whether the brain area involved is outside of the SCN like the MASCO and FEO.

Multiple oscillator system

The multiple oscillator system is the idea that different oscillators can work together to create a stable oscillation and coordinate the timing of behavioral output. The existence of an FEO and MASCO support the idea that behavior is not only driven by the SCN as they can both modulate behavior. Other oscillators outside of the SCN can take over the role of main oscillator in certain conditions. The SCN, FEO and MASCO can all entrain the phases of peripheral oscillators, this means that they are at the top of the hierarchy in terms of a network of pacemakers. The data of Yamazaki

et al shows that; the SCN, FEO and MASCO together can drive behavior¹. To put this in an evolutionary framework, the existence of multiple oscillators to drive behavior can help optimize timing of day to be active. Hereby the animal could obtain the most food and reward, while minimizing energy expenditure by being active at the optimal moments off the day ¹.

Research question

In this research we are investigating the possible link between the SCN and maintaining metabolic balance. We want to know if the SCN is involved in the shift towards diurnality when mice are subjected to simulated food scarcity. To do this the SCN is lesioned and the animals are placed on the WFF protocol.

Ad libitum 12:12 LD-cycle (21 days)	Surgery + Recovery (7 days)	Ad lib DD (14 days)	Baseline AD lib pellets LD (7 days)	Low WL WFF LD (14 days)	High WL WFF LD (21 days)	High WL WFF LL (14 days)	AD lib LL (14 days)	AD lib chow LD (21 days)	Cholera retina injections (7 days)
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Figure 3. Timetable of all different procedures. LD = Light dark, DD = complete darkness, WL = workload, WFF = work for food, LL = complete light.

Materials and Methods

Animals and housing

All procedures were approved by the Animal Experimentation Committee of the University of Groningen (DEC 6545F). Male CBA/CaJ mice (n=21) from our breeding colony were housed in individual running wheel cages for the whole experiment. The mice were around 2 months old at the start of the experiment. The mice were kept on a 12:12 LD cycle in climate control rooms with a temperature of around 20° degrees Celsius. Water was accessible ad libitum throughout the experiments. Food as indicated for the different phases of the experiment. With the exemption of the first 4 days of post-surgery recovery, wheel-access was unrestricted and running data was obtained and stored in 2 min bins by the CAMS system (Circadian Activity Monitoring System, Lyon, Cooper-lab).

Surgeries; brief introduction.

After 14 days of habituation mice received surgery. The individuals were divided into two groups: sham (n=8) versus SCNx (n=13). The surgeries were done by bilateral thermos-electric lesions of the SCN, the coordinates can be found in the Appendix #1.

Groups

We have two groups of animals, all mice were randomly assigned to each group. The SHAM (n=6) group consists of animals that underwent the whole surgery with the only exception that the electricity was not turned on to place the lesion. Two animals were anesthetics (SHAM*) control as there were difficulties during the operation. n=2), They underwent the process of shaving and being placed in the stereotact while being anesthetized but they did not receive a lesion and the skull was still intact.

The SCN lesioned mice (n=16) went through the surgery and this time the lesion was placed, for the full protocol see Appendix #1, surgery protocol.

Behavior recording

The recording of running wheel activity was continuous and stored in two minute bins with the CAMS system. The food pellets were given by a food dispenser system; Med Associates Inc., St. Albans VT, USA). In this system the amount of revolutions and workload were observed and delivered a food pellet at the time when the animal made enough revolutions for one pellet.

Surgery

The surgeries were performed when the mice were around 2,5-3 months old. Both the SHAM and the SCN lesioned group underwent surgery with the only difference that the electricity was turned on to place the thermostatic lesions. A brief description of the procedures: the animals were anesthetized using isoflurane. A 1.5-2cm incision in the scalp was made rostro-caudally along the midline of the head of the mouse, to expose the skull. Lidocaine was dripped as local anesthetic on the site of the incision. The animals were leveled into the stereotact and the coordinates of the lesion were adapted for the bregma-lambda distance that we measured. A hole was drilled into the skull and bilateral lesions were placed using a tungsten teflon coated electrode (total diameter of 1.1 mm) the current used was 1,1mA for 20 seconds. The scalp was closed by using medical glue. The animals received 1,0 cc of saline with 30% glucose and 0,1 cc of fentanyl to minimize pain and compensate dehydrating effects of the surgery.

Recovery

After the surgery the animals were placed back

in their homecage, which was heated by an electrical blanket to restore body temperature after the surgery. Extra nesting material was placed in the cage so the animals could maintain body temperature better during recovery. A couple of hours after the surgery the heating blanket was removed and the animals were put back on their original place in the climate chamber. The following days the animals were closely monitored for signs of suffering. Body mass was measured daily at random times of the day. The running wheel was put back after the animal seemed recovered; this was approximately around 3 to 4 days after the surgery.

Lesion analysis

To assess whether the animals were successfully lesioned the animals were kept in complete darkness (DD) for 14 days. A light pulse of 16 hours was given the first day of starting the DD period. Lesions in the SCN are known to lead to arrhythmicity, we analyzed this by looking at the recordings of the running wheel behavior. By making an periodogram analysis of the DD period we determined whether there was still a significant circadian component visible in the behavior recordings.

Work for food protocol

In the work for food (WFF) protocol, mice had to run in their wheel to earn food pellets of 45mg/612J per pellet (F0165; Bio-Serv, Frenchtown NJ, USA). During this protocol the amount of revolutions to earn a pellet was gradually increased (workload). The mice were able to work and receive food at all times of the day and they received one pellet at a time.

Baseline measurements

Prior to starting the WFF protocol we switched the diet of the mice from normal chow to the dispenser pellets (unlimited access). Of this 1 week baseline period the average running

wheel activity was calculated over the last five days. This spontaneous activity level under ad libitum feeding was used to determine the starting workload.

Workload

Dividing the average amount of activity by 150 gave the starting workload for each animal. At the starting workload a mouse would thus earn 150 pellets when it retained its spontaneous activity-level, which is more than the ad-lib intake (which is ~120 pellets, previous experiments). Gradually the workload increases with 5-10 revolutions a day, depending on the amount of pellets earned and body weight changes. Body weight was monitored closely during the full duration of the experiment, to ensure a gradual decrease of body mass. Mice were kept above 75% of their initial body mass and 18 gr.

Complete light WFF

After the animals were on a high workload with an LD-cycle of 12:12, the animals were placed in complete light (LL) (see figure 3). The workload remained high and was adapted for body weight loss or gain. After 14 days of high workload in LL the animals were switched to ad libitum feeding of the dispenser pellets. The workload was set to the starting workload and daily checks ensured that there was always food available in the cage. The ad libitum measurement in LL were done for another 14 days and then the animals switched towards the 12:12 LD cycle again with normal chow.

Sacrifice/ Histology

The animals were sacrificed in their homecage by an overdose of CO₂ inhalation. The brain was then taken out and fixated by 4% paraformaldehyde. The brains were cryo-sliced in 50µm slices. The brain slices were mounted

on polylysine glasses with a buffer of 0,1 M PBS and closed wet with a cover slip. The glass was made airtight by the use of transparent nail polish.

Statistics

The data was corrected with a running two hours smooth to clarify possible ultradian rhythmicity and to lower the noise-ratio. Periodogram analysis was done by Actoview,

with a significance level of $p=0,001$. The relative dQp value is calculated by the periodogram analysis of the two hour smooth data for 10 days in each condition. Periodogram analysis measures the strength of timing of behavior and plots this for each period (τ)²². The relative dQp is a measurement for the strength of the peak found in the periodogram analysis. The lesion and sham group were analyzed using Statistix and a student T-test.

Results

To analyze the success of the lesions the animals were kept in complete darkness for 14 days. The periodogram analysis of the last 10 days in complete darkness showed that 12 out

of the 13 SCN lesioned animals were arrhythmic. The relative dQp measurements are plotted in table 1. for the DD period

SCNx	Rel dQp	Tau (h)	Succesfull lesion?
SCNx11	0,7	00:52	Yes
SCNx2	0,63	01:00	Yes
SCNx 16	0,9	00:18	Yes
SCNx 5	0,72	02:56	Yes
SCNx 9	1,13*	23:00	Partial
SCNx 1	0,82	23:48	Yes
SCNx 19	1,10*	22:16	Partial
SCNx 20	0,94	01:26	Yes
SCNx 6	0,65	22:58	Yes
SCNx 15	2,34* ¹	22:58	No
SCNx 22	0,67	00:56	Yes
SCNx 10	0,86	02:56	Yes
SCNx 13	0,8	00:58	Yes
SHAM 21	4,57*	23:24	-
SHAM 4	4,42*	22:42	-
SHAM 18	4,12*	23:14	-
SHAM 14	3,95*	23:30	-
SHAM 3	4,74*	23:42	-
SHAM 8	4,48*	22:52	-
SHAM 7	4,08*	22:42	-
SHAM 17	3,52*	22:36	-

Table 1. Relative dQp values of the periodogram analysis of 10 days in complete darkness (DD).

*The data was smoothened with a running average of two hours. the peak of this rhythm by Tau, the strength by the relative dQp. The significance threshold for the dQp value is $dQp > 1,00 = *$.*

In Table 1 it is shown that most of the SCN lesioned animals do not have a significant circadian peak, as relative dQp < 1,00. Three animals that do show significant peaks are the animals; SCNx 9 (rel dQp = 1,13), SCNx 19 (rel dQp = 1,10) & SCNx 15 (rel dQp = 2,34). SCNx 9 & 19 show a significance of just above 1,00, for that reason they are still included in further analysis. Mouse SCNx 15 however shows a peak well above the significance level and is

rhythmic under complete darkness and excluded in our further analysis. Histological analysis criteria will be used to validate the exclusion of mouse SCNx 15. The average of the relative dQp is 0,83 with a standard deviation of 0,17. All of the 8 SHAM lesioned animals show a significant circadian peak with an average rel dQp of 4,24 and has a standard deviation 0,39.

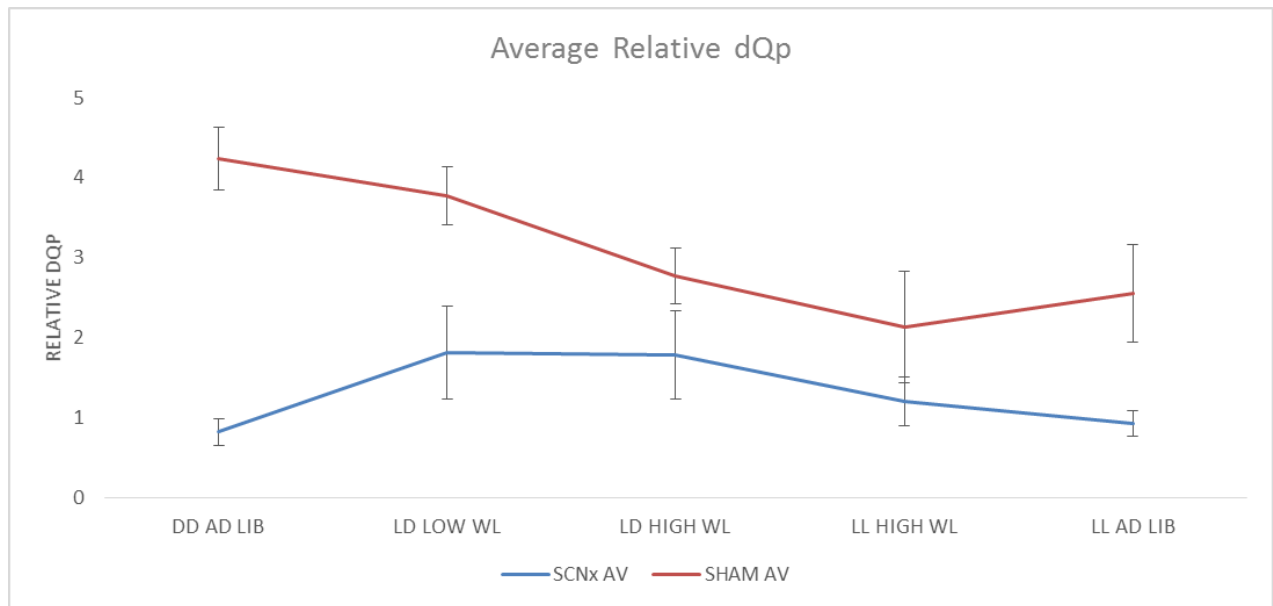


Figure 4. Average relative dQp measurements and standard deviation (SD) plotted for the lesioned group (SCNx) and the control group (SHAM). The different light and feeding conditions are plotted on the x-axis in chronological order of the experiment. DD refers to complete darkness, LD is the light schedule of 12h hours light and 12h dark.. LL is when the animals are kept in complete light.

Figure 4. confirms table 1, in a way that the SCNx animals are arrhythmic under complete darkness with ad libitum feeding (dQp= 0,83; SD= 0,17). Reintroducing the light-dark cycle restores some rhythmicity in the lesioned animals, this means that there is still light information processed in the brain that entrains behavior. Under a high workload the animals show a stronger rhythmicity as seen by

the difference between the LL ad libitum and the LL high workload group. The SHAM group loses strength of rhythmicity under a high workload in the WFF protocol. In complete light the SHAM animals lose strength of rhythmicity, as the SCN becomes weaker due to constant input of light. When switched to ad libitum feeding during complete light, the strength of rhythmicity is for a part restored

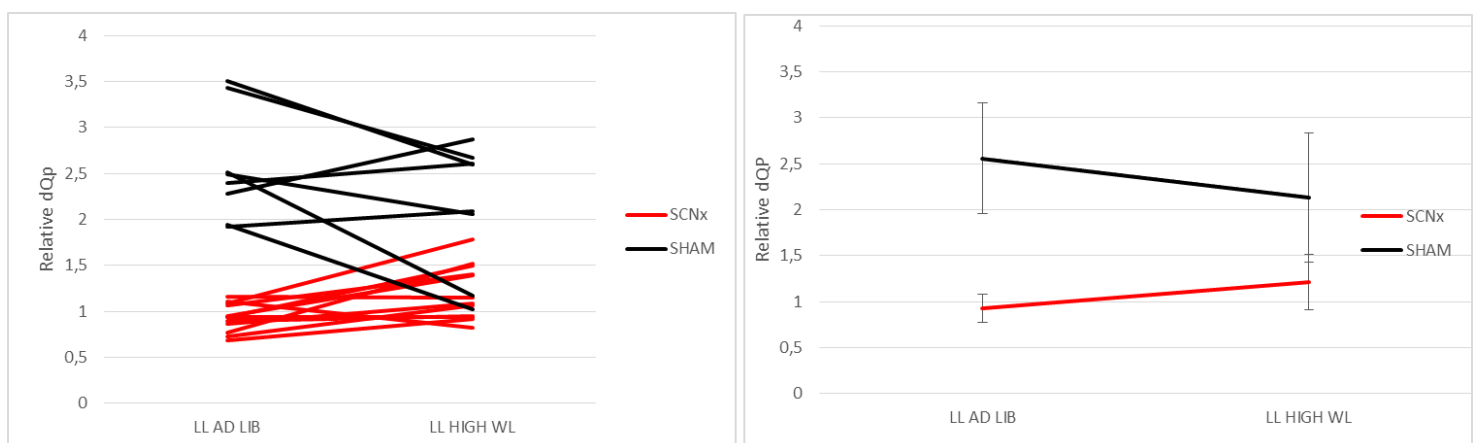


Figure 5. The relative dQp values are shown for the experiment in constant light, where the animals are on ad libitum feeding and high workload respectively. The left graph shows the individual changes in dQp, whereas the right graph shows the group averages of the lesioned animals and the Sham-control mice with the standard deviation.

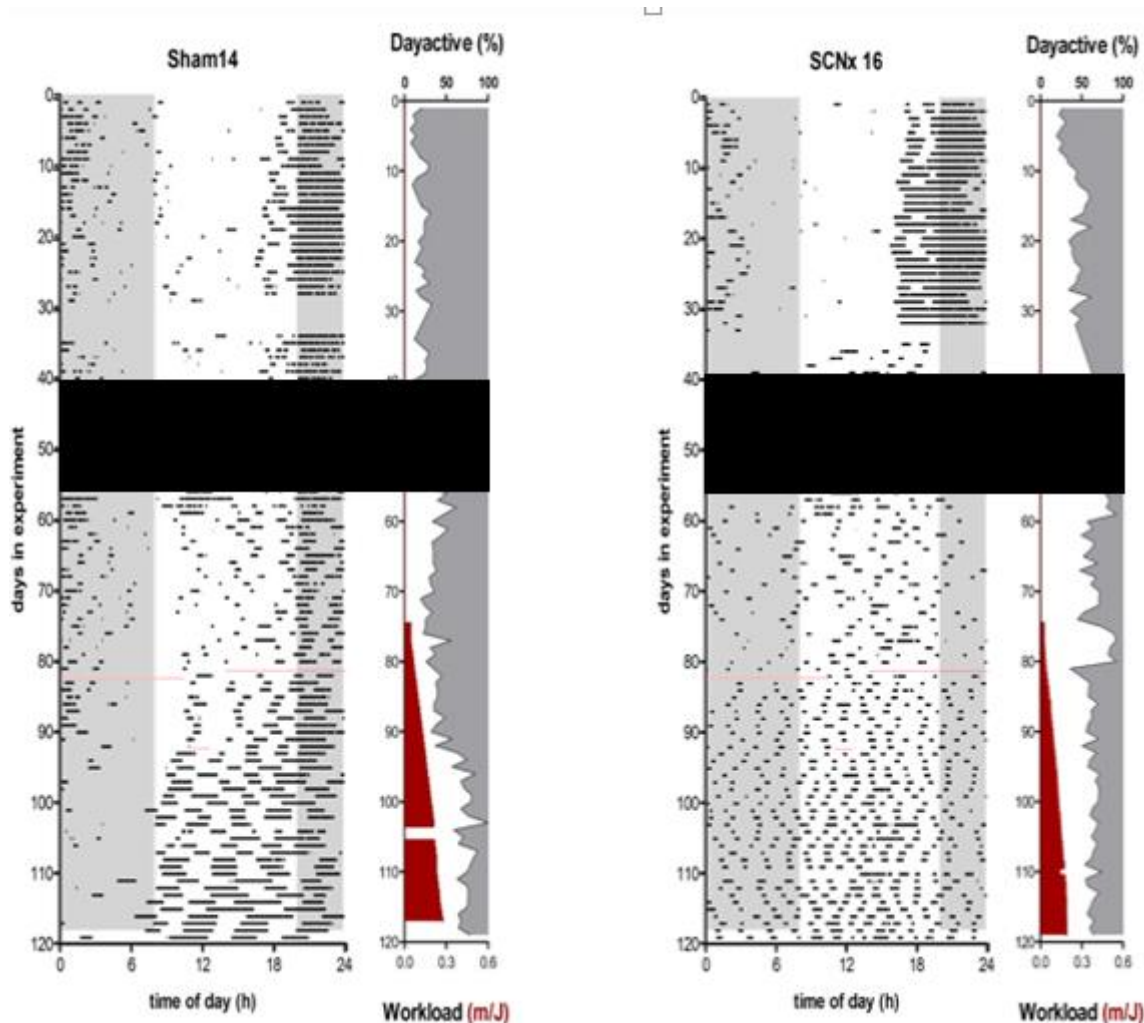


Figure 6. a) Actogram of a SHAM lesioned animal, plotted over 24 hours. Next to the actogram the diurnality is plotted in white and the workload in red. b) Actogram of a SCN lesioned animal. Next to the actogram the diurnality (percent of the activity in the light phase) is plotted in white and the workload in red. The black bar was added because this was the complete dark period and therefore no diurnality index can be calculated from that data.

Figure 6a. shows that the SHAM lesioned animals increase the proportion of activity during the day with increasing workload. This is in accordance with previous research where the animals also showed a diurnal pattern of activity during a high workload ⁷. The SCNx mouse in figure 6b. does not show an increase in diurnal activity with increased workload. The diurnal stays around 60%, independent of

the workload. In figure 7., the graph shows that for the SHAM's diurnality does increase with workload, but this is not the case for the SCNx lesioned animals. The Pearson correlation test shows that the SCNx lesioned animals have a negative correlation between increasing workload and diurnality of -0.77. The SHAM's show a strong positive correlation 0.96.

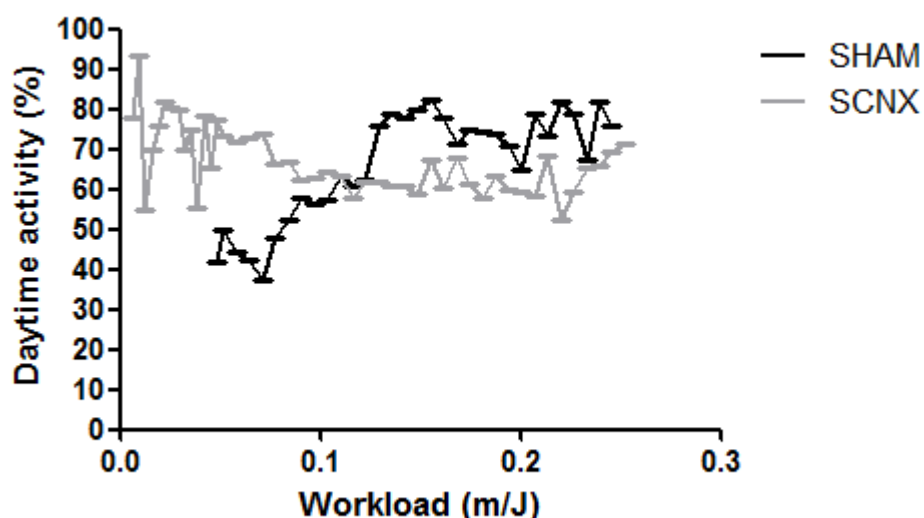


Figure 7. Diurnality (% of activity during the light phase) and workload (m/J), plotted over the consecutive days of the experiment. *Diurnality is expressed in the percentage of activity spent during the hours of light. The workload is the amount of meters the animals need to run in their running wheel to obtain one pellet of food (612J).*

The SHAM lesioned animals show a higher percentage of daytime activity when they have to run far to obtain food. The daytime activity increases until a certain maximum seems to be arrived at around 80% of the activity during the

day. The SCN lesioned animals start off with a high daytime activity in the absence of a high workload. With increasing workload the animals become a little bit less active during the day.

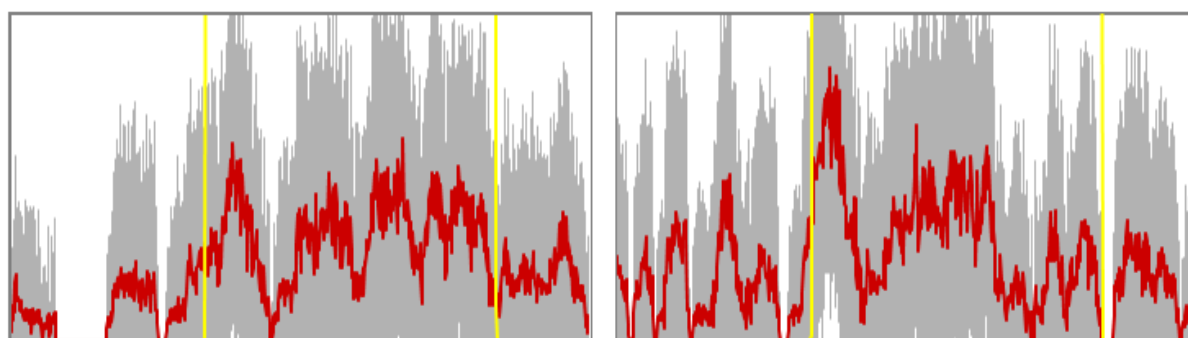


Figure 8. Activity profile of mice SHAM #21 (left graph) and SCN#2 (right graph), over a period of 10 days on a high workload in LD. *The yellow lines represent the on and-offset of light. The activity profile calculates relative amplitude of activity over the 10 days and is plotted with the standard error in grey. Both animals show light masking as light modulates behavior. When the lights go on (first yellow line) locomotor activity increases, positive light masking. When the lights go off (second yellow line) locomotor activity decreases, negative light masking.*

Discussion

Becoming day active under simulated food scarcity saves energy and can partially restore metabolic balance. This is exactly what the SHAM operated mice show, as expected. The suprachiasmatic nucleus is necessary for the phenotypic flexibility shown under simulated food scarcity. Mice with a lesioned SCN do not show the shift towards diurnality that is observed in the SHAM lesioned animals.

Diurnality

The actograms in figure 6a show that when the workload increases the activity spent during the day also increases for the SHAM mice. In the SCN-lesioned mice such correlation is not found and diurnality does not significantly increase with workload. This data is summarized in figure 7, where the average daytime activity is plotted for both groups. Additionally figure 7 shows the SCNx mice show a high percentage of daytime activity when the workload is low and this remains when the workload increases.

Lesion analysis

The relative dQp measurements in figure 4 represents the strength of the circadian rhythm for the five different conditions. In complete darkness the SCN lesioned animals no longer show a significant circadian peak except for one animal (CAMS 293, table 1). As far as we can conclude from the behavioral data the lesions were highly successful. 85% of the animals that underwent the SCN lesion show arrhythmicity during complete darkness. One animal was sacrificed after surgery due to unsuccessful recovery and another animal still showed a significant circadian peak in complete darkness which indicates that the SCN is probably missed or partially lesioned in this animal.

Light-masking

The reintroduction of the light-dark cycle, the SCNx animals regain circadian rhythmicity. This means that light information still enters the brain and has an impact on the behavioral output. Figure 8 shows that light still has an effect on activity. Both animals shown positive light masking when the light are turned on and show negative light masking when light are turned off. This behavior is compatible with a diurnal profile, becoming more active when lights are turned on and less active when lights are turned off²³. That light information still has an effect on locomotor activity is probably due to that besides the SCN there are also other brain areas that receive retinal light input like for instance the intergeniculate leaflet.

High workload and dQp

Gradually the workload of the animals increases, for the SCNx animals this has no impact on the strength of the circadian and the daytime activity. In the SHAM animals being under a high workload decreases the strength of the circadian peak. This is probably due to a more bimodal peak of activity distribution. Under a high workload the SHAM animals shift their normal nocturnal behavior to a diurnal pattern. Probably maintaining the metabolic balance is more important than the phase of entrainment under these circumstances.

Constant light

The animals are kept in constant light, this is done to diminish the role of the SCN for both groups. In constant light the SCN receives constant light input and is thereby weakened in strength²⁴. This weakening of the SCN reflects in the reduction in relative dQp measurements

of the SHAM animals. Under constant light the circadian rhythm is weakened, this can in part be restored by ad libitum feeding. The SCN animals show a significant peak of circadian rhythmicity when on a high workload during constant light. The rhythmicity found during the high workload is lost when the SCN animals are switched to ad libitum feeding while remaining in constant light. High workload seems to increase rhythmicity in SCN animals, when in complete light.

Slave oscillator

Increase of rhythmicity during a high workload in LL in the SCN, could indicate a slave oscillator, outside of the SCN, that can partially restore rhythmicity when challenged with food scarcity. The slave oscillator probably takes over the role of the SCN as the main circadian clock when it is necessary to maintain metabolic balance.

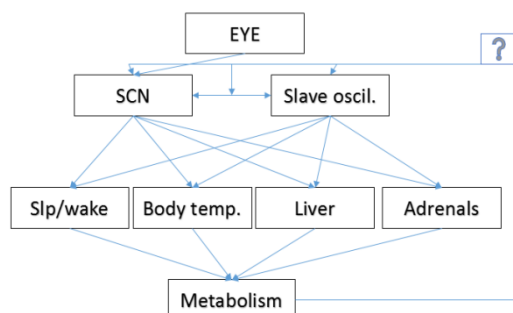


Figure 1. Schematic overview of how the SCN and Slave oscillator might be linked to metabolism.

The above scheme represents a possible overview of how the sensory input is linked to the oscillators and how they control behavior and metabolism. The focus in this research how metabolism can influence the two different oscillators discussed here. Metabolism or metabolic balance could have an effect on three different places in the pathway; on the SCN directly, on the slave oscillator or on the connection between the SCN and the slave oscillator.

The slave oscillator is most likely to be an ultradian oscillator, as the actograms show activity bouts that reoccur with a semi-stable rhythmicity smaller than 24 hours. In figure 6a from day 80 when the workload starts increasing the activity pattern of the SCN animal becomes more fragmented and spread out over the whole 24 hours. This could simply be an oscillator that is involved in the digestive system where the animal runs for a couple of hours and receives some food pellets, eats the pellets and rests for a while until it gets hungry again. It would be very interesting for further research to find out how this ultradian rhythm is organized and why it would be adaptive when faced with food scarcity.

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Appendices

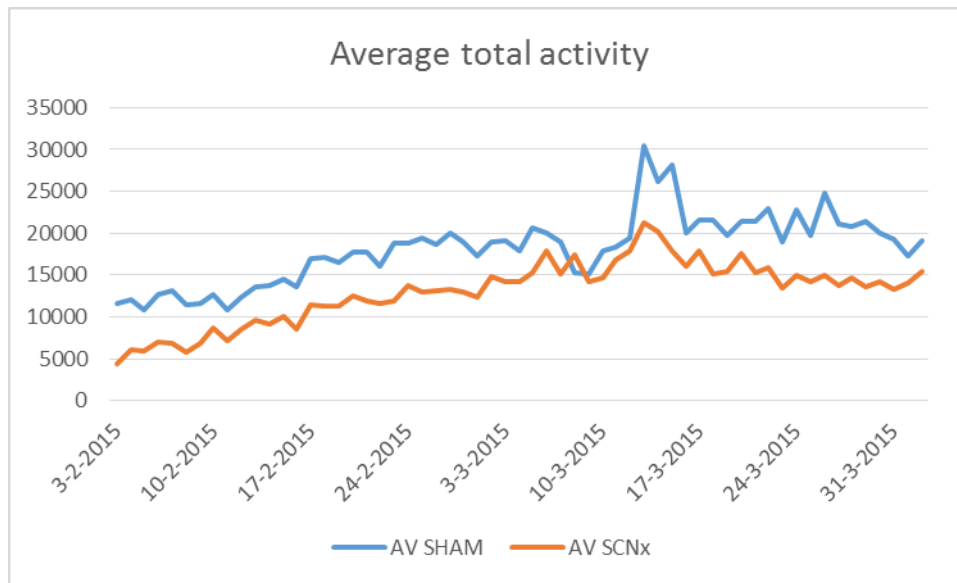


Figure 8. Shows the total amount of activity under the time of LD where the animals went from a low workload to a high workload. The increase of activity is seen in both the groups although the SCN group has a overall lower level of activity.

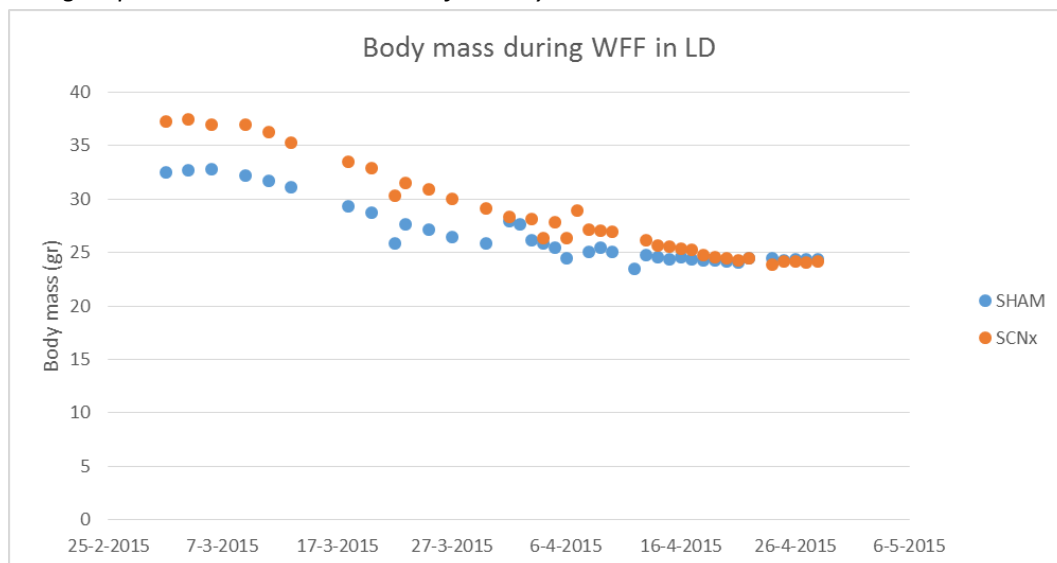


Figure 9. Body mass during WFF in LD, with increasing workload. Increasing workload is decreasing the bodymass in both groups. The SCNx are heavier under a low workload, but become around the same weight as the SHAM mice under a high workload. The SCNx mice are less active then the SHAM mice, this might explain the weight difference during the start of the protocol.

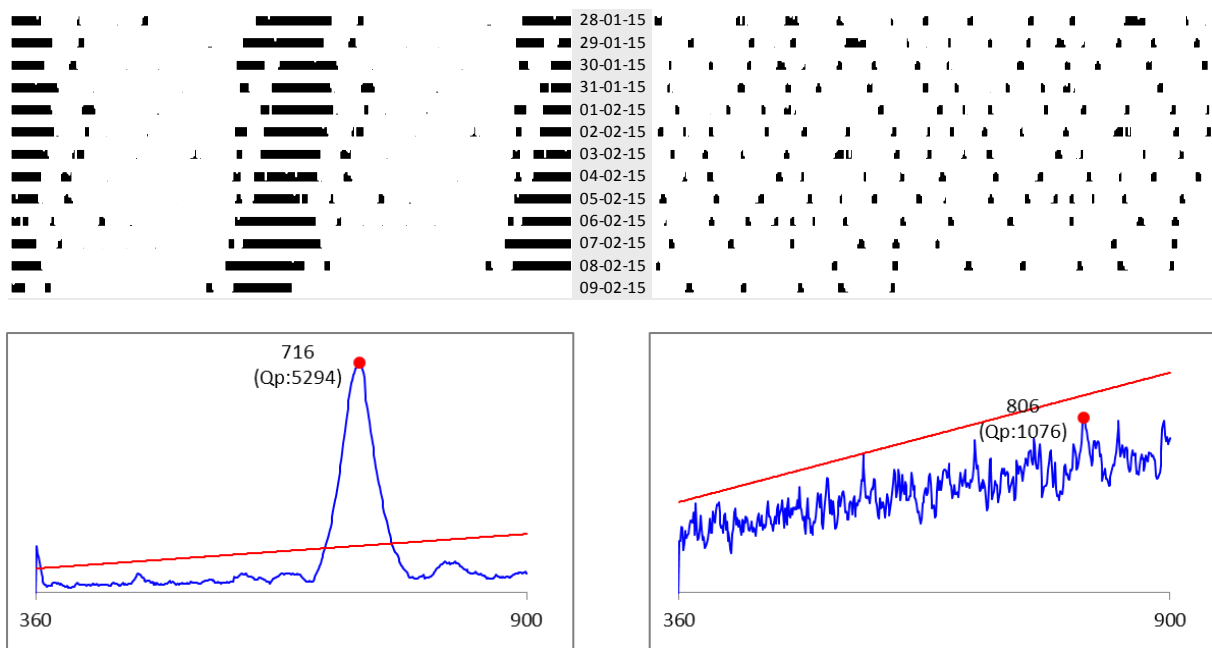


Figure 9. Complete darkness actograms and periodogram analysis. *In the upper right graph there is an example of an actogram of a SHAM lesioned animal in complete darkness, in the upper right graph there is an actogram of the same condition in a SCN lesioned animal. In the lower two graphs the periodogram analysis is shown for the two animals. The Qp value and time of peak in half hours are given in the plot. The actograms and periodograms are conducted with a 2 minute bin interval data collection.*

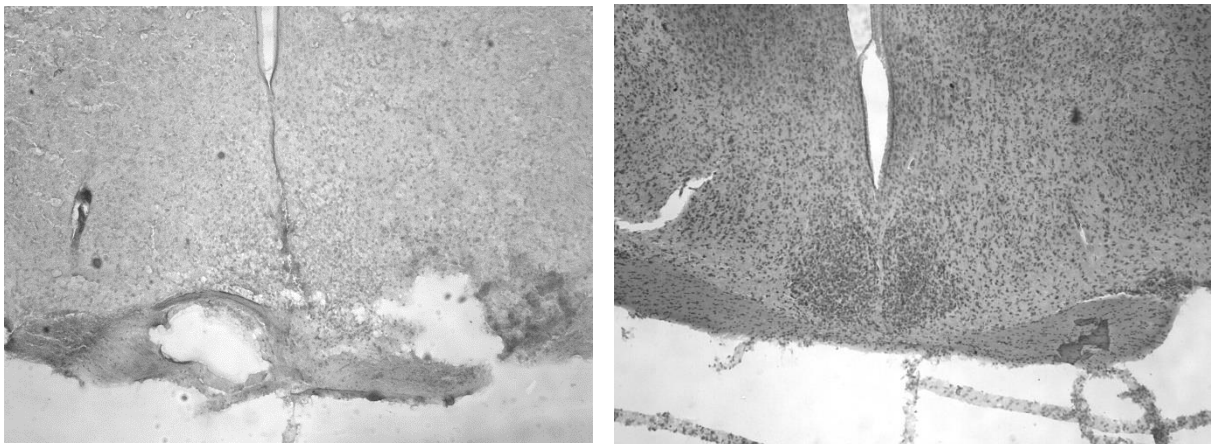


Figure 9a & b. Microscopic images (40x magnification) *a) Animal SCNx #12 (sacrificed due to unsuccessful recovery). b) Animal SCNx pilot #3, unsuccessful lesion of the SCN.*

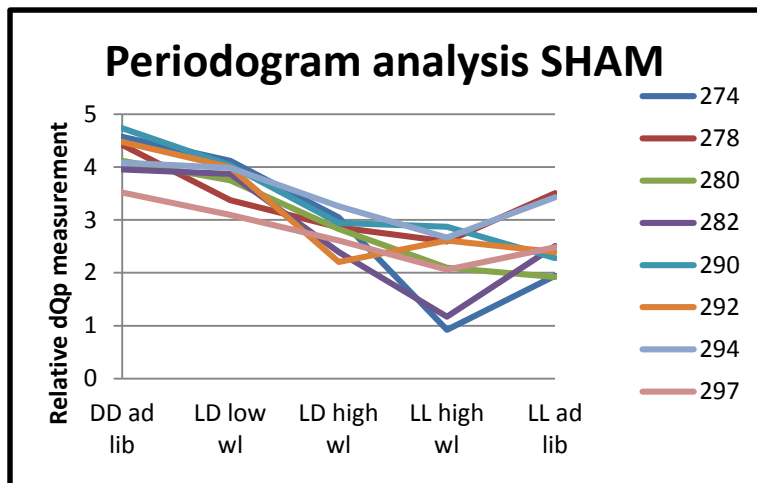


Figure 10A.

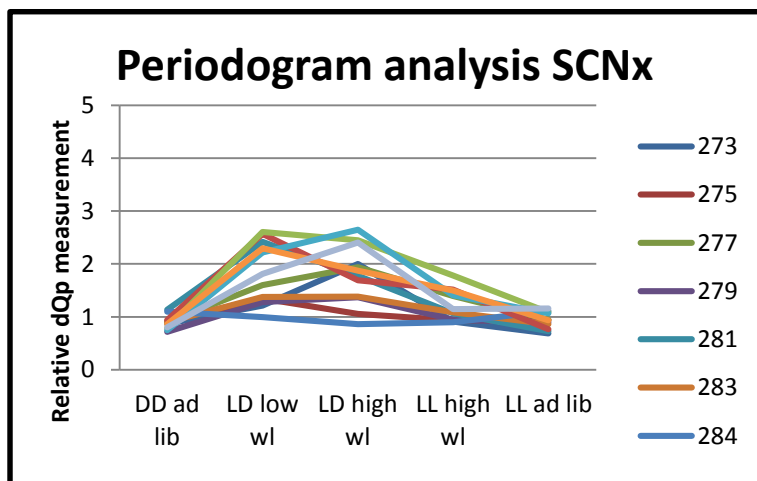


Figure 10A & 10B. The relative dQp measurements from the periodogram analysis are plotted for the lesioned group in figure 10A (SCNx) and the control group in figure 10B (SHAM). The individual values are plotted and the CAMS number of the animal is in the legend. The different light and feeding conditions are plotted on the x-axis in chronological order of the experiment. DD revere to complete darkness, LD is the regular light schedule of 12:12 hours light

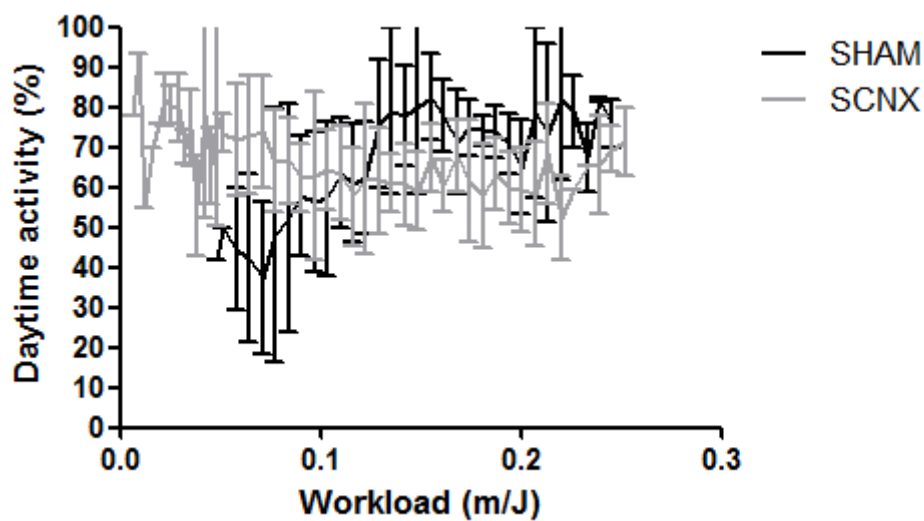


Figure 11. The diurnality and workload (m/J) are plotted over the consecutive days of the experiment. The diurnality is expressed in the percentage of activity spent during the hours of light (with SD/SE?). The workload is the distance per Joule.

Surgical procedures SCN lesions

Equipment

- Electrode; 1,1 mm, coated
- Metal needle as ground electrode
- Stereotact
- Surgical tools
- Magnifying glass/ binocular
- Intraperitoneal injection needle
- Isoflurane
- Cotton swabs
- Lidocaine
- Finedyne
- Drill; 0,8 mm diameter
- Shaving device
- Surgical glue
- Betadine
- Glucose/NaCl solution PBS(0,9% NaCl + 4% glucose)
- 70% EtOH
- Heating pad
- Vita-Pos® salve (Ursapharm) eye cream
- Scale

Anesthetics

Animals were anaesthetized by inhalation of 3% Isoflurane and 5 units of oxygen. After the animal was fully sedated, as checked by toe pinch or tail reflex, isoflurane was turned back to 1.5% to maintain anaesthetized state. Regular breathing and hart rate were monitored during the full procedure.

Preparing the animal:

Bring an animal that is at least 12 weeks old (but no older than 52 weeks) to the surgery room, in its cage. Weigh the animal, note down the DOB number, Fill in the surgery report sheet and write animal number and date of surgery on the information card of the animal.

Anesthetize the animal as described above

Shave the top of the animal head from slightly behind its ears until between its eyes, making sure not to trim any whiskers. Apply the eye salve (Vita-POS) and smear it out with a cotton swap, in a way that the whole eye is covered.

Fixation of the animal

Hold the animal by its snout, and place its right ear around the right hand ear bar, inside the animal ear is a small hole that leads to a small hole in the skull. This is where the ear bar has a strong anchor point. Make sure to enter the ear bar far enough into the small hole, a 'click' sound should be heard. Continue holding the animal by its snout, making sure the animal's head is not held at an angle. Slide the left hand ear bar into the animal's left ear and into the small hole inside the ear. Make sure the animal's head can no longer move to the left or right, only up and down, rotating around the ear bar axis. Tighten the left ear bar so it can no longer move. Use a pair of tweezers to lift the animal's incisors into the tooth bar. Tighten the tooth bar screw so the tooth bar cannot move. Tighten the tooth bar cover while pressing it down onto the animal's snout. Make sure the animal's head cannot move in any direction.

Surgery

Disinfect the shaved area by the use of betadine and 70% ethanol. Carefully cut a small mid-line incision over the top of the scalp, starting caudal to rostral using surgical scissors.. Numb the periosteum by applying lidocaine (2-3 drops) Let it resolve for around 0.5 minute and then expose the skull by removing the periosteum with a scalpel.. The Bregma- Lambda (BL) distance was measured to determine the SCN coordinates corrected for the size of the individual animal (see Table 2 for the conversion table used). The height of the skull was measured at various points to ensure that the animal was completely level in the stereotact. Holes were drilled in the skull at the mediolateral and anterior-posterior coordinates corresponding to the SCN loci on both sides of the sagittal suture. Procedures were halted until the bleeding associated with piercing the skull stopped, before moving on to the other side or sequential steps. Place the grounding electrode intraperitoneal and make sure the connections are wet with PBS, for better conductance. After the holes were drilled a coated Tungsten electrode with a diameter of 1.1 (exposed tip of 0.5 mm) was gently inserted into the brain until the right depth was reached (see Table 2, DV coordinates). Start the lesion (1.1mA; 20 sec; 115 Volt). Let the electrode cool down for 1 minute while leaving it in the same position before pulling it out slowly (2nd scale, table, 0.1 mm/sec). Repeat the procedure for both sides of the SCN.

After each lesion the electrode was carefully inspected to ensure it had remained straight. Any deviations were carefully noted in the surgical notes. After both sides had been lesioned the skin was closed with surgical glue.

Postoperative care

After the surgery was completed, an subcutaneous injection containing 0,1 ml Finedyne as analgesic and 1.0 ml saline/2,5%glucose to compensate for blood-loss and help recovery.

Animals were allowed to recover on an electric heat-pad for 1-3 hours. All animals were placed back in their individual homecage after surgery with standard chow pellets on the cage floor. On the first post-operative day behavior will be assessed for suffering, if this is the case we will inject them with another 0.1 ml finedyne injection. Posture, behavior and fur condition, as well as bodyweight were monitored closely for the ten day recovery period. The animals were individually housed in cages equipped with a running wheel, the running wheel was put back in the cage after 4 days of recovery. Running wheel behavior was recorded using CAMS software.

Bregma-Lambda (B-L)	Dorsoventral location (DV)	Anteroposterior location (AP)
3,8	5,20	0,30
3,9	5,22	0,30
4	5,25	0,31
4,1	5,27	0,31
4,2	5,29	0,31
4,3	5,32	0,32
4,4	5,34	0,32
4,5	5,37	0,33
4,6	5,39	0,33
4,7	5,42	0,33
4,8	5,44	0,34
4,9	5,46	0,34
5	5,49	0,34

Table 2. SCN coordinates conversion table to adjust for skull sizes measured by the bregma-lambda distance. *B-L: bregma-lambda distance in mm. AP: anteroposterior location, distance in mm posterior from bregma. DV: dorsoventral location, depth of the inserted electrode in mm, measured from the top of the skull. Mediolateral distance, bilateral distance in mm measured from the sagittal fissure was kept constant for all animals at 0,250 mm.*