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“The interplay of central and peripheral circadian clocks in white adipose function and metabolic homeostasis”

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Declaration

Herewith, I confirm that I have written the present PhD thesis independently and with no other sources and aids than quoted.

Lübeck, Mai 2017
Isa Kolbe

“Zeit. Es gibt Kalender und Uhren, um sie zu messen, aber das will wenig besagen, denn jeder weiß, dass einem eine einzige Stunde wie eine Ewigkeit vorkommen kann, mitunter kann sie aber auch wie in einem Augenblick vergehen – je nachdem, was man in dieser Stunde erlebt. Denn Zeit ist Leben”

Michael Ende

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Summary

The rotation of the Earth around its axis has driven the evolution of endogenous internal time keepers that run with a period of approximately 24-hours, called *circadian* (from the Latin *circa* – approximately and *dies* - day) clocks. In mammals, these circadian clocks are ubiquitously expressed and drive the rhythmic transcription of tissue-specific clock-controlled output genes to adapt to daily recurring changes in environmental factors. While principally self-sustained, circadian clocks are entrained by external time cues or *zeitgebers* (German for time-giver) to stay in sync with the 24-hour day. A master clock residing in the suprachiasmatic nucleus (SCN) of the hypothalamus is reset by the external light-dark cycle and coordinates other central and peripheral rhythms. Disturbed circadian rhythms, e.g. in clock gene mutant animals or due to shift work, are associated with metabolic disturbances and overweight. Whether this condition is a consequence of central SCN disruption or due to misalignment of clocks in peripheral metabolic tissues, is still unknown.

To address this question, I studied the interaction of the central pacemaker and white adipose tissue clocks in the regulation of energy homeostasis. In the first part, I analyzed epididymal white adipose tissue (eWAT) circadian transcriptome rhythms in animals with a genetically ablated SCN clock (SCN-KOs). White adipocyte clock function was preserved in these mice, but metabolic transcript rhythms were strongly dampened – in line with an abrogation of food intake rhythms – compared to control mice. At the same time genes associated with innate immunity gained rhythmicity in the absence of a functional SCN, suggesting a regulation by local adipose clocks. Next, I studied the impact of SCN and peripheral clock function on long-term energy homeostasis. Under rhythmic light-dark conditions, when peripheral clock synchrony in SCN-KO mice was preserved, body weight regulation and glucose metabolism were unaltered. In constant darkness, however, adipocyte clock rhythms deteriorated, leading to increased body weight and impaired glucose handling. Metabolic functions were restored by

rhythmic feeding in SCN-KO mice suggesting that peripheral clock function is essential for the maintenance of metabolic health.

Zusammenfassung

Die Erdrotation hat die Evolution von internen Tageszeit-Messern gefördert. In Säugetieren sind diese *zirkadianen* (lat. *circa* – ungefähr, *dies* – Tag) Uhren ubiquitär exprimiert. Sie kontrollieren gewebsspezifische transkriptionelle Programme und adaptieren so den Organismus an sich täglich wiederholende Umweltveränderungen. Prinzipiell sind die inneren Uhren selbsterhaltend, reagieren aber auf externe Zeitgeber und passen sich so an den 24h-Tag/Nacht-Zyklus an. Im *Nucleus suprachiasmaticus* (SCN) befindet sich der Schrittmacher des Körpers. Er wird durch den Licht/Dunkel-Rhythmus reguliert und koordiniert periphere Uhren untereinander und mit der Tageszeit. Gestörte zirkadiane Rhythmen, sei es durch Uhrengenmutationen oder Schichtarbeit, sind mit metabolischen Störungen und Übergewicht assoziiert. Ob dies primär die Folge einer Störung zentraler oder peripherer Uhren ist, ist noch ungeklärt.

Im Fokus dieser Arbeit stand die Interaktion von SCN- und Adipozytenuhren in der Regulation des Energiestoffwechsels. Zunächst habe ich das zirkadiane Transkriptom des epididymalen Fettgewebes in Mäusen mit einer genetisch ausgeschalteten SCN-Uhr (SCN-KO) untersucht. Die Uhren im weißen Fettgewebe dieser Tiere waren weiterhin funktional, aber die Rhythmen metabolischer Transkripte waren im Vergleich zu Kontrolltieren stark gedämpft – analog zum Verlust der rhythmischen Nahrungsaufnahme. Zeitgleich erlangten Gene, die im Zusammenhang mit angeborerener Immunität stehen, rhythmische Expression in Abwesenheit der Hauptuhrfunktion, was auf eine Regulation durch lokale Uhren hindeutet. Weiterhin habe ich SCN-KOs auf ihre Langzeit-Gewichtshomöostase hin untersucht. Unter normalen Licht/Dunkel-Zyklen, in denen die Rhythmik peripherer Uhren in SCN-KOs bestehen bleibt, waren Gewicht und Glukosemetabolismus unverändert. In konstanter Dunkelheit führt die Abwesenheit peripherer Uhrenrhythmen jedoch zu Übergewicht und Glukoseintoleranz. Diese metabolischen Veränderungen konnten durch tagesrhythmische Fütterung in SCN-KOs wiederhergestellt werden, was für eine

essentielle Rolle peripherer Uhren in der Aufrechterhaltung des metabolischen Gleichgewichts spricht.

Abbreviations

ACTH	adrenocorticotropic hormone
al	*ad libitum*
AMPK	AMP-activated protein kinase
AngII	angiotensin II
ANS	autonomic nervous system
aP2	Adipocyte protein 3 / FABP4
ARC	arcuate nucleus
AT	adipose tissue
ATP	Adenosine triphosphate
AVP	arginine vasopressin
BAT	brown adipose tissue
BBB	blood brain barrier
BMAL1	brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL)
BNST	bed nucleus of the stria terminalis
bp	base pairs
C3ar1	complement component 3a receptor 1
CalB	calbindin
CalBII	calreinin
CAMKII	calcium/calmodulin kinase II
cAMP	cyclic adenosine monophosphate
CCG	clock-controlled gene
Ccl19	chemokine (C-C-motif) ligand 19
cGMP	cyclic guanosine monophosphate
CLC	cardiotrophin-like cytokine
Clock	circadian locomotor output cycles kaput
cre	cre recombinase
CRE	cAMP response element
CREB	cAMP response element binding protein
CRH	corticotrophin releasing hormone
Cry	cryptochrome
Dbp	albumin d-box binding protein
DD	dark/dark
DMH	dorsomedial nucleus of the hypothalamus
DNA	deoxyribonucleic acid
DNL	de novo lipogenesis
DRN	dorsal raphe nucleus
E4BP4	basic leucine zipper transcription factor E4 promoter-binding protein 4
eef1α	eukaryotic elongation factor alpha
Elovl6	ELOVL fatty acid elongase 6
eWAT	epididymal white adipose tissue
fc	fold change
Fcer1g	high-affinity immunoglobulin epsilon receptor subunit gamma
FFAs	free fatty acids
FOV	force equal field of view
GC	glucocorticoid
Glut2	glucose transporter type 2 (SLC2A2)
Glut4	glucose transporter type 4 (SLC2A4)
GO	gene ontology
GRP	gastrin releasing protein

GTT	glucose tolerance test
HFD	high-fat diet
HPA	hypothalamic-pituitary-adrenal
HSL	hormone-sensitive lipase
IGL	intergeniculate leaflet
IL-6	interleukin 6
INN	met-enkephalin
ip	intraperitoneal
ipRGC	intrinsically photosensitive retinal ganglion cell
Itgam	integrin alpha-M
Itgb2	integrin beta-2
ITT	insulin tolerance test
IVC	individually ventilated cage
kDa	kilo Dalton
LD	light/dark
LEPRb/OBRb	long isoform of the leptin receptor
LPL	lipoprotein lipase
LS	lateral septum
MAPK	mitogen-activated protein kinase
MBH	mediobasal hypothalamus
min	minutes
MRN	median raphe nucleus
NAMPT	nicotinamide phosphoribosyltransferase
Nckap1l	NCK-associated protein 1-like
NE	norepinephrine
nm	nanometer
NPY	neuropeptide y
NT	neurotensin
PACAP	pituitary adenylate cyclase-activating polypeptide
PCR	polymerase chain reaction
Per	period
PGKII	cGMP-dependent protein kinase II
PK2	prokineticin 2
POA	preoptic area
PTT	pyruvate tolerance test
PVN	paraventricular nucleus of the hypothalamus
qPCR	quantitative polymerase chain reaction
RER	respiratory exchange ratio
Rev-erbα/β	reverse-erythroblastosis virus alpha/beta
RF	restricted feeding
RHT	retino-hypothalamic tract
ROI	region of interest
Ror	retinoic acid receptor-related orphan receptor
rpm	rounds per minute
Scd1	stearoyl-coA desaturase 1
SCN	suprachiasmatic nucleus
SIRT1	sirtuin 1
SNR	signal-to-noise ratio
SPVZ	subparaventricular zone of the hypothalamus
Sub	subcutaneous
Syt10	synaptotagmin 10
TE [ms]	time-to-echo
TGFα	transforming growth factor α

TGs	triglycerides
TI [ms]	inversion time
TNF-α	tumor necrosis factor alpha
TR [ms]	time to repeat
UCP1	uncoupling protein 1
VIP	vasoactive intestinal polypeptide
Vis	visceral
VTA	ventral tegmental area
WAT	white adipose tissue
τ	tau (period length)

1 Introduction

1.1 Biological Rhythms

Regular recurrences in time are a natural phenomenon and can be seen in a wide variety of frequencies. Biological rhythms vary from seconds and days up to several years and are often distinct among different species and dependent on the location and the natural habitat. The rotation of the Earth around its axis creates one of the most prominent rhythms, the 24-hour day and night cycle. In connection with the alternating light and dark phases, we experience oscillations in temperature, availability of natural food resources and predatory perils. Thus, circadian (from the Latin *circa* - approximately and *dies* - day) rhythms are prominent throughout the kingdom of life. Organisms of all phyla can adapt to these time-dependent variations in the external environment by reacting directly to occurring changes or anticipating them by internal timekeeping systems or clocks. The first internal clocks have most likely appeared very early in the evolution of life, as even simple organisms like cyanobacteria contain them (Johnson, Golden et al. 1996). It has been hypothesized that they initially served to avoid UV light-induced DNA damage during DNA replication by confining them to the night (Pittendrigh 1993). Corresponding to this theory, cryptochrome genes, which are an essential part of the vertebrate and non-vertebrate circadian timing systems, have evolved from light-induced DNA repair enzymes (Gehring and Rosbash 2003, Lin and Todo 2005).

The majority of species on Earth have a circadian clock. Whether these clock systems originate from a common progenitor is not known, but is very likely due to the high degree of conservation of genes constituting the molecular clockwork. Still, clocks between bacteria and eukaryotes, but also between fungi and plants, and even in-between the animal kingdom display high variations and a convergent evolution is likely (Bell-Pedersen, Cassone et al. 2005, Rosbash 2009).

In so-called *resonance* studies, organisms with different endogenous rhythms were exposed to alternating external periods. They showed convincingly that

resonating internal and external rhythms result in an evolutionary advantage over endogenous rhythms that differed from the external period length. For example, hamster with a highly shortened circadian period (*tau* hamster) show decreased longevity compared to their wild-type counter parts (Hurd and Ralph 1998). Other investigations concluded that an intrinsic and functional circadian clock improves competitive growth, fertility, and life span in cyanobacteria, *Drosophila*, chipmunks and mice (Pittendrigh and Minis 1972, DeCoursey, Walker et al. 2000, Beaver, Gvakharia et al. 2002, Green, Tingay et al. 2002, Dodd, Salathia et al. 2005). However, potential pleiotropic effects of such genetic clock mutations might have influenced the study outcomes (Yerushalmi and Green 2009). The advantage of an intrinsic rhythm that is aligned to external periods is reasonable, as the temporal association of food availability, predator activity, and temperature changes is directly affecting an organism's chance of survival. Interestingly, mammals in arctic latitudes express much weaker circadian behavior (van Oort, Tyler et al. 2005, Lu, Meng et al. 2010) while animals which are not exposed to diurnal variations still express functional circadian clocks and have the capability to adapt to external time signals, like the subterranean blind mole rat that lives most of its life underground (Avivi, Oster et al. 2002, Oster, Avivi et al. 2002). Thus, an intrinsic circadian system enables the synchronization of the body's physiology to external conditions, but without a direct selective pressure on its self-sustainment (Roenneberg and Merrow 2002).

1.2 Properties of circadian clocks

Many biological reactions in nature are rhythmic, but not all of them have the properties of true oscillators. Many rhythmic processes are direct reactions to repeated stimuli like light or temperature changes and, thus, "mask" the behavior of a true oscillator. In most living organisms, light is the main source for temporal information and a potent *zeitgeber* (German for time giver) for organisms with the ability of light detection. However, circadian rhythms are self-sustained and oscillate also in the absence of external time information. These self-sustained rhythms were already observed in 1729 by the French astronomer Jean-Jacques

d´Ortous de Mairan in plants. He exposed the heliotroph *Mimosa pudica* to a constant light/dark (LD) routine and observed the diurnal opening and closing of the leaves even after the plants were transferred to constant darkness (DD) conditions. Endogenous rhythms in humans were closer characterized by Jürgen Aschoff. He conducted the so-called “bunker” experiments, where students were monitored in isolation from the outside world. Under these “free-running” conditions, without any external time information, all subjects continued to display regular sleep-wake and body temperature rhythms (Aschoff 1965) with an individual, genetically programmed internal period (τ).

These intrinsic periods are usually not exactly 24 hours long. Humans typically display a longer, mice a shorter period than 24 hours. This time discrepancy is the reason why the internal clocks of organisms constantly need to be reset to stay aligned to the external geophysical time. This constant entrainment also enables organisms to adapt their circadian clocks to seasonal changes and to different time zones after trans-meridian travel. The time the inner clock needs to adapt to a new time zone is often accompanied by disturbances in sleep, physiology and cognitive functions. It was speculated that these “jetlag” symptoms of misaligned inner and outer time actually are part of the avian navigation system on long-distance migration flights for the depiction of their east-west position (Chernetsov, Kishkinev et al. 2008).

Light is not the only entrainment factor. Other, non-photic *zeitgeber* are food (Honma, von Goetz et al. 1983) and temperature (Aschoff and Tokura 1986, Francis and Coleman 1997). Temperature changes may have an enormous effect on the function of poikilothermic organisms. At the same time circadian clocks compensate non-rhythmic temperature fluctuations and are independent in their period of external temperature (Pittendrigh 1954, Hastings and Sweeney 1957) – a remarkable phenomenon, since temperature is known to influence the frequency and reaction rate of many biochemical reactions. The temperature regulation in mammals gets particularly interesting the moment mammalian cells are handled *ex vivo*. It was suggested that temperature stabilization is mediated via

temperature insensitive clock protein phosphorylation by kinases, like casein kinase I (CKI) (Isojima, Nakajima et al. 2009), but the molecular basis for temperature compensation in mammals is still unknown.

1.3 Molecular mammalian circadian timing system

Circadian gene expression rhythms can be defined as the regulated repetition of strong to weak transcription rates with an approximately 24-hour period. Molecular clocks that can be found in all nucleated cells in the mammalian body drive these cell internal oscillations. At the molecular level, the mammalian circadian clock apparatus is based on interlocked transcriptional-translational feedback loops (TTLs). The very core of the clock apparatus comprises two transcription factors brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1, also known as ARNTL or MOP3) and circadian locomotor output cycles kaput (CLOCK), three period (*Per1-3*), and two cryptochrome (*Cry 1-2*) genes. BMAL1 and CLOCK constitute the positive arm of the core TTL. They form heterodimers (BMAL1:CLOCK) that bind to *E-Box* elements within the promoters of *Per* and *Cry* and thereby activate their transcription (Gekakis, Staknis et al. 1998, Hogenesch, Gu et al. 1998, Yoo, Ko et al. 2005, Fustin, O'Neill et al. 2009). Following transcriptional activation by BMAL1:CLOCK, PER and CRY proteins accumulate in the cytoplasm and translocate back into the nucleus where they interfere with BMAL1:CLOCK DNA binding. This interaction leads to a dissociation of BMAL1:CLOCK from the DNA and stops the transcription of *Pers* and *Crys* (Kume, Zylka et al. 1999). Thus, PER and CRY proteins represent the negative feedback arm of the core oscillator. Before BMAL1:CLOCK heterodimers can be reactivated, PER and CRY proteins need to be degraded. The enrichment of PER and CRY proteins in the cytoplasm takes several hours, resulting in a delay between the activation of transcription and PER:CRY-mediated dissociation of the BMAL1:CLOCK complex, which is essential for the generation of temporal oscillations of around 24 hours.

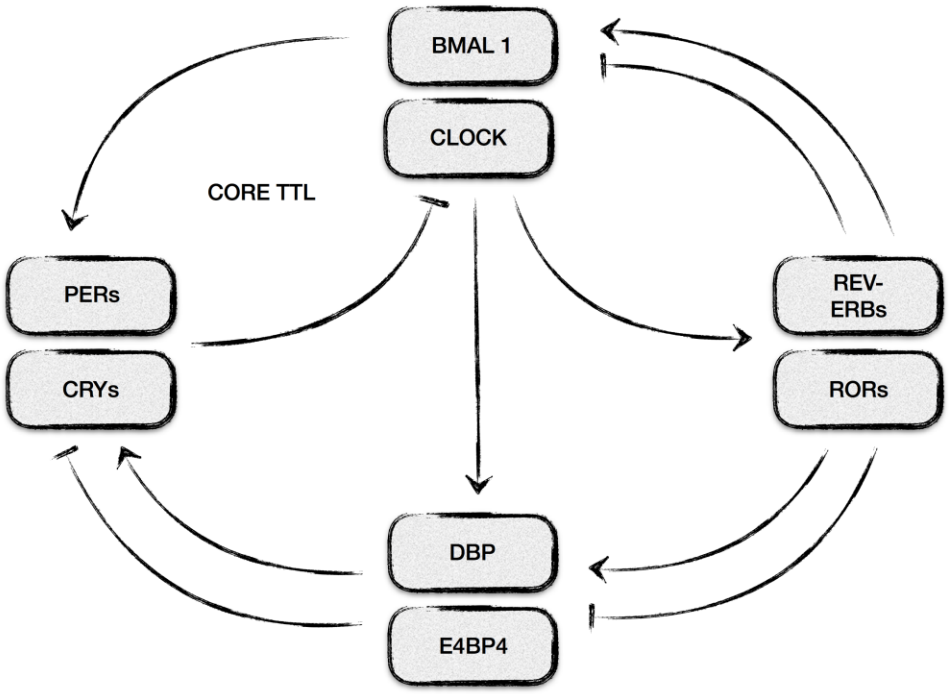


Figure 1: The molecular basis of the circadian clock, transcriptional/translational feedback loops (TTLs)

Heterodimers of circadian locomotor output cycles kaput (CLOCK) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) activate the transcription of period (*Per*) and cryptochrome (*Cry*) genes by E-box binding. PER and CRY proteins form heterodimers and translocate back into the nucleus where they inhibit BMAL1:CLOCK mediated transcriptional activation. Reverse-erythroblastosis virus alpha/beta (*Reverbα*/β) and retinoic acid receptor-related orphan receptors (*Rorα*/β/γ) are part of an auxiliary loop, regulating *Bmal1* and E4 promoter-binding protein 4 (*E4pb4*) transcription. Albumin d-box binding protein (*Dbp*) transcription is activated by BMAL1:CLOCK and activates *Per1* transcription by *D-Box* binding. E4BP4 represses *Per2* expression.

For the temporal stabilization of its 24-h oscillation the core loop is interlocked with several other feedback loops. One of these *auxiliary* TTLs contains the reverse-erythroblastosis virus alpha/beta (REV-ERBα/β or NR1D1/2) and retinoic acid receptor-related orphan receptors (RORα/β/γ). The transcription of *Rev-erb* and *ROR* genes is activated by the BMAL1:CLOCK heterodimer which binds to *E-box*

elements in the promoters of these genes (Sato, Panda et al. 2004, Triqueneaux, Thenot et al. 2004). RORs and REV-ERBs compete for the same binding sides in the *Bmal1* promoter region, the retinoic acid-related orphan receptor response elements (*RORE*s) (Guillaumond, Dardente et al. 2005). While the feedback of the REV-ERB proteins on *Bmal1* transcription is negative, RORs have a stimulatory effect. The stabilizing effects of REV-ERBs are essential for *Bmal1* oscillation, RORs are considered to have a more modulatory function and are not essential for rhythmic transcription of *Bmal1* (Liu, Tran et al. 2008). Another additional feedback loop consists of the basic leucine zipper transcription factor E4 promoter-binding protein 4 (E4BP4, also known as NFIL3) and the proline and acidic-rich basic leucine zipper transcription factor albumin d-box binding protein (DBP). These two transcription factors display circadian mRNA levels and are antiphasic to each other (Mitsui, Yamaguchi et al. 2001). While *Dbp* transcription is activated by BMAL1:CLOCK and activates *Per1* transcription, E4BP4 is regulated by REV-ERB/ROR, represses the transcription of *Per2*, and stabilizes the *Per2* mRNA rhythm in alliance with BMAL1:CLOCK (Yamaguchi, Mitsui et al. 2000, Ohno, Onishi et al. 2007).

1.4 The master clock in the *suprachiasmatic nucleus* (SCN)

For the optimal alignment of rhythms in the different tissues of an organism, the circadian clock network is arranged in a hierarchical order. In mammals, the master clock resides in the *suprachiasmatic nucleus* (SCN). The SCN is a paired structure directly above the optic chiasm in the ventral hypothalamus. SCN transplantation experiments between hamsters with a short period (τ) and wild-types revealed that the genotype of the SCN-donating hamster determines the period of circadian locomotor activity (Ralph, Foster et al. 1990). Total ablation of the SCN by operative lesioning results in a complete loss of rhythms in circadian drinking behavior, locomotor activity, and also endocrine regulation (Stephan and Zucker 1972). The SCN aligns itself with the external light/dark cycle. Light information reaches the SCN *via* the retino-hypothalamic tract (RHT) (Johnson,

Moore et al. 1988, Levine, Weiss et al. 1991, de Vries, Treep et al. 1994). A subset of specialized retinal ganglion cells expresses melanopsin (OPN4), a photo-pigment which enables these retinal ganglion cells to become light-sensitive (Schmutz, Wendt et al. 2011). Further, these intrinsically photosensitive retinal ganglion cells (ipRGCs) project directly to the SCN (Berson, Dunn et al. 2002, Provencio, Rollag et al. 2002). Additionally, light information can be transmitted *via* rods and cones as some of them also project to the ipRGCs. Both systems can substitute each other, therefore a deletion of one is not sufficient to disrupt circadian light entrainment (Freedman, Lucas et al. 1999, Panda, Sato et al. 2002). Destruction of the melanopsin retinal ganglion cells and the rods and cones or the ablation of ipRGCs results in a complete loss of light entrainment (Hattar, Lucas et al. 2003, Guler, Ecker et al. 2008). IpRGCs have large dendritic fields with a relatively high activation threshold and display tonic activation by long light pulses, in this way preventing the reaction to light noise (Berson, Dunn et al. 2002, Provencio, Rollag et al. 2002).

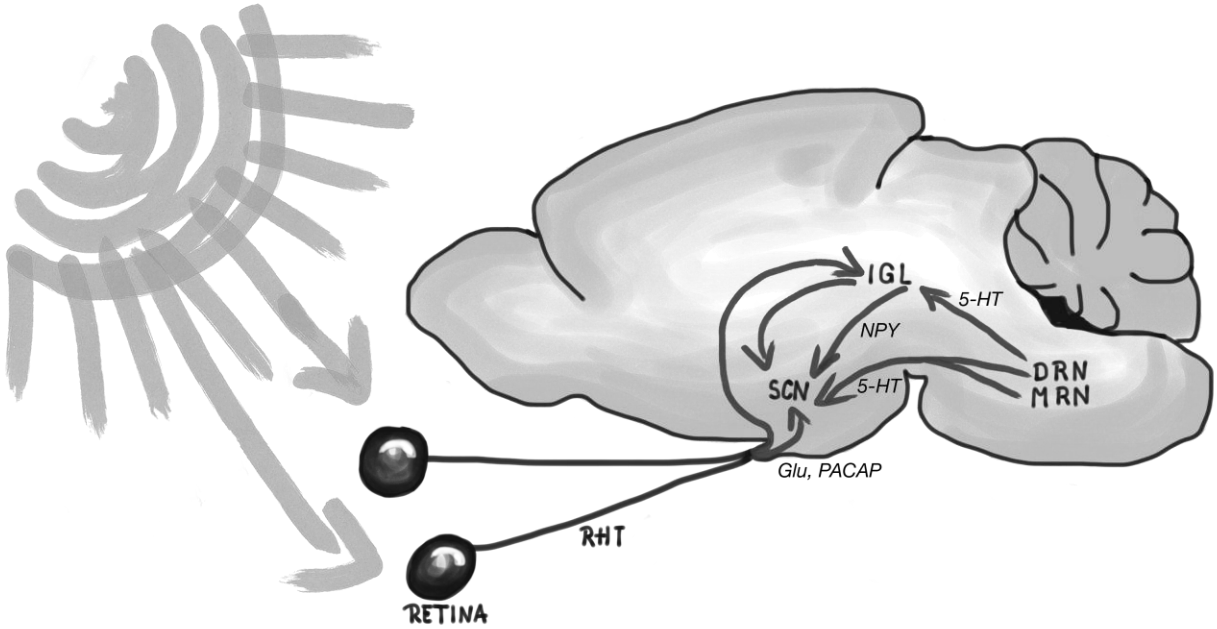


Figure 2: SCN entrainment
Light input to the SCN is directly transmitted *via* the retino-hypothalamic tract (RHT) and indirectly by the intergeniculate leaflet (IGL). RHT stimulates SCN neurons *via* glutamate (Glu) and pituitary adenylate cyclase-activating polypeptide (PACAP) release. Non-photic input is mainly transmitted from the dorsal and median raphe nuclei (DRN, MRN) and from the IGL *via* serotonergic (5HT) and neuropeptide Y (NPY) neurotransmission.

The spectral sensitivity of phase shifts in the circadian system *in vivo* fits to the maximal light sensitivity of melanopsin to blue light at a wavelength of around 480 nm (Takahashi, DeCoursey et al. 1984, Berson, Dunn et al. 2002). RHT synapses mainly target the ventral SCN and use glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) as neurotransmitters (Hirota and Fukada 2004). Binding of glutamate and PACAP receptors activates intracellular signaling cascades in SCN neurons, e.g. calcium/calmodulin kinase II (CAMKII), mitogen-activated protein kinase (MAPK) or cGMP-dependent protein kinase II (PGKII). All pathways ultimately lead to the phosphorylation of the transcription factor cAMP response element binding protein (CREB). Phosphorylated CREB induces the expression of *Per* genes by binding to cAMP response elements (CREs) in their promoter regions (Hirota and Fukada 2004). Interestingly, the clock resetting response to a light pulse followed by CREB activation and subsequent *Per* induction only occurs during the natural night/dark hours and not during the light

phase. The circadian gating of this response is partly mediated by the rhythmic sensitivity of glutamate receptors in SCN neurons (Pennartz, Hamstra et al. 2001). Photic input to the SCN can be modulated by the raphe nuclei via serotonin. Additionally, light signals can be transmitted to the SCN via neuropeptide Y (NPY) neurons of the intergeniculate leaflet (IGL) (Jacob, Vuillez et al. 1999, Muscat, Tischler et al. 2005). Non-photic, serotonergic input is received from the dorsal and median raphe nuclei (DRN, MRN) and also transmitted by NPY neurons from the IGL, that again receive input from the DRN (Dibner, Schibler et al. 2010). (Fig.: 2) The IGL receives light information and serotonergic input from the raphe nuclei, thus merging photic and non-photic temporal information. Retrograte and anterograde labeling in rats provided evidence for further afferents from thalamic, hypothalamic and limbic structures to the SCN (Moga and Moore 1997).The SCN is objective to different time signals which partly have time dependent efficiencies in their clock resetting capacities. Hence, the time of day is an issue in regard to experimental designs.

1.4.1 SCN anatomy

Each of the murine SCN nuclei consists of around 10,000 cells, mostly small and densely packed neurons (Welsh, Takahashi et al. 2010). Rhythmic electrical activity is autonomously generated in SCN neurons, but for a precise output cells need to synchronize with each other (Welsh, Logothetis et al. 1995, Herzog, Aton et al. 2004). Neuronal coupling stabilizes SCN output as seen in studies using murine clock mutants (Liu, Welsh et al. 2007). Interestingly, reduced coupling leads to a decrease in rhythmic cell numbers implying that coupling is necessary for some cells to remain rhythmic. The period and phase of single SCN neurons is quite diverse. SCN explants of hamsters in free-running conditions showed that cells of the dorsal SCN are phase advanced in comparison to the ventral part (Hamada, Antle et al. 2004). Not only the phase, but also local neuropeptide expression differs between SCN regions. The neurons of the ventrolateral core region are characterized by vasoactive intestinal polypeptide (VIP) expression, while the surrounding SCN shell consists of neurons expressing arginine

vasopressin (AVP) (Welsh, Takahashi et al. 2010). The murine SCN core also contains the neurotransmitters GABA, calbindin (CalB), VIP, calretinin (CalBII), gastrin releasing peptide (GRP) and neurotensin (NT), while the shell contains GABA, CalB, AVP, angiotensin II (AngII) and met-enkephalin (INN) positive cells (Abrahamson and Moore 2001). Afferent and efferent connections differ for the two parts of the SCN. The SCN core is highly targeted by RHT fibers and input from the IGL and raphe nuclei is also mainly directed towards the core region. The SCN shell is targeted by potentially more non-photic stimuli from brain areas like the basal forebrain, the hippocampus and other hypothalamic nuclei (Moga and Moore 1997, Abrahamson and Moore 2001).

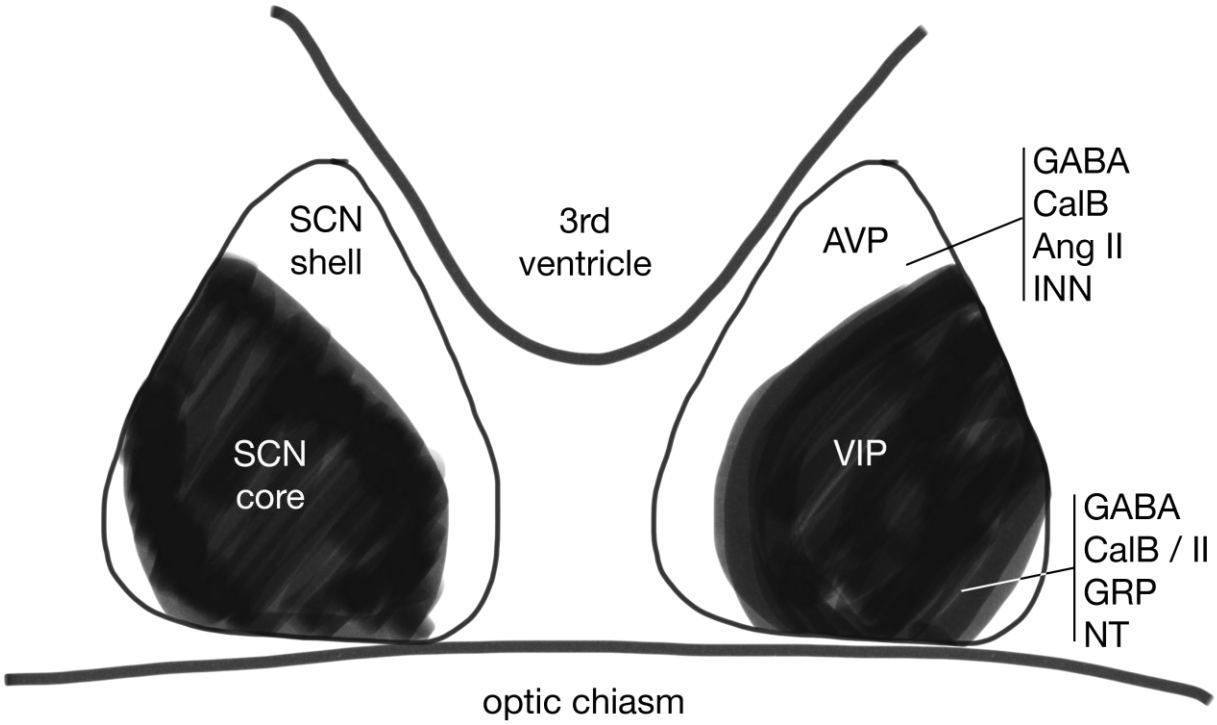


Figure 3: SCN structure is subdivided into a core and shell region
The murine SCN core region expresses VIP, GABA, calbindin (CalB), calretinin (CalBII), gastrin releasing peptide (GRP) and neurotensin (Nt), while the shell region expresses arginine vasopressin (AVP), GABA, CalB, met-enkephalin (INN) and angiotensin II (AngII).

Dense projections within the SCN reach from the core to the shell, while only few shell neurons innervate the core region (Leak, Card et al. 1999). With this bimodal distribution of internal signaling it can be assumed that the core region receives photic information, which is then transmitted to the shell. Corresponding to this

idea, a light pulse first increases the firing rate in VIP-positive cells and induces *cFos* and *Per1/2* expression in the core before the signal is passed on to the shell (Yan and Silver 2002, Kuhlman, Silver et al. 2003, Hamada, Antle et al. 2004). This observation is in line with jetlag experiments where the core SCN aligns with the phase advanced light cycle faster than the shell (Nagano, Adachi et al. 2003, Nakamura, Yamazaki et al. 2005, Davidson, Castanon-Cervantes et al. 2009). Still, *Per* induction limited to the core is not sufficient to shift the behavioral phase. To do so the signal of the core first needs to spread to the shell (Yan and Silver 2002). The core region forwards temporal information to the shell but also stabilizes the rhythm of the shell neurons. Observations of rhythmic AVP release from SCN slices in culture revealed that the period of AVP release corresponds to the period of locomotor activity of free-running animals. Meanwhile, after surgical separation of ventral and dorsal SCN, the period of AVP release shortens in the dorsal neurons and the cells desynchronize faster (Yamaguchi, Isejima et al. 2003, Noguchi, Watanabe et al. 2004).

1.4.2 SCN output

There are still many open questions when it comes to the mechanisms by which SCN rhythms are transferred into circadian behavior and physiology. So far it is clear that, both, neuronal and humoral signals are needed to forward time signals to peripheral tissues. Humoral SCN signals are sufficient to restore circadian locomotor activity in SCN lesioned animals. It was shown in transplantation experiments that SCN grafts surrounded by a semipermeable membrane is able to restore rhythmicity by diffusible signals (Silver, LeSauter et al. 1996). In culture, the synchronizing effect of humoral signals from SCN cells and explants can be measured in cells over a distance of several millimeters (Allen, Rappe et al. 2001, Prolo, Takahashi et al. 2005). There are several humoral factors, released by the SCN in a circadian fashion, like AVP, transforming growth factor α (TGFα), prokineticin 2 (PK2), and cardiotrophin-like cytokine (CLC). While AVP-mutant mice and rats display normal circadian locomotor activity (Li, Burton et al. 2009, Loh, Dragich et al. 2011), infusion of TGFα, PK2 or CLC into the third

ventricle results in locomotor activity suppression (Leak, Card et al. 1999, de la Iglesia, Meyer et al. 2000, Kramer, Yang et al. 2001).

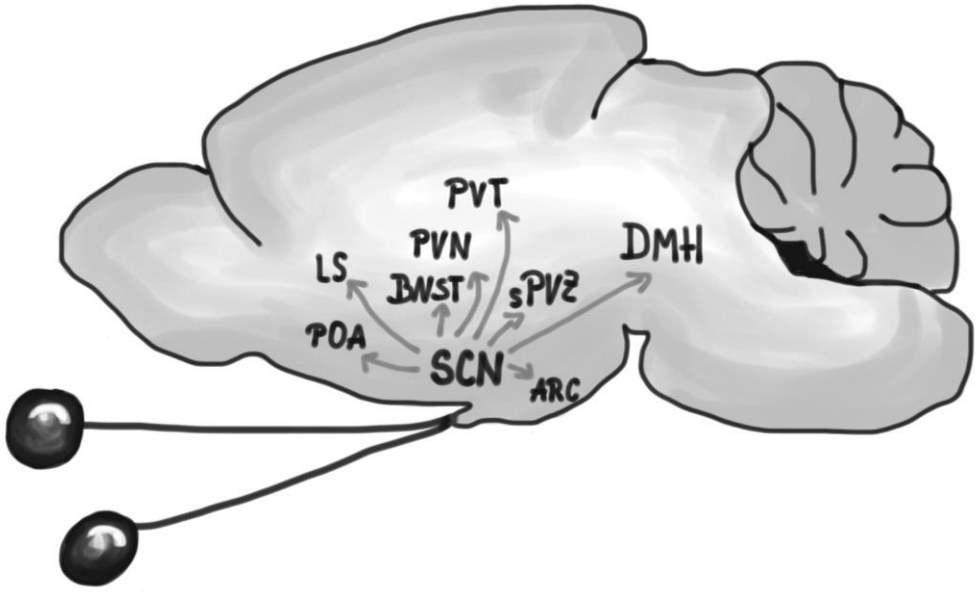


Figure 4: SCN efferents
The SCN mainly targets hypothalamic nuclei like the paraventricular nucleus of the hypothalamus (PVN), the subparaventricular zone of the hypothalamus (SPVZ), the dorsomedial nucleus of the hypothalamus (DMH), the arcuate nucleus (ARC), the lateral septum (LS), the bed nucleus of the stria terminalis (BNST) and the preoptic area (POA). Also other parts of the brain are directly innervated by SCN efferents, e.g. the paraventricular nucleus of the thalamus (PVT).

Still, humoral SCN signals alone are not enough to restore endocrine rhythms (Meyer-Bernstein, Jetton et al. 1999). Cutting SCN efferences (Stephan and Nunez 1977) or preventing SCN neurons from firing results in arrhythmic behavior (Ruby, Burns et al. 1999), suggesting that both humoral and neuronal signals are necessary. SCN neurons project mainly into the medial hypothalamus. There, the paraventricular nucleus of the hypothalamus (PVN), the sub-paraventricular zone of the hypothalamus (SPVZ), the dorsomedial nucleus of the hypothalamus (DMH), the arcuate nucleus (ARC), the lateral septum (LS), the bed nucleus of the stria terminalis (BNST), and the preoptic area (POA) are targeted (Leak and Moore 2001). Further, the SCN projects to the paraventricular nucleus of the thalamus

(PVT) (Buijs and Kalsbeek 2001). While the main neurotransmitters are GABA and glutamate, SCN neurons additionally release neuropeptides like AVP, VIP, GRP and somatostatin (Buijs and Kalsbeek 2001).

1.5 Peripheral clocks

Circadian clock genes are expressed throughout all mammalian tissues and organs. Early cell culture experiments already indicated that cells of other origins than the SCN harbor intrinsic clocks and can sustain rhythmic circadian clock gene expression *ex vivo* (Balsalobre, Damiola et al. 1998). Tissue cultures from transgenic animals in which a luciferase reporter was coupled to a circadian promoter display strong circadian clock gene expression for several days in culture (Yamazaki, Numano et al. 2000, Yoo, Yamazaki et al. 2004). Still, in comparison to SCN cells or explant cultures these peripheral oscillators appear to be less robust and desynchronize much faster (Nagoshi, Saini et al. 2004). *In vivo* peripheral organs display internal desynchronization after SCN lesion, supporting the theory that the SCN is not the driver for peripheral rhythms, but rather coordinates the correct phasing and synchrony between the organs (Yoo, Yamazaki et al. 2004, Dibner, Schibler et al. 2010). Circadian microarray and RNA sequencing studies found that about 5-10% of gene transcripts in each tissue oscillate with a circadian period. The identities of these clock-controlled output genes and their proteins, however, are highly tissue specific (Kornmann, Preitner et al. 2001, Duffield, Best et al. 2002, Kita, Shiozawa et al. 2002, Storch, Lipan et al. 2002, Reddy, Karp et al. 2006, Young 2006).

1.6 Circadian clock hierarchy

In the early 70s, the SCN region was identified as the master pacemaker in rodents. Upon bilateral lesion of the SCN, animals become arrhythmic in activity, drinking behavior, body temperature and corticosterone release (Moore and Eichler 1972, Stephan and Zucker 1972, Lehman, Silver et al. 1987, Ralph, Foster et al. 1990, Refinetti, Kaufman et al. 1994, Silver, LeSauter et al. 1996, Sujino, Masumoto et al. 2003). Likewise, peripheral gene expression rhythms dampen

after SCN lesion in constant darkness (DD) and rhythmic light-dark (LD) conditions (Sakamoto, Nagase et al. 1998, Akhtar, Reddy et al. 2002, Guo, Brewer et al. 2005, Guo, Brewer et al. 2006). This observation fits with the idea that the SCN drives peripheral circadian rhythms. Circadian behavior in SCN-lesioned animals can be rescued by SCN transplantation, but these animals follow only their intrinsic period and are not able to adapt to external LD rhythms (Lehman, Silver et al. 1987, Ralph, Foster et al. 1990). This incapability of light entrainment might result from the interrupted RHT innervation of SCN transplants. Interestingly, such transplantation studies revealed that some, like behavior, but not all rhythms are restored. Prominent endocrine rhythms like corticosterone and melatonin stay arrhythmic (Meyer-Bernstein, Jetton et al. 1999) and clock gene rhythms in the periphery reveal non-uniform results. Gene expression rhythms in liver, kidney and muscle tissue are rescued, while expressions in heart, spleen and adrenal tissues stay arrhythmic (Guo, Brewer et al. 2005, Guo, Brewer et al. 2006) indicating that the SCN clock uses different ways for transferring temporal information to the periphery. Local tissue clocks stay functional after SCN lesion, as *ex vivo* measurements of *Period2::Luc* reporter mice tissues suggest (Yoo, Yamazaki et al. 2004). To answer the question if peripheral clock rhythms persist without a functional SCN clock *in vivo*, mice with a genetically ablated SCN clock function were created (Husse, Zhou et al. 2011, Izumo, Pejchal et al. 2014). In contrast to SCN-lesioned animals, these animals have an intact RHT tract and synchronizing light signals can pass through the SCN region. Subsequently, peripheral clocks in these animals are directly synchronized by light under LD conditions. The moment this external time clues are lost, e.g. after releasing the animals into DD, peripheral clock rhythms dampen (Husse, Leliavski et al. 2014).

1.6.1 Peripheral clock synchronization

Peripheral circadian rhythms are the result of, both, local tissue clocks and systemic factors derived directly or indirectly from the SCN (Vollmers, Gill et al. 2009). The SCN can influence peripheral rhythms through ANS innervation, by controlling feeding and activity behavior, fluctuations of body temperature, or

circulating endocrine and metabolite rhythms. Physiological parameters like endocrine rhythms are often controlled by several regulators in parallel. A strong and prominent endocrine rhythm is seen in the glucocorticoid corticosterone, which peaks before the onset of activity (Oster, Damerow et al. 2006). The rhythmic release of corticosterone is centrally as well as peripherally regulated. The SCN projects to corticotropin-releasing hormone (CRH) releasing PVN neurons that stimulate the release of adrenocorticotropic hormone (ACTH) from the pituitary (Kalsbeek and Buijs 2002). As part of the hypothalamic-pituitary-adrenal (HPA) axis, ACTH induces the release of corticosterone from the adrenal glands. The sensitivity to ACTH in the adrenal cortex is further regulated by local circadian clocks (Oster, Damerow et al. 2006).

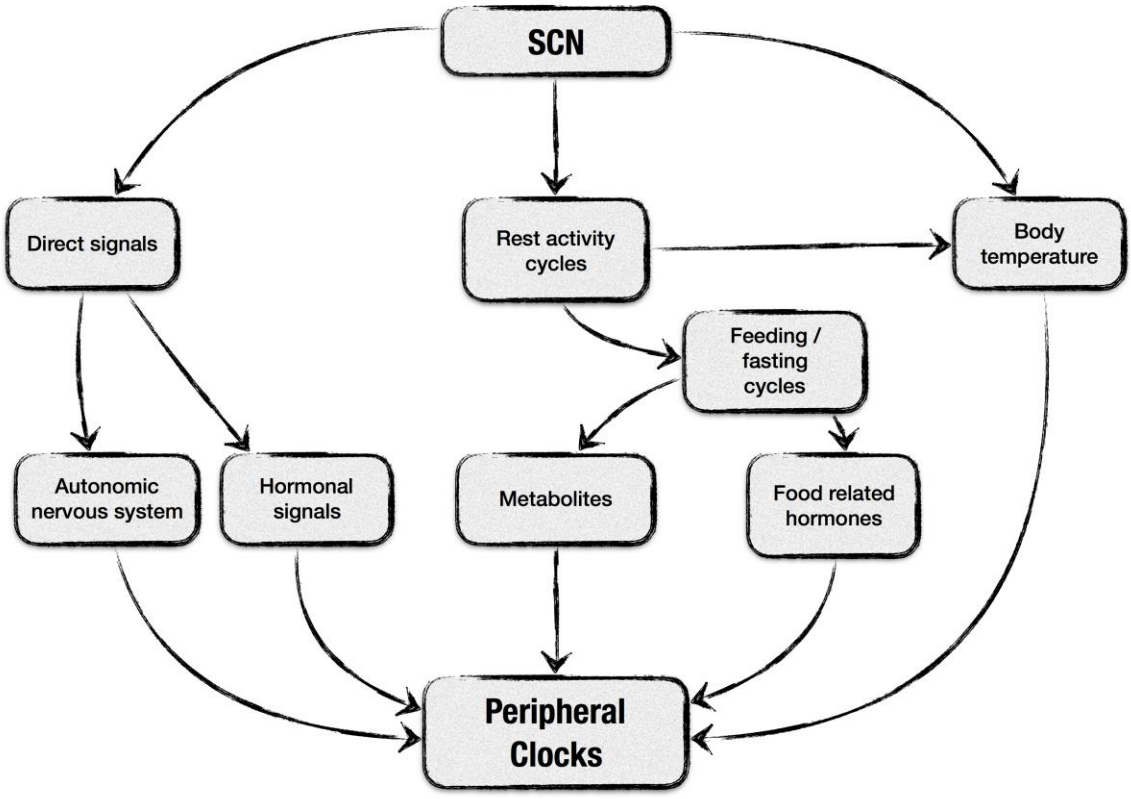


Figure 5: Peripheral clocks entrainment pathways

The SCN synchronizes peripheral clocks by neuronal (ANS) and humoral signals. Additionally, body temperature is influenced directly by SCN signaling and indirectly by setting rest/activity cycles. Activity influences food intake and this, in turn, entrains peripheral clocks through food-related hormones and metabolites. Modified from (Dibner, Schibler et al. 2010).

Besides the HPA axis, autonomic signals from the splanchnic nerve seem to stimulate the release of corticosterone directly and adrenal denervation results in dampened corticosterone rhythms (Jasper and Engeland 1994, Ulrich-Lai, Arnhold et al. 2006). This interplay of central and peripheral clock regulation can refine and stabilize the endocrine rhythms, but this complex interaction exacerbates the dissection of local from systemic regulation. The different synchronizing signals have weighted influences in their entraining capability of peripheral clocks, with food access as the strongest entrainment factor for metabolic tissues. Time restricted feeding (RF) can totally uncouple peripheral clocks from the SCN. When food is only provided during the normal rest phase, the phase relationships of peripheral and central clocks are inversed (Damiola, Le Minh et al. 2000, Stokkan, Yamazaki et al. 2001).

1.7 Circadian clocks and metabolism

Diurnal variations in many metabolites and hormones associated with energy homeostasis like insulin, glucose, corticosterone, leptin, and triglycerides imply a direct connection of metabolism with circadian rhythms (De Boer and Van der Gugten 1987, Ahima, Prabakaran et al. 1998, La Fleur, Kalsbeek et al. 1999, Rudic, McNamara et al. 2004). Especially the observation that some of these rhythms are lost upon SCN lesion suggests an involvement of the master clock in metabolic homeostasis (La Fleur, Kalsbeek et al. 1999, Kalsbeek, Fliers et al. 2001). In humans, the connection between disrupted rhythms and metabolic disorders has been observed in epidemiological studies. Shift workers display a higher prevalence for the development of the metabolic syndrome and cardiovascular diseases (Karlsson, Knutsson et al. 2001, De Bacquer, Van Risseghem et al. 2009, Pan, Schernhammer et al. 2011). In a forced desynchrony protocol in humans, participants followed an artificial 28-h rhythm. In this experiment activity behavior dissociated from intrinsic circadian rhythms and the participants developed hyperglycemia, hyperinsulinemia and hypoleptinemia

(Scheer, Hilton et al. 2009). The importance of the timing of food intake was also seen in mice that had access to food only during the light phase, their normal rest phase. These mice show increased body weight compared to their *ad libitum* or night time restricted food controls (Arble, Bass et al. 2009, Fonken, Workman et al. 2010). Meanwhile in another study, mice fed a high-fat diet (HFD) restricted to their normal active phase did not gain weight and stayed healthy in contrast to *ad libitum* HFD controls (Hatori, Vollmers et al. 2012). HFD itself dampens molecular and behavioral rhythms (Kohsaka, Laposky et al. 2007). Metabolic state and the clock machinery are linked on the molecular level. AMP-activated protein kinase (AMPK), a sensor for AMP/ATP ratios, promotes CRY1 degradation upon high AMP levels and thereby directly affects the circadian TTL (Lamia, Sachdeva et al. 2009). Furthermore, the transcriptional activity of BMAL1:CLOCK complexes can be directly influenced by the NAD^+-dependent deacetylase Sirtuin 1 (SIRT1) (Asher, Gatfield et al. 2008, Nakahata, Kaluzova et al. 2008). Then again, the transcription of nicotinamide phosphoribosyltransferase (*Nampt*), encoding the main enzyme for NAD^+ regeneration, is under circadian control and thereby feeds back of SIRT1 (Nakahata, Sahar et al. 2009, Ramsey, Yoshino et al. 2009). In conclusion, metabolic state sensing pathways connect the circadian clock and metabolism on the molecular level and feedback works in both directions. Therefore, it is not surprising that model organisms of genetic clock defects display metabolic alterations (see next chapter) and that clock alterations can be found in metabolically challenged (e.g. obesity, type-2 diabetes) conditions.

1.7.1 Metabolic phenotypes of circadian clock gene mutant mice

Under metabolically healthy and stress-free conditions levels of triglycerides (TGs), free-fatty acids (FFAs), glucose and insulin in the blood oscillate with a circadian period (Sukumaran, Xue et al. 2010). Glucose and lipid metabolism and lipid storage are frequently altered when circadian rhythms are disturbed. Genetically modified mice with a targeted deletion or mutation of individual clock genes and clock-targeting drug administration experiments provide further insight into the actual extent of the influence of the clock machinery on energy metabolism.

1.7.1.1 Bmal1

Global disruption of the core clock gene *Bmal1* (Bmal1-KO) in mice, results in a complete loss of rhythmic behavior and of blood glucose and TGs oscillations (Bunger, Wilsbacher et al. 2000, Rudic, McNamara et al. 2004). Targeted *Bmal1* mutation in liver or adipose tissue promotes obesity in young mice and hyperphagy during the light phase (Lamia, Storch et al. 2008, Guo, Chatterjee et al. 2012). *Bmal1*-KO mice fail to develop substantial adipose stores, but have increased levels of circulating lipids and more ectopic fat deposition in liver and skeletal muscle (Shimba, Ogawa et al. 2011). The expression of adipokines like adiponectin and resistin is decreased in these mice and they display glucose intolerance and increased insulin sensitivity due to low insulin levels (Rudic, McNamara et al. 2004, Lamia, Storch et al. 2008, Hemmers and Rudensky 2015). They also suffer from a premature aging phenotype, which averts the study of metabolic defects at old age (Kondratov, Kondratova et al. 2006).

1.7.1.2 Clock

Mutant mice in which the exon 19 of the *Clock* transcript is excised during splicing (ClockΔ19) display impaired DNA-binding and trans-activational activity of the BMAL1:CLOCK heterodimer (Vitaterna, King et al. 1994, King, Zhao et al. 1997, Gekakis, Staknis et al. 1998, Katada and Sassone-Corsi 2010). These animals become behaviorally arrhythmic in DD and display blunted feeding rhythms already in LD conditions. Their body weight and hepatic lipid accumulation are increased, blood glucose and lipid levels elevated, while transcripts of food intake-regulating neuropeptides like orexin and ghrelin are down-regulated (Turek, Joshu et al. 2005). *Clock*$^{-/-}$ mutants, however, have minor behavioral phenotypes with normal food intake, but still display increased body weight gain (Debruyne, Noton et al. 2006, Eckel-Mahan, Patel et al. 2012).

1.7.1.3 Period

Despite a normal glucocorticoid response to stress, *Per2*$^{-/-}$ mice show no circadian corticosterone rhythm (Yang, Liu et al. 2009). On standard chow, they

consume similar amounts of food as wild-type (WT) control animals, but weigh slightly less (Grimaldi, Bellet et al. 2010). However, if challenged with HFD, *Per2*$^{-/-}$ mice accumulate more adipose tissue (AT) than controls and show an altered lipid metabolism with hypotriglyceridemia (Yang, Liu et al. 2009, Grimaldi, Bellet et al. 2010) and increased food consumption (Yang, Liu et al. 2009, Dallmann and Weaver 2010). *Per3* KO mice display normal feeding rhythms, but still have increased AT mass (Dallmann and Weaver 2010); *Per1/2/3* triple mutants are overweight under HFD conditions compared to WT controls (Dallmann and Weaver 2010).

1.7.1.4 Cryptochrome

Cry1/2 double deficient mice gain more weight under HFD than controls and become hyperglycemic and hyperinsulinemic (Barclay, Shostak et al. 2013). Under LD conditions and with normal chow, however, they show reduced body size and weight (Bur, Cohen-Solal et al. 2009). Still, their glucose metabolism is disturbed displaying decreased glucose tolerance (Lamia, Papp et al. 2011). Besides decreased blood triglyceride levels, *Cry* mutants are drawn to develop liver steatosis, implying defects in hepatic triglyceride metabolism (Cretenet, Le Clech et al. 2010).

1.7.1.5 Nuclear receptors

Mice lacking clock genes from the auxiliary loops, like *Rorα* or *Rev-erbα* show impaired circadian rhythms of locomotor activity and blunted clock gene expression (Preitner, Damiola et al. 2002, Sato, Panda et al. 2004). After treatment of obese wild-type mice with REV-ERBα and REV-ERBβ activating drugs, they lose weight due to increased energy expenditure and suppression of lipogenic genes (Solt, Wang et al. 2012).

1.7.2 SCN lesion and metabolism

The role of the master pacemaker in glucose metabolism and metabolic homeostasis was investigated in SCN lesioned animals. SCN surgical ablation leads to a loss of rhythmic locomotor activity, diurnal feeding rhythms and

arhythmicity of metabolic factors like leptin or blood glucose. In LD and with normal chow, SCN lesioned mice develop a pre-diabetes phenotype with abolished insulin rhythms and hyperinsulinemia. Due to an increased fat mass in comparison to their sham operated controls, lesioned animals also gain significantly more weight, which might contribute to systemic insulin resistance (Coomans, van den Berg et al. 2013). Their diurnal rhythms of blood leptin and glucose concentrations are abolished (La Fleur, Kalsbeek et al. 1999), while the overall concentration of leptin is elevated after thermal SCN ablation, which might be due to the increase in adipose tissue (AT) mass (Kalsbeek, Fliers et al. 2001). However, in these lesion experiments not only SCN efferents were disrupted, but also those of neighboring regions, which might have influenced the experimental outcome.

1.8 Peripheral clocks as metabolic regulators

Many other rhythmic physiological processes are connected to peripheral tissue function. Blood pressure, pulse rate (Veerman, Imholz et al. 1995), and also xenobiotic detoxification in the liver (Gachon, Olela et al. 2006) and major aspects of glucose and lipid metabolism (Rudic, McNamara et al. 2004) display diurnal rhythms. 5-10 % of gene transcripts in each tissue display a circadian expression pattern, including genes encoding rate-limiting enzymes and regulators in metabolic pathways. These clock-controlled genes (CCGs) directly link the molecular tissue clock to metabolism (Panda, Antoch et al. 2002, Storch, Lipan et al. 2002). Additionally, many CCGs are transcription factors which transmit circadian physiological output to their downstream targets (Yang, Downes et al. 2006, Guillaumond, Grechez-Cassiau et al. 2010, Jeyaraj, Haldar et al. 2012, Jeyaraj, Scheer et al. 2012). The physiological importance of peripheral clocks was demonstrated by deleting *Bmal1* specifically in the liver (Lamia, Storch et al. 2008). In these animals glucose homeostasis is altered since fasting periods are no longer counterbalanced by hepatic glucose export (Lamia, Storch et al. 2008). The glucose transporter 2 (GLUT2; SLC2A2), which is involved in glucose export from liver cells into the blood stream results to be a direct target of the hepatocyte clock. Its mRNA expression peaks during the inactive/fasting phase when animals have

to draw from their energy reservoirs. Deletion of clock function in pancreatic β-cells leads to an insensitivity towards glucose and impaired insulin secretion, resembling a pre-diabetic state (Marcheva, Ramsey et al. 2010).

1.9 Adipose tissue

Adipose tissues (ATs) consist of a conglomerate of adipocytes and connective tissue. In mammals, the bulk mass of AT is depicted by white adipose tissue (WAT) while a smaller part (species dependent) consists of brown adipose tissue (BAT). Both differ in their morphology and function. BAT adipocytes contain multiple small lipid droplets and a large number of mitochondria. BAT contributes to body heat production via non-shivering thermogenesis. BAT adipocytes express high levels of uncoupling protein 1 (UCP1) in the inner membrane of mitochondria. UCP1 enables uncoupling of proton transport leaking into the mitochondrial matrix during oxidative phosphorylation. This proton leak leads to production of heat instead of adenosine triphosphate (ATP) (Cannon and Nedergaard 2004).

White adipocytes store energy in form of cholesterol and triglycerides and, in combination with their endocrine function, play a major role in energy homeostasis. White adipocytes are characterized by unilocular lipid droplets that make up to 95% of the cell volume. In times of energy demand TGs in the lipid droplet are hydrolyzed by lipolysis and energy in form of FFAs is released into the bloodstream (Arner 2005).

1.9.1 WAT innervation

Central to peripheral communication is in part conducted via autonomic nervous system (ANS) signaling and the neurotransmitter norepinephrine (NE) (Slavin and Ballard 1978, Bartness, Shrestha et al. 2010). Different WAT depots are diversely innervated and only 2-3% of adipocytes are directly innervated by the ANS (Slavin and Ballard 1978, Bamshad, Aoki et al. 1998, Bartness, Shrestha et al. 2010). Still, ANS activation induces lipolysis in WAT and local denervation increases WAT mass as a consequence of enhanced adipocyte proliferation (Cousin, Casteilla et al. 1993, Bamshad, Aoki et al. 1998, Bowers, Festuccia et al.

2004, Shi, Song et al. 2005, Foster and Bartness 2006). Retrograde tracing experiments revealed that WAT innervations originate in multiple regions in the brain stem, midbrain, forebrain and the spinal cord, including regions involved in the regulation of energy homeostasis like the ARC, the dorsomedial hypothalamic nucleus and the PVN. WAT and the SCN are connected with each other via multisynaptic pathways (Bamshad, Aoki et al. 1998). Retrograde pseudorabies tracer studies in rat WAT in combination with simultaneous autonomic denervation revealed intense neuronal labeling in the dorsal motor nucleus of the vagus (DMV) (Kreier, Fliers et al. 2002). The DMV is the main cranial motor nucleus for the vagal nerve, which again is one of the major parasympathetic nerves and the neuronal labeling of this nucleus implies the innervation of WAT by the parasympathetic nervous system (PNS). The PNS innervation of AT and its possible role for metabolism is still on debate as deviating results in this context were achieved. For example chemical denervation experiments in hamsters could not confirm a PNS innervation of WAT and immunohistochemical markers of parasympathetic innervations are absent in AT (Giordano, Song et al. 2006).

1.9.2 Fatty acid metabolism

Lipids, in form of triglycerides, are a compact energy storage form and contain twice as many kilojoules per gram than proteins or carbohydrates. The majority of the body's energy resources are stored in adipose tissue depots. Triglycerides are composed of a glycerol molecule and three fatty acids. To sustain energy homeostasis TGs are synthesized and stored in time of energy surplus (lipogenesis) and utilized in times of energy demand (lipolysis). Neutral lipids within adipocytes congregate in large lipid droplets with a hydrophobic triglyceride and sterol ester core surrounded by an amphiphilic phospholipid monolayer. To utilize the chemical energy of triglycerides they have to be disassembled into fatty acids and glycerol (Walther and Farese 2012).

1.9.2.1 Lipogenesis

Increased glucose uptake into adipocytes is regulated by insulin which up-regulates glucose transporter 4 (GLUT4) expression on adipocyte membranes.

This increases glucose uptake and promotes fatty acid biosynthesis (Dimitriadis, Mitrou et al. 2011). Carbohydrates are converted into pyruvate *via* glycolysis. Insulin further stimulates lipogenesis by activating pyruvate dehydrogenase, an enzyme that converts pyruvate to acetyl-CoA (Rutter, Diggle et al. 1992, Denton, McCormack et al. 1996), and acetyl-CoA carboxylase by phosphorylation. Acetyl-CoA carboxylase is one of the rate-limiting enzymes of fatty acid biosynthesis, converting acetyl-CoA to malonyl-CoA (Ninni, Sarappa et al. 1978). Food-derived FFAs can also be directly esterified to yield chemically neutral triglycerides and stored in lipid droplets.

1.9.2.2 Lipolysis

While lipogenesis occurs in times of energy surplus, stored energy needs to be mobilized in times of energy demand – a sequence that naturally occurs during the daily feeding and fasting cycle. While in most mammals metabolism during active hours runs mainly on carbohydrates, during the resting phase lipid utilization is enhanced. This fuel difference can be measured *via* indirect calorimetry, where the respiratory quotient (volume of produced CO_2 / volume of absorbed O_2) approximates a value of 1.0 for carbohydrate and 0.7 for lipid metabolization. Lipid mobilization (or lipolysis) is regulated via the ANS and several hormones (Bartness, Shrestha et al. 2010). While epinephrine, NE, and cortisol induce lipolysis, local autocrine and paracrine factors have stimulating (TNF-α) or inhibiting (adenosine) properties (Fruhbeck, Mendez-Gimenez et al. 2014). In order to be metabolized TGs undergo hydrolysis into free fatty acids (FFAs) and glycerol, which are released into the circulation and taken up by metabolically active tissues like liver or muscle. Inside the cells, FFAs are subjected to β-oxidation in the mitochondrial matrix. The carbon chains of FAs are split into acetyl-CoA which enters the Krebs cycle for ATP production. Glycerol can either enter gluconeogenesis or glycolysis to produce glucose or pyruvate, respectively. During times of starvation excess amounts of acetyl-CoA are further utilized to ketone bodies which serve as an alternate energy substrate for the brain (Hasselbalch, Knudsen et al. 1994).

1.9.4. Endocrine function of adipose tissue

Besides its role in answering to the organism's energy demands by storing and releasing lipids, adipose tissue is an active endocrine organ that secrets a large variety of peptide hormones, so called adipo(cyto)kines (Henry and Clarke 2008). Best known are leptin, adiponectin, visfatin and resistin, but to date several hundred adipokines have been identified with functions in lipid metabolism, energy homeostasis, insulin sensitivity, immunity, angiogenesis and blood pressure (Lau, Dhillon et al. 2005) and many display circadian oscillations in transcription or in protein levels in humans or rodents (see Table 1).

Table 1: Rhythmic adipokine proteins (P) or RNAs (R) in rodents and humans with their peak phase and function. Modified from (Kiehn, Tsang et al. 2017)

Rhythmic adipokine	Peak phase rodents	Peak phase humans	Function	References
Leptin	night (P)	(early) night (P)	proinflammatory, food intake, reproduction, angiogenesis, immunity, ↓lipogenesis, ↑lipolysis	(Bray and Young 2007, Benedict, Shostak et al. 2012)
Adiponectin	night (R)	day (P)	anti-inflammatory, ↑FA oxidation, ↑insulin sensitivity	(Oliver, Ribot et al. 2006, Scheer, Chan et al. 2010, Gomez Abellan, Gomez Santos et al. 2011)
Resistin	night (R)	n/a	proinflammatory, mediator T2DM and cardiovascular disease, ↑TNF- α, ↑IL-6, ↑SOCS-3, ↓insulin sensitivity	(Oliver, Ribot et al. 2006)
Visfatin	evening (R)	mid-day (P)	proinflammatory, ↓insulin sensitivity	(Bray and Young 2007, Benedict, Shostak et al. 2012)
Apelin	evening (P)	n/a	↓insulin sensitivity, ↑angiogenesis	(Butruille, Drougard et al. 2013)
Chemerin	day (P)	no variation	proinflammatory, ↑lipolysis, adipocyte differentiation, ↑leptin, ↑adiponectin	(Parlee, Ernst et al. 2010, Chamberland, Berman et al. 2013)
Tumor necrosis factor alpha (TNF-α)	day (P)	n/a	proinflammatory, ↓insulin sensitivity, ↑FFA release, ↓adiponectin synthesis	(To, Irie et al. 2009)
Interleukin 6 (Il-6)	day (P)	early morning (P)	proinflammatory, ↓adiponectin synthesis, ↓gluconeogenesis	(Guan, Vgontzas et al. 2005, Vgontzas, Bixler et al. 2005, Cano, Cardinali et al. 2009)
Plasminogen activator inhibitor-1 (PAI-1)	early night (P)	morning (activity) (P)	proinflammatory, vascular homeostasis	(Angleton, Chandler et al. 1989, Oishi 2009)

1.9.4.1 Leptin

The best studied adipokine is leptin, a 16-kDA protein secreted from white adipocytes. In healthy subjects, the amount of circulating leptin is directly proportional to body fat mass and an indicator for the energy state of the organism (Maffei, Halaas et al. 1995). Leptin is involved in energy homeostasis regulation and its blood levels decrease upon fasting. Leptin acts in the brain where it promotes energy expenditure and decreases food intake by signaling in the mediobasal hypothalamus (MBH), the ventral tegmental area (VTA) and the nucleus of the solitary tract (NTS). Neurons of the MBH, the main regulator of

energy homeostasis, express the long isoform of the leptin receptor (LEPRb/OBRb) which contains an intracellular C-terminus mediating downstream signaling. Leptin binding to LEPRb activates anorexigenic pro-opiomelanocortin neurons while simultaneously inhibiting orexigenic neuropeptide Y or agouti related neuropeptide (AgRP) expressing neurons in the MBH (Friedman and Halaas 1998). The influence of leptin on energy expenditure was extensively studied in two complementary mouse models. *Ob/ob* mice carry a nonsense mutation in the second exon of the leptin gene (*Lep*) and, thus, do not produce leptin (Zhang, Proenca et al. 1994). In contrast, *db/db* mice have a loss-of-function mutation in the leptin receptor gene (*LepR*) which prevents intracellular leptin signaling (Lee, Proenca et al. 1996). Both mouse lines display an obese, hyperphagic phenotype with reduced locomotor activity. Leptin levels in *db/db* mice are high, but the lack of leptin signaling and the missing metabolic feedback to the hypothalamus promotes overconsumption just like the absence of leptin in *ob/ob* mice (Ingalls, Dickie et al. 1950, Hummel, Dickie et al. 1966). Obesity is further promoted as the missing leptin signaling decreases energy expenditure as well.
Other leptin functions were described for stress responses, thermoregulation, locomotor activity and reproduction (Cannon and Nedergaard 2004, Lu, Kim et al. 2006). The loss of leptin action in obese subjects is in most cases not due to mutated leptin or to non-functional leptin receptors, but because of an acquired central leptin resistance (Myers, Heymsfield et al. 2012). Obese individuals have permanently high serum concentrations of leptin, but leptin's function of suppressing appetite is reduced. The underlying mechanism is still unknown, but an impaired transport across the blood-brain barrier (BBB) or suppressed leptin receptor signaling in the hypothalamus have been proposed (Henry and Clarke 2008).

1.9.5. The adipose clock

Circadian clocks were identified in basically all tissues, where they govern tissue-specific rhythmic transcription. Cell based experiments indicate an important connection between adipose function and clock genes. During

adipogenesis *Bmal1* is upregulated and *Bmal1* deficiency leads to disturbed differentiation of mature adipocytes (Shimba, Ishii et al. 2005, Guo, Chatterjee et al. 2012). The clock gene *Rev-erbα* has a dual role in adipogenesis. It initially promotes adipocyte differentiation and is required for mitotic cell division, while at later stages it detains TG accumulation *via* its inhibitory effect on the gene expression of *Pparγ* (Wang and Lazar 2008). PPARγ is also targeted by *Per2* which prevents the binding of PPARγ protein to target genes (Grimaldi, Bellet et al. 2010). The connection between circadian rhythms and adipose physiology can be observed in diurnal oscillations of circulating adipose tissue-derived hormones like leptin, adiponectin, and visfatin in healthy humans (Sinha, Ohannesian et al. 1996, Gavrila, Peng et al. 2003, Benedict, Shostak et al. 2012). mRNA expression of most of these adipokines is rhythmic in murine AT and these rhythms are abolished under obese or diabetic conditions, once more connecting metabolic state and clock gene function (Ando, Yanagihara et al. 2005). Rhythmic clock gene expression can be found in human and mouse ATs (Ando, Yanagihara et al. 2005, Zvonic, Ptitsyn et al. 2006, Otway, Mantele et al. 2011) and hundreds of gene transcripts with a circadian expression pattern were identified in murine brown and white ATs (Ptitsyn, Zvonic et al. 2006). Of note, in these studies it was not specifically addressed if these rhythms were controlled on a systemic or on a local level. The majority of rhythmic AT genes are involved in metabolic processes and adipose clock gene regulation is sensitive to the timing of food intake. Temporal food restriction to the normal rest phase of animals results in a phase shift of adipose clock genes, but also many other rhythmic transcripts (Zvonic, Ptitsyn et al. 2006).

1.9.5.1 AT specific clock disruption and metabolism

For the investigation of peripheral clock function and to elucidate the importance of tissue-specific clocks in metabolic regulation, systemically and locally controlled rhythms need to be dissected. Genetic deletion of *Bmal1* completely abolishes circadian clock function in affected tissues and a number of tissue-specific *knock-out* animals were created in the last decade (Jeffery, Berry

et al. 2014). For the deletion of *Bmal1* in adipocytes different drivers for the *Cre-loxP* system were used (Paschos, Ibrahim et al. 2012). Animals with an *adipocyte protein 2* (*ap2*) driven disruption of the adipose clock display altered diurnal feeding rhythms and adiposity (Paschos, Ibrahim et al. 2012). *aP2* is expressed in white and brown adipocytes and in macrophages and *ap2*-driven transgene activity has further been detected in a number of other tissues including the brain (Tsang, Astiz et al. 2017). The *adiponectin (Adipoq)* promotor is more specific to drive recombinase activity to adipocytes. $Adipoq^{Cre}$ x $Bmal1^{flox}$ animals display a similar obesogenic phenotype as $ap2^{Cre}$ x $Bmal1^{flox}$ animals. A possible explanation for the overeating phenotype of both AT clock knock-outs are decreased levels of poly-unsaturated fatty acids. Poly-unsaturated fatty acids are able to suppress food intake by crossing the blood brain barrier (BBB) and acting on MBH neurons (Lam, Pocai et al. 2005, Cintra, Ropelle et al. 2012). In mice with disrupted AT clocks, blood levels of poly-unsaturated fatty acids are decreased due to a down-regulation of enzymes involved in their biosynthesis. ELOVL fatty acid elongase 6 (*Elovl6*) and stearoyl-coA desaturase 1 (*Scd1*) harbor *E-boxes* in their promoter regions and are direct targets of the adipose clock (Hogenesch, Gu et al. 1998, Paschos, Ibrahim et al. 2012). Low poly-unsaturated fatty acid blood levels lead to elevated appetite during rest phase hours and thereby to an increased obesity risk. Still, these animals have normal systemic and local insulin sensitivity in adipose tissues, suggesting that the adipose tissue clock is not involved in insulin sensitivity regulation (Paschos, Ibrahim et al. 2012).

1.9.5.2 Regulation of AT by clock regulated hormones

Circadian regulation in AT is governed *via* neuronal innervation, but also endocrine factors. Several hormones affecting adipose physiology display diurnal variations in blood. They may contribute to the temporal regulation and fine-tuning of AT rhythms in anticipation of diurnal energy demands. Disruption of circadian rhythms is linked to altered adipose function and disrupted endocrine rhythms might impinge on this outcome by enhancing or impairing rhythmic AT physiology.

Clock-regulated hormones like GCs or insulin influence a vast spectrum of biological processes and also affect adipose tissue physiology.

1.9.5.3 Glucocorticoids

Glucocorticoids are best known for their involvement in stress-related responses. In stress the adrenal cortex is activated by adrenocorticotropic hormone (ACTH) to release glucocorticoids – mainly cortisol in humans and corticosterone in nocturnal rodents – within minutes. During an acute response GC levels peak to high concentrations, but an underlying diurnal rhythm of GC release can be found under non-stressed conditions. This diurnal GC rhythm is regulated by clocks along the HPA axis regulating autonomic activity and ACTH responses (Oster, Damerow et al. 2006, Oster, Challet et al. 2017). GCs negatively feed back to the hypothalamus and anterior pituitary to inhibit the production of corticotrophin releasing hormone (CRH) and ACTH. This feedback response results in a pulsatile secretion pattern of HPA axis hormones overlaying acute and circadian GC regulation. Chronic stress subverts this feedback control mechanism and GC baseline levels become permanently increased, promoting elevated blood glucose levels, hyperinsulinemia and insulin resistance (Spiga, Walker et al. 2014).

1.9.5.4 Insulin

Insulin is a metabolic key hormone with a broad spectrum of functions, but best known for its regulative activity in blood glucose levels. It is also a potent regulator of lipid metabolism and circulating insulin levels as well as tissue insulin sensitivity display circadian variations (La Fleur, Kalsbeek et al. 1999, Rudic, McNamara et al. 2004, Lamia, Storch et al. 2008, Shi, Ansari et al. 2013). While tissue specific disruption of the positive TTL arm (*Bmal1* or *Clock*) results in hypoinsulinemia (Marcheva, Ramsey et al. 2010, Sadacca, Lamia et al. 2011), the deletion of negative components (*Pers* or *Crys*) leads to hyperinsulinemia (Barclay, Husse et al. 2012, Zhao, Zhang et al. 2012). Insulin secretion from pancreatic beta cells can be further modulated by the circadian clock as a number of CCGs are associated with peptide secretory pathways (Perelis, Marcheva et al. 2015, Saini, Petrenko et al. 2016). Through activation of the mitogen-activated protein kinase

and phosphoinositide 3 kinase (PI3K) pathways in hepatocytes, insulin mediates food-induced liver clock resetting by stimulating *Per1* and *Per2* expression (Tahara, Otsuka et al. 2011, Yamajuku, Inagaki et al. 2012). The canonical insulin signaling pathway that subserves most of the metabolic insulin functions involves the activation of PI3K and serine-threonine protein kinase (AKT) (Dimitriadis, Mitrou et al. 2011). In consequence, glucose uptake is promoted by increased surface expression of GLUT4 and glycolysis in adipocytes and muscle cells due to stimulation of hexokinase and 6-phosphofructokinase activity (Garvey, Maianu et al. 1998, Dimitriadis, Mitrou et al. 2011).

High insulin levels inhibit adipose lipolysis by reducing hormone sensitive lipase (HSL) activity *via* protein phosphatase-1 activation and subsequent dephosphorylation of HSL and reduced cAMP signaling. Simultaneously, TG uptake for fatty acid biosynthesis is increased by lipoprotein lipase (LPL) activation (Coppack, Patel et al. 2001, Frayn 2002, Dimitriadis, Mitrou et al. 2006). *De novo* lipogenesis (DNL) is promoted by insulin by upregulating the expression of carbohydrate-responsive element-binding proteins (ChREBPs or Mlxipl) (Eissing, Scherer et al. 2013), but has different outcomes in different tissues. While in liver DNL causes increased lipid accumulation and increases the risk for non-alcoholic fatty liver disease with insulin resistance, steatohepatitis and elevated serum TGs (Postic and Girard 2008, Hudgins, Parker et al. 2011, Despres 2012). Increased DNL in AT is associated with improved insulin sensitivity (Roberts, Hodson et al. 2009), which additionally is modulated by the insulin-sensitizing fatty acid species palmitoleate (Cao, Gerhold et al. 2008). Interestingly, local clock deletion in adipocytes does not influence insulin sensitivity, while SCN-lesioned mice and mice with a global clock impairment display systemic insulin resistance (Rudic, McNamara et al. 2004, Paschos, Ibrahim et al. 2012, Coomans, van den Berg et al. 2013, Shi, Ansari et al. 2013). It is, thus, possible that the SCN clock directly governs insulin sensitivity in adipose tissue, as ATs are directly connected to the SCN via ANS projections (Bartness, Shrestha et al. 2010).

1.10 Aims of this work

Circadian clocks can be found in every nucleated cell in the mammalian body. They temporally organize physiology by orchestrating and priming cellular processes, adapting them to predictable environmental changes. The circadian master clock in the SCN regulates daily activity and rest phases and aligns itself to the external light and dark cycle. Without external time cues it continues to oscillate with its endogenous period and transfers this temporal information to all other tissues. Hereby, the SCN synchronizes peripheral organ clocks amongst themselves and with external time. While global clock dysfunction and operative SCN lesioning is known to have negative effects on glucose and lipid metabolism, both manipulations lead to a global ablation of rhythms, thus making it difficult to distinguish between systemic and local clock effects on tissue function. In this project, I aimed at closing this gap, specifically studying the interplay of central SCN with peripheral clock function in white adipose tissue with a focus on metabolic regulation.

To address this question, I used a mouse line with a tissue-specific genetic clock deletion in the master pacemaker to delineate the influence of SCN clock function on circadian adipose clock physiology. I first analyzed circadian transcriptome regulation in white adipose tissue. Second, I studied the long-term effects of dysfunctional master and adipose clocks on metabolic homeostasis and glucose metabolism.

2 Material and Methods

2.1 Animal experiments

All animal experiments were done with prior permission by the “Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume Schleswig-Holstein (MELUR)” with the personal file reference of V 312-7224.122-4 of the state of Schleswig-Holstein and in accordance with the German Law of Animal Welfare.

2.1.1 Animal housing and breeding

Mice were housed in transparent, individually ventilated cages (IVCs) with filter tops. Up to five animals in one cage were maintained under constant temperature (23 °C) and humidity (55-60 %) conditions. If not stated otherwise, mice were provided with breeding chow (Altromin, Lage, Germany) and water *ad libitum* and kept in LD cycles with 12 hours "lights on" (300 lux white fluorescent light) and 12 hours "lights off" (12:12 LD). “Lights on” was defined as *Zeitgeber* time (ZT) 0 and “lights off” as ZT 12. At the minimum age of 7 weeks animal breedings were established ether in pairs or triplets with two females and one male. Pups were weaned at the age of 3-4 weeks and their ears marked for individual identification. Ear biopsies were used for genotype analysis.

2.1.3 SCN mutant (SCN-KO) mouse breeding

A mouse line with a tissue-specific deletion of the clock component *Bmal1* (*Arntl*) in the SCN region was generated by controlling CRE recombinase expression under the synaptotagmin 10 (*Syt10*) promoter.

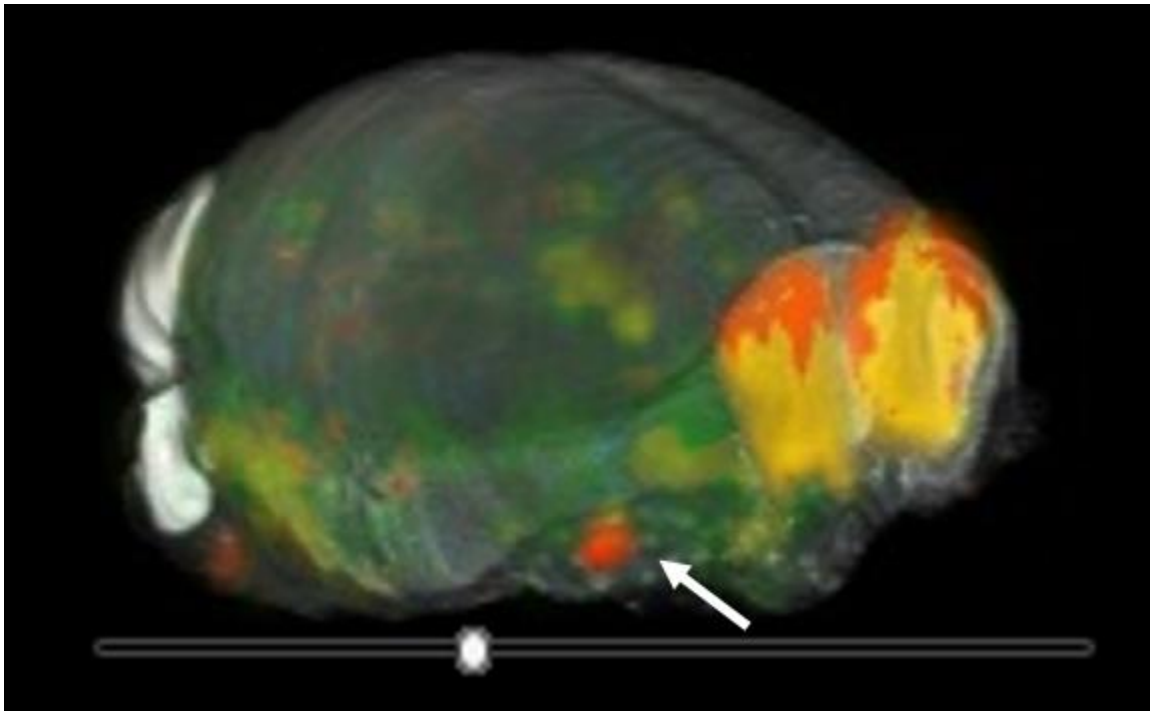


Figure 6: Synaptotagmin 10 expression distribution in the murine brain

Synaptotagmin 10 (*Syt10*) expression (green = low, red = high) throughout the murine brain (Allen Brain Atlas, brain-map.org). *Syt10* is highly expressed in the SCN region (white arrow).

By crossing *Syt10*^{Cre} mice to animals with a *loxP*-flanked *Bmal1* allele animals were generated in which *Bmal1* was conditionally deleted in SCN neurons. *Syt10*$^{Cre/Cre}$ x *Bmal1*$^{flox/-}$ mice have previously been shown to contain a dysfunctional SCN clock and display arrhythmic locomotor activity under constant darkness conditions (Husse, Zhou et al. 2011). *Syt10*$^{Cre/Cre}$ x *Bmal1*$^{+/-}$ littermates were used as rhythmic controls. All animals were kept on a C57BL/6J genetic background.

2.1.4 Wheel-running analysis

To record wheel-running activity, mice were singly housed in transparent plastic cages (Tecniplast 1155M) equipped with a steel running-wheel (11.5 cm diameter, Trixie 6083, Trixie GmbH, Germany). Rotations of the running-wheel

were measured by a magnetic switch and the signal transmitted to a computer running the activity counting software ClockLab (Actimetrics, Austin St. Evanston, USA). Running-wheel cages were changed approximately every two weeks. Running-wheel data analysis was performed using the ClockLab analysis software plug-in (Actimetrics) for MatLab (The Mathworks). The first seven days of measurement were designated as adaptation time for the animals and excluded from analysis. The magnitude of periods were determined by $\chi 2$ periodogram analysis using GraphPad Prism software (GraphPad, La Jolla, USA).

2.1.5 Energy expenditure

Oxygen consumption and carbon dioxide production were investigated under standard housing conditions at 23 °C in an open-circuit indirect calorimetry system (TSE Systems, Bad Homburg, Germany). Mice were singly housed in standard cages prior to the experiment and for a better transition adapted one week in advance to the drinking bottles of the measurement chambers. Water and food were provided *ad libitum* throughout the experiment and their consumption measured in 10-min intervals. Mice were allowed to acclimatize to the system for three days and then oxygen consumption was first measured for two days in LD followed by three days in DD conditions. Only the last two days of DD were used for analysis. Body weight was measured daily and oxygen consumption normalized to the individual body weight.

2.1.6 Temperature measurement

Animal body temperature was determined using a FLIR (Flir, Wilsonville, USA) thermal imaging camera. The animals were singly housed and for measurements put on top of their home cages. Thermal images were taken from the ear, a body region where the measured temperature is representative for the core body temperature. Ear temperatures on images were determined using FLIR Tools analysis software.

2.1.7 Tissue collection and blood collection

Animals were sacrificed at indicated time points by cervical dislocation. If animals were killed during the dark phase, they were handled under red light and after killing eyes were quickly removed to prevent acute light effects on gene expression. Tissues were collected and snap-frozen on dry-ice or liquid nitrogen. All tissues were kept at -80 °C until further processing.

To analyze serum leptin concentrations trunk blood was collected after mice were killed by cervical dislocation. Blood samples were left 30 min at room temperature to clot and then centrifuged (500 x g, 4 °C, 30 min). Supernatant was stored at -80 °C until use.

2.1.9 Restricted feeding

In order to test the synchronizing effects of timed food intake on peripheral clocks in DD conditions and their role in metabolic homeostasis chow food access was limited to 12 hours per day. The food access time in DD was congruent to the dark phase prior to the experiment lasting from 8 p.m. in the evening to 8 a.m. in the morning.

2.2 Immunobiological experiments

2.2.1 Leptin enzyme-linked immunosorbent assay (ELISA)

All reagents used for the sandwich ELISA, with the exception of demineralized water for dilutions, were provided by the Mouse Leptin ELISA Kit (Millipore, Billerica, USA) and stored at 2-8 °C until used. Immediately before usage all reagents were pre-warmed to room temperature. Mouse leptin standard (30 ng/ml) was diluted with assay buffer (0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide, 0.05% Triton X-100 and 1% BSA) to 30.00, 15.00, 7.50, 3.75, 1.88, 0.94, 0.47 and 0.23 ng/ml for standard curve generation.

10x HRP wash buffer (50 mM Tris Buffered Saline containing Tween-20) was diluted with demineralized water and the *Leptin Microtiter Plate* washed three times

with 300 µl wash buffer. 30 µl assay buffer and 10 µl rat/mouse leptin matrix solution (0.08% sodium azide) were added to background, standard and quality control wells and 40 µl assay buffer to sample wells. 10 µl of standards, quality controls, assay buffer for background and samples were added to the according wells with 50 µl rat/mouse leptin antiserum (pre-titered anti-rodent leptin serum), sealed with an adhesive plate sealer and incubated for two hours at room temperature on a plate shaker at 400 rpm. Then the plate was washed three time with 300 µl wash buffer, 100 µl rat/mouse leptin detection antibody (pre-titered biotinylated anti-mouse leptin antibody) added and the sealed plate incubated at room temperature for an hour on a plate shaker at 400 rpm. The plate is washed again three times with 300 µl wash buffer and 100 µl enzyme solution (streptavidin-horseradish peroxidase conjugate) added and the sealed plate incubated at room temperature for 30 min on a plate shaker at 400 rpm. Then the plate was washed six times 300 µl wash buffer, 100 µl substrate solution (3, 3',5,5'-tetramethylbenzidine) added and 100 µl stop solution (0.3 M HCl) added after 5 – 20 min when high concentrated leptin standards displayed a deep blue color.

Stop solution changed the blue color to yellow and the absorbance was determined at 450 and 590 nm with the Epoch microplate spectrometer (BioTek, Winooski, USA). The reference curve was calculated from leptin standards and leptin concentration in quality controls and samples calculated accordingly.

2.3 Molecular biological experiments

2.3.1 DNA extraction from mouse ear biopsies

Ear biopsies (Ø 2mm) were taken at the age of weaning (3-4 weeks) and digested in 21 µL extraction buffer (50 mM KCl, 10 mM Tris/HCl (pH 8.3), 2.5 mM MgCl2, 0.1 mg/mL gelatine, 0.45 % v/v NP40, 0.45 % v/v Tween 20, 0.015 µg/µL proteinase K, Roche) at 55 °C for 1 h. 500 µL of demineralized water were added and proteinase K was inactivated by heating the samples up to 95 °C for 10 min. The samples were centrifuged (500 x g, 1 min) and stored at 4 °C and the supernatant was used for genotype analysis.

2.3.2 Genotyping PCRs

2.3.2.1 Bmal1-flox genotyping

Setup for one reaction:

1 µL DNA

2 µL 10x ammonium buffer (Thermo Scientific, Waltham, USA)

1 µL dNTPs (dNTP mix, Thermo Scientific)

1.5 µL $MgCl_2$ (Thermo Scientific)

1 µL primer forward (Bmal-flox-F)

2 µL primer reverse (Bmal-flox-R)

1 µL primer forward (Bmal-KO-F)

0.1 µL Taq polymerase (Taq DNA Polymerase, Ampliqon, Stenhuggervej, Denmark)

10. 4 µL demineralized water

Σ 20 µL reaction volume

Program for thermocycler:

3:00 min 94 °C

0:30 min 94 °C ┐

0:30 min 59 °C x 35

1:00 min 72 °C ┘

7:00 min 72 °C

4 °C

Wild-type band: 327 bp / *Bmal1-flox* band: 431 bp / *Bmal1* deletion band: 570 bp

2.3.2.2 Synaptotagmin10-Cre genotyping

Setup for one reaction:

1 µL DNA

2 µL 10x ammonium buffer (Thermo Scientific)

1 µL dNTPs (Thermo Scientific)

1.5 µL $MgCl_2$ (Thermo Scientific)

2 µL primer forward (Syt 10F)

1 µL primer reverse (Syt 10R)

1 µL primer reverse (Syt 10Kir)

0.1 µL Taq polymerase (Ampliqon)

10.4 µL demineralized water

Σ 20 µL reaction volume

Program for thermocycler:

3:00 min 94 °C

0:30 min 94 °C ┐

0:30 min 64 °C x 38

1:00 min 72 °C ┘

7:00 min 72 °C

4 °C

Wild-type band: 426 bp / mutant band: 538 bp

Table 2: Genotyping primer sequences

Primer	DNA sequence
Bmal-flox-F	5´-ACTGGAAGTAACTTTATCAAACTG-3´
Bmal-flox-R	5´-CTGACCAACTTGCTAACAATTA-3´
Bmal-KO-F	5´-CTCCTAACTTGGTTTTTGTCTGT-3´
Syt 10F	5´-AGACCTGGCAGCAGCGTCCGTTGG-3´
Syt 10R	5´-AAGATAAGCTCCAGCCAGGAAGTC-3´
Syt 10Kir	5´-GGCGAGGCAGGCCAGATCTCCTGTG-3´

2.3.3 mRNA isolation and cDNA synthesis

mRNA was isolated from frozen (-80 °C) tissues using TRIzol (Invitrogen Carlsbad, CA), according to the manufacturer´s protocol. Isolated mRNA was dissolved in ddH_20 and the concentration determined with the Epoch microplate spectrometer (BioTek). Samples were stored at -80 °C until further analysis. cDNA was synthesized from mRNA samples with the MultiScribe Reverse Transcription Kit (Applied Biosystems, Foster City, USA).

Setup for one reaction (all reagents provided by the MultiScribe Reverse Transcription Kit (Applied Biosystems)):

2 µL 10x RT buffer

0.8 µL 25x dNPT mix

2 µL 10x random primers

1 µL MultiScribe RT

4.2 µL nuclease-free water

Σ 10 µL reaction volume

2-3 µg RNA of samples were aliquoted into PCR tubes, filled up to 10 µL with nuclease-free water and 10 µL reaction volume added.

Program for thermocycler:

10:00 min 25 °C

120:00 min 37 °C

05:00 min 85 °C

4 °C

cDNA was diluted 1:20 with nuclease-free water (Applied Biosystems) and stored at -20°C.

2.3.4 Quantitative real-time PCR (qPCR)

Gene expression was measured by quantitative real-time PCR, which was performed on a iCycler thermocycler (Bio-Rad, Hercules, USA) with iQ-SYBR Green Supermix (Bio-Rad) according to the manu-facturer´s protocol. Primer pairs (Table 3) were designed with Primer3 (http://frodo.wi.mit.edu/ cgibin/ primer3/primer3www.cgi) with an approximate amplicon size of 200 bp using cDNA sequences from NCBI Entrez Gene (www.ncbi.nlm.nih.gov). To avoid possible cross reactions on homology sites, primer-gene specificity was evaluated with BLASTn (www.ncbi.nlm.nih.gov/blast). To determine the amplification efficiency a standard curve with a cDNA dilution series of 1:10, 1:40, 1:160, 1:640 and 1:2,560 was generated. Data analysis was performed using Excel (Microsoft, Redmond, USA) software by fitting a linear regression. The amplification efficiency indicates the amount of cycles needed for doubling the amount of cDNA. Ideal doubling rates approximate 1 cycle (efficiency = 100 %). Only primer pairs with an experimentally determined efficiency of 90-110 % were used. For the analysis 5 µl of 1:40 diluted cDNA samples were applied in duplicates to a 96-well qPCR plate containing 10 µl DNA Master SYBR Green I mix (Bio-Rad), and 5 µl primer mix (1.4 µM) in each well.

Program for thermocycler:

5:00 min 94 °C
0:15 min 94 °C ┐
0:15 min 60 °C x 40
0:20 min 72 °C ┘
5:00 min 72 °C

To control for primer pair specificity a melt curve was determined with a temperature gradient (65-95 °C) with positive increments of 0.5 °C per 10 sec. CT (threshold cycling) values were exported from the iCycler software (Bio-Rad) and relative expression values were obtained by ΔΔCT normalization against the house keeping gene, eukaryotic elongation factor 1 alpha (*Eef1α). Eef1α* is consistently and at constant levels expressed in all evaluated tissues. All target gene values were normalized against the average of each circadian profile for each gene.

Table 3: qPCR primer sequences

Primer	DNA sequence
Ef1α	Forward primer 5'-CACATCCCAGGCTGACTGT-3' Reverse primer 5'-TCGGTGGAATCCATTTTGTT-3'
Bmal1	Forward primer 5'-ATCAGCGACTTCATGTCTCC-3' Reverse primer 5'-CTCCCTTGCATTCTTGATCC-3'
Per2	Forward primer 5'-GCCAAGTTTGTGGAGTTCCTG-3' Reverse primer 5'-CTTGCACCTTGACCAGGTAGG-3'
Dbp	Forward primer 5'-AATGACCTTTGAACCTGATCCCGCT-3' Reverse primer 5'-GCTCCAGTACTTCTCATCCTTCTGT-3'

2.4 Metabolic experiments

2.4.1 Glucose, insulin and pyruvate tolerance tests

Application solutions for the glucose (GTT), insulin (ITT) and pyruvate (PTT) tolerance tests were all prepared with isotonic 0.9 % NaCl solution (Fresnius Kabi, Bad Homburg, Germany). For the glucose and pyruvate solutions powdered D(+)-glucose (ROTH, Karlsruhe, Germany) or sodium pyruvate (SIGMA) were directly

dissolved in 0.9 % NaCl, while for insulin human insulin stock solution (1000 U/mL) (SIGMA, St. Louis, USA) was diluted with saline to the final working concentration (see Table 4). Mice were fasted before and during the test (see Table 4). Fasting for the GTT and PTT in the LD experiment started at ZT 12 and the test was conducted at ZT 4 the following day. ZT 4 corresponded to 10:00 a.m. and with the same fasting period the DD *ad libitum* group was tested at this time. ITTs were conducted for all groups at ZT 4 after 4 hours of fasting. For the RF group the normal fasting period of 12 hours was prolonged to 16 hours and animals tested at 0:00 a.m.. The initial blood glucose value (mg/dL) was directly measured with a glucometer (ACCU-CHEK Aviva, Roche, Basel, Switzerland). Therefore, the mice were fixed in a mouse securer and blood drawn from the tail tip. Afterwards the substrate (see Table 4) was applied via intraperitoneal (ip) injection and the blood glucose concentration measured 15, 30, 90 and 120 minutes after injection.

Table 4: Tolerance test stock solution, final concentrations and fasting periods

	Stock solution concentration	Final substrate concentration	Fasting period duration
GTT	0.125 g/mL	1.5 g/kg	16 h
ITT	10^{-3} U/mL	1 U/kg	4 h
PTT	0.1 g/mL	2 g/kg	16 h

2.5 Microarray hybridizations

Isolated mRNA from eWAT was sent to the microarray core facility of the University of Göttingen, where cRNA synthesis and labeling was performed according to standard protocols. cRNA was hybridized to GeneChip Mouse Gene 2.x ST Arrays (Affymetrix, Santa Clara, CA). Raw fluorescent intensity values were normalized using the MAS5 algorithm and Affymetrix Expression Console software.

2.5.1 Circadian rhythm analysis

Gene expression data of 4 time points was analyzed for circadian rhythmicity by sine/cosine curve fitting using the CircWave software (EUCLOCK, Munich, Germany).

$$f(t) = a_0 + \sum_{i=1}^{\infty} \left(p_i \sin i 2\pi \frac{t}{\tau} + q_i \cos i 2\pi \frac{t}{\tau} \right)$$

Only fits with a p-value lower than 0.05 and a minimum relative circadian amplitude of 25 % were considered rhythmic. Peak times were calculated from the curve fitting (equation 2.5.1). To compare the rhythmic properties of specific genes between genotypes 24-h sine wave fits were compared with the GraphPad Prism software (GraphPad).

2.5.2 Gene enrichment analysis

Gene set enrichment analysis was performed using the open source web based tool WebGestalt (Zhang, Kirov et al. 2005) that determines statistical enrichment of gene sets by comparing them to the Gene Ontology (GO) database. Gene sets are tested against the whole-array set (mmusculus_affy_mogene_ 2_0_st-v1) using hypergeometric analysis with Benjamini-Hochberg correction for multiple testing ($p < 0.05$; $n \geq 2$). Only GO categories with fewer than 500 entries were considered to avoid too broad assertions.

2.6 Magnetic resonance imaging

For quantification of adipose tissue mass and origin, animals were sacrificed by cervical dislocation and measured in a small animal MRI scanner (Aspectimaging, Hevel Modi´in, Israel). To improve signal to noise ratio (SNR) mice were placed centrally in the tightest fitting coil (Mouse Body L50 D30 Serial 1, Serial Number 71014549, Aspectimaging) and for optimal fat/lean mass distinction in soft tissue measured with T1-weighted settings (TE/TR = 10/322.434,

FOV = 60x30 mm, 21 slices of 1 mm, measurement time 5:59 min). To distinguish between subcutaneous and epididymal origin adipose tissue regions of interest (ROIs) were manually marked in every slide and the tissue volume interpolated by using the Aspectimaging quantification software (Aspectimaging).

2.7 Statistical analysis

Data are expressed as means ± SEMs. Statistical comparisons were made using Graph Pad Prism software. Student´s t-tests were used for one-to-one comparisons, one-way ANOVAs for comparison of more than two groups. For time and group interactions two-way ANOVAs with Bonferroni post-test were used. P-values < 0.05 depict significant effects.

3 Results

3.1 Behavior and metabolic phenotype of SCN-KO mice

In order to investigate brain-adipose communication in regards to circadian rhythmicity we genetically deleted the molecular clock function in the murine master pacemaker (Husse, Zhou et al. 2011). The SCN clock controls daily activity rhythms and perpetuates them also in the absence of external time clues (Stephan and Zucker 1972, Ralph, Foster et al. 1990). To test the circadian behavior of SCN-KO, I analyzed the circadian locomotor activity with running wheels under different light conditions. Under a 12 hours light, 12 hours dark (LD) cycle SCN-KO mice displayed normal 24 h entrainment to the light cycle comparable to *Syt10*$^{Cre/Cre}$*Bmal1*$^{+/-}$ littermate controls (Con), displaying their main activity during the dark hours (Fig. 7A). After 8 days in LD, light conditions were changed to constant darkness (DD) to investigate the intrinsic free-running periods of the animals. While Con animals displayed activity periods of around 24 h (23.78 ± 0.09 h), no period could be fitted to the running-wheel behavior of SCN-KO animals in DD due to their arrhythmicity (Fig. 7B, right panel).

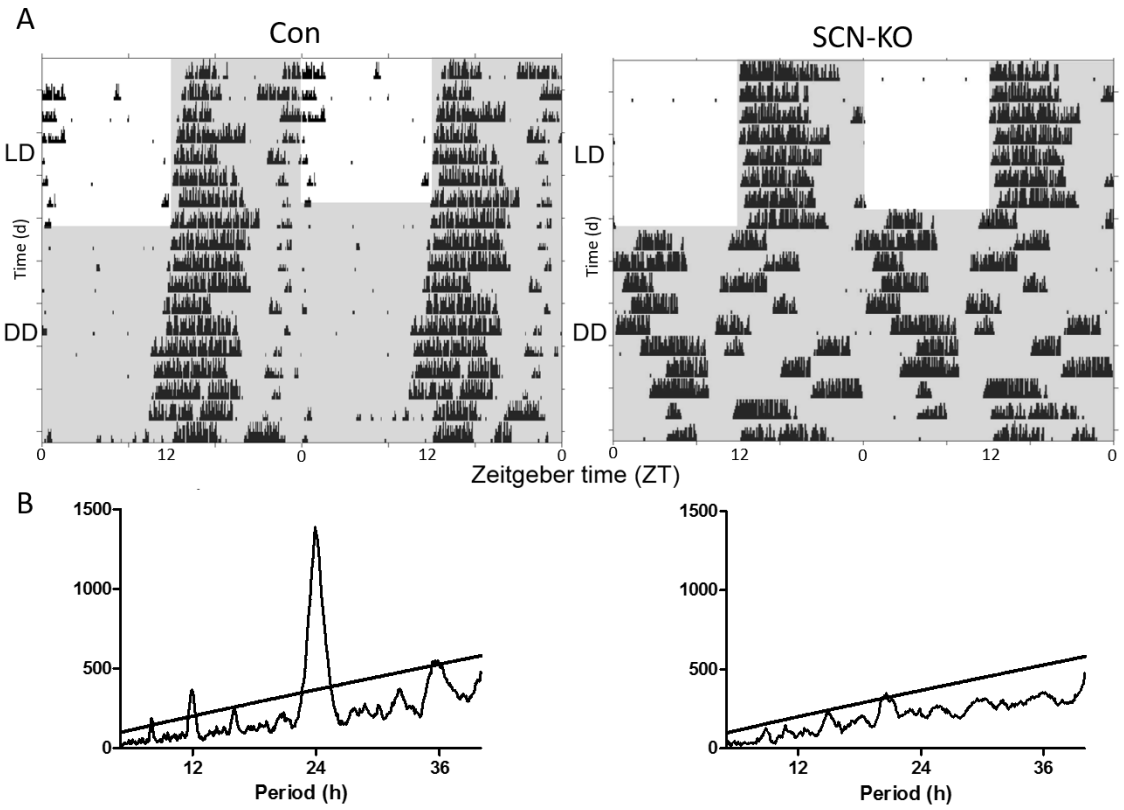


Figure 7: Wheel running behavior of SCN-KO mice becomes arrhythmic in DD

(A) Representative actograms of Con and SCN-KO animals in LD (day 1-8) and DD (day 9-18). White and grey shaded areas indicate light and dark phases, respectively. (B) Representative periodogram of Con (left) and SCN-KO (right) in DD. Magnitude of periods were determined by χ2 analysis (n=6).

Food intake is a strong synchronizing factor for peripheral tissues and can uncouple peripheral clocks from the SCN if restricted to the normal rest phase (Damiola, Le Minh et al. 2000). To test if this possible entrainment factor is still rhythmic in SCN-KO mice, food intake was measured every 12 hours for ten days in LD. The diurnal pattern with the majority of calories consumed in the dark phase was consistent in Con and SCN-KO animals, but with a blunted day/night difference in SCN-KO (Fig. 8, left panel). Total food intake under LD conditions did not differ between genotypes (Fig. 8, middle panel). For food rhythms in DD, mice were entrained to a 12-hour light-dark cycle for at least seven days and then released into DD. SCN-KO animals displayed rhythmic food intake under LD

conditions, but lost this rhythm in the absence of external light clues in DD. Food was measured every 12 hours for ten days and one representative day chosen for analysis (Fig. 8), obtaining values for the subjective day and night phases. While Con animals kept their intrinsic rhythm of consuming most of their food during the subjective night, the intake rhythm of SCN-KO animals was already abolished on the second day in DD (Fig. 8 right panel). However, total food consumption in DD did not differ between genotypes.

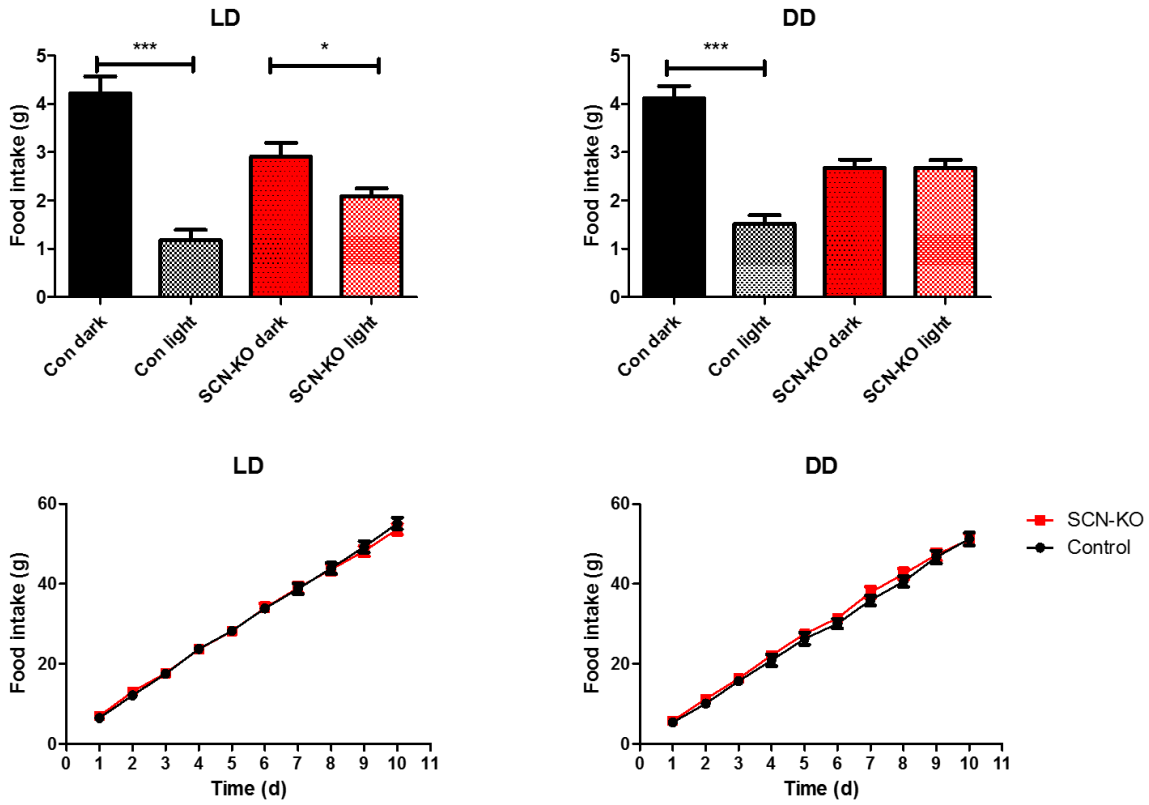


Figure 8: SCN-KO mice consume equal amounts of food but loose feeding rhythms in DD

Food intake profile of Con (black) and SCN-KO (red) mice in the light (ZT0-12) and dark (ZT12-24) phase of one day in g (left panel). The cumulative food intake of Con (black) and SCN-KO (red) mice for ten consecutive days in g (middle panel). Food intake profile on the second day in DD of Con (black) and SCN-KO (red) mice (right panel)., t test, ***: $p < 0.001$, *: $p < 0.05$, n = 10 / genotype

Rhythmic behavior and metabolic rhythms are deeply linked as the daily division of activity and rest phases can not only be monitored on the behavioral, but also

on the metabolic level. During the active phase mammalian organisms preferably utilize carbohydrates, while rest and fasting phases are characterized by increased lipid utilization. The used energy substrate can be determined from the respiratory exchange ratio (RER), which is monitored by indirect calorimetry in metabolic cages. For the calculation the volumes of produced CO_2 and consumed O_2 are measured and the ratio ($RER = \frac{VCO2}{VO2}$) determined. Due to the different quantities of oxygen needed for the energy utilization of lipids and carbohydrates, RER values around 0.7 depict lipid and values around 1.0 carbohydrate metabolism. The RER of wild-type mice switches from values around 0.7 in the light (inactive) phase to around 1.0 in dark (active) phase. To determine if this light cycle-induced behavior had metabolic consequences I measured oxygen consumption and CO_2 production in Con and SCN-KO animals via indirect calorimetry. After acclimatization for several days in LD, CO_2 production and O_2 consumption were determined in 10-min intervals for 4 days in LD, followed by a period of 4 days in DD. Con animals showed a clear diurnal metabolic rhythm with lower (lipid) RER values during the day and higher (carbohydrate) values during the night (Fig. 9 A). These metabolic rhythms were preserved in Con animals in DD conditions (Fig. 9 B). SCN-KO mice displayed a blunted, but still rhythmic diurnal pattern of RER in LD with lower RER values during the night and higher values during the day (Fig. 9 A). In DD the mean RER values of SCN-KO are higher in comparison to LD conditions towards glucose metabolism representing values and no difference between the subjective light and dark phase could be measured (Fig. 9 B).

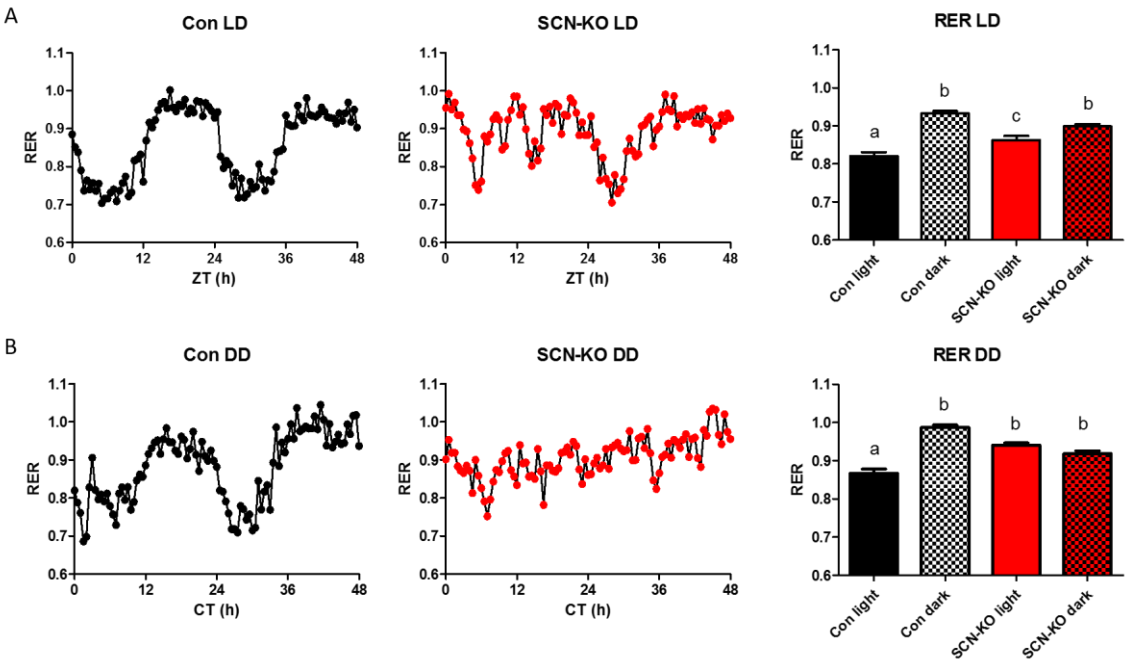


Figure 9: Dampened substrate utilization shifts between lipid and carbohydrate metabolism in Syt10$^{Cre/Cre}$Bmal1$^{flx/-}$(SCN-KO) animals.

(A) Representative diurnal RER profiles for two days in LD of Con (black) and SCN-KO (red) mice and average of RER for light (ZT0-12) and dark (ZT12-24) hours (n=6 per genotype). (B) Representative RER course starting at the second day of DD for Con (black) and SCN-KO (red) mice and average of RER for the subjective light (CT0-12) and dark (CT12-24) hours. One-way ANOVA, n = 6 per genotype.

Another important metabolic parameter that displays diurnal variations is body temperature, which daily oscillation is very robust and displays a nadir during subjective light hours and peaks during the dark phase in mice (Solarewicz, Angoa-Perez et al. 2015, Dispersyn, Sauvet et al. 2017). I tested Con and SCN-KO mice in both light conditions, LD and DD, to evaluate if the synchronizing effects of light (Husse, Zhou et al. 2011) influences daily temperature patterns in SCN-KO mice. Ear temperature, which reassembles body core temperature, of Con mice was rhythmic in both conditions with lowest body temperature during the day in LD (37.7 ± 0.2 °C) and the subjective day in DD (37.6 ± 0.5 °C) and elevated temperatures during the active (LD: 38.3 ± 0.3 °C; DD: 38.4 ± 0.3 °C) phase in both lightning conditions (Fig. 10). SCN-KO mice showed no significant

temperature variation over the day. Their body temperature remained constant in LD (38.1 ± 0.5°C) and DD (38.1 ± 0.3°C).

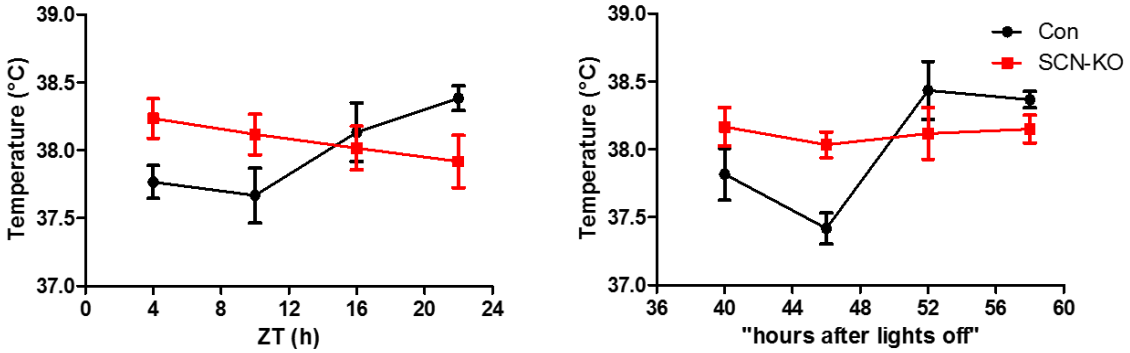


Figure 10: SCN-KO animals display no detectable diurnal temperature oscillation

Ear temperature of Con and SCN-KO animals was measured with an infrared camera in 6 hour intervals over a period of 24 h starting at either ZT 4 in LD (left panel) or 40 hours after "lights off" in DD (the second day of DD, right panel). 2 way ANOVA analysis revealed temperature changes over time for Con in LD and DD and no significant temperature changes for SCN-KO animals in LD and DD Data are shown as mean ± SEM, n=6/genotype.

I next analyzed body composition as an additional important parameter that is changed in SCN lesioned mice in LD conditions (Coomans, van den Berg et al. 2013). As visceral AT depots have more prominent endocrine activity than subcutaneous depots and ATs contributes to the metabolic glucose homeostasis, I analyzed the body composition of 10-week old Con and SCN-KO mice by magnetic resonance imaging (MRI) to distinguish between subcutaneous and visceral AT depots (Fig. 11 A-C). Animals were held in normal LD husbandry conditions with *ad libitum* chow and Con and SCN-KO animals did not differ in weight (Fig. 11 D). Con and SCN-KO animals had more visceral than subcutaneous AT volume, but no difference in AT volumes was observed between genotypes (Fig. 11 E).

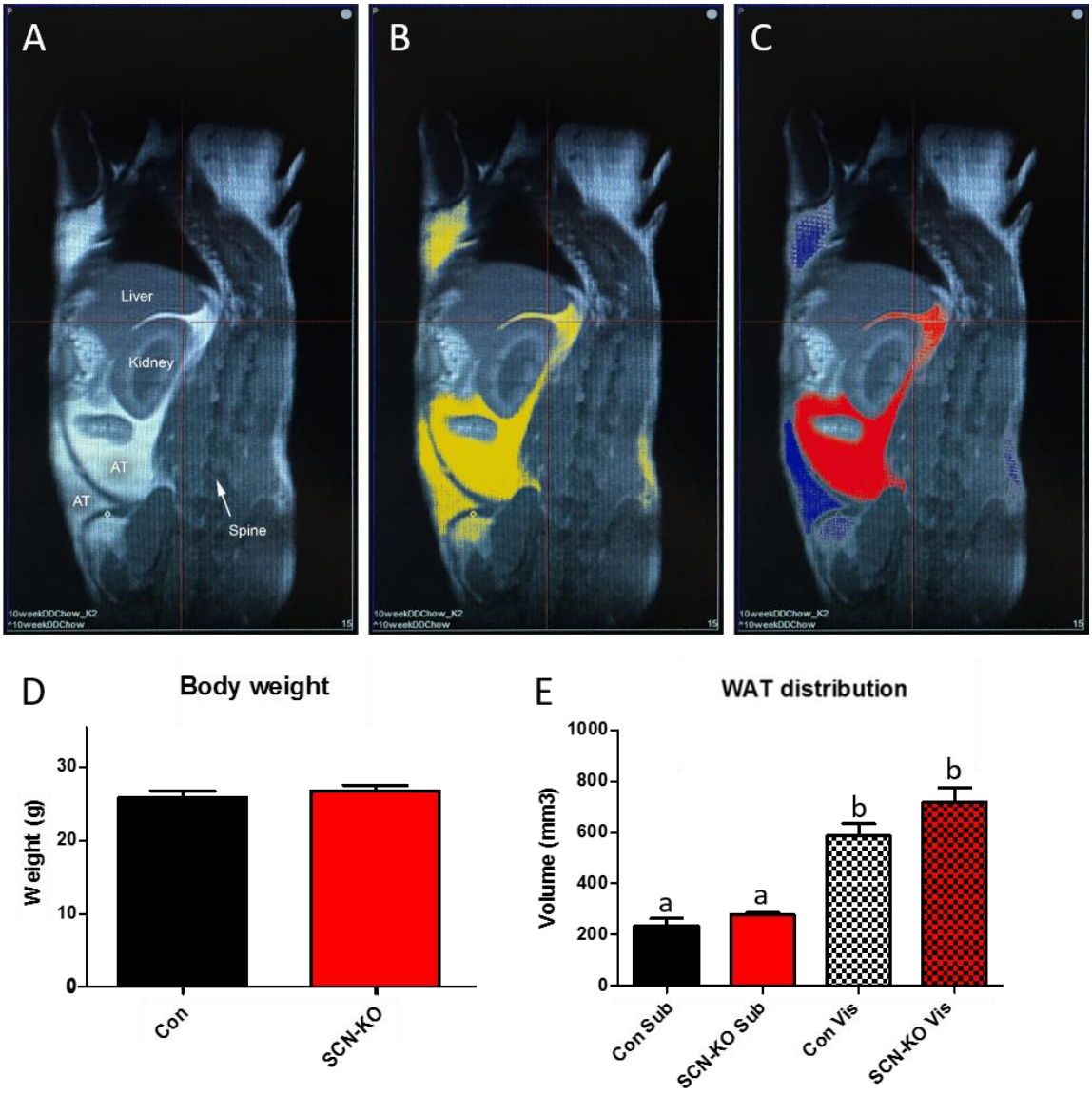


Figure 11: SCN-KO mice at 10 weeks of age have no alteration in weight or body fat distribution

(A-C) Scheme of body fat evaluation. (A) Raw T1 weighted MRI scan of mouse torso. Water containing tissues appear dark, AT containing tissues appear white. (B) Auto RIO scan and colorization of suggested AT areas in MRI image. (C) Manual AT distribution into subcutaneous and visceral depots. (D) Animal weight at the age of 10 weeks. Con and SCN-KO mice (n = 4 per genotype) that were used for the MRI scan had no difference in body weight, t test. (E) Volume of AT depots interpolated out of all sagittal MRI slides per mouse. The volume of visceral and subcutaneous tissues does not differ between genotypes, one-way ANOVA (n = 4 per genotype).

3.2 White adipose tissue microarray analysis

SCN-KO animals displayed alterations in body temperature rhythms and behavior in DD (Fig. 8-10). To get an insight if metabolic consequences are of central or of peripheral origin I chose to further elucidate the circadian transcriptome of SCN-KO animals in an metabolically active organ, white adipose tissue. Epididymal white adipose tissue (eWAT) depots are easy to dissect and are part of visceral (Vis) white adipose tissues. To avoid light-induced masking effects I sampled tissues of LD entrained Con and SCN-KO mice on the second day of DD in 6-hour intervals, starting at 40 hours after “lights off”. The expression levels of 34,390 genes were analyzed in parallel by GeneChip hybridization. Of all analyzed genes 1,955 qualified as rhythmic in Con and 668 in SCN-KO eWAT, so the total number of rhythmic transcripts declined from a total of 5.7 % in Con to 1.9% in SCN-KO (Fig. 12 A). Only a small number of 164 genes was rhythmic in Con as well as in SCN-KO mice (Fig. 12 A). The overall decline in rhythmic transcripts in SCN-KO was accompanied by 504 transcripts which gained rhythmicity in the absence of a master pacemaker (Fig. 12 A). Comparing all phase sorted rhythmic transcripts of Con to the corresponding gene expressions in SCN-KO animals displayed an altered phase distribution (Fig. 12 B). The analysis of peak times of rhythmic transcripts revealed a bimodal distribution in Con animals (Fig. 12 C), with one peak during the subjective day and one in the subjective night. This day-night distribution was lost in SCN-KO animals, where transcript peaks clustered towards the end of the subjective night.

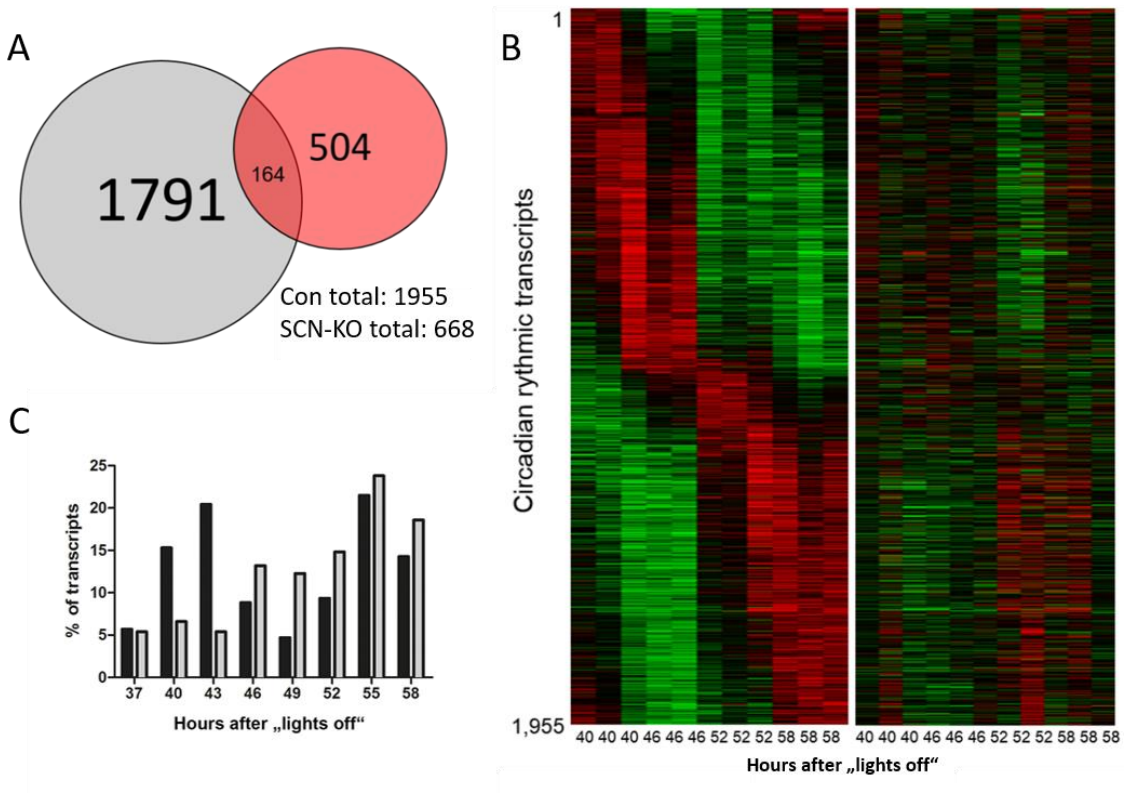
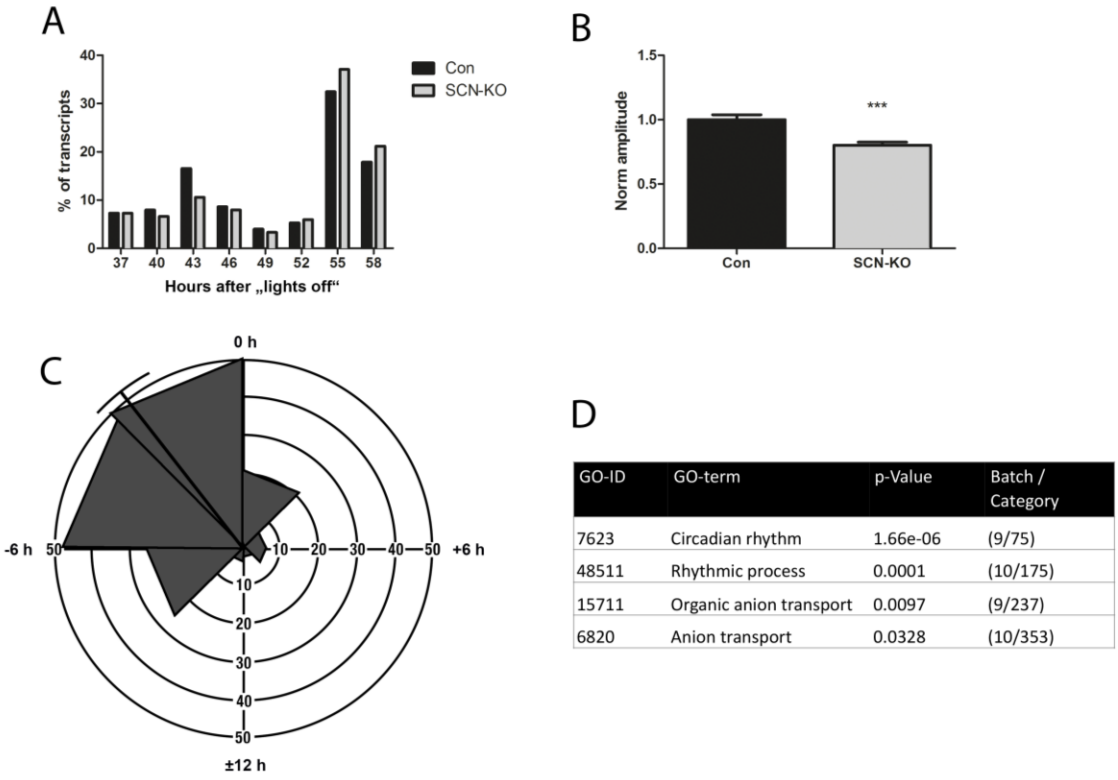


Figure 12: The rhythmic transcriptome of epididymal white adipose tissue in SCN-KO and Con mice

(A) Venn diagram of rhythmic transcripts in eWAT of Con (grey) and SCN-KO (red) animals. (B) Heat map of rhythmic genes in Con animals (right panel) sorted by phase and their counterpart in the SCN-KO gene expression (left panel) with red representing high and green representing low expression. (n = 3 per time point and genotype) (C) Peak time distribution in Con (black) and SCN-KO (grey) animals calculated over 24h in 3 hour bins (modified from (Kolbe, Husse et al. 2016)).

For a more detailed look on the interaction of SCN and local adipose clocks, the three different rhythmic genes groups (rhythmic in both, rhythmic in Con only, rhythmic in SCH-KO only) (Fig. 12 A) were analyzed in detail. The robust transcripts that were found to be rhythmic in both genotypes displayed a similar peak distribution as was seen for the whole rhythmic transcriptome (Fig 13 A). Rhythmic Con transcripts showed a bimodal peak distribution with the first peak during the subjective day and a second during the subjective night (Fig 13 A). In SCN-KO animals, this bimodal distribution was blunted and a higher proportion of peaks shifted towards the subjective late night. A comparison of expression rhythm

amplitudes between Con and SCN-KO revealed an average 20% reduction in fold change in SCN-KO eWAT. By directly comparing the peak times of Con and SCN-KO transcripts with each other, I found that phases were on average advanced for 2.0 h in SCN-KO (Fig. 13 C). Gene ontology enrichment analysis of the 164 transcripts that displayed expression rhythms in Con and SCN-KO revealed a strong overexpression of genes associated with “circadian rhythm”. This strongly enriched category was followed by less specifically defined and higher gene number containing groups like “rhythmic process”, “organic anion transport” and “anion transport” (Fig. 13 D).



GO-ID	GO-term	p-Value	Batch / Category
7623	Circadian rhythm	1.66e-06	(9/75)
48511	Rhythmic process	0.0001	(10/175)
15711	Organic anion transport	0.0097	(9/237)
6820	Anion transport	0.0328	(10/353)

Figure 13: Robust oscillating genes have blunted rhythms and are phase advanced in SCN-KO mice

(A) Peak time distribution of robustly oscillating eWAT transcripts in Con (black) and SCN-KO (grey) animals over a 24-hour distribution in 3-h bins. (B) Normalized circadian amplitudes of rhythmic genes in Con (black) and SCN-KO (grey) animals, n = 164; t test, ***p< 0.001. (C) Rose plot of peak shifts of SCN-KO phases in comparison to Con phases (0 h represents the original Con peak time). Longitudes represent the number of transcripts and angles the phase difference (in 3-h bins). (D) Gene ontology enrichment analysis for robust rhythmic transcripts in Con and SCN-KO mice. Modified from (Kolbe, Husse et al. 2016).

The outcome of the GO enrichment analysis revealed that genes involved in circadian rhythms were significantly overrepresented in the group of genes which were robustly rhythmic in Con and in SCN-KO mice. To see if clock gene rhythms are preserved in eWAT of the SCN-KO mice I determined core clock expression profiles from the array data. Significant expression rhythms were seen in the members of the positive (*Bmal1*, *Clock*) and negative (*Per 1-3*, *Cry 1*) arm of the core TTL and in gene transcripts of the auxiliary clock loops (*Dbp*, *Reverbα/β*) (Fig. 14 A). Rhythm amplitudes in SCN-KO were dampened for *Per2/3*, *Reverbα/β* and *Dbp* (Fig. 14 A) and for all clock genes, with the exception of *Clock,* the phases were advanced in SCN-KO eWAT transcripts (Fig. 14 A). Comparison of the normalized amplitude of all rhythmic clock genes revealed an overall reduction of 30% in SCN-KO amplitudes (Fig 14 B) and a mean phase advance of 4.8 h in SCN-KO eWAT (Fig. 14 C).

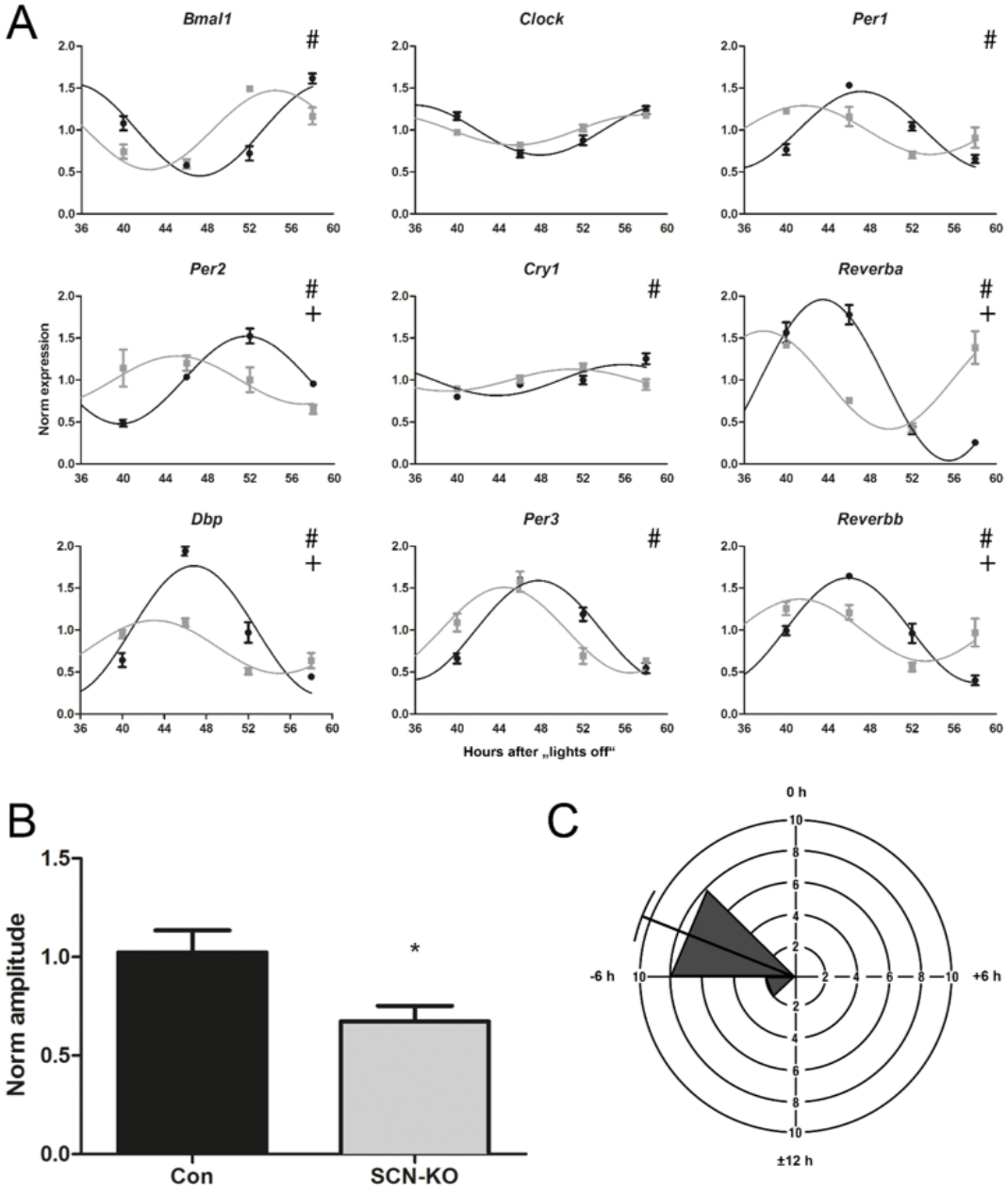


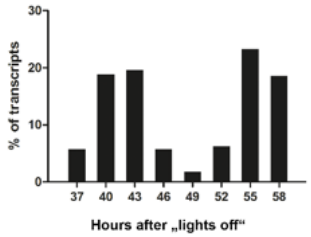
Figure 14: Clock gene rhythms are dampened and phase advanced in SCN-KO eWAT

(A) mRNA expression profiles of clock genes in Con (black) and SCN-KO (grey) animals. 24-h sine wave fittings were compared between genotypes, n = 3 per genotype and time point. #: $p < 0.05$ for phase shift; +: $p < 0.05$ for amplitude difference; extra-sum-of-squares *F* test. (B) Normalized amplitudes of clock genes in Con (black) and SCN-KO (grey) animals; n= 10; *: $p < 0.05$, t test. (C) Rose plot of peak shifts of SCN-KO phases in comparison to Con phases (0 h represents the original Con peak time). Longitudes

represent the number of transcripts and angles the phase difference (in 3-h bins). From (Kolbe, Husse et al. 2016).

Rhythmic gene transcripts that are dependent on a functional SCN clock and lose their rhythmicity in SCN-KO animals displayed a bimodal distribution of their rhythm phases. Rhythm phases peaked during the subjective day (43 h after lights off) and towards the end of the subjective night (55 h after lights off) (Fig. 15 A). For these genes batch GO enrichment analysis revealed a strong overrepresentation of genes associated with energy metabolism. In the ten GO categories with the strongest enrichment I found associations with lipid or carbohydrate metabolism (Fig. 15 B). 36% of the genes in the GO category “cholesterol biosynthetic process” displayed rhythms in eWAT of Con animals. Despite the majority of cholesterol being synthesized in the liver, cholesterol biosynthesis can be also found in adipocytes and various steroidogenic tissues (Panini, Sexton et al. 1986, Heikkila, Kahri et al. 1989). The main starting substrate for cholesterol synthesis is acetyl-CoA which in a rate limiting step is converted into HMG-CoA by HMG-CoA synthetase (encoded by *Hmgsc1*). HMG-CoA is further modified in a multi-enzymatic process into cholesterol (Fig. 15 C). In eWAT of Con mice the transcripts of positive regulatory genes of cholesterol synthesis peaked towards the end of the subjective night, while the negative regulators *Prkaa1* and *Por* peaked towards the end of the subjective day (Fig. 15 C, D upper panel). The rhythm and phase coherences of this process regulating genes was lost in SCN-KO mice (Fig.15 D, lower panel). In summary, a large group of genes in eWAT is dependent on a functional SCN clock to maintain rhythmicity. Especially genes associated with metabolic processes lost their rhythmic expressing which correlates to the previously observed loss in locomotor activity and food intake in DD (Fig. 7).

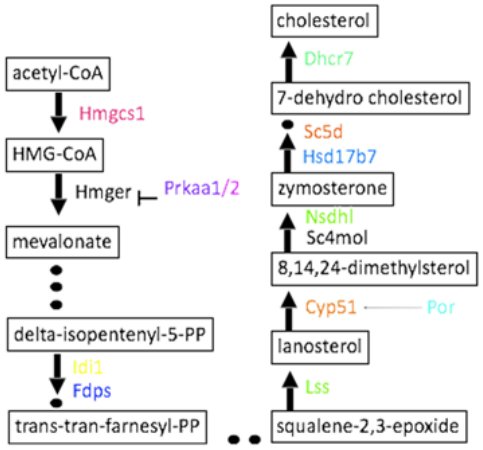
A



B

GO-ID	GO-term	p-Value	Batch / Category
8203	cholesterol metabolic process	2.51e-07	(25/98)
16125	sterol metabolic process	2.51e-07	(26/105)
8610	lipid biosynthetic process	2.74e-06	(57/417)
6066	alcohol metabolic process	1.68e-05	(39/252)
16126	sterol biosynthetic process	1.77e-05	(14/42)
6695	cholesterol biosynthetic process	1.77e-05	(13/36)
44723	single-organism carbohydrate metabolic process	3.99e-05	(55/434)
8202	steroid metabolic process	9.51e-05	(35/234)
44283	small molecule biosynthetic process	0.0002	(47/370)
19318	hexose metabolic process	0.0004	(30/200)

C



D

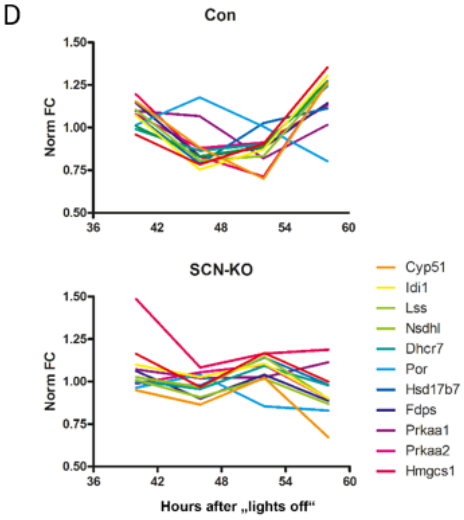


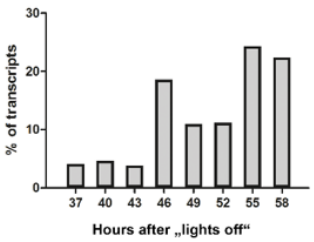
Figure 15: Transcripts that depend on a functional SCN-clock are involved in metabolic regulation

(A) Peak time distribution of transcripts in eWAT that oscillate only in Con animals, calculated over 24 h and represented in 3 h bins. (B) GO enrichment analysis for solei in Con rhythmic eWAT transcripts. (C) Flow chart of the cholesterol biosynthesis pathway depicting the involvement of rhythmic transcripts. (D) Normalized circadian profiles (fold change, FC) of rhythmic transcripts in the cholesterol biosynthesis in Con (upper panel) and SCN-KO (lower panel) mice. From (Kolbe, Husse et al. 2016).

Interestingly a large number (n = 504) of transcripts gained circadian rhythmicity in eWAT in the absence of the master pacemaker clock (Fig. 16). Most transcripts displayed their peaks in transcription towards later phases of the day, starting in the subjective late light phase with the maximum towards the end of the subjective night (Fig.16 A). GO enrichment analysis revealed that genes involved in innate

immunity function were over represented in this group (Fig. 16 B). Out of the 50 genes registered for the GO category “neutrophil chemotaxis” 6 gained rhythmicity in eWAT of SCN-KO animals. These genes are involved in regulating neutrophil migration, such as complement component 3a receptor 1 (*C3ar1*), integrin beta-2 (*Itgb2*), integrin alpha-M (*Itgam*) and high-affinity immunoglobulin epsilon receptor subunit gamma (*Fcer1g*), or in neutrophil adhesion like the NCK-associated protein 1-like (*Nckap1l*). These transcripts displayed minor and not significant oscillations in Con eWAT with elevated expression in the early subjective night (Fig 16 D, left panel). In SCN-KO mice these transcripts gained significant rhythmicity with peak phases around 52 hours after light off. In contrast, the neutrophil adhesion-associated chemoattractant gene chemokine (C-C-motif) ligand 19 (*Ccl19*) was completely phase shifted with a peak time during the subjective midday in SCN-KO mice (Fig. 16 D, right panel). In summary, I found a large group of gene transcripts involved in immune cell regulation that gained defined rhythmic expression in the absence of a functional master pacemaker clock.

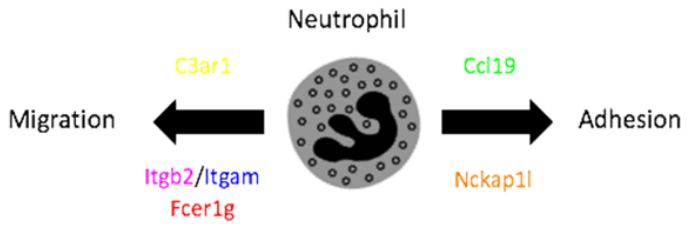
A



B

GO-ID	GO-term	p-Value	Batch / Category
45087	innate immune response	6.69e-05	(18/294)
10324	membrane invagination	0.0013	(5/22)
50900	leukocyte migration	0.0016	(11/163)
30595	leukocyte chemotaxis	0.0017	(9/108)
32103	positive regulation of response to external stimulus	0.0023	(10/142)
34341	response to interferon-gamma	0.0023	(6/44)
32101	regulation of response to external stimulus	0.0027	(16/356)
2690	positive regulation of leukocyte chemotaxis	0.0031	(6/47)
30593	neutrophil chemotaxis	0.0042	(6/50)
7229	integrin-mediated signaling pathway	0.0045	(7/76)

C



D

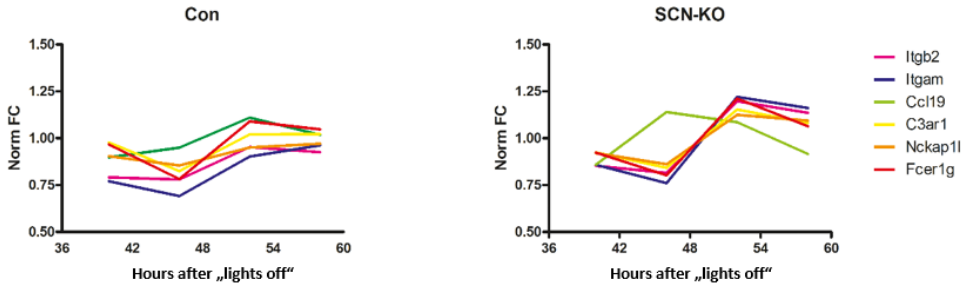


Figure 16: Newly rhythmic transcripts in SCN-KO mice are involved in innate immunity

(A) Peak time distribution of transcripts in eWAT that gained rhythmicity in SCN-KO animals, calculated over 24 h and represented in 3 h bins. (B) GO enrichment analysis for solei in SCN-KO rhythmic eWAT transcripts. (C) Scheme of genes involved in neutrophil chemotaxis in either migration (*C3ar1*, *Itgb2*, *Itgam*, *Fcer1g*) or adhesion (*Ccl19*, *Nckap1l*). (D) Normalized circadian profiles (fold change, FC) of rhythmic transcripts involved in neutrophil chemotaxis in Con (left panel) and SCN-KO (right panel) mice. from (Kolbe, Husse et al. 2016).

AT-derived hormones, so-called adipokines, have multiple functions in the mammalian body and many of them display diurnal differences in their expression (see Table 1). In eWAT of Con, the transcription of *Leptin* peaked during the active

dark hours. This circadian rhythm was abolished in SCN-KO mice and the expression levels remained constant over the course of the day (Fig. 17 A). Visfatin is involved in glucose homeostasis and insulin sensitivity and inhibits neutrophil apoptosis. *Visfatin* expression was rhythmic in Con and SCN-KO animals. However, a comparison of sine curve fits revealed a phase shift of SCN-KO *Visfatin* expression towards the late subjective day instead of the late subjective night (Fig. 17 B). Pai-1 is an inhibitor of fibrinolysis with its natural peak at the beginning of the active phase. It has a suspected role in cardiac diseases and its concentrations rise with the mass of AT. The oscillating expression of *Pai-1* was preserved in SCN-KO eWAT, but its expression rhythm was dampened and phase-advanced in comparison to Con animals (Fig. 17 C). Nesfatin, which is also involved in metabolic homeostasis and glucose metabolism, was not rhythmically expressed in Con or SCN-KO animals, but the overall expression was elevated in SCN-KO eWAT (Fig. 17 D, 2-way ANOVA).

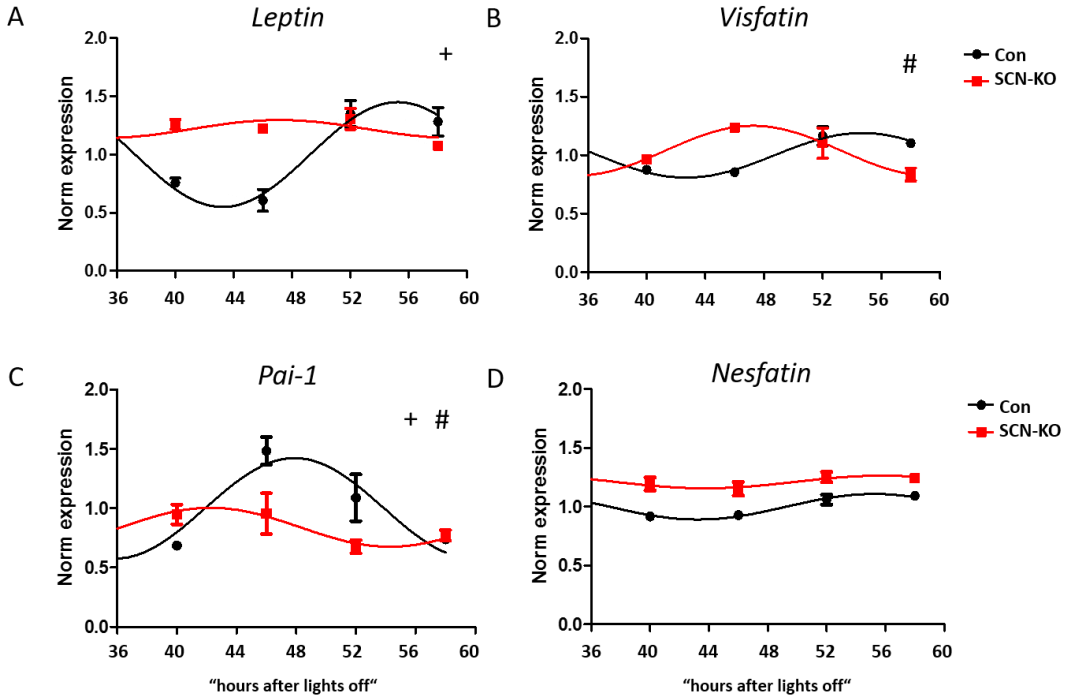


Figure 17: Rhythmic adipokine expressions are altered phase and amplitude in SCN-KO animals

Adipokine mRNA expression in eWAT on the second day of DD in Con (black) and SCN-KO (red) animals. (A) *Leptin* (B) *Visfatin* (C) *Pai-1* (D) *Nesfatin*. Data are shown as mean ± SEM. 24-h sine wave fittings were compared between Con (black) and SCN-KO (red) animals. N=3, #: $p < 0.05$ for phase shift and +: $p < 0.05$ for amplitude difference, ***: Genotype difference 2-way ANOVA, n = 3 per genotype and time point.

The loss of rhythmic *Leptin* transcription in eWAT of SCN-KO (Fig. 17 A) animals in DD prompted me to examine serum leptin levels in LD (Fig. 18 A) and on the second day of DD conditions (Fig. 18 B). Independent of genotype, leptin concentrations were rhythmic and peaked during the early dark phase in LD and the subjective early dark phase in DD (Fig. 18).

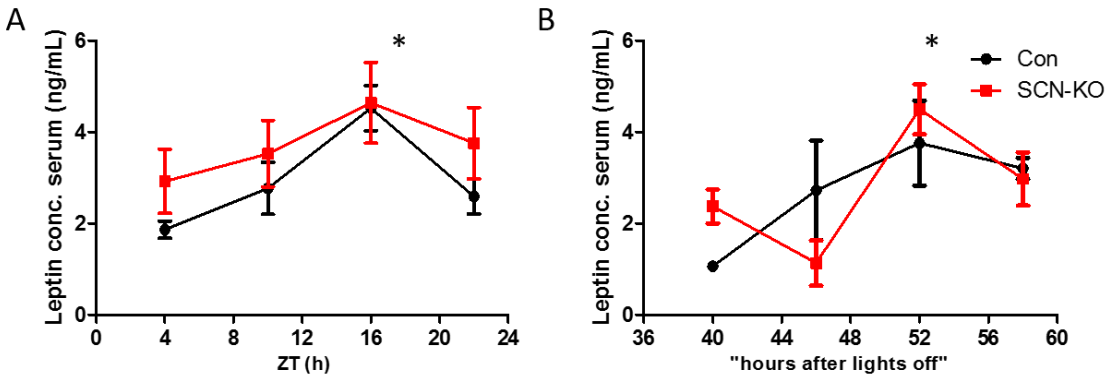


Figure 18: Serum leptin rhythms persist in DD conditions in Con and SCN-KO mice

(A) Leptin serum concentration Con (black) and SCN-KO (red). (B) Leptin serum concentration Con (black) and SCN-KO (red) mice on the second day of DD. Data are shown as mean ± SEM, *: difference over time, 2-way ANOVA, Bonferroni post-test, n = 5 per genotype and time point.

In summary, a high number of metabolic transcripts in epididymal white adipose tissue lost their rhythmicity in DD while the local clock machinery contains rhythmicity, while being dampened and phase advanced. Adipokine transcription revealed distinct changes for different genes in the absence of the master clock function, but these changes in transcription for *Leptin* could not be seen in the respective serum levels.

3.3 Influence of SCN and peripheral clocks on body weight and glucose homeostasis

In the second part of this project I wanted to elucidate the differential effects of central and peripheral clock function on body weight and glucose homeostasis in mice. Therefore I measured the food intake and weight gain of Con and SCN-KO mice for 10 weeks in LD (SCN arrhythmic, periphery rhythmic) and DD (SCN and periphery arrhythmic) conditions and subsequently tested glucose metabolism and analyzed body composition. At the beginning of the experiment all mice were 10 weeks of age (Fig. 11). Until the start of the experiment, mice were held in LD and

with chow and water *ad libitum*. In LD conditions Con and SCN-KO animals consumed the same amount of chow in total and did not differ in weight development (Fig. 19 A & B). Body composition revealed more visceral AT mass than subcutaneous in both genotypes, but no origin specific significant difference was detected between SCN-KO and Con (Fig. 19 C). In DD, when SCN-KO became arrhythmic the total food intake was still comparable between Con and SCN-KO animals, but SCN-KO animals showed a higher body weight at the end of the experiment (Fig. 19 D & E). Visceral AT volume was again larger than subcutaneous depots in both genotypes. Visceral AT volume in SCN-KO was increased in comparison visceral AT in Con and in comparison to both subcutaneous depots (Fig. 19 F).

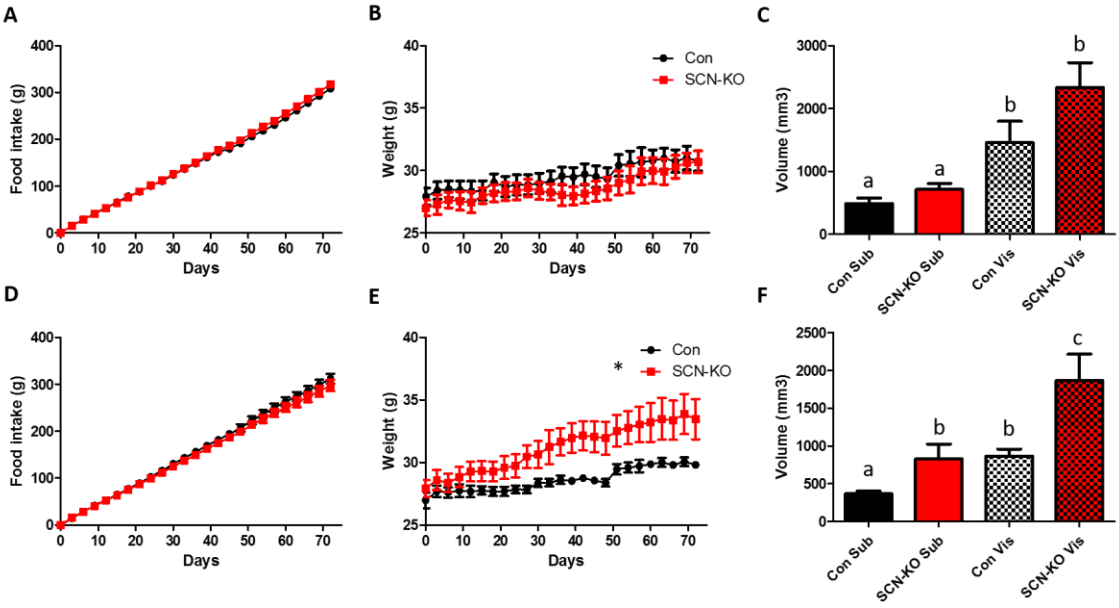


Figure 19: SCN-KO animals gained visceral adipose tissue mass in DD

(A) Cumulative chow intake over 10 weeks in Con and SCN-KO animals in LD. (B) Con and SCN-KO animal weight development starting at an age of ten weeks. Data are shown as mean ± SEM, 2-way ANOVA, Bonferroni post-test, n = 6 per genotype. (C) Volume of AT depots in Con and SCN-KO mice. Data are shown as mean ± SEM, 1-way ANOVA test, n = 6 per genotype. (D) Cumulative chow intake over 10 weeks in Con and SCN-KO animals in DD. (E) Weight development starting from ten weeks of age in Con and SCN-KO animals. Data are shown as mean ± SEM, *: genotype difference, 2-way ANOVA, Bonferroni post-test, n = 7 per genotype. (F) Volume of AT depots in Con and SCN-KO mice. 1-way ANOVA, n = 7 per genotype.

Impaired metabolic homeostasis is often linked with an impaired glucose metabolism. To test if possible alterations in glucose homeostasis due to the missing SCN clock function or the de-synchrony of peripheral organs, I conducted glucose and insulin tolerance tests (GTT and ITT) after ten weeks of LD or DD husbandry. As well as AT, liver is a key organ in glucose homeostasis and an impaired circadian liver function is tightly linked to a defective glucose metabolism (Lamia, Storch et al. 2008). Therefore I also tested hepatic gluconeogenesis function with a pyruvate tolerance test.

After 10 weeks in LD and chow provided *ad libitum* SCN-KO mice did not display an impaired reaction to glucose or insulin administration (Fig. 20 A & B). In both tests the areas under the GTT and ITT curves were comparable between Con and SCN-KOs. Interestingly, hepatic glucose release after pyruvate administration was reduced in SCN-KO mice (Fig. 20 C). After 10 weeks in DD SCN-KO mice also displayed an impaired glucose tolerance with an increased area under the curve in the GTT (Fig. 20 D). The responses to insulin and pyruvate administration did not differ from Con mice (Fig. 20 E & F).

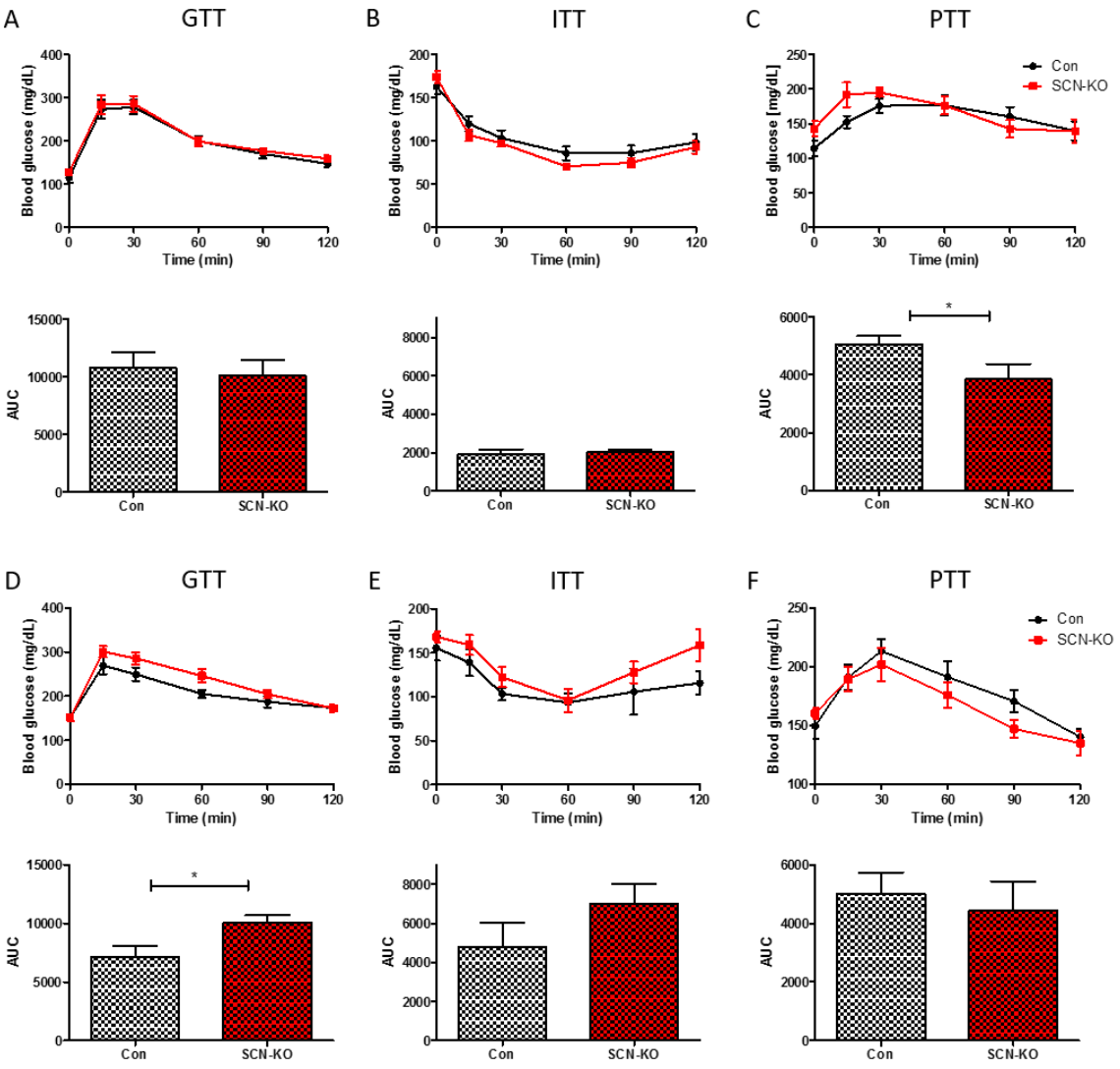


Figure 20: Altered glucose and pyruvate response in SCN-KO mice

Blood glucose levels in LD after (A) glucose, (B) insulin or (C) pyruvate administration at 0, 15, 30, 60, 90 and 120 min with corresponding areas under the curve (AUC) for (A) glucose, (B) insulin or (C) pyruvate administration. Data are shown as mean ± SEM, t test, *: $p < 0.05$, n = 6 animals per genotype. Blood glucose level in DD after (D) glucose, (E) insulin or (F) pyruvate administration at 0, 15, 30, 60, 90 and 120 min with according areas under the curve (AUC) for (D) glucose, (E) insulin or (F) pyruvate administration. Data are shown as mean ± SEM, t test, *: $p < 0.05$, n = 7 animals per genotype.

The natural rhythm of food intake was abolished in SCN-KO mice in DD. In this condition the behavioral masking and the entraining properties of light signaling are lost, but peripheral clocks can entrain to food rhythms (Saini, Suter et al. 2011).

I used this paradigm to synchronize peripheral clocks by restricted feeding and confined the food availability to the subjective dark phase.

At the age of 10 weeks animals were transferred from LD and *ad libitum* chow to DD conditions with the access to food restricted to the subjective night time. *SCN*-KO animals had a decreased cumulative food intake in these 10 weeks of DD (Fig. 21 A), but body weight development was comparable to Con (Fig. 21 B). In line with body weight, AT volumes were not different between genotypes (Fig. 21 C).

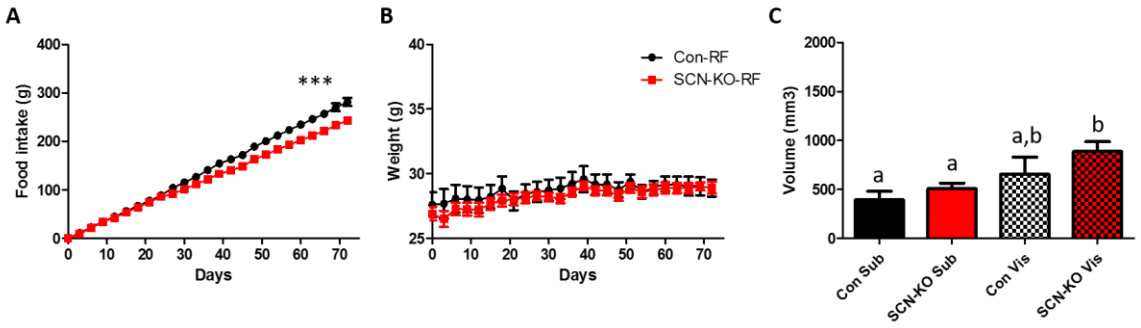


Figure 21: Restricted feeding decreases food intake and reestablishes metabolic homeostasis in DD in SCN-KO

(A) Cumulative chow intake over 10 weeks. *: $p > 0.05$, 2-way ANOVA, Bonferroni post-test (n = 6 animals per genotype). (B) Con and SCN-KO animal weight development starting at the age of ten weeks, n = 6 animals per genotype. (C) Volumes of AT depots in Con and SCN-KO, mice .1-way ANOVA, n = 6 animals per genotype.

Restricted feeding with decreased food intake led to a weight normalization in SCN-KO animals and AT depot volumes, which was comparable to Con animals (Fig. 21 B). To test if glucose metabolism was also normalized under restricted feeding conditions GTTs, ITTs and PTTs were performed. Glucose clearance and responses to insulin administration were similar between Con and SCN-KO animals (Fig. 22 A & B). Also hepatic reaction to pyruvate administration were restored (Fig. 22 C).

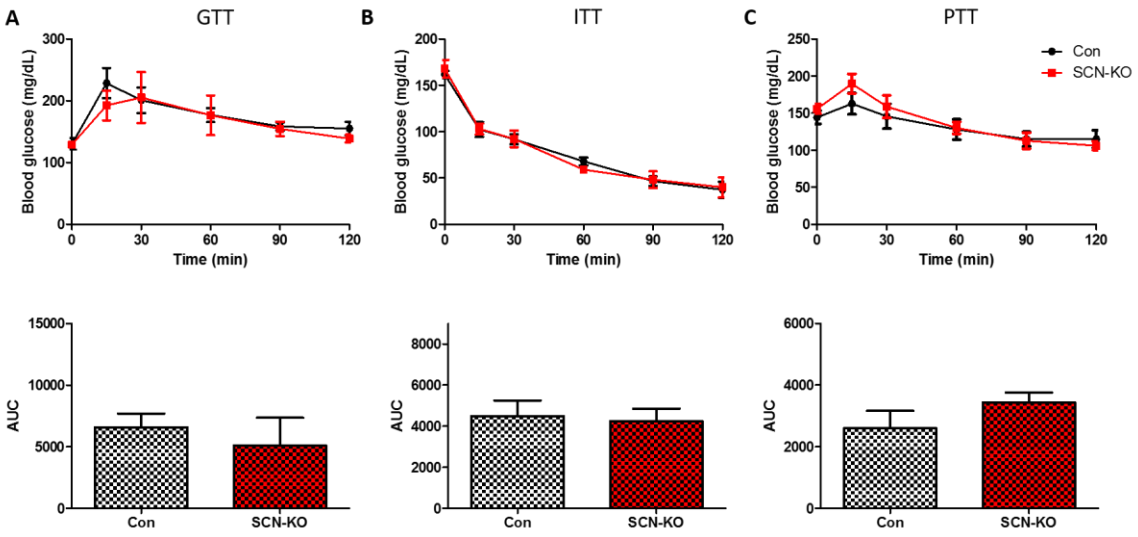


Figure 22: Restricted feeding adjusts SCN-KO glucose response

Blood glucose levels under DD-RF conditions after (A) glucose, (B) insulin or (C) pyruvate administration at 0, 15, 30, 60, 90 and 120 min with corresponding areas under the curve (AUC) for glucose, insulin or pyruvate administration. t test, n = 6 animals per genotype.

Thus synchronizing peripheral clocks by rhythmic food intake rescued the metabolic and homeostatic phenotype of constant DD conditions.

4 Discussion

The circadian timing system in mammals is hierarchically organized with a master pacemaker localized in the SCN, a paired hypothalamic nucleus with densely packed rhythmic neurons (Welsh, Takahashi et al. 2010). The SCN neurons receive direct light information *via* the RHT, entraining their firing rhythms to the 24-h light-dark cycle (Hattar, Lucas et al. 2003, Guler, Ecker et al. 2008). From the SCN temporal information is passed on *via* multiple output mechanisms to other central and peripheral circadian oscillators to synchronize those with each other (Yoo, Yamazaki et al. 2004, Dibner, Schibler et al. 2010). These semi-autonomous subordinate clocks can be uncoupled from the central signaling by mistimed food intake, which is a potent *zeitgeber* for peripheral clocks (Damiola, Le Minh et al. 2000, Stokkan, Yamazaki et al. 2001). Antiphasic feeding resets peripheral clock rhythms, while the SCN clock remains synchronized to the light-dark cycle (Damiola, Le Minh et al. 2000, Yamazaki, Numano et al. 2000). By targeting the essential clock gene *Bmal1* (Bunger, Wilsbacher et al. 2000) with the Cre-lox-system the clock function in the Cre recombinase expressing tissues is disrupted (Storch, Paz et al. 2007, Lamia, Storch et al. 2008). To create animals with a SCN specific clock deletion we crossed mice with the Cre recombinase under the control of the Synaptotagmin 10 (*Syt10*) promoter (Husse, Zhou et al. 2011) with $Bmal1^{flx/flx}$ mice (Storch, Paz et al. 2007). Syt10 is highly expressed in the SCN region (Fig.6) and thereby the central clock is targeted without affecting the molecular clocks in the periphery as *Syt10* is only expressed centrally and in the testis (Husse, Zhou et al. 2011). Still the Cre recombinase activity in $Syt10^{Cre/Cre}Bmal1^{flx/flx}$ mice was not sufficient enough to abolish clock function in the SCN and an additional deletion of one Bmal1 allele was introduced to create $Syt10^{Cre/Cre}Bmal1^{flx/-}$ (SCN-KO) mice (Husse, Zhou et al. 2011). By manipulating the two timing systems of peripheral clocks - SCN output and food access - in mice, I studied the interplay of the central pacemaker and eWAT clocks on adipose physiology and energy homeostasis.

4.1 Metabolic phenotype of SCN-KO mice

Light is the main *zeitgeber* synchronizing the SCN clock, which coordinates circadian rhythms in physiology and behavior. Interestingly, the genetic ablation of SCN clock function in our SCN-KO animals did not disrupt rhythmic running-wheel activity in alignment to the light-dark cycle (Fig. 7 A). This rhythmic behavior in combination with rhythmic food intake indicates an overall synchronization and it was indeed shown that in LD clock genes in SCN-KO display circadian oscillations comparable to Con in peripheral tissues like liver, adrenal, heart and lung (Husse, Leliavski et al. 2014). However, behavioral rhythms in SCN-KO mice are not self-sustained and do, thus, not entrain to the LD cycle but directly react to the light stimulus. When Con mice are exposed to unnaturally short LD cycles, to which their endogenous clock cannot adapt, they follow their intrinsic rhythm and are active in light episodes that fall into their circadian activity period and display reduced activity in dark phases which occur in their intrinsic rest period (Husse, Zhou et al. 2011). In contrast, the activity of SCN-KO animals in these lightning conditions is suppressed during all light phases and, therefore, their behavior is directly masked by the light stimulus (Husse, Zhou et al. 2011). Interestingly operative lesioning of the SCN results in arrhythmic behavior even in LD (Coomans, van den Berg et al. 2013). In SCN-KOs arrhythmic behavior appears only upon release into DD (Fig. 7 A). Without external time clues the masking effect is gone and the absence of a functional SCN clock results in arrhythmicity. Izumo et al. used a genetic approach similar to ours to delete SCN clock function by using the forebrain-specific *Camk2-Cre+Bmal1*$^{flx/flx}$ line (Forebrain-KO). Forebrain-KO mice display the same behavioral phenotype as SCN-KO mice with light masking and arrhythmic behavior in constant conditions (Izumo, Pejchal et al. 2014). These findings support the idea that intact neuronal connections – but not functional SCN clocks – are necessary for the transfer of temporal light information to the periphery.

Like activity, food consumption in SCN lesioned mice is arrhythmic in LD and total food intake reduced in comparison to their sham-operated controls (Coomans, van

den Berg et al. 2013). In contrast, our SCN-KO mice showed no reduced total food intake, neither in LD nor in DD.

To analyze if light conditions might also affect energy metabolism in SCN-KO mice I tested their RER in LD and DD conditions. In LD no differences between Con and SCN-KO animals were observed. Both utilized mainly carbohydrates during the active and lipids during the inactive phase (Fig. 9 A). In contrast to these findings, SCN lesioned animals do not display differences in substrate utilization between light and dark phase and tend to an overall carbohydrate-fueled metabolism (Coomans, van den Berg et al. 2013). However – and in line with what was seen for behavior and feeding - an abrogation of day-night differences was observed in SCN-KO mice and a development towards elevated RER values and carbohydrate utilization and in DD (Fig. 9 B). In DD SCN-KO mice displayed non-rhythmic food intake without long fasting periods. As lipid-based metabolism is a marker of fasting times, when stored TGs in adipocytes undergo lipolysis to sustain the energy demands of the organism (Cahill, Herrera et al. 1966, Cahill 1970). The continuous food intake in SCN-KO may have disturbed this metabolic fuel exchange. In line with this, overall increased RER values can be found in various other mouse models of genetic clock disruption (Huijsman, van de Par et al. 2009, Eckel-Mahan, Patel et al. 2012). Together, my data indicate that SCN-KO mice are conditional model for the investigation of clock disruption, with functional (peripheral) clocks and rhythms in LD and ablated circadian organization in DD conditions.

Body temperature in rodents displays a diurnal variation with an elevated temperature during the active and a lower temperature during the rest phase. It is centrally regulated and diurnal body temperature rhythms continue in DD, even if sleep is deprived or disrupted (Solarewicz, Angoa-Perez et al. 2015, Dispersyn, Sauvet et al. 2017). A recent study suggests that coordination of circadian and metabolic signaling within the hypothalamus regulates body temperature rhythms involving the SCN and the ARC (Guzman-Ruiz, Ramirez-Corona et al. 2015). SCN-lesioned mice do not display rhythmic body temperature (Coomans, van den

Berg et al. 2013) and in SCN intact animals restricted feeding can uncouple temperature oscillations from the SCN (Sen, Raingard et al. 2017). SCN ablation in rats leads to a loss of temperature rhythms as well (Scheer, Pirovano et al. 2005, Guzman-Ruiz, Ramirez-Corona et al. 2015), but after an extended recovery time they regain rhythmic body temperature under LD conditions. Temperature rhythms in these animals are not detectable in DD, which indicates a direct light-driven regulation *via* alternative pathways and brain regions (Scheer, Pirovano et al. 2005). The missing temperature rhythmicity in our SCN-KO mice indicates the importance of SCN clock function – rather than the light-dark cycle – for temporal temperature regulation. However, the temporal resolution of 6-h measurement intervals and the measurement technique itself might not be sensitive enough to detect dampened temperature rhythms in SCN-KO mice.

Under LD conditions SCN-KO mice show a normal body weight phenotype, with no weight changes compared to Con (Fig. 19 A). Coomans et al. observed weight gain and increased adipose tissue mass in SCN lesioned animals. This was accompanied by metabolic and endocrine deregulation (Coomans, van den Berg et al. 2013). Prompted by these findings, I investigated not only the total adipose content but also the volumes of different adipose depots in SCN-KOs. In line with the unaltered body weight and rhythmic behavior SCN-KO mice showed similar amounts of subcutaneous and visceral AT in LD conditions compared to Con. In mice, epididymal white adipose tissue (eWAT) is an anatomically well-defined visceral AT depots and to obtain a detailed insight of the impact of SCN clock function on the transcriptional circadian regulation of adipose tissue, I compared circadian rhythms in the eWAT transcriptome between Con and SCN-KO mice.

4.2 Transcriptional alteration in eWAT in the absence of the master pacemaker SCN

The rhythmic regulation of tissue-specific transcriptional programs is the main output of the circadian clock gene machinery. I gained further insight into the influence of central and local adipose tissue clocks on rhythmic gene expression

patterns by analyzing over 34,000 gene transcripts for their rhythmic expression in Con and SCN-KO eWAT samples. In Con animals, around 6% of gene transcripts displayed circadian oscillations (Fig. 12 A). This percentage is comparable to results from other transcriptome studies in other peripheral tissues such as the liver and the adrenal (Panda, Antoch et al. 2002, Oster, Damerow et al. 2006, Kornmann, Schaad et al. 2007). Depending on the cut-off criteria that are set for detection algorithms and sampling resolution, however, the percentage of detected rhythmic transcripts can reach much higher values (Zhang, Tong et al. 2014). Our relatively low sampling resolution (6-hour intervals) might promote false results as curve fitting might miss significant results or create false positive results due to stochastic noise. To avoid accumulation of artifacts and to focus on physiological meaningful rhythms an additional fold-change cut-off of 25 % was introduced.

When comparing the rhythms of genes with rhythmic expression in Con and SCN-KO eWAT, an overall phase shift in SCN-KO transcript rhythms was observed (Fig. 12 B). It was shown before that clock gene expression in the periphery is dampened and phase-advanced in SCN-KO mice on the second day in DD (Husse, Leliavski et al. 2014). In line with a regulation by the local clock gene machinery, overall dampened and phase-shift oscillations were observed for this relatively small number (n = 164) of potential direct adipose CCGs in SCN-KO eWAT.

The observed bimodal distribution of gene expression peak times over 24-hour in Con (Fig. 12 C) fits to earlier studies were the clustering of peripheral transcription phases lead to one peak during the active and another peak during the inactive phase (Akhtar, Reddy et al. 2002, Archer, Laing et al. 2014). Transcripts in the group with higher expressions during the rest phase belong to state-dependent transcripts that represent the actual rest state (Anafi, Pellegrino et al. 2013) in the rhythmic Con mice. The transcription of genes peaking during the active phase is presumably influenced by activity and food intake (Zvonic, Ptitsyn et al. 2006). Since peripheral clocks in SCN-KO mice are entrained by the light-dark cycle (Husse, Leliavski et al. 2014), tissue collection was performed on the second day

of DD to avoid possible masking effects from light-driven behavioral rhythms. On the second day in DD, the 164 genes, which were rhythmic in both genotypes, showed a similar bimodal rhythm phase distribution, but with decreased amplitudes and a phase advance in SCN-KO animals (Fig. 13 A, B). Besides control by local adipose clocks, these rhythms could partly be influenced by synchronizing systemic signals like glucocorticoids (Dickmeis 2009), which continue to oscillate for several days in SCN-KO in DD, but with a dampened amplitude and advanced phase (Husse, Leliavski et al. 2014). The fact that most of the stably rhythmic eWAT transcripts were phase-advanced in SCN-KO mice supports the hypothesis that peripheral rhythms in the absence of a mediating SCN clock show an earlier phase due to the impact of direct, unprocessed photic synchronization signals (Husse, Leliavski et al. 2014). Gene enrichment analysis revealed an overrepresentation of genes associated with circadian rhythm (Fig. 13 D) and numerous clock genes were present in this group. Rhythmic but dampened and phase-advanced expression was seen before in other peripheral organs like heart, liver, adrenal and kidney in the absence of a master clock (Husse, Leliavski et al. 2014).

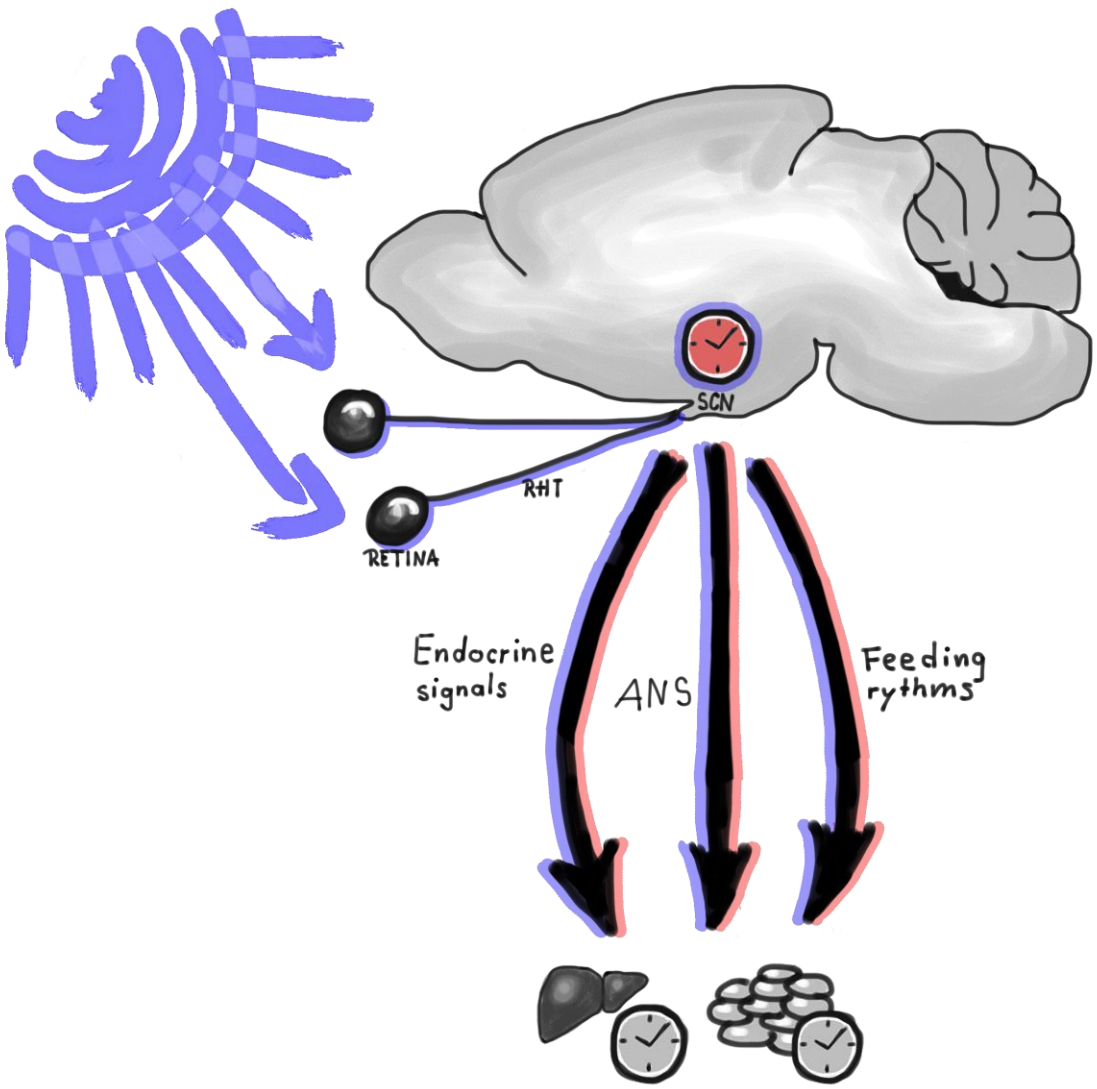


Figure 23: Hierarchical clock network and synchronization pathways

Peripheral clocks are synchronized by the SCN *via* the autonomic nervous system (ANS), endocrine signals and indirectly *via* feeding rhythms. Light signals reach the SCN through the retino-hypothalamic tract (RHT) and this light information can pass the SCN region also without a functional pacemaker clock to directly synchronize peripheral clocks (blue). Without this external light information a functional SCN clock is needed to sustain signaling to the periphery and maintain synchrony (red).

Gene transcripts that were rhythmic only in Con (1,791 genes) were mostly associated with metabolic processes. Genes associated with cholesterol biosynthesis were among the overrepresented pathways (Fig. 15 B). WAT is the

major storage organ for cholesterol and actively involved in the stabilization of cholesterol plasma levels (Kovanen and Nikkila 1976). Furthermore, low cholesterol plasma concentrations can induce cholesterol production in WAT (Lakshmanan, Berdanier et al. 1977), although the liver is the main organ for cholesterol *de novo* biosynthesis (Back, Hamprecht et al. 1969, Gupta, Sexton et al. 1989). Comparable to rat liver transcripts (Jurevics, Hostettler et al. 2000) most of the biosynthesis-associated genes peaked in the subjective night in Con animals while the rhythmicity of the corresponding transcripts in SCN-KO mice was abolished (Fig. 15).

The overall number of rhythmic transcripts in SCN-KO declined dramatically compared to controls, but interestingly some transcripts (504 genes) gained rhythmicity in the absence of SCN function. The gain of rhythm in these genes in amplitude strength is comparable to the loss of amplitude in the transcripts that lose their rhythm in SCN-KO and should, therefore, not be a statistical artifact due to rhythms at the limit of significance. It is possible that, in a functional circadian clock system, these gene transcription oscillations are absent as a result of local clock and systemic counter-regulation. In consequence, local rhythms become unmasked in the moment the orchestrating pacemaker loses rhythmicity and by that systemic influence is weakened. A similar mechanism was already described in hepatic blood glucose regulation where systemic signals like food intake and local liver clock signaling interact to balance blood glucose levels. While hepatocytes react directly to changes in circulating glucose (Klover and Mooney 2004), the release of glucose into the bloodstream is clock-regulated to counterbalance the lack of glucose during rest (fasting) hours (Lamia, Storch et al. 2008). If the liver clock is ablated this buffer function is lost and glucose levels become rhythmic following the rhythm of food intake (Lamia, Storch et al. 2008).

Enrichment analysis of the genes that were only rhythmic in SCN-KO revealed an association with immune functions (Fig. 16 B). Adipose tissue harbors large quantities of immune cells that permanently transmigrate through the tissue and can constitute 5 to 50 % of the total cell mass (Weisberg, McCann et al. 2003,

Ortega Martinez de Victoria, Xu et al. 2009). The daily blood concentration rhythm of free neutrophils has its maximum around midday (Brown and Dougherty 1956), a time when neutrophil numbers in adipose tissue are low. In line with this, the expression profile of neutrophil chemotaxis genes in SCN-KO displayed their nadir (Fig. 16 D). As pointed out earlier, for the counterbalance of systemic and local signals seen for glucose homeostasis (Lamia, Storch et al. 2008), the phenomenon of counter regulatory action of local and systemic rhythms might also apply to immune cell migration in WAT. As here systemic signals are absent in SCN-KO mice (Fig. 8, right panel) and local rhythms become unmasked. An increased immigration of neutrophils into WAT is a first indication for metabolic inflammation (Mraz and Haluzik 2014). Shift work is a risk factor for the development of metaflammation (Sookoian, Gemma et al. 2007) and an underlying mechanism might be a disruption of circadian neutrophil migration behavior. In turn, the manipulation of the temporal migration of innate immune cells may represent a novel approach for metaflammation treatments. An involvement of food intake and the missing feeding/fasting cycle could further amplify the migration rhythms in SCN-KO WAT. While dietary lipids themselves can increase the immune cell content in AT, fasting induces lipolysis and the release of FFAs from adipocytes into the blood stream (Kosteli, Sugaru et al. 2010). The rhythm of FFA release, which is controlled by adipocyte clocks (Shostak, Meyer-Kovac et al. 2013), opposes neutrophil cell recruitment phases and could obliterate the rhythmic expression of neutrophil chemotaxis genes in Con. In the modulation of immune response and infection, GCs interfere with bronchiolar secretory cell clocks and adjust their immune response in a diurnal fashion (Gibbs, Ince et al. 2014). GC rhythms are preserved in SCN-KO animals on the second day of DD and, thus might affect the expression of immune relevant genes due to their anti-inflammatory properties (Rhen and Cidlowski 2005). Finally, it is most likely that a combination of different systemic factors is mediating the rhythmic translational outcome and dissecting these factors – by genetic or pharmacological approaches – is indicated for further studies.

The missing rhythmic input from the SCN clock has diverse outcomes on the expression of adipokine transcripts in SCN-KO eWat. *Leptin* transcription directly reacts to feeding and fasting cycles (Zhang, Matheny et al. 2002), and as feeding rhythms were lost in SCN-KOs the metabolic state of the adipocytes did no longer vary over the day. As a result, rhythmic leptin transcription was abolished (Fig. 17 A). Visfatin is also involved in neutrophil migration and adhesion and the phase of its transcription is phase-advanced in SCN-KO animals, which is in line with what was observed for other stably rhythmic eWAT transcripts and indicative or a direct regulation by local adipocyte clocks (Fig. 13 & 14). Pai-1 was previously suspected to be regulated *via* the circadian clock (Schoenhard, Smith et al. 2003) and circulating PAI-1 oscillations in mice are independent of feeding rhythms (Oishi, Ohkura et al. 2017). The dampening and phase alteration of *Pai-1* transcripts in SCN-KO mice fits to these assumptions (Fig. 17 C), but also indicates that *Pai-1* transcription may further be regulated by other factors. The overall increased transcription of the hunger-suppressing *Nes1* and the alterations seen in innate immune transcripts (Fig. 17 D) match findings in humans that Nesfatin levels are increased during inflammation, e.g. in cystic fibrosis (Cohen, Ginsberg et al. 2013).

Contrary to transcript levels, leptin serum concentrations in LD and DD conditions remained rhythmic in SCN-KO mice (Fig. 18), suggesting an involvement of local adipose clocks in leptin translation and/or release. Such rhythmic leptin release was found in cultured murine adipocytes (Otway, Frost et al. 2009). The glucocorticoid analogue dexamethasone also increases the release of leptin from adipocytes (Considine, Nyce et al. 1997) and as GC levels are still rhythmic on the second day of DD this might also influence rhythmic leptin serum concentrations (Husse, Leliavski et al. 2014).

In conclusion, an immediate change in the adipose tissue transcriptome after the removal of external light information could be observed and presumably the rhythmic transcription would dampen further in prolonged DD conditions. It was shown that dampened or disrupted rhythmicity is often coherent with metabolic disturbances (Knutson et al., 2007; Knutson and Van Cauter, 2008 JH). Therefore,

I next analyzed if SCN or global circadian disruption lead to metabolic impairments using the SCN-KO model.

4.3 Body weight regulation and glucose homeostasis in synchronized and unsynchronized conditions

Peripheral rhythms and the synchrony among tissues adapt the organism to daily metabolic fluctuations. The SCN as the master pacemaker has various ways of orchestrating tissue rhythms between themselves and with external time. Surgical SCN ablation leads to desynchronization among tissues and is followed by metabolic disturbances like increased weight gain and a disturbed glucose metabolism in mice (Coomans, van den Berg et al. 2013). In a similar way, global clock disruptions are known to lead to adverse metabolic consequences (Rudic, McNamara et al. 2004, Turek, Joshu et al. 2005, Dallmann and Weaver 2010). In SCN-KO mice with a genetically ablated SCN clock function, peripheral tissue clocks retain rhythmicity in LD, presumably driven by rhythmic light input driving rhythmic GC signaling, sleep-wake behavior and food intake. All these factors are arrhythmic even in LD in SCN lesioned animals (Coomans, van den Berg et al. 2013). As nerve connections from the retina to the SCN and further to downstream structures are intact in our model, light signals are directly passed through the SCN region onto the periphery. Apparently, processing of the SCN clock in Con mice leads to a delay in photic signaling, which might explain the overall phase advance in transcript rhythms of clock genes and CCGs in peripheral tissues of SCN-KO mice (Husse, Leliavski et al. 2014). This light masking effect, however, is lost in the moment external time clues are turned off. In DD conditions the direct readout of a missing master pacemaker is arrhythmic locomotor and feeding behavior. On the transcriptional level, already on the second day, a decline in the number of rhythmic eWAT transcripts can be observed and endocrine rhythms like GC secretion dampen continuously over several days until no rhythm is measurable anymore (Husse, Leliavski et al. 2014).

SCN-KO mice gained more weight and fat mass than Con animals in DD although the food intake was comparable (Fig. 19 E, D). It was shown before that the SCN is involved in energy homeostasis in mice (Phan, Chan et al. 2011) and rats (Angeles-Castellanos, Salgado-Delgado et al. 2010) and weight was also seen in SCN lesioned mice (Coomans, van den Berg et al. 2013). These mice gained already more weight in LD due to an increased fat mass, but if neighboring brain regions, like PVN and VMH were damaged mice became obese and as lesions exclusive to the SCN region are rare (20-30% of all operated animals) the effects of lesion studies always have to be reviewed carefully (Kalsbeek, Fliers et al. 2001, Coomans, van den Berg et al. 2013).

In clock mutants the local adipose clock is genetically impaired and often the diurnal lipid mobilization is inhibited (Oishi, Atsumi et al. 2006, Kennaway, Owens et al. 2007, Guo, Chatterjee et al. 2012). In this case the concentration of FFAs in the blood is reduced and the organism mainly utilizes carbohydrates for its energy demands (Wu, Wang et al. 2012). The preference for a carbohydrate-driven metabolism in clock mutants and in SCN-KO can be a hallmark for an impaired metabolic flexibility (Kelley, He et al. 2002). A suppressed lipid mobilization can promote weight gain due to increased TG storage in AT (Shostak, Meyer-Kovac et al. 2013) as TGs that are synthesized during the time of energy surplus are stored but not utilized. Glucocorticoids increase lipolysis in AT and could stabilize the lipolysis/lipogenesis rhythm in LD conditions, but in prolonged DD conditions SCN-KO mice have no detectable GC rhythm anymore (Husse, Leliavski et al. 2014), which is in line with the increased AT volume in DD. As discussed before the SCN-KO animals display reduced lipid utilization directly upon the release into DD in the subjective rest phase (Fig. 9 B). During that measurement period the GC rhythms are still rhythmic. Therefore, GC rhythms were unlikely to drive increased AT storage, but impaired lipolysis might explain the increase in visceral adipose depot size in SCN-KO in DD.

GC rhythms also influence glucose homeostasis (Pasieka and Rafacho 2016). Glucose metabolism – like GC rhythms – was largely unaltered in SCN-KO mice

in comparison to controls in LD conditions (Fig 20 A-C). Some differences, however, were observed for the pyruvate tolerance test, indicating that SCN function might impact on liver gluconeogenesis already in LD. In contrast to the results obtained in LD, glucose tolerance was decreased in SCN-KOs in DD conditions (Fig. 20 D), while sensitivity to insulin was still normal. This indicates that the pancreatic insulin release in response to glucose administration (Rakshit, Qian et al. 2015) is impaired. This could be due to peripheral desynchronization as pancreatic beta-cells display a circadian gating in insulin release in synchronized conditions (Perelis, Marcheva et al. 2015). Additionally, impaired metabolic flexibility, which was observed in SCN-KO mice in DD, has been associated with impaired insulin sensitivity in humans (Corpeleijn, Saris et al. 2009).

Food is an important entrainment factor for peripheral clocks (Honma, von Goetz et al. 1983, Damiola, Le Minh et al. 2000, Stokkan, Yamazaki et al. 2001). By reinstating rhythmic food intake I aimed at recovering peripheral rhythmicity and normalize body weight homeostasis in SCN-KO mice in the absence of rhythmic light information. First the temporal food restriction led to a decreased food intake in SCN-KO mice and normalized weight gain relative to controls (Fig. 21). A pair-fed experiment with equal amounts of food intake in both genotypes or the determination of the remaining energy in feces could be follow up experiments to further investigate this difference in body weight-to-food intake ratio difference. Restricted feeding also rescued the response to glucose administration in SCN-KO mice. Pancreatic cells harbor intrinsic circadian clocks and it was shown before that the insulin response is gated in a circadian fashion (Perelis, Marcheva et al. 2015). As clocks in beta-cells are sensitive to food resetting (Rakshit, Qian et al. 2015), the restricted access to food could have shifted the glucose sensitivity in SCN-KO animals and influence metabolic outcome. In conclusion, prolong arhythmicity and missing internal synchrony lead to disturbances in weight and glucose homeostasis. This metabolic alterations are presumably the consequences of internal de-synchrony and not of the missing SCN signaling per se, as all alterations were rescued by alternative peripheral synchronization.

4.4 Conclusion

In summary, clock function in the SCN is not needed to stay aligned with the external light-dark cycle. In the presence of rhythmic light input no differences in behavior, body weight or composition and metabolic activity are found in mice with non-functional SCN clocks - with the exception of a dampened body temperature rhythm. In constant (or, likely unstable) environmental conditions, the importance of a functional SCN emerges, as SCN-KO animals display impairments in behavioral rhythms associated with weight gain and altered glucose homeostasis. Interestingly, by restoration of peripheral rhythms metabolic homeostasis can be rescued in SCN-KO mice, suggesting peripheral clock function as promising target for the treatment of metabolic disorders.

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Microarray gene list of rhythmic transcripts

Table 5: Gene transcripts rhythmic in Con mice with gene symbols. List of rhythmic transcripts with Affimetrix identification number, the according gene symbol, normalized fold change (Norm FC) and the phase. Genes with a red marked Gene symbol are also rhythmic in SCN-KO animals.

Con					Con			
Affimetrix-ID	Gene Symbol	Norm FC	Phase		Affimetrix-ID	Gene Symbol	Norm FC	Phase
17211174	A830018L16Rik	0.27	6		17380222	Pck1	0.66	9
17211198	Sulf1	0.58	19		17380464	2210418O10Rik	0.53	19
17211258	Rdh10	0.31	5		17380518	Gm14295	0.41	19
17212087	Npas2	0.98	24		17380549	Gm14420	0.63	17
17212138	Map4k4	0.28	4		17380604	9030418K01Rik	0.26	21
17212518	Wdr75	0.34	23		17381630	Pfkfb3	0.74	7
17212908	Plcl1	0.48	11		17381752	Fam188a	0.26	22
17212982	Aox1	0.40	7		17381779	Rsu1	0.27	20
17213189	Gm20257	0.31	7		17382729	Tmem141	0.30	21
17213578	Gm20342	0.45	7		17383129	Snora17	0.43	7
17213608	Adam23	0.44	4		17383835	Uck1	0.30	4
17213687	Ccnyl1	0.27	23		17383858	Dnm1	0.33	19
17213765	Crygf	0.31	9		17383886	1110008P14Rik	0.31	23
17213880	Rpe	0.26	22		17384994	Orc4	0.29	24
17214368	Cyp27a1	0.40	20		17385422	Acvr1c	0.54	6
17214685	Acsl3	0.46	21		17385719	Dpp4	0.70	21
17214924	Sp140	0.27	13		17385853	Grb14	0.30	24
17214979	Sp100	0.27	6		17386479	Mir5115	0.33	8
17215029	2810459M11Rik	0.25	7		17386481	Ola1	0.31	24
17215395	Dgkd	0.40	8		17386514	Gpr155	0.25	21
17215605	Cxcr7	0.30	21		17386569	Chn1	0.30	19
17216012	Sept2	0.27	21		17386700	Nfe2l2	0.36	19
17216563	Mki67ip	0.31	22		17387517	Serping1	0.42	20
17217048	5430435G22Rik	0.44	20		17387674	Olfr1061	0.28	14
17217171	Gm7241	0.28	18		17387774	Olfr1137	0.27	12
17217283	Ppp1r15b	0.29	10		17387928	Olfr1231	0.28	9
17217619	Phlda3	0.62	20		17388041	Ptprj	0.28	22
17217846	Mir181b-1	0.79	21		17388332	Creb3l1	0.40	24
17218006	B3galt2	0.56	7		17388435	Tspan18	0.47	2
17218027	Uchl5	0.29	6		17388499	Accs	0.36	7
17218927	Dpt	0.46	21		17388573	Hsd17b12	0.30	6
17219005	Creg1	0.39	20		17388687	Prr5l	0.28	8
17219196	Dusp12	0.34	7		17389051	Elp4	0.33	2
17220034	Sccpdh	0.26	5		17389578	Atpbd4	0.49	22
17220475	Dusp10	0.72	23		17389775	A430105I19Rik	0.33	18
17220489	Rab3gap2	0.31	8		17390810	Gatm	0.27	5
17221010	Mir3962	0.80	5		17390879	Fbn1	0.65	23
17221014	Cd34	0.40	21		17391781	Gm14057	0.40	7
17221360	Lactb2	0.39	23		17391883	Cenpb	0.27	8
17221490	Ube2w	0.43	22		17391920	Rassf2	0.30	23
17221662	Tram2	0.39	21		17392047	Lrrn4	0.73	21
17221792	Fam135a	0.31	5		17392209	Esf1	0.29	22
17222429	Txndc9	0.28	24		17392255	Flrt3	0.38	21
17223169	Ankrd44	0.37	3		17392568	Thbd	0.37	21
17223429	Als2cr12	0.50	2		17392676	2310001A20Rik	0.28	5
17223574	Sumo1	0.45	18		17392690	Acss1	0.25	19
17223903	Acadl	0.30	7		17393131	Snta1	0.39	21
17224071	Fn1	0.54	20		17393755	Blcap	0.46	7
17224180	Igfbp5	0.39	22		17394153	Slpi	0.71	20

17224702	Farsb	0.27	23		17394405	Elmo2	0.32	1
17224724	BC035947	0.25	4		17394668	Kcnb1	0.39	23
17224971	Pid1	0.31	17		17394679	Ptgis	0.32	19
17225113	Gm19524	0.30	8		17395165	Atp5e	0.30	5
17225169	Snora75	0.63	10		17395354	Gm14405	0.31	19
17225179	Pde6d	0.27	21		17395398	C330013J21Rik	0.44	10
17225506	Per2	1.04	16		17395408	Zfp931	0.35	19
17225999	Pign	0.26	3		17395462	Ppp1r3d	0.50	21
17226593	Cxcr4	0.65	19		17395488	Psma7	0.42	6
17227077	Prelp	0.31	17		17395629	Gata5	0.30	18
17227094	Adora1	0.26	10		17395707	Nkain4	0.36	21
17227320	Ipo9	0.26	4		17395928	Sox18	0.30	7
17228353	Mr1	0.35	22		17396003	Pkia	0.26	21
17228362	BC034090	0.26	21		17396143	Car13	0.37	22
17228743	Cacybp	0.54	21		17396162	Car2	0.31	5
17229036	Fmo2	1.15	18		17396260	Cp	0.29	2
17229178	Atp1b1	0.57	20		17396638	Skil	0.32	23
17229454	Rgs4	0.26	8		17396733	Actl6a	0.38	22
17229466	Hsd17b7	0.32	23		17396996	4932438A13Rik	0.26	3
17229481	Ddr2	0.27	21		17397333	Larp1b	0.32	7
17229607	Fcgr2b	0.31	20		17397426	Ccrn4l	0.28	24
17229665	Ndufs2	0.27	5		17397439	Naa15	0.27	22
17229681	Ppox	0.27	5		17397475	Mgst2	0.48	8
17230045	Ifi204	0.78	20		17397511	Lhfp	0.27	23
17230078	Mnda	0.27	19		17398043	Vmn2r1	0.33	9
17230111	Ifi205	0.49	19		17398082	Tiparp	0.30	1
17230127	Grem2	0.81	10		17399044	Smg5	0.46	22
17230131	Rgs7	0.46	5		17399808	S100a5	0.26	17
17230279	AI503316	0.39	8		17399812	S100a6	0.52	20
17230408	Adck3	0.27	8		17400000	S100a10	0.32	20
17230735	Mosc2	0.26	7		17400759	Acp6	0.26	4
17230750	Mir1981	0.39	9		17400813	Notch2	0.25	23
17231163	Sertad4	0.27	14		17401086	Sike1	0.31	20
17231248	G0s2	0.52	21		17401335	Slc16a1	0.61	23
17231287	Cd46	0.30	8		17401364	St7l	0.25	21
17231366	Plekhg1	0.36	24		17401447	Ovgp1	0.27	9
17231461	Esr1	0.31	11		17401480	Dennd2d	0.38	9
17231625	Shprh	0.31	5		17402113	Dpyd	0.31	7
17231784	Cited2	0.59	6		17402193	Arhgap29	0.27	1
17231844	Perp	0.30	21		17402305	Bcar3	0.36	4
17231891	Map3k5	0.31	6		17402558	Elovl6	1.04	23
17232189	Taar7d	0.38	17		17402604	Gm11425	0.28	18
17232593	Lama4	0.31	3		17402936	Slc39a8	0.51	21
17233078	Prep	0.26	23		17403138	Eif4e	0.34	22
17233613	Spock2	0.50	21		17403150	Tspan5	0.28	22
17233669	Pcbd1	0.73	20		17403282	Ccbl2	0.25	6
17233720	Ppa1	0.49	6		17403653	Usp33	0.30	7
17233751	Aifm2	0.41	7		17403866	Ptger3	0.50	20
17234339	Adora2a	0.51	6		17403879	Ankrd13c	0.31	20
17234552	Lss	0.45	24		17404058	Pag1	0.29	7
17234612	Slc19a1	0.38	23		17404126	Zfand1	0.36	8
17234955	Fstl3	0.29	18		17404259	Armc1	0.29	23
17235002	Prtn3	0.25	11		17404474	1600012F09Rik	0.31	21
17235227	Cirbp	0.31	6		17404750	Mccc1	0.33	6
17235360	Onecut3	0.25	16		17404796	Anxa5	0.40	20
17235408	Dot1l	0.36	5		17404941	5430434I15Rik	0.38	17

17235941	Chst11	0.28	6		17404997	6430590A07Rik	0.45	9
17236003	Tcp11l2	0.31	7		17405005	Pgrmc2	0.36	6
17236077	Ric8b	0.36	2		17405075	Pcdh18	0.38	20
17236339	Gnptab	0.35	23		17405285	Nbea	0.26	6
17236378	Arl1	0.25	20		17405513	Dhx36	0.28	4
17236604	Ntn4	0.42	21		17405795	Kpna4	0.28	23
17236800	Dcn	0.29	21		17406091	Etfdh	0.27	5
17236882	Dusp6	0.38	22		17406285	D930015E06Rik	0.30	5
17237142	Ppp1r12a	0.34	4		17406399	Gm15535	0.27	7
17237502	5330438D12Rik	0.38	6		17406514	Fcrls	0.32	20
17238394	Pan2	0.31	7		17406908	Fdps	0.32	1
17238934	Syne1	0.27	3		17407049	Dcst1	0.35	9
17239113	Sash1	0.33	23		17407098	Flad1	0.26	4
17239227	Rab32	0.68	21		17407457	Lce1g	0.25	11
17239401	Pex3	0.26	6		17407578	Tuft1	0.30	20
17239493	Heca	0.26	7		17407669	Psmd4	0.31	6
17239817	Enpp3	0.30	23		17407775	Anxa9	0.38	22
17240226	Marcks	0.34	3		17407829	Adamtsl4	0.29	19
17240303	G630090E17Rik	0.28	24		17407850	Ecm1	0.51	20
17240330	Slc16a10	0.36	20		17407934	Gm129	0.91	10
17240357	Gtf3c6	0.62	22		17408015	Hist2h3c2	0.44	19
17240379	Cdc40	0.30	6		17408067	Txnip	0.27	7
17240989	Sept10	0.28	6		17408074	Polr3gl	0.51	9
17241032	Ddit4	0.64	9		17408483	Ptgfrn	0.33	21
17241130	Slc29a3	0.25	20		17408793	Capza1	0.27	20
17241234	Gm19912	0.26	5		17408940	2010016I18Rik	0.97	10
17241637	Nrbf2	0.37	22		17409075	Csf1	0.54	21
17241692	Cdk1	0.32	9		17409284	Celsr2	0.48	9
17241934	Gstt1	0.37	19		17409406	Stxbp3a	0.28	3
17241946	Gstt4	0.25	11		17409445	Prpf38b	0.39	6
17241954	Gstt2	0.83	9		17409621	S1pr1	0.32	9
17242177	Col6a1	0.32	22		17409753	Agl	0.28	2
17242516	Pdxk	0.35	19		17409826	Snx7	0.29	1
17242842	Gamt	0.54	21		17409858	Rwdd3	0.31	8
17243247	Mrpl54	0.54	23		17410063	Snhg8	0.27	5
17243450	Gm3055	0.30	20		17410239	5730508B09Rik	0.29	24
17243493	Glt8d2	0.38	19		17410251	Enpep	0.33	1
17243644	Cry1	0.45	20		17410343	Sec24b	0.38	8
17244378	Metap2	0.28	6		17410376	Ostc	0.36	22
17244514	Nudt4	0.35	22		17410400	Cyp2u1	0.25	20
17244705	Acss3	0.34	7		17410410	Sgms2	0.33	21
17244864	Nav3	0.31	3		17410492	Tet2	0.37	6
17245231	Lyz1	0.60	20		17410578	Gm16500	0.26	16
17245248	Mdm2	0.25	23		17410617	Dapp1	0.38	21
17245264	Slc35e3	0.31	22		17410809	Lmo4	0.31	7
17245624	Usp15	0.27	5		17411103	Lphn2	0.31	4
17246038	Gpr182	0.28	10		17411141	Rpsa	0.26	6
17246043	Rdh7	0.25	18		17411262	St6galnac3	0.32	4
17246284	Suox	0.29	22		17411492	LOC621549	0.51	9
17246390	Olfr766	0.34	10		17411716	4930412C18Rik	0.32	7
17246438	Olfr811	0.26	11		17411885	C430048L16Rik	0.37	4
17247039	Aebp1	0.49	20		17411939	Tmem55a	0.26	5
17247389	Egfr	0.33	21		17411961	Tmem64	0.29	1
17247533	Rab1	0.32	21		17412123	Ccnc	0.32	23
17247948	Efemp1	0.36	20		17412272	Ndufaf4	0.27	20
17247962	Pnpt1	0.34	23		17413403	Npr2	0.29	9

17248288	Sh3pxd2b	0.29	22		17413466	A630077J23Rik	0.31	9
17248304	Ubtd2	0.36	21		17413789	Ncbp1	0.29	23
17248486	Mir103-1	0.30	20		17413821	Anp32b	0.29	9
17248971	Olfr1388	0.26	13		17413866	Col15a1	0.30	2
17249036	Gfpt2	0.86	20		17414017	Tmeff1	0.35	6
17249206	Adamts2	0.25	22		17414277	Zfp462	0.26	1
17250365	4933439F18Rik	0.35	22		17414331	BC026590	0.60	21
17250530	Tmem11	0.38	7		17414351	Palm2	0.26	23
17250533	Map2k3	0.29	15		17415380	Mtap	0.36	22
17250682	Pigl	0.28	7		17415778	Alg6	0.26	4
17250740	Snord49b	0.30	7		17415922	0610043K17Rik	0.27	8
17250744	Snord65	0.56	6		17416643	Zcchc11	0.27	5
17250849	B430319H21Rik	0.63	7		17416761	Kti12	0.31	21
17250852	B130011K05Rik	0.84	7		17417407	Pik3r3	0.39	5
17251222	Gas7	0.35	21		17418128	Trit1	0.49	9
17251527	Per1	0.88	11		17418177	Heyl	0.27	4
17251607	Trappc1	0.28	22		17418403	Rspo1	0.39	20
17251677	Sat2	0.26	5		17418904	Ak2	0.29	7
17252070	Psmb6	0.27	5		17418916	Rnf19b	0.26	10
17252626	Tax1bp3	0.28	22		17419263	Snord85	0.30	6
17252767	1300001I01Rik	0.31	5		17419407	Snora44	0.78	8
17252827	Tsr1	0.27	23		17419553	Map3k6	0.33	16
17252878	Mir212	0.28	16		17419812	D4Wsu53e	0.38	8
17253215	Slc6a4	0.34	22		17419890	Il22ra1	0.32	21
17253235	Ssh2	0.37	6		17420016	Id3	0.28	20
17253258	Coro6	0.26	7		17420108	9130020K20Rik	0.27	8
17253297	Abhd15	0.35	8		17420646	Ubr4	0.27	7
17253304	Taok1	0.38	8		17420777	Aldh4a1	0.31	5
17253835	Mir193	0.39	21		17420897	Sdhb	0.26	5
17253837	Mir365-2	0.32	1		17421074	Hspb7	1.08	18
17253937	Rhbdl3	0.27	22		17421312	Dhrs3	0.31	24
17253996	Tmem98	0.27	21		17421394	Gm13034	0.41	23
17254249	Taf15	0.27	7		17421444	Gm13251	0.28	20
17254295	Expi	0.29	16		17421491	Gm13152	0.33	21
17254508	Car4	0.45	9		17421550	Mthfr	0.27	23
17254633	Rnft1	0.32	23		17421694	Srm	0.44	20
17254974	Msi2	0.26	7		17421972	Errfi1	0.28	20
17255058	Tmem100	0.68	21		17422704	Ccnl2	0.26	7
17256138	Thra	0.43	4		17423713	Manea	0.29	21
17256522	Coasy	0.29	5		17424077	B4galt1	0.33	18
17256537	Mlx	0.58	22		17424163	Ubap2	0.27	18
17256549	Tubg1	0.27	22		17424742	Gba2	0.28	7
17256673	Psme3	0.34	22		17424907	Zbtb5	0.32	8
17256870	Cd300lg	0.30	8		17424933	2900093L17Rik	0.25	12
17257067	Acbd4	0.31	6		17424998	Xpa	0.26	7
17257405	Tanc2	0.40	24		17425043	Trim14	0.29	7
17257554	Psmc5	0.33	5		17425228	Mrpl50	0.32	23
17257937	Kcnj2	0.37	21		17425278	Rnu1b1	0.51	8
17258131	Slc9a3r1	0.28	20		17425496	Tmem245	0.29	9
17258708	2810008D09Rik	0.34	8		17425521	Mir32	0.29	5
17258714	Sec14l1	0.29	20		17425631	Svep1	0.39	10
17259235	Baiap2	0.26	2		17426365	Tnc	0.43	8
17260364	Purb	0.28	6		17426497	Megf9	0.30	24
17260699	Cobl	0.26	20		17427181	Gm12669	0.41	3
17260815	Meis1	0.25	22		17427746	Prkaa2	0.25	24
17260916	Ugp2	0.28	23		17428017	Zyg11a	0.89	10

17261107	Rel	0.48	22		17428702	Mmachc	0.26	23
17261865	Ccng1	0.30	21		17429285	Olfr1333	0.42	17
17262259	Btnl9	0.34	7		17429858	Utp11l	0.28	5
17262277	Cnot6	0.25	24		17430140	Ncdn	0.35	22
17262355	Mir804	0.29	15		17430222	Gjb4	0.27	20
17262491	Rmnd5b	0.26	22		17430288	Adc	0.27	8
17262589	Ppp2ca	0.28	21		17430397	Hdac1	0.31	21
17262621	Hspa4	0.26	23		17430645	Nkain1	0.33	19
17262848	Lyrm7	0.30	21		17430729	Epb4.1	0.26	10
17263189	Olfr317	0.34	13		17430861	Sesn2	0.26	6
17263416	Zfp867	0.29	4		17431496	Pnrc2	0.35	21
17263511	Rasd1	0.82	8		17431607	C1qb	0.28	19
17263925	A530017D24Rik	0.29	6		17431861	Nbl1	0.48	19
17264036	Ubb	0.43	9		17432440	Pdpn	0.54	21
17264330	Myh8	0.26	17		17433040	Pgd	0.51	24
17264824	Mpdu1	0.37	6		17433145	Rbp7	0.75	10
17265365	Inca1	0.38	7		17433328	Per3	1.06	12
17265657	Smtnl2	0.50	21		17433888	Tmem88b	0.42	9
17266038	Fam101b	0.49	4		17433934	Tnfrsf18	0.33	8
17266079	Glod4	0.31	5		17434973	Sema3c	0.53	22
17266095	Nxn	0.29	1		17435493	4831440E17Rik	0.35	6
17266563	Wsb1	0.27	2		17435748	Hadhb	0.26	6
17266698	Myo1d	0.25	21		17436237	Fosl2	0.57	21
17266952	Ccl9	0.28	19		17436328	Yes1	0.31	6
17266960	Ccl6	0.48	19		17436438	4933407H18Rik	0.27	7
17267237	Ints2	0.44	23		17436907	Afap1	0.28	24
17267393	Dhx40	0.27	7		17436942	D5Ertd579e	0.52	1
17267702	Hlf	0.26	13		17437043	Cytl1	0.27	21
17268120	Pdk2	0.29	11		17437129	Cpeb2	0.66	22
17268598	Pip4k2b	0.29	22		17437765	Klb	0.63	2
17268884	Nr1d1	1.52	8		17437887	Limch1	0.25	22
17269368	Krt19	0.48	20		17437998	Guf1	0.31	4
17269391	Krt14	0.41	19		17438035	Atp10d	0.33	7
17269556	Dnajc7	0.26	6		17438246	Pdgfra	0.35	22
17270761	Ccdc47	0.25	3		17438882	Gm9958	0.29	6
17271968	Mif4gd	0.43	22		17438969	Pf4	0.29	24
17271984	Slc25a19	0.39	22		17439037	Parm1	0.41	1
17272770	Timp2	0.45	20		17439060	G3bp2	0.33	4
17272857	Cbx4	0.27	20		17439094	Art3	0.33	9
17272913	Sgsh	0.27	22		17439239	LOC100862150	0.33	16
17273804	Gm6682	0.54	21		17439388	Fras1	0.27	21
17274021	Osr1	0.47	21		17439481	Bmp2k	0.29	22
17274099	Fam49a	0.30	22		17439669	Arhgap24	0.32	21
17274415	Asap2	0.25	3		17439687	Ptpn13	0.33	20
17274471	Iah1	0.26	6		17439901	Lrrc8b	0.37	22
17274744	Fam150b	0.59	1		17440054	A830010M20Rik	0.30	6
17274766	Fam110c	0.44	22		17440618	Mir701	0.51	22
17274875	Nampt	0.31	19		17440754	Rps28	0.30	8
17275869	Gm10166	0.29	18		17441490	Fbxo21	0.68	10
17276020	Frmd6	0.26	24		17441671	Slc24a6	0.32	7
17276139	Dact1	0.38	20		17441949	Aldh2	0.54	7
17276352	Snapc1	0.25	22		17442046	Hvcn1	0.47	21
17276565	Hspa2	0.39	19		17442413	Ccdc62	0.25	8
17276674	Gphn	0.34	6		17442823	Ran	0.30	4
17276921	Mir3067	0.28	7		17442834	Gpr133	0.67	21
17277043	Dcaf4	0.29	5		17442966	4930579G22Rik	0.33	10

17277146	Acot4	0.41	10		17442970	Vkorc1l1	0.28	19
17277294	Isca2	0.33	21		17443286	Por	0.37	10
17277625	1810035L17Rik	0.26	5		17443342	Hspb1	0.31	18
17277694	Gm2046	0.33	19		17443380	Upk3b	0.71	21
17277788	Flrt2	0.38	21		17443539	Ephb4	0.42	8
17277907	Psmc1	0.27	6		17443562	Gm8066	0.26	10
17278073	Golga5	0.29	4		17443901	Fam20c	0.62	19
17278261	Serpina3b	1.32	8		17444106	Lfng	0.52	20
17278268	Serpina3f	0.26	8		17444304	0610040B10Rik	0.42	6
17278305	Serpina3j	0.33	9		17444346	Daglb	0.43	21
17278321	Serpina3m	0.59	5		17444664	Zfp655	0.30	6
17278775	AF357355	0.36	23		17444806	Rasl11a	0.28	22
17278884	Mir541	0.34	20		17444876	Pomp	0.32	3
17279230	Zfyve21	0.32	24		17445044	B3galtl	0.25	1
17279509	Crip1	0.47	20		17445308	Cyp51	0.56	1
17279584	Esyt2	0.26	22		17446307	Rheb	0.26	21
17279737	Rab10	0.28	20		17446767	Cgref1	0.25	17
17280125	Pqlc3	0.37	20		17446793	Slc5a6	0.93	10
17280287	9030624G23Rik	0.34	10		17447081	Spon2	0.28	1
17280296	Cys1	0.59	17		17447365	Htra3	0.43	19
17280452	Sntg2	0.25	11		17447868	Tapt1	0.37	8
17280498	Dld	0.27	2		17448001	Ppargc1a	0.35	6
17280806	Arl4a	0.28	7		17448064	Lgi2	0.27	22
17280867	Mir1938	0.26	5		17448264	Rfc1	0.25	5
17280897	Stxbp6	0.26	20		17448924	Kdr	0.33	4
17281637	Map4k5	0.25	3		17448960	Clock	0.52	24
17282090	Max	0.26	19		17449417	Sult1e1	0.48	21
17282216	Zfp36l1	0.26	21		17449673	Naaa	0.35	20
17282732	0610007P14Rik	0.36	22		17449710	Cxcl9	0.29	7
17282743	Tgfb3	0.39	20		17449725	Cxcl11	0.38	8
17282980	4930534B04Rik	0.27	6		17449827	C87414	0.34	8
17283155	Eml5	0.50	8		17449939	Antxr2	0.31	21
17283463	Itpk1	0.39	22		17449989	Rasgef1b	0.30	10
17283725	Clmn	0.45	10		17450196	Agpat9	0.60	9
17283742	4831426I19Rik	0.56	9		17450319	Slc10a6	0.32	19
17284002	Hsp90aa1	0.25	21		17450515	Gbp11	1.12	9
17284037	Brp44l	0.38	21		17450718	Fam69a	0.35	4
17284135	Xrcc3	0.33	1		17450741	Atp5k	0.26	7
17284963	Marcksl1-ps4	0.44	16		17451390	Cmklr1	0.31	18
17285056	Idi1	0.55	23		17451443	Coro1c	0.31	21
17285097	Dip2c	0.27	6		17451568	Gltp	0.29	19
17285687	Hist1h4m	0.25	6		17451661	Unc119b	0.27	4
17285746	Hist1h2bn	0.51	12		17451721	Gatc	0.51	21
17285846	LOC100862646	0.68	18		17451816	Hspb8	0.32	20
17285851	Hist1h2bc	0.36	20		17452562	LOC100503186	0.45	8
17285859	Hist1h1c	0.41	24		17452689	Rsrc2	0.27	5
17285879	Hist1h1a	0.35	9		17452705	Niacr1	0.61	7
17286190	Prl2a1	0.29	17		17452709	Gpr81	0.31	8
17286295	Irf4	0.34	9		17452957	Scarb1	0.27	8
17286412	Serpinb9f	0.26	20		17452978	Ubc	0.31	4
17286462	Nqo2	0.32	21		17453154	Chchd2	0.29	9
17286487	Bphl	0.27	5		17453347	Gtf2ird1	0.42	2
17286717	Mir5124	0.29	21		17453573	Hip1	0.35	21
17286732	Tmem14c	0.34	4		17453617	Tmem120a	0.35	8
17286783	BC024659	0.26	21		17453819	Serpine1	0.80	12
17287066	Rnf144b	0.40	4		17454226	Mcm7	0.33	6

17287115	Bicd2	0.39	21		17454278	Gm454	0.39	9
17287148	Ecm2	0.43	21		17454414	Mir339	0.37	15
17287160	Aspn	0.47	19		17454416	Zfand2a	0.39	21
17287175	Ogn	0.27	24		17454804	Spdyb	0.25	12
17287206	Iars	0.29	22		17454944	Tecpr1	0.32	5
17287325	S1pr3	0.42	4		17455281	Lnx2	0.31	22
17287642	Prelid1	0.27	21		17455507	Hsph1	0.43	20
17287827	Tgfbi	0.58	20		17455554	N4bp2l1	0.64	9
17287984	Dapk1	0.58	1		17456545	Lep	0.75	19
17288160	Cdk20	0.29	22		17457184	Cald1	0.26	20
17288548	Slc12a7	0.33	7		17457232	3110062M04Rik	0.37	7
17288716	Glrx	0.67	23		17457436	Luc7l2	0.42	6
17289344	Gfm2	0.35	3		17457462	Gm20022	0.33	6
17289372	Enc1	0.26	19		17457498	Jhdm1d	0.25	16
17289497	Ccdc125	0.26	24		17457680	Tcrb-J	0.27	17
17289602	Adamts6	0.26	10		17457872	Tas2r139	0.27	16
17289660	Dimt1	0.44	22		17457876	Gstk1	0.29	22
17290003	Mir449b	0.27	17		17457981	Olfr448	0.33	13
17290139	4833420G17Rik	0.27	6		17458110	Rny3	0.36	7
17290173	Hmgcs1	0.55	1		17458682	Creb5	0.38	18
17290242	Net1	0.56	9		17458771	Gars	0.33	5
17290301	Akr1e1	0.28	24		17458813	Aqp1	0.27	9
17290324	Pfkp	0.47	23		17459621	Tmem150a	0.25	23
17290565	Mtr	0.27	5		17460185	Dysf	0.45	7
17290770	Mrpl32	0.39	3		17460826	Klf15	0.41	10
17290781	5033411D12Rik	0.29	5		17460941	Slc6a6	0.65	10
17290997	Hist1h2bl	0.25	15		17461023	Nr2c2	0.28	7
17291174	1700024h08rik	0.33	8		17461124	Arl6ip5	0.31	22
17291195	Hist1h1e	0.29	20		17461132	Mitf	0.27	10
17291525	Sox4	0.40	1		17461414	Bhlhe40	1.37	20
17291767	Tubb2a	0.45	21		17461423	Arl8b	0.26	21
17291881	F13a1	0.45	22		17461684	Il17re	0.28	21
17291904	Ssr1	0.29	23		17461793	6720456B07Rik	0.31	21
17291953	Muted	0.27	4		17462145	8430408G22Rik	0.49	12
17291964	Eef1e1	0.33	21		17462149	Cxcl12	0.39	8
17292157	Dtnbp1	0.27	7		17462318	Kdm5a	0.35	5
17292262	Tpmt	0.42	22		17462373	Cecr2	0.27	6
17292634	Nfil3	0.61	20		17462451	Slc6a13	0.34	19
17292677	Drd1a	0.29	8		17462663	Mfap5	0.50	21
17292871	Dbn1	0.29	19		17462729	Clec4a1	0.25	20
17292941	Fam193b	0.29	8		17462905	Lpcat3	0.29	23
17293085	Hnrnpa0	0.29	5		17463020	Ing4	0.34	8
17293313	Gas1	0.28	21		17463031	Acrbp	0.26	8
17293475	Gm19792	0.28	12		17463727	Apold1	0.29	7
17293534	Gm5665	0.39	7		17463747	Gprc5a	0.44	20
17293675	Cdc14b	0.25	6		17463909	Mgst1	0.30	8
17293706	Ctsl	0.35	20		17464165	Etnk1	0.25	4
17293968	Zfp459	0.27	9		17464473	Ergic2	0.31	7
17294458	Rhobtb3	0.29	4		17464492	4833442J19Rik	0.36	11
17294477	Rfesd	0.29	5		17464530	Gm20559	0.28	6
17294489	Arsk	0.26	20		17464588	Sgce	0.26	21
17294723	Cox7c	0.29	24		17464614	Pon1	0.87	5
17294738	Vcan	0.31	22		17464718	Asns	0.33	21
17295136	Iqgap2	0.30	19		17464836	Ndufa4	0.33	6
17295266	Plp2	0.28	18		17465332	Lrrc4	0.31	9
17295503	Mccc2	0.26	5		17465341	Rbm28	0.28	20

17295796	Pik3r1	0.31	19		17465365	Prrt4	0.25	23
17295944	Ppwd1	0.32	6		17465484	Ube2h	0.34	8
17296082	Apoo	0.46	22		17465608	AB041803	0.37	8
17296174	Map3k1	0.27	2		17465772	2010107G12Rik	0.42	19
17296312	Snx18	0.41	22		17466452	Fam115a	0.29	4
17297072	Rpp14	0.32	22		17466488	Tpk1	0.25	7
17297305	Ngly1	0.29	4		17466505	Rbpj	0.32	21
17297391	Nid2	0.29	2		17466507	Ezh2	0.26	6
17297495	Fut11	0.56	8		17466599	Lrrc61	0.39	20
17297497	Chchd1	0.25	4		17466996	Fkbp14	0.28	21
17297750	Ppif	0.34	23		17467209	Fam13a	0.78	10
17297759	Anxa11	0.30	20		17467624	Kdm3a	0.27	6
17297913	Arf4	0.32	21		17467664	Ptcd3	0.27	1
17298041	Arhgef3	0.27	18		17467782	Elmod3	0.29	11
17298126	Lrtm1	0.37	4		17468486	Pcyox1	0.36	21
17298139	Actr8	0.33	6		17468573	Antxr1	0.43	21
17298262	Mustn1	0.38	17		17468690	Cnbp	0.30	3
17298750	Gm5460	0.27	9		17468828	Plxna1	0.34	22
17299329	Lgals3	0.32	19		17469136	Adamts9	0.48	5
17299465	Naa30	0.31	4		17469217	Lrig1	0.51	6
17299575	Rnase4	0.40	20		17469879	Timp4	0.57	19
17299893	Trav14d-3-dv8	0.27	11		17469894	Raf1	0.28	22
17300181	Trav9d-3	0.52	4		17469967	Plxnd1	0.39	24
17300279	Mmp14	0.27	2		17470031	Alox5	0.29	21
17301147	Fam124a	0.26	16		17470580	Apobec1	0.25	23
17301245	Mir598	0.29	11		17470597	Slc2a3	0.29	16
17301256	Sox7	0.47	9		17471107	Plekhg6	0.26	10
17301414	Scara5	0.28	21		17471222	Ccnd2	0.31	21
17301440	Ccdc25	0.31	23		17472192	Mgp	0.33	20
17301452	Clu	0.27	20		17472422	Slco1a6	0.27	18
17301854	Rcbtb2	0.26	5		17472530	Kcnj8	0.30	2
17302061	Nufip1	0.26	21		17473061	Dennd5b	0.31	7
17302333	4930474H20Rik	0.27	15		17473402	Isoc2a	0.27	6
17302483	Cln5	0.33	21		17473455	Zfp784	0.27	8
17302834	Ubac2	0.30	3		17473477	U2af2	0.29	3
17303024	Gm10021	0.53	5		17474181	Prkd2	0.31	8
17303400	Pdhb	0.36	2		17474235	Psg16	0.26	15
17303625	Nr1d2	1.24	10		17474480	Six5	0.26	7
17304186	Plac9	0.41	19		17474844	Vmn1r169	0.26	17
17304193	D14Ertd449e	0.52	20		17475295	4732471J01Rik	0.25	9
17304646	Phf7	0.26	9		17475695	Gm10046	0.29	16
17304860	Timm23	0.30	23		17475787	Dyrk1b	0.29	9
17305034	Sncg	0.60	19		17475863	Il28b	0.29	17
17305685	Ddhd1	0.30	4		17475956	Hnrnpl	0.41	6
17306104	Ear11	0.29	17		17475989	Lgals4	0.36	8
17306362	Prmt5	0.26	23		17476521	C630016N16Rik	0.46	8
17306477	Slc7a8	0.47	1		17476535	Dmkn	0.27	21
17306690	Dcaf11	0.32	8		17476728	Cebpa	0.27	8
17306696	Fam158a	0.27	8		17477811	Gys1	0.27	6
17306793	Tinf2	0.25	4		17477897	Bcat2	0.32	7
17306960	Mcpt4	0.61	20		17477979	Dbp	1.50	11
17307305	Gm6907	0.28	6		17478044	Tmem143	0.31	6
17307588	Fdft1	0.26	18		17478263	Tmem86a	0.51	8
17307791	Scara3	0.38	20		17478745	Atp10a	0.41	20
17307837	Ephx2	0.37	7		17479706	Whamm	0.33	7
17307905	Dpysl2	0.31	20		17479875	Stard5	0.26	20

17308257	Sorbs3	0.27	20		17480205	Rab30	0.32	21
17308727	Gm4285	0.46	8		17480243	Odz4	0.34	6
17308772	9030625A04Rik	0.53	22		17480290	Mir708	0.64	2
17308842	Dgkh	0.30	7		17480312	Gab2	0.38	3
17309262	Slain1	0.25	17		17480327	Alg8	0.36	22
17309304	Gm17066	0.38	4		17481169	Olfr583	0.30	11
17309310	Rbm26	0.29	4		17481252	Olfr635	0.30	15
17309397	Tgds	0.30	2		17481504	Olfr713	0.30	16
17309580	Dock9	0.30	10		17481550	Olfml1	0.29	21
17309907	Prkaa1	0.28	5		17481743	Wee1	0.75	16
17310187	Nadkd1	0.27	6		17481936	Tead1	0.34	16
17310259	Prlr	0.35	4		17481960	Arntl	1.03	23
17310368	Adamts12	0.68	6		17482000	Spon1	0.28	6
17310673	Ank	0.53	20		17482230	Acsm5	0.32	12
17310816	Cmbl	0.35	8		17482307	Ppp1cc	0.27	22
17310872	Sdc2	0.26	21		17482332	2610020H08Rik	0.60	8
17311179	Fzd6	0.33	8		17482508	Vwa3a	0.36	6
17311315	Ttc35	0.38	21		17483110	Ypel3	0.27	9
17311350	Pkhd1l1	0.83	21		17483383	Snora30	0.67	9
17311512	Mal2	0.32	20		17483546	Fus	0.41	5
17311652	Wdr67	0.33	7		17483842	Tacc2	0.26	11
17311807	Sqle	0.50	24		17484578	Olfr539	0.31	11
17311846	Myc	0.43	6		17484881	Taldo1	0.43	6
17311909	Efr3a	0.39	2		17484952	Tspan4	0.54	15
17312032	Ndrg1	0.29	10		17485226	Lsp1	0.39	19
17312236	Gpihbp1	0.34	10		17485357	Dhcr7	0.38	23
17312939	Polr2f	0.44	21		17485880	Hspbp1	0.28	23
17313276	Rbx1	0.40	5		17486011	Gm19933	0.28	19
17313376	Tef	0.62	12		17486261	Vmn2r41	0.40	16
17313504	Srebf2	0.27	23		17486455	Vmn1r68	0.28	9
17313677	Pnpla3	1.02	22		17486469	Zscan4a	0.28	19
17313739	Prr5	0.39	23		17487161	Qpctl	0.26	23
17313811	Fbln1	0.41	21		17487369	Apoc4	0.26	21
17313862	Ppara	0.27	7		17487374	Apoc1	0.56	6
17313926	Gramd4	0.38	2		17487643	Vmn1r159	0.27	16
17314011	Pim3	0.53	8		17487982	Bckdha	0.40	7
17314190	Shank3	0.37	8		17488032	Axl	0.50	20
17314222	Acr	0.31	7		17488057	Cyp2s1	0.28	18
17314556	Slc48a1	0.31	22		17488094	Cyp2b23	0.26	15
17314679	Tuba1b	0.59	22		17488148	Mir3101	0.36	3
17315007	Mettl7a3	0.45	11		17488325	1700049G17Rik	0.36	6
17315200	Krt7	0.28	20		17488458	Zfp36	0.36	10
17315272	Tenc1	0.44	10		17489279	Tmem147	0.29	3
17315305	Igfbp6	0.69	21		17489304	Ffar2	0.52	6
17315519	Hoxc6	0.30	20		17489384	Lsr	0.30	19
17315558	Copz1	0.26	22		17489431	Fxyd3	0.29	18
17315860	Slc1a3	0.35	9		17490011	Gm6871	0.28	12
17316057	Sub1	0.27	22		17490149	Cd33	0.34	22
17316700	Azin1	0.25	23		17490608	Snord33	0.29	5
17316754	Lrp12	0.26	6		17490622	Snord35a	0.31	6
17317046	Tnfrsf11b	0.41	20		17490624	Snord34	0.58	7
17317225	Zhx1	0.27	20		17490791	Snrnp70	0.26	7
17317266	Fbxo32	0.27	7		17491026	Emp3	0.56	21
17317296	Tmem65	0.32	20		17491716	Mir344e	0.35	11
17317313	Tatdn1	0.46	24		17492244	Ntrk3	0.36	21
17317486	Fam49b	0.57	23		17492431	Anpep	0.33	20

17318502	Dgat1	0.25	8		17492870	Bnc1	0.47	21
17318548	Cpsf1	0.25	6		17492905	Il16	0.31	20
17318704	C030006K11Rik	0.29	4		17493212	Prss23	0.49	20
17318967	Gm20024	0.35	5		17493292	Tmem126a	0.46	22
17318983	Tst	0.52	5		17493643	Mogat2	0.36	23
17319050	Mfng	0.35	9		17493658	Serpinh1	0.40	22
17319491	Gm5218	0.28	12		17493681	Snord15b	0.54	7
17319529	Tob2	0.28	13		17493689	Slco2b1	0.41	9
17319619	Ndufa6	0.30	6		17493756	Ppme1	0.33	21
17319736	Mir3080	0.38	21		17493916	Inppl1	0.29	7
17320073	7530416G11Rik	0.25	15		17494449	Lamtor3	0.30	21
17320711	Zcrb1	0.26	21		17494507	Smpd1	0.29	20
17321227	Kansl2	0.31	3		17494894	St5	0.27	23
17321474	Tuba1a	0.79	20		17495097	Lyve1	0.47	19
17321626	Gpd1	0.33	22		17495172	Galntl4	0.25	5
17322113	Krt8	0.65	20		17495207	2310014F06Rik	0.26	11
17322146	Csad	0.40	6		17495309	4933406I18Rik	0.29	7
17322327	Cbx5	0.26	21		17495320	Cyp2r1	0.36	2
17322359	Zfp385a	0.38	21		17495368	Nup35	0.39	22
17322429	Ppp1r1a	0.32	7		17495586	Gde1	0.41	8
17322476	Nat15	0.31	18		17495667	Thumpd1	0.25	6
17322536	Vasn	0.27	19		17495692	Dcun1d3	0.27	18
17323061	Rrn3	0.28	22		17495821	Cdr2	0.64	21
17323755	Cldn5	0.54	4		17496367	Slx1b	0.28	5
17323838	Klhl24	0.41	7		17496947	Fgfr2	0.48	7
17324859	Tnk2	0.33	3		17497076	Chst15	0.28	7
17324932	Lrch3	0.27	6		17497223	Dhx32	0.63	22
17324998	Osbpl11	0.29	10		17497421	Bnip3	0.30	9
17325291	Kpna1	0.25	21		17498041	Dusp8	0.29	17
17325514	Fstl1	0.32	20		17498467	Ano1	0.46	22
17325592	Popdc2	0.26	20		17498695	2900053A13Rik	0.30	23
17326069	Retnla	0.51	20		17498750	Cd209g	0.53	20
17326166	Cd47	0.26	20		17498791	Map2k7	0.26	6
17326183	Gm6936	0.34	19		17498810	Gm14378	0.32	8
17326700	Mir99a	0.39	2		17498874	Gpi1	0.26	18
17326725	4930478L05Rik	0.26	16		17498962	Col4a2	0.34	4
17326756	Ncam2	0.33	8		17499560	Agpat5	0.35	22
17326887	ORF63	0.48	19		17499602	Defb6	0.29	8
17327909	Ppl	0.29	19		17499622	Defb7	0.28	8
17328220	Mir1945	0.34	10		17499637	4930467E23Rik	0.28	7
17328225	Cpped1	0.33	21		17499773	LOC100505096	0.44	9
17328333	LOC100504755	0.44	16		17500097	Tm2d2	0.25	21
17328937	Comt	0.53	21		17500108	Fgfr1	0.25	3
17329149	Gm15760	0.31	5		17500270	Zfp703	0.46	8
17329151	Alg3	0.40	21		17500441	Purg	0.27	7
17329167	Clcn2	0.37	9		17500548	Ppp1r3b	0.35	20
17329833	Fbxo45	0.29	7		17500605	6430573F11Rik	0.47	5
17330359	Pla1a	0.30	22		17500694	Gm2085	0.30	16
17330427	Upk1b	0.78	21		17500744	B430010I23Rik	0.51	8
17330463	Gm19522	0.59	21		17500922	Pdlim3	0.26	20
17330577	Boc	0.33	1		17500992	Actg1	0.28	19
17330602	Gtpbp8	0.25	21		17500996	Acsl1	0.27	9
17330625	Cd200	0.41	20		17501104	Dctd	0.28	21
17331044	Tfg	0.42	22		17501191	Gpm6a	0.91	20
17331114	Tbc1d23	0.29	5		17501230	Gm19464	0.28	15
17331173	St3gal6	0.26	8		17501544	Npy1r	0.30	17

17331247	Olfr191	0.26	16		17501652	Atp6v1b2	0.28	23
17331284	Crybg3	0.25	1		17501672	Zfp930	0.25	1
17331692	Cyyr1	0.39	8		17501810	Rfxank	0.56	22
17331705	Adamts1	0.58	20		17501962	Ell	0.30	7
17331720	Adamts5	0.75	21		17502001	Jund	0.29	20
17331731	Ltn1	0.27	4		17502191	Mrpl34	0.33	22
17331918	Tiam1	0.26	18		17502233	Slc27a1	0.44	10
17332122	Tmem50b	0.35	21		17504074	Cpne2	0.31	3
17333186	Qk	0.31	8		17504572	Ces2g	0.41	21
17333206	Park2	0.26	11		17504707	Nol3	0.30	19
17333505	2210404J11Rik	0.47	10		17505060	Pla2g15	0.41	24
17333707	Mirlet7e	0.41	9		17505069	Slc7a6	0.31	24
17333709	Ncrna00085	0.26	8		17505197	Cirh1a	0.26	3
17333854	Ppp2r1a	0.27	21		17505252	Cyb5b	0.41	24
17334102	Pkmyt1	0.32	8		17505599	Vac14	0.27	24
17334147	Mir5125	0.60	8		17505907	Dynlrb2	0.30	11
17334241	Rnps1	0.25	22		17506042	Cdh13	0.28	8
17334365	Zfp598	0.36	10		17506081	Osgin1	0.60	21
17334419	Sepx1	0.37	22		17506270	Gm10614	0.34	9
17334904	Luc7l	0.28	3		17506273	Cox4i1	0.26	6
17334932	Ergic1	0.29	23		17506477	2810013P06Rik	0.27	9
17334956	A930001N09Rik	0.30	8		17506732	Galnt2	0.41	21
17335364	Mapk14	0.25	19		17506754	Cog2	0.31	5
17335467	Cdkn1a	1.17	23		17506800	Arv1	0.26	3
17335506	Pi16	0.65	19		17506824	Gnpat	0.32	3
17335952	Cyp4f17	0.34	6		17506987	Gm6921	0.25	3
17335966	Cyp4f16	0.30	8		17507184	Cd209d	0.61	20
17336172	Rab11b	0.36	7		17507221	Cd209f	0.73	21
17336190	Ndufa7	0.30	4		17507374	Irs2	0.50	8
17337100	H2-Q4	0.39	7		17507377	Col4a1	0.28	5
17337152	H2-Q10	0.85	10		17507559	Grtp1	0.33	22
17337197	Cdsn	0.41	22		17507584	Dcun1d2	0.28	21
17337228	Ier3	0.43	21		17507637	Rasa3	0.33	7
17337314	Ppp1r10	0.38	20		17507872	2610005L07Rik	0.27	6
17337375	Gm6034	0.27	15		17508134	Agpat6	0.40	9
17337545	Ubd	0.26	11		17508416	Bag4	0.49	8
17337591	Olfr121	0.32	15		17508544	Adrb3	0.33	8
17338286	Usp49	0.32	7		17508591	Snord13	0.62	7
17338434	AI314976	0.26	7		17508735	Tnks	0.27	6
17338637	Zfp959	0.40	5		17508787	Lonrf1	0.25	14
17339013	Emr1	0.35	20		17508846	Mir383	0.32	17
17339390	Gm16519	0.35	18		17509013	Adam34	0.28	9
17339549	Ypel5	0.36	8		17509069	Cyp4v3	0.31	19
17339574	Ehd3	0.70	20		17509189	Ccdc111	0.38	6
17339604	Slc30a6	0.31	23		17509217	Stox2	0.26	2
17340050	Plekhh2	0.29	22		17509274	Ing2	0.28	7
17340120	Ppm1b	0.27	8		17509721	Tufm	0.25	4
17340220	Rhoq	0.26	19		17509976	Slc25a42	0.28	9
17340232	Cript	0.34	21		17510072	2810428I15Rik	0.40	24
17340435	Pisd-ps2	0.51	7		17510338	Plvap	0.32	8
17340609	Ezr	0.52	20		17510667	Tmem184c	0.33	22
17340654	Ttll2	0.47	9		17510735	Smad1	0.26	6
17340657	Gm9992	0.97	9		17510749	Abce1	0.38	22
17340673	Fndc1	0.53	20		17510885	Elmod2	0.36	6
17341000	Gm11166	0.39	10		17511389	Itfg1	0.28	21
17341150	Gm3417	0.36	11		17511731	Ces1f	0.46	8

17341161	9030025P20Rik	0.48	10		17511825	Nudt21	0.29	6
17341269	Has1	0.30	18		17511878	Pllp	0.29	6
17341326	Vmn2r109	0.30	16		17511887	Ciapin1	0.28	23
17341412	Zfp944	0.39	24		17512023	Prss54	0.27	16
17341440	Zfp945	0.25	4		17512103	Got2	0.25	21
17341589	Paqr4	0.37	11		17512434	Tppp3	0.63	20
17342051	Eme2	0.27	23		17512628	Dpep2	0.69	11
17342340	Msln	0.80	20		17512666	Smpd3	0.49	21
17342581	Decr2	0.29	22		17512716	Terf2	0.31	5
17342689	2900010M23Rik	0.54	21		17512868	Chst4	0.86	20
17342868	Fkbp5	0.38	16		17512990	Ddx19b	0.34	6
17343387	Akap8l	0.27	7		17513026	9430091E24Rik	0.29	6
17343628	Angptl4	0.63	6		17513389	Mphosph6	0.52	22
17343710	H2-K1	0.26	9		17513534	1190005I06Rik	0.41	8
17343724	Ring1	0.26	6		17513590	Fbxo31	0.46	9
17344126	Hspa1b	0.42	18		17514078	Agt	0.29	5
17344405	Ddr1	0.30	21		17514447	Casp12	0.30	19
17344835	Olfr92	0.39	9		17514832	Taf1d	0.31	7
17345038	Cd2ap	0.31	21		17514871	Chordc1	0.36	20
17345586	Rpl7l1	0.47	20		17514936	Zfp317	0.26	21
17346094	Sh3gl1	0.31	21		17515045	Ppan	0.30	21
17346317	Lonp1	0.28	6		17515315	Ldlr	0.89	20
17346349	Rfx2	0.32	19		17515335	Gm6484	2.03	21
17346666	4930405O22Rik	0.35	6		17515371	2310047B19Rik	0.28	23
17346774	Twsg1	0.28	22		17515819	Ets1	0.29	6
17346812	Gm10495	0.30	4		17515921	Cdon	0.57	22
17346975	Emilin2	0.47	20		17516300	Olfr981	0.29	15
17347913	4833418N02Rik	0.35	9		17516365	Hspa8	0.37	20
17348126	Mtpap	0.27	23		17516383	Snord14e	0.59	19
17348398	Mir1b	0.39	5		17516469	Usp2	0.68	15
17348400	Gata6	0.38	22		17516777	Fxyd6	0.33	21
17348435	Cables1	0.35	23		17516871	Sik3	0.27	5
17348840	Rnf125	0.47	3		17516997	Gm5617	0.26	22
17348933	Mapre2	0.26	19		17517142	Fdxacb1	0.26	22
17349139	Sap130	0.29	8		17517222	Arhgap20	0.51	22
17349181	Wdr33	0.25	5		17517424	Ireb2	0.31	4
17349361	2410004N09Rik	0.42	7		17517456	Psma4	0.26	4
17349721	Slc35a4	0.26	20		17517634	Sin3a	0.27	6
17349731	Tmco6	0.29	8		17517723	Rpp25	0.33	20
17349993	Rnf14	0.31	21		17518191	Itga11	0.36	20
17350369	Ap3s1	0.33	6		17518378	Dennd4a	0.64	22
17350378	4833403I15Rik	0.28	21		17518526	Clpx	0.42	3
17350437	Gm5506	0.42	10		17518664	Ppib	0.39	20
17350824	Slc12a2	0.32	8		17518672	Fam96a	0.43	21
17351053	Csf1r	0.36	21		17519112	Aldh1a2	0.42	21
17351111	Csnk1a1	0.27	4		17519364	BC031353	0.71	8
17351426	Gnal	0.26	8		17519503	4933433G15Rik	0.28	9
17351444	Chmp1b	0.29	5		17519509	Leo1	0.39	22
17351465	Tubb6	0.32	21		17519738	Cd109	0.31	21
17351497	Seh1l	0.28	23		17520085	Zfp949	0.29	6
17351837	BC031181	0.26	22		17520425	Pcolce2	0.42	19
17352120	Pard6g	0.47	8		17520687	Cep70	0.54	8
17352462	Kif5b	0.26	21		17520905	Slco2a1	0.37	20
17352571	Mpp7	0.28	10		17521159	Twf2	0.31	23
17352763	Ankrd29	0.39	18		17521194	Dusp7	0.34	22
17352824	Zfp521	0.28	20		17521541	Uba7	0.38	6

17352907	4921533I20Rik	0.28	14		17521762	Usp19	0.26	4
17352985	B4galt6	0.45	23		17521887	Prkar2a	0.27	22
17353337	Gypc	0.49	23		17522779	Cmtm6	0.44	4
17353663	Tmem173	0.27	23		17522958	Ctdspl	0.30	9
17353752	Ndufa2	0.27	24		17522969	Mir26a-1	0.41	8
17353957	Spry4	0.69	23		17523398	Snrk	0.26	9
17353962	Fgf1	0.42	21		17523566	Clec3b	0.37	22
17354101	Dpysl3	0.36	20		17524087	Ankrd49	0.34	23
17354367	Lox	0.39	23		17524311	Muc16	0.86	21
17354755	Mir378	0.43	6		17524451	Col5a3	0.49	3
17354784	E330013P06	0.27	10		17524760	Kank2	0.27	22
17354992	Amd1	0.35	21		17524914	Ecsit	0.30	4
17355463	Ctif	0.30	8		17524969	Anln	0.28	9
17355862	1700034H14Rik	0.29	5		17525288	Tmem45b	0.41	19
17355955	1810055G02Rik	0.33	22		17525329	St3gal4	0.48	10
17356279	Rbm4b	0.27	6		17525471	Gm3434	0.26	3
17356369	Cd248	0.42	22		17525803	Gramd1b	0.27	1
17356412	4930481A15Rik	0.28	9		17525955	Sc5d	0.57	22
17356613	Znhit2	0.28	5		17526102	Oaf	0.25	3
17357072	Pla2g16	0.26	7		17526127	Gm10687	0.59	7
17357083	Hrasls5	0.28	9		17526315	Phldb1	0.36	8
17357255	Lrrn4cl	0.27	19		17526982	Gm684	0.37	19
17357444	Fads3	0.42	17		17527332	Nrg4	0.41	13
17357640	Ms4a4a	0.28	19		17527353	Etfa	0.27	7
17357671	Ms4a6c	0.32	21		17527460	Snx33	0.28	6
17357688	Ms4a6b	0.25	16		17527661	Islr	0.49	20
17357815	Fam111a	0.29	21		17527808	Gm20199	0.25	3
17358098	E030003E18Rik	0.49	18		17528229	Ptplad1	0.45	22
17358103	Aldh1a1	0.44	20		17528430	Tpm1	0.29	22
17358324	Kank1	0.28	4		17528617	Fam63b	0.32	24
17358368	Dmrt2	0.46	20		17529029	Dppa5a	0.27	12
17358375	Smarca2	0.34	17		17529316	Fam46a	0.41	19
17358503	Rcl1	0.32	7		17529549	Snhg5	0.45	5
17358638	Rpl31-ps20	0.30	21		17529699	1190002N15Rik	0.34	19
17358690	Papss2	0.26	17		17530141	Pccb	0.30	5
17358876	Rpp30	0.29	23		17530633	Ppm1m	0.32	7
17359113	O3far1	0.28	3		17530881	Cish	0.39	20
17359160	Plce1	0.41	18		17531168	Dag1	0.27	22
17359299	LOC100504786	0.31	19		17531281	Ccdc72	0.41	21
17359411	Zfp518a	0.29	24		17531987	Tgfbr2	0.31	22
17359520	Pi4k2a	0.25	24		17532045	Plcd1	0.32	19
17359531	Marveld1	0.37	17		17532063	Acaa1b	0.45	8
17359608	Entpd7	0.31	22		17532137	Myd88	0.25	24
17359689	Scd2	0.68	24		17532694	Pisd-ps3	1.04	10
17359902	Nolc1	0.31	20		17532923	Wdr45	0.27	8
17360501	Tcf7l2	0.33	8		17533952	Gm2863	0.34	18
17360877	Fam45a	0.30	23		17534074	Lonrf3	0.42	20
17360890	Grk5	0.30	24		17534122	Ube2a	0.33	23
17361032	Ndufs8	0.34	22		17534352	Mcts1	0.26	1
17361338	Rbm4	0.37	7		17534408	Xiap	0.32	8
17361764	Fam89b	0.34	22		17534665	Arhgap36	0.39	23
17361771	Sssca1	0.35	21		17534673	2610018G03Rik	0.30	22
17361805	Neat1	0.34	5		17534960	Fhl1	0.41	20
17361855	Pola2	0.27	7		17535245	Aff2	0.26	18
17361915	Mrpl49	0.31	22		17535390	Prrg3	0.26	20
17362240	Stip1	0.31	22		17535434	Nsdhl	0.44	23

17362453	Slc3a2	0.27	21		17535448	Zfp185	0.37	23
17362595	Fads2	0.29	1		17536170	Tab3	0.25	6
17362616	Gm98	0.61	21		17536187	Gm14762	0.29	8
17362973	Ms4a6d	0.29	19		17536230	Gm8954	0.27	17
17363086	Dtx4	0.68	24		17536233	Gm5941	0.40	10
17363239	Vps13a	0.27	20		17536339	Zxdb	0.31	5
17363407	Anxa1	0.50	20		17536383	F630028O10Rik	0.26	22
17363505	Smc5	0.28	6		17536922	Gm5166	0.36	18
17363533	Mamdc2	0.28	20		17537003	Uprt	0.31	20
17363559	Fam189a2	0.27	9		17537112	Lpar4	0.33	23
17363670	2610016A17Rik	0.28	18		17537190	Sh3bgrl	0.30	20
17364098	Acta2	0.33	19		17537260	2010106E10Rik	0.54	10
17364139	Slc16a12	0.51	22		17537592	Srpx2	0.77	19
17364208	Ppp1r3c	1.04	21		17538590	Pfkfb1	0.35	9
17364246	4931408D14Rik	0.45	10		17539125	Rps6ka3	0.35	1
17364280	Myof	0.27	20		17539303	Phka2	0.28	7
17364367	Noc3l	0.33	22		17540127	Ssxb8	0.30	17
17364474	Pdlim1	0.37	19		17540240	Mid1ip1	0.36	22
17364762	Exosc1	0.25	23		17540544	Uxt	0.34	22
17364828	Crtac1	0.49	15		17540589	Klhl13	0.29	24
17364932	Got1	0.33	21		17541008	Snora69	0.51	7
17365421	Nt5c2	0.29	22		17541010	Upf3b	0.26	5
17365444	Pcgf6	0.27	24		17541036	Ndufa1	0.37	6
17365992	Hspa12a	0.40	23		17541147	Rhox11	0.25	9
17366141	Prdx3	0.25	5		17541383	Zdhhc9	0.26	20
17366241	Csprs	0.28	16		17541440	Zfp280c	0.27	5
17366536	Ccdc3	0.28	20		17541612	Rap2c	0.28	3
17366756	Mir669g	0.30	10		17541719	Mir450-2	0.29	7
17366760	Mir669j	0.29	19		17542021	Mir505	0.29	6
17366922	Mir669m-1	0.50	15		17542267	Xlr3a	0.46	16
17366930	Mir466g	0.33	17		17543104	Meg3	0.50	21
17366932	Mir466h	0.50	16		17543196	Pdk3	0.33	22
17366938	Mir297a-4	0.38	14		17543281	Gm7061	0.26	11
17367004	Il15ra	0.44	8		17543284	Zxda	0.31	5
17367102	Mrc1	0.45	23		17543315	Asb12	1.09	23
17367390	Etl4	0.27	6		17543407	Ophn1	0.41	5
17367436	Gm13363	0.27	20		17544002	Fnd3c2	0.28	10
17367536	Apbb1ip	0.47	22		17544333	Rps12	0.25	5
17367576	Acbd5	0.27	5		17544396	4930570D08Rik	0.26	7
17367868	Rnf208	0.26	17		17544618	Tceal6	0.26	10
17368171	Bmyc	0.40	22		17544878	Morc4	0.61	24
17368646	Olfm1	0.40	20		17544939	Tsc22d3	0.57	17
17368666	Mrps2	0.28	22		17545865	Txlng	0.38	7
17368714	Tsc1	0.31	5		17545885	Syap1	0.27	19
17368834	Med27	0.25	23		17545979	Mospd2	0.25	24
17369235	Ppp2r4	0.31	4		17546109	Tlr7	0.28	22
17369721	Pomt1	0.33	2		17546762	Mid1	0.26	23
17370043	Angptl2	0.29	13		17546834	Ddx3y	0.28	6
17370309	Ptgs1	0.26	22		17547468	1700040F15Rik	0.37	17
17370745	Rif1	0.34	8		17547515	Gm19894	0.65	22
17370799	Cacnb4	0.31	8		17547581	Gm10387	0.31	23
17370947	Gm13544	0.26	9		17547620	Ftl1	0.26	5
17371101	Tank	0.43	22		17547694	2900097C17Rik	0.37	6
17371201	Gca	0.26	2		17547719	Ahnak2	0.60	20
17371310	B3galt1	0.35	5		17547877	Deptor	0.34	21
17371756	Metap1d	0.26	6		17547913	LOC100505027	0.46	21

17371800	Itga6	0.30	4		17547951	Gm4416	0.25	20
17372197	Osbpl6	0.33	8		17547965	Cbr1	0.34	7
17372621	Slc43a1	0.75	9		17547980	Olfr177	0.57	20
17373028	Fnbp4	0.25	4		17548030	Ddx39b	0.28	9
17373450	Phf21a	0.26	6		17548053	Gm19939	0.30	9
17373484	Pex16	0.33	5		17548094	Gnpnat1	0.50	21
17373521	Chst1	0.55	11		17548112	Morf4l2	0.36	22
17373550	Trp53i11	0.64	4		17548123	Ehd1	0.60	21
17373680	Pamr1	0.29	19		17548193	Vim	0.29	20
17373825	Cd59a	0.31	19		17548244	Gm19816	0.26	24
17374295	2900064A13Rik	0.30	21		17548250	Gm19343	0.30	5
17374421	Mir1951	0.34	10		17548334	Ptges3	0.43	19
17374939	Mapkbp1	0.33	7		17548377	Gm19313	0.26	20
17375694	Dtwd1	0.27	21		17548417	Gm19648	0.66	9
17375742	Ap4e1	0.28	22		17548573	4931406P16Rik	0.34	9
17376063	Zc3h6	0.47	7		17548585	Twf1	0.36	21
17376252	Nop56	0.26	21		17548638	Fam58b	0.27	13
17376549	Prnd	0.51	9		17548671	Gm19274	0.51	21
17376805	Ankrd5	0.73	7		17548738	LOC100503226	0.50	9
17376977	Snrpb2	0.26	20		17548746	Bace1	0.28	8
17377144	Slc24a3	0.29	24		17548750	Cryab	0.29	20
17377170	BC039771	0.30	7		17548779	1700024F20Rik	0.30	8
17377177	Rin2	0.26	6		17548781	Ywhaq	0.40	18
17377360	Gzf1	0.30	6		17548806	Polr2k	0.47	8
17377675	Csnk2a1	0.29	3		17548850	Arpc1b	0.31	20
17378348	Trp53inp2	0.52	20		17548916	LOC100862127	0.29	9
17378827	Lbp	0.51	19		17548928	Zfp330	0.27	6
17379606	Mmp9	0.45	3		17548973	Pten	0.37	17
17379864	1500012F01Rik	0.36	5					

Table 6: Gene transcripts rhythmic in SCN-KO mice with gene symbols. List of rhythmic transcripts with Affimetrix identification number, the according gene symbol, normalized fold change (Norm FC) and the phase. Genes with a red marked Gene symbol are also rhythmic in Con animals.

SCN-KO					SCN-KO			
Affimetrix-ID	Gene Symbol	Norm FC	Phase		Affimetrix-ID	Gene Symbol	Norm FC	Phase
17211174	A830018L16Rik	0.397196	11		17403866	Ptger3	0.368839	10
17212087	Npas2	0.803968	20		17404337	Cpa3	0.645281	19
17212229	Il18r1	0.418684	23		17404432	Fndc3b	0.304675	23
17213213	Casp8	0.419796	22		17404464	Tmem212	0.27245	11
17213578	Gm20342	0.270647	2		17404796	Anxa5	0.274611	21
17214948	Sp100	0.311846	21		17405062	Pabpc4l	0.266444	4
17216469	Serpinb10	0.330148	18		17406492	Kirrel	0.440785	23
17217619	Phlda3	0.378068	12		17406686	Mir9-1	0.308889	15
17218349	Glul	0.337205	10		17407489	Lce3a	0.3018	7
17218861	F5	0.255912	20		17407509	Tdpoz1	0.706838	16
17219005	Creg1	0.327494	19		17407531	Gm10696	0.315859	15
17219418	Cd84	0.378207	19		17407850	Ecm1	0.264865	19
17220432	Tlr5	0.279343	18		17408052	Terc	0.27442	21
17224071	Fn1	0.416183	21		17408940	2010016I18Rik	0.568331	6
17224154	Ankar	0.259533	16		17408960	Cd53	0.493581	19
17224180	Igfbp5	0.34484	20		17409284	Celsr2	0.259367	9
17225929	Pam	0.290486	21		17410410	Sgms2	0.504303	23
17226346	Insig2	0.270143	23		17410581	Bank1	0.290899	19
17227348	Nav1	0.256004	21		17410617	Dapp1	0.392277	20

17227696	Cfh	0.393401	21		17411014	Gm10636	0.306231	15
17227828	Pla2g4a	0.360268	20		17411147	Ifi44	0.487152	23
17227892	Prg4	0.743253	20		17415973	Leprot	0.255836	24
17228293	Cacna1e	0.546653	21		17416009	Gm12794	0.330038	15
17229389	Uck2	0.395621	1		17416967	Spata6	0.284853	23
17229658	Fcer1g	0.408912	19		17419287	Laptm5	0.356914	19
17231040	Nenf	0.333806	3		17419437	Ptafr	0.349932	20
17231366	Plekhg1	0.29029	19		17419553	Map3k6	0.297957	7
17231423	Akap12	0.298379	22		17421222	Gm13083	0.321078	18
17231694	Plagl1	0.276782	1		17421444	Gm13251	0.327969	21
17231717	Fuca2	0.307331	21		17421476	Gm13139	0.522327	20
17232332	Arhgap18	0.327516	22		17421488	Gm13248	0.293365	1
17232910	Mir3473b	0.330412	16		17422832	Fam132a	0.258041	10
17233226	Lilrb4	0.533849	19		17423479	Osgin2	0.321063	24
17233323	Pln	0.280607	9		17423792	Pnrc1	0.280336	24
17233384	Fabp7	0.35497	18		17424742	Gba2	0.271409	1
17233534	Sowahc	0.27192	6		17425276	Amd1	0.373815	14
17234339	Adora2a	0.346197	24		17425523	Snora17	0.337078	4
17234647	Itgb2	0.382482	19		17427051	Ptplad2	0.369742	20
17235029	Kiss1r	0.254921	22		17429581	Gm12888	0.606009	16
17235941	Chst11	0.295403	23		17429600	Gm12887	0.268288	17
17236003	Tcp11l2	0.369578	2		17430576	Tinagl1	0.262329	23
17238718	B020014A21Rik	0.348465	9		17431174	Cd52	0.271993	20
17240098	Gm3258	0.278376	15		17431181	Sh3bgrl3	0.317892	22
17241799	1700049L16Rik	0.259485	10		17431332	Man1c1	0.275632	21
17243644	Cry1	0.253947	15		17431607	C1qb	0.321478	18
17244949	Krr1	0.270355	16		17432674	Tnfrsf1b	0.324701	20
17245223	Lyz2	0.314938	20		17433145	Rbp7	0.576015	23
17245231	Lyz1	0.557738	19		17433328	Per3	0.937391	8
17247039	Aebp1	0.369831	19		17434224	Ccl19	0.261032	13
17247948	Efemp1	0.353449	21		17434973	Sema3c	0.505746	22
17248288	Sh3pxd2b	0.29748	20		17435577	Gm17695	0.306893	15
17248309	Stk10	0.261446	21		17437686	Fam114a1	0.302249	24
17248476	Pank3	0.368965	9		17437765	Klb	0.306228	22
17248911	Olfr56	0.311201	21		17439164	Sept11	0.293939	21
17249980	Igtp	0.459908	21		17439388	Fras1	0.285506	21
17249990	Irgm2	0.466252	21		17440826	Acacb	0.358881	11
17250533	Map2k3	0.393616	10		17442719	Aacs	0.684466	11
17250585	Mfap4	0.288343	23		17442834	Gpr133	0.40071	20
17251527	Per1	0.523157	6		17442985	Tpst1	0.272849	22
17252497	Atp2a3	0.392648	21		17443185	Mlxipl	0.325313	10
17253996	Tmem98	0.418334	21		17443486	Cldn15	0.405523	22
17254289	Gm11428	0.46684	19		17444682	Cyp3a57	0.258822	17
17255556	Mir196a-1	0.320069	14		17444806	Rasl11a	0.400838	21
17257085	Hexim1	0.308876	13		17444961	Alox5ap	0.360649	19
17258738	Sept9	0.287138	22		17446793	Slc5a6	0.411886	8
17260761	Plek	0.335149	19		17447081	Spon2	0.28537	22
17261650	Dock2	0.34008	19		17447868	Tapt1	0.264674	4
17262241	9930111J21Rik2	0.3316	20		17448960	Clock	0.35452	21
17262250	Tgtp2	0.725436	21		17449749	Scarb2	0.279903	24
17262702	Rad50	0.328962	23		17450049	Tmem150c	0.27209	10
17263511	Rasd1	0.299746	6		17450448	Gbp9	0.486081	21
17264835	Cd68	0.506562	18		17450461	Gbp4	0.858675	22
17265229	Alox15	0.493142	20		17450501	Gbp10	0.323859	21
17266095	Nxn	0.314672	21		17451437	Selplg	0.312568	20
17266196	Mir423	0.293595	4		17451568	Gltp	0.269605	21

17266368	Tlcd1	0.307068	17		17453714	Sh2b2	0.459027	11
17266372	Snord42b	0.264011	9		17454977	Baiap2l1	0.306039	20
17266520	Lgals9	0.260848	22		17455386	Slc46a3	0.329428	11
17266563	Wsb1	0.314892	23		17456121	Mdfic	0.258601	22
17266960	Ccl6	0.423672	20		17456772	Fam40b	0.318846	9
17268884	Nr1d1	0.986515	2		17456963	Mir335	0.265161	15
17274099	Fam49a	0.288803	22		17457232	3110062M04Rik	0.455198	24
17274408	Gm17758	0.297634	5		17458308	Atp6v0e2	0.254382	23
17274875	Nampt	0.387572	11		17458520	Mpp6	0.285293	21
17274889	Gdap10	0.37139	8		17458813	Aqp1	0.28274	6
17277251	2900006K08Rik	0.263107	13		17458924	Vmn1r4	0.304034	13
17278395	D430019H16Rik	0.377686	4		17460185	Dysf	0.278809	23
17278696	Mir665	0.295463	3		17460826	Klf15	0.405079	7
17279317	A530016L24Rik	0.324385	10		17460941	Slc6a6	0.253525	10
17279349	Adssl1	0.253749	15		17462566	Mug2	0.277725	16
17279670	Rapgef5	0.255048	22		17462663	Mfap5	0.311831	21
17280640	Cdhr3	0.290549	11		17462738	Clec4a3	0.401707	17
17280913	Nova1	0.286499	23		17463465	Clec2g	0.271082	15
17282929	Gm2035	0.340026	16		17464367	Ppfibp1	0.343944	22
17282956	Gm7104	0.253197	17		17464654	Pdk4	0.760075	23
17283088	1700019M22Rik	0.434096	15		17466452	Fam115a	0.267509	4
17283380	Fbln5	0.447783	22		17467209	Fam13a	0.4182	9
17283445	Lgmn	0.331947	20		17468018	Hk2	0.345971	10
17285746	Hist1h2bn	0.378606	17		17468183	Actg2	0.280115	7
17286403	Serpinb9e	0.527797	16		17468702	H1fx	0.461875	10
17287901	Ntrk2	0.267925	24		17469365	Frmd4b	0.298676	19
17288716	Glrx	0.517388	19		17470597	Slc2a3	0.297058	9
17289527	Cd180	0.458219	19		17470616	C3ar1	0.311136	19
17289928	Ppap2a	0.353394	22		17471541	Clec7a	0.329622	20
17291053	Hist1h4i	0.267016	16		17472903	Pthlh	0.255225	6
17292316	Zfp169	0.259081	22		17473061	Dennd5b	0.273777	24
17292634	Nfil3	0.481582	16		17473734	Vmn1r79	0.259153	15
17293237	Golm1	0.274691	19		17473948	Gm4745	0.279941	15
17293607	Ptch1	0.320247	23		17474547	Rtn2	0.324124	23
17294222	Rpl9	0.341788	23		17474726	Vmn1r143	0.326805	10
17295987	Rgs7bp	0.493024	8		17474730	Vmn1r104	0.287548	11
17296558	LOC100861753	0.256594	18		17474759	Vmn1r130	0.282441	10
17297660	1700112E06Rik	0.440522	15		17474765	Vmn1r135	0.367316	10
17300127	A630038E17Rik	0.307185	17		17474785	Vmn1r148	0.435849	12
17300279	Mmp14	0.25612	21		17474789	Vmn1r152	0.274403	11
17301670	Loxl2	0.255606	22		17474795	Vmn1r107	0.26915	10
17301968	Lcp1	0.308688	19		17475487	Cyp2a12	0.340868	15
17302936	Itgbl1	0.256608	22		17475536	Mir1191	0.339772	14
17303625	Nr1d2	0.700373	5		17476364	Tyrobp	0.389392	19
17304155	Plac9	0.509222	16		17477979	Dbp	0.726885	7
17305508	Gm16506	0.305223	17		17478222	Gm5331	0.255336	15
17305520	Ear1	0.313365	18		17478263	Tmem86a	0.275251	3
17306477	Slc7a8	0.588077	23		17480290	Mir708	0.308545	19
17306678	Gm8894	0.259146	2		17480312	Gab2	0.251559	23
17306960	Mcpt4	0.405997	18		17480485	Lrrc32	0.277483	22
17307093	C030013D06Rik	0.261193	1		17481743	Wee1	0.6369	10
17307290	D14Ertd668e	0.445318	20		17481960	Arntl	0.893598	18
17307588	Fdft1	0.317053	13		17483098	Gdpd3	1.231542	18
17307860	Ptk2b	0.327232	22		17483180	Kctd13	0.258828	2
17309935	Dab2	0.257846	19		17483577	Itgam	0.460051	19
17309981	Fyb	0.28629	18		17483912	Htra1	0.295308	23

17310808	Dap	0.267505	23		17485226	Lsp1	0.265469	21
17311551	Col14a1	0.498619	21		17486099	Zim1	1.042876	23
17311909	Efr3a	0.260607	21		17486554	Vmn2r56	0.250721	4
17312759	Cyth4	0.278657	20		17487607	Vmn1r142	0.255324	11
17313376	Tef	0.569059	8		17489508	Gm5329	0.330807	15
17315198	Mir1941	0.322285	14		17489645	4931406P16Rik	0.320581	2
17315272	Tenc1	0.279127	4		17490589	Fcgrt	0.349135	12
17315438	Prr13	0.418728	22		17490978	Kcnj14	0.288494	17
17315570	Nckap1l	0.263409	19		17491026	Emp3	0.278819	19
17317393	9930014A18Rik	0.30805	22		17492314	Mfge8	0.284667	1
17317533	Adcy8	0.26787	11		17492417	Pex11a	0.252972	10
17318312	Nrbp2	0.503753	11		17492431	Anpep	0.272704	19
17318428	Oplah	0.314579	11		17493212	Prss23	0.366825	21
17318950	Csf2rb2	0.375327	20		17493556	Acer3	0.365188	20
17318983	Tst	0.64611	12		17494168	Olfr601	0.262088	11
17319529	Tob2	0.250714	9		17494643	Gvin1	0.361221	21
17319736	Mir3080	0.322229	13		17494651	Gm8989	0.393709	22
17322750	Abat	0.296913	20		17494656	Gm4070	0.305772	22
17323302	Pkp2	0.470479	24		17494662	Gm17757	0.330435	22
17323755	Cldn5	0.41425	22		17494664	Gm8979	0.397308	22
17324471	Mir28	0.26216	16		17494674	Gm8995	0.287781	18
17325206	Adcy5	0.451141	23		17495821	Cdr2	0.325937	21
17326069	Retnla	0.345561	21		17495839	Igsf6	0.427136	19
17328842	Prodh	0.468148	10		17496651	Sephs2	0.377307	10
17331247	Olfr191	0.29918	22		17497724	Ifitm6	0.408333	22
17331284	Crybg3	0.378964	21		17499155	Mcf2l	0.268752	3
17333696	Vmn2r90	0.292357	18		17500662	Zdhhc2	0.420567	23
17333731	Fpr2	0.378933	16		17500679	Vps37a	0.250537	22
17337110	H2-Q5	0.543488	21		17504572	Ces2g	0.350368	19
17337133	H2-Q7	0.451373	22		17507374	Irs2	0.385568	1
17337197	Cdsn	0.25619	23		17508325	Htra4	0.356683	24
17337618	Olfr128	0.258185	6		17508336	Plekha2	0.328653	22
17337796	Pla2g7	0.27682	19		17508787	Lonrf1	0.315212	9
17339460	Lpin2	0.455576	24		17508850	Msr1	0.363049	19
17339574	Ehd3	0.501893	22		17509013	Adam34	0.286785	15
17340050	Plekhh2	0.307531	21		17509069	Cyp4v3	0.311215	20
17340177	Prkce	0.328264	10		17512654	Pla2g15	0.262596	16
17340654	Ttll2	0.251696	10		17513672	Cyba	0.333363	21
17340657	Gm9992	0.417431	9		17514435	Casp4	0.307944	20
17340673	Fndc1	0.398164	21		17515786	Arhgap32	0.290968	7
17342027	Snora78	0.296572	4		17515921	Cdon	0.366762	20
17342403	Rhbdl1	0.324183	10		17516280	Olfr150	0.251976	16
17342868	Fkbp5	0.271084	9		17516469	Usp2	0.592732	11
17343628	Angptl4	0.56803	23		17516777	Fxyd6	0.382224	21
17343856	Btnl5	0.318513	16		17517105	Il18	0.257171	21
17344637	Gm8815	0.262103	15		17517222	Arhgap20	0.534651	21
17344835	Olfr92	0.336038	16		17517723	Rpp25	0.344026	20
17348282	Colec12	0.310413	19		17518278	Lctl	0.683838	21
17349209	Lims2	0.288023	1		17519364	BC031353	0.418237	4
17350925	Iigp1	0.566746	22		17519738	Cd109	0.44795	21
17351053	Csf1r	0.296146	19		17521541	Uba7	0.313226	22
17351837	BC031181	0.299866	23		17523650	Ccr2	0.261687	21
17353378	Epb4.1l4a	0.262394	24		17523921	Arhgap42	0.300712	22
17357810	Mpeg1	0.362753	19		17524338	Olfr853	0.270882	17
17357938	Olfr1494	0.353971	5		17524969	Anln	0.261578	23
17359285	Cyp2c29	0.497874	15		17525288	Tmem45b	0.371325	21

17360890	Grk5	0.34718	20		17525465	Gm5916	0.250265	13
17362616	Gm98	0.378928	20		17526707	Zbtb16	0.589765	4
17362696	Tmem216	0.271098	2		17527332	Nrg4	0.412212	10
17362874	Ms4a8a	0.290704	19		17528405	Rab8b	0.309929	20
17363058	Olfr1424	0.253648	18		17529582	9330159M07Rik	0.287229	4
17363086	Dtx4	0.544551	20		17530059	Mras	0.255426	20
17363407	Anxa1	0.403506	23		17530311	Tmem108	0.394215	22
17363670	2610016A17Rik	0.35546	22		17530653	Alas1	0.388399	11
17364139	Slc16a12	0.499696	9		17533553	Phf16	0.493251	22
17364163	Mir107	0.32966	12		17534074	Lonrf3	0.321341	15
17364565	Blnk	0.250393	18		17534240	Rhox2d	0.291852	16
17366012	4930506M07Rik	0.430409	21		17534489	1110059M19Rik	0.779462	22
17366352	Sp110	0.288638	21		17535208	Fmr1	0.393318	1
17367216	Plxdc2	0.375504	21		17535600	Dusp9	0.285981	23
17367536	Apbb1ip	0.392468	20		17536390	Heph	0.255685	5
17369235	Ppp2r4	0.258715	1		17537409	Astx	0.278777	10
17370043	Angptl2	0.26906	8		17537592	Srpx2	0.500667	21
17370309	Ptgs1	0.26567	21		17537906	Kir3dl1	0.312216	18
17370455	Nek6	0.25636	23		17538425	Snora35	0.304915	16
17371101	Tank	0.258205	20		17538704	Phf8	0.310098	23
17372797	Olfr1034	0.422131	4		17539064	Acot9	0.274196	1
17372970	Olfr1265	0.309704	9		17540154	Cybb	0.478142	19
17373550	Trp53i11	0.399264	23		17540589	Klhl13	0.376972	22
17374488	Thbs1	1.694747	22		17541681	Gpc3	0.295917	20
17374939	Mapkbp1	0.320373	24		17541760	Mospd1	0.267276	23
17376423	Hspa12b	0.275235	23		17541806	Xlr	0.38523	14
17376649	Crls1	0.283621	23		17541926	Fgf13	0.251424	20
17378898	Adig	0.372068	2		17542220	Gabra3	0.368977	19
17380222	Pck1	0.587415	1		17542948	Prrg1	0.28453	24
17382101	Etl4	0.264502	21		17543407	Ophn1	0.358471	24
17385422	Acvr1c	0.253187	1		17543562	Gm614	0.27578	17
17385719	Dpp4	0.645068	21		17543572	Il2rg	0.328189	21
17386479	Mir5115	0.406905	4		17544878	Morc4	0.346165	22
17387517	Serping1	0.366372	20		17545488	Kctd12b	0.270184	24
17387846	Olfr1196	0.252103	15		17546119	Prps2	0.359433	21
17387888	Olfr1219	0.295948	13		17546251	LOC100862032	0.362478	13
17388332	Creb3l1	0.352277	22		17546522	Ssty1	0.257117	12
17388687	Prr5l	0.277045	1		17546997	LOC100039753	0.382041	15
17389775	A430105I19Rik	0.290299	11		17547223	LOC382133	0.420289	10
17390879	Fbn1	0.519382	23		17547231	LOC100039574	0.321956	10
17392056	Fermt1	0.297741	10		17547420	Ssty2	0.372832	10
17392805	LOC100125594	0.250515	14		17547428	MGC107098	0.258629	10
17393839	9430008C03Rik	0.291897	9		17547436	LOC100861873	0.561428	14
17394297	Pltp	0.329044	20		17547505	Col6a1	0.452646	23
17395091	Pmepa1	0.374227	8		17547827	Snrpn	0.45761	21
17395389	Gm14406	0.261299	23		17547859	BC056474	0.334172	23
17397511	Lhfp	0.282202	23		17547929	A930007A09Rik	0.267602	24
17397575	Postn	0.444791	22		17547933	Bod1	0.272945	19
17400000	S100a10	0.280549	20		17547939	Ndufa11	0.448702	11
17400048	Rorc	0.322985	14		17547953	Gm6958	0.301752	15
17401335	Slc16a1	0.436622	20		17548018	Lnpep	0.253586	4
17401480	Dennd2d	0.37255	8		17548277	Gm19382	0.491579	22
17402193	Arhgap29	0.28552	21		17548354	Llph	0.252781	16
17403224	Gbp7	0.266996	21		17548597	E330027M22Rik	0.251311	23
17403864	Mir186	0.334053	7		17548748	LOC100504898	0.911618	6

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