Adiponectin mediates metabolic feedback to the mediobasal hypothalamic circadian clocks

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Göttingen, December 2014 Anthony Hiu King Tsang

Content

Summary

The rotation of the Earth around its axis results in a repetitive succession of day and night. The profound environmental changes associated with the day-night cycle drove most organisms to evolve endogenous timekeepers to reliably anticipate predictable events at particular times of day and adjust their behaviors and physiology accordingly. Such endogenous timekeeping machineries are known as circadian (from Latin *circa diem* – about a day) clocks. In mammals, the cellular time-keeping machinery is comprised of an interlocked transcriptional-translational feedback loop (TTL) that during the daytime the transcriptional activating BMAL1/CLOCK complexes activate their own repressors PERs and CRYs which will then be degraded during the night. This molecular clockwork regulates local cellular physiology and is shared among the central circadian pacemaker – the suprachiasmatic nucleus (SCN) and other tissues in the brain and in the periphery. Recent studies have highlighted an extensive crosstalk between metabolism and circadian clock. For example, circadian misalignments contribute to metabolic disorders and *vice versa*. However, the mechanism of this link is still poorly understood.

The mediobasal hypothalamus (MBH) is an assembly of hypothalamic nuclei which together play a major role in regulating behavioral rhythms such as feeding/fasting and sleep/wake cycles. It has been documented that the autonomous cellular clockwork exists in multiple MBH nuclei and regulates the local physiology such as electrophysiological properties and appetite-regulating neuropeptides (NP) expression, hinting for the role of molecular clock in appetite regulation. One of the most important features of the MBH is its ability to integrate information carried by circulating metabolic hormones to regulate energy homeostasis of the body. I therefore hypothesize that there are metabolic hormones that can modulate the molecular clock in the MBH and thereby regulating feeding rhythms.

To search for metabolic hormones that can reset the MBH clock, I engineered a hypothalamic neuronal cell line to stably express a circadian reporter and used it as a model to screen for metabolic signals that are capable of resetting neuronal clocks. In a small scale screening, I identified an adipokines – adiponectin as a

novel mediobasal hypothalamic cellular clock modulator. As it is known that circulating adiponectin levels are regulated by the metabolic status of the body, I further hypothesize that adiponectin is a mediator that can feed back to the MBH clocks according to the metabolic status of the body.

I demonstrated that adiponectin possesses a phase-resetting effect in multiple *in vitro models* of MBH neurons and an induction effects on *Bmal1* transcription in MBH neurons both *in vitro* and *in vivo*. Further molecular analyses revealed that these circadian effects of adiponectin are, at least in part, mediated by a adiponectin receptor 1 (AdipoR1), peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) and RAR-related orphan receptor alpha (RORα) dependent mechanism.

Using adiponectin deficient (Adipoq KO) mice as a model, I investigated the role of adiponectin in circadian behavioral rhythms. Adipoq KO mice have largely normal circadian locomotor activity rhythms and photic entrainment of the circadian clock. However, they show significant dampened 24-hr feeding rhythms associated with altered diurnal profiles of clock and appetite-regulating gene expression in the MBH. Moreover, the mutants also show abnormal food entrainment of the locomotor activity under a time-restricted feeding (RF) regime - known as food anticipatory activity (FAA). Conversely, compared to *ad libitum* fed animals, the RF regime significantly enhances the circadian oscillation of plasma adiponectin, upregulates the diurnal expression of adiponectin receptors and *Pgc1α* clock genes associated with a profound reorganization of the diurnal expression patterns of appetite-regulating genes in the MBH of wild-type mice. Furthermore, central delivery of an antagonist of RORα in wild-type mice could recapitulate the impaired FAA phenotypes of Adipoq KO mice. These data thus provide evidence to support the role of central adiponectin signaling in food entrainment of MBH clocks and feeding rhythms.

Together, these data reveal a novel metabolic feedback mechanism to the central circadian clocks.

List of abbreviations

3'-UTR	3’ untranslated region
aCSF	artificial cerebrospinal fluid
AgRP	agouti-related peptide
AMPK	adenosine monophosphate-activated protein kinase
And	adiponectin
ARC	arcuate nucleus
BMAL1	Brain and muscle Arnt-like protein-1
Bmal1-luc	Bmal1::LUCIFERASE
CART	cocaine and amphetamine regulated transcript
CCG	clock controlled genes
CLOCK	Circadian Locomotor Output Cycles Kaput
CNS	central nervous system
CRY	Cryptochrome
Ct	threshold cycle
DBP	D site of albumin promoter (albumin D-box) binding protein
DD	constant darkness
Dex	dexamethasone
DIV	day *in vitro*
dKD	double knockdown
DMH	dorsomedial nucleus of the hypothalamus
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FAA	food anticipatory activity
FAA	food anticipitatory activity
fAdn	full-length adiponectin
FEO	food entrainable oscillator
Fors	forskolin
gAdn	globular adiponectin
GC	glucocorticoid
GFP	green fluorescent protein
HBSS	Hank's balanced salt solution
HCRT	hypocretin
HFD	high fat diet
HMW	high molecular weight

i.c.v.	Intracerebroventricular
i.v.	intravenous
IFU	infection unit
IgG	immunoglobulin G
IGL	intergeniculate leaflet
ipRGC	intrinically photosensitive retinal ganglionic cells
KCl	potassium chloride
KD	knockdown
KO	knockout
LD	12:12 hr light-dark
LH	lateral hypothalamus
MBH	mediobasal hypothalamus
MC3R	melanocortin-3 receptor
MEF	murine embryonic fibroblast
mRNA	messenger ribonucleic acid
$NaCl_2$	sodium chloride
NAD	nicotinamide adenine dinucleotide
NaF	sodium flouride
NaH2PO4	sodium phosphate monobasic monohydrate
NaHCO3	sodium hydrogen carbonate
$NaPP_i$	sodium pyrophosphate
$NaVO_5$	sodium orthovandate
ND	normal diet
neuropeptide	NP
NPY	neuropeptide Y
ORF	open reading frame
PER	Period
Per2-luc	PER2::LUCIFERASE
PFC	prefrontal cortex
PGC1α/β	peroxisome proliferator-activated receptor gamma coactivator 1 alpha/beta
PGK	phosphoglycerate kinase 1
POMC	pro-opiomelanocortin
PPARα	peroxisome proliferator-activated receptor alpha
PPARγ	peroxisome proliferator-activated receptor gamma
PRC	phase response curve
PVN	paraventricular nucleus of hypothalamus

qPCR	Quantitative real-time polymerase chain reaction
RHT	retinal-hypothalamic tract
RORE	retinoic acid-related orphan receptor response element
RORα	RAR-related orphan receptor alpha
SCN	suprachiasmatic nucleus
SDS-PAG	sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SEM	standard error mean
shRNA	short hairpin ribonucleic acid
SIRT	sirtuin
TBS	Tris-buffer saline
TNFα	tumor necrosis factor alpha
TTL	transcriptional-translational feedback loop
VPLO	ventrolateral preoptic nucleus
WT	wild-type
α-MSH	alpha-melanocyte-stimulating hormones

1. Introduction

In modern industrialized societies, there is an increasing prevalence of professions requiring irregular work hours. Extended shiftwork has been identified as a risk factor predisposing individuals to metabolic disorders (1, 2). Exposure to mistimed environmental time-cues (i.e., Zeitgebers) such as food intake leads to desynchrony among endogenous physiological rhythms and the external light/dark cycles and is believed to be one of the culprits that contributes to the adverse effects of shiftworking. Recent studies have highlighted an extensive crosstalk between metabolism and the circadian clock (3, 4). However, our understanding of the mechanistic links between these two systems is still largely incomplete.

1.1. Mammalian circadian system

The rotation of the Earth around its axis results in a repetitive succession of day and night. The profound environmental changes associated with the day-night cycle drove most organisms to evolve endogenous timekeepers to reliably anticipate predictable events at particular times of day and adjust their behaviors and physiology accordingly (5). Such endogenous timekeeping machineries are known as circadian (from Latin *circa diem* – about a day) clocks. In mammals, the cellular timekeeping machinery is comprised of a set of clock genes intertwined in a delayed interlocking transcriptional-translational feedback loop (TTL). Transcriptional activating Brain and muscle Arnt-like protein-1/ Circadian Locomotor Output Cycles Kaput (BMAL1/CLOCK) complexes activate the expression of their own repressors, *Periods* and *Cryptochromes* (*Pers* and *Crys*), during the day while high levels of PERs/CRYs protein accumulated in the nucleus during the night inhibit the activity of BMAL1/CLOCK and hence their own transcription. Progressive degradation of PERs and CRYs towards the end of the late night releases the inhibition and thus allows for the resumption of a new cycle of oscillation. This molecular clockwork regulates cellular physiology via controlling the rhythmic expression of E-box containing genes and is shared among the central circadian pacemaker – the suprachiasmatic nucleus (SCN) – and other tissues in the brain and in the periphery (Fig. 1).

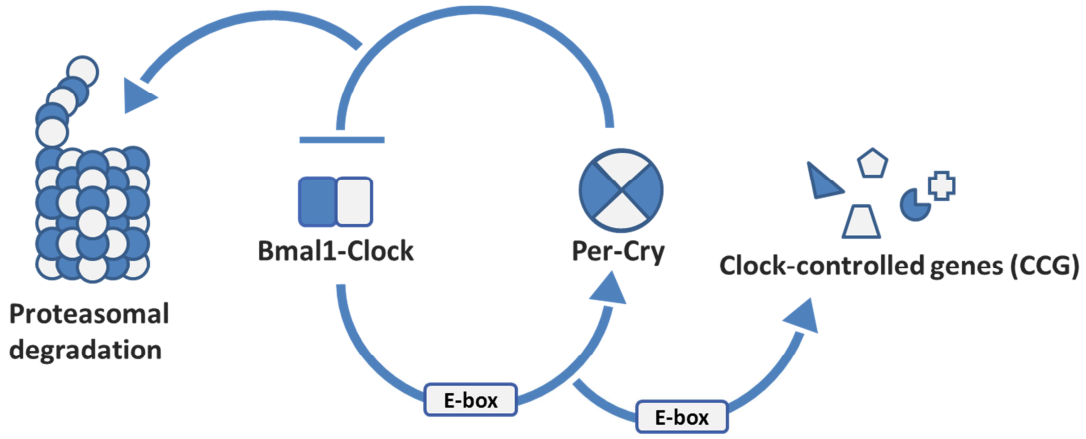

Figure 1. Molecular make-up of the mammalian cellular circadian clockwork

In mammals, the cellular timekeeping machinery is comprised of a set of clock genes intertwined with a delayed interlocking transcriptional-translational feedback loop (TTL). BMAL1/CLOCK complexes transcriptionally activates their own repressors *Per1-3* and *Cry1-2* via the E-box elements in their promoters during the day while the high level of PERs/CRYs accumulated in the nucleus during the night inhibits the activity of BMAL1/CLOCK and hence their own transcriptions. Progressive degradation of PERs and CRYs towards the end of the late night releases the inhibition and thus allows for the resumption of a new cycle of oscillation. This molecular clockwork regulates cellular physiology via controlling the rhythmic expression of other E-box containing clock control genes (CCGs).

In order to produce coherent physiological rhythms, the mammalian circadian system is organized in a hierarchical fashion in which the SCN synchronizes the circadian clocks of different physiological structures in both the periphery and the central nervous system (CNS) to the external light-dark cycle via multiple routes including direct neural connections, neuro-endocrine secretion and behavioral regulations (5) (Fig. 2).

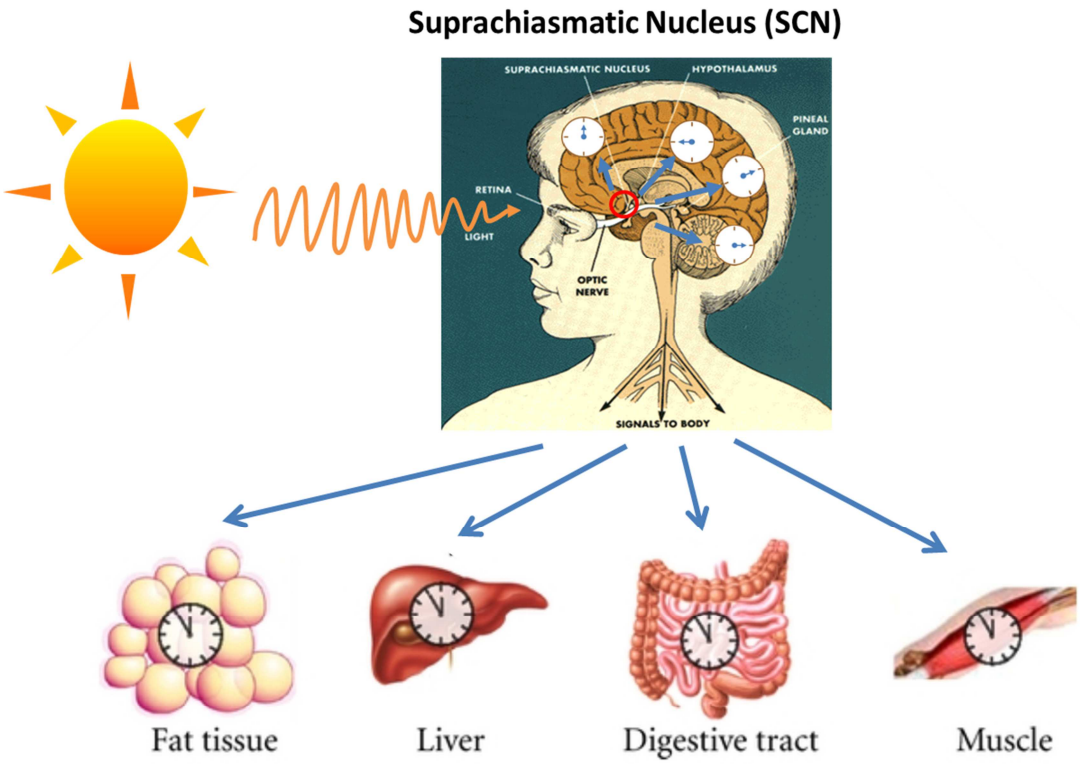

Figure 2. Schematic diagram of the hierarchical organization of mammalian circadian system. In order to generate coherent physiological and behavioral circadian rhythms, the local clocks residing in the periphery and the CNS are synchronized to the external day-night cycle by the SCN. The SCN clock is reset by the photic inputs transmitted from intrinsically photosensitive retinal ganglion cells (ipRGC) of the retina via the retinohypothalamic tract (RHT). The SCN clock then entrains the local clocks via multiple routes including direct neural connections, neuromodulators (in the CNS), hormones (in the periphery) and behavioral regulation

1.2. Interaction between metabolism and the circadian clock

Recently, metabolic signaling has been shown to impinge on the circadian system at various levels. Circadian clocks regulate feeding and metabolic rhythms of animals in sync with the environmental day/night cycle. Animals with mutations in clock genes are predisposed to develop feeding and metabolic deregulation (6, 7) (Table 1). Polymorphisms of clock genes have also been shown to associate with metabolic disorders in humans (8). Molecular clocks regulate a large array of metabolic pathways in various organs (5). Furthermore, many metabolic hormones such as ghrelin, glucocorticoids (GC), insulin and leptin are known to be regulated by the circadian clock on the one hand, and, on the other hand, are also known to feed back to the circadian system (9). Food is a potent Zeitgeber which can entrain and reset various non-SCN clocks

while having little effect on the SCN. Restricted feeding during the normal rest phase can uncouple peripheral tissue clocks from the SCN (10). The exact food-regulated clock resetting mechanisms in different tissues are still not completely understood (7). However, at the molecular level a number of important metabolic sensors such as adenosine monophosphate-activated protein kinase (AMPK), cellular nicotinamide adenine dinucleotide redox state (i.e., NAD^+/NADH ratio), peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α and β) and sirtuins (SIRT1 and 3) have been shown to impinge on molecular clock rhythms (7). However, the upstream physiological signals that couple to these metabolic pathways are still elusive. Together, not surprisingly many studies have demonstrated that compromised energy homeostasis of the body promotes circadian disruption (6). Feeding on diet that is rich in fat content (i.e., high fat diet; HFD) has been shown to disrupt circadian activity and local physiological rhythms even before the development of obesity (11). Moreover, mistimed feeding (i.e., eating during the normal rest phase) disturbs metabolic homeostasis (12, 13). When food access is temporally restricted to a particular time window of the day during the normal rest phase, animals will adapt by developing anticipatory behaviours and functions such as increased locomotor activity (i.e., food anticipatory activity; FAA), body temperature, glucocorticoid secretion just prior to the scheduled feeding. FAA rhythms represent a form of food entrainment of the circadian system as they persist even when the scheduled feeding condition is lifted (i.e., they free-run) (14). Food anticipation is believed to be advantageous for survival as it allows animals to organize their physiology for foraging in anticipation of a predictable food availability time window (15). Both the anatomical and molecular make-ups of the food entrainable oscillator (FEO) that is underlying FAA are still largely unknown. Of note, the SCN itself has been shown to suppress FAA (15, 16 and unpublished data). Some controversy exists about whether canonical clock gene function is necessary for FAA (15, 17, 18). Both the CNS and periphery are involved in the development of FAA (15, 19). In the CNS, the hypothalamus, the reward circuitry and the cerebellum have been shown to regulate FAA rhythms while peripheral metabolic hormones are known to have modulatory roles (15).

Table 1. Summary of the impacts of clock gene alterations on feeding and metabolic regulations

Gene target	Metabolic/feeding phenotype	Food anticipatoty activity	Refs
BMAL1	Loss of feeding rhythm (arrhythmic)	Maintained	(88)
CK1	Altered activity, feeding, and metabolic rhythms in $CK1^{tau}$	Maintained	(89)
$CLOCK^{\Delta 19}$	Hyperphagic, altered feeding rhythm, obese	Maintained	(76)
CRY1/CRY2	Loss of feeding rhythm (arrhythmic), hyperglycaemic	Maintained	(90,91)
Per1	—	Maintained	(92)
Per2	Altered feeding rhythms, lean, altered lipid metabolism	Attenuated in $Per2^{brdm}$	(92.93)
Per1/2	— —	Maintained in $Per1^{ldc}Per2^{ldc}$ Attenuated in $Per1^{-/-}$ / $Per2^{brdm}$	(63,94)
Per3	Exacerbation of diet-induced obesity	—	(95)
Reverbα	Obese, exacerbation of diet-induced obesity, altered lipid metabolism	—	(96)
Reverbα/β	Arrhythmic, altered lipid metabolism	—	(63)
RORa	Hyperphagic, lean, resistant to DIO	—	(97)

Adapted from Ref. 46

1.3. Extra-SCN hypothalamic clocks in the mediobasal hypothalamus

The mediobasal hypothalamus (MBH) is an assembly of hypothalamic nuclei, which controls body homeostasis and also plays a major role in regulating behavioral rhythms such as feeding/fasting and sleep/wake cycles (20). Despite the SCN has direct connections to multiple nuclei of the MBH, it has also been documented that autonomous cellular clocks exist in the MBH that regulate local physiology such as electrophysiological properties and the expression of appetite-regulating neuropeptides (NPs) (21, 22) (Fig. 3). Diurnal expression rhythms of major NPs in the arcuate nucleus (ARC) – neuropeptide Y (NPY), agouti-related peptide (AgRP), pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) – have been documented (23-25). The importance of these appetite-regulating NPs in feeding rhythms was revealed in a number of studies using pharmacological and genetic approaches. For example, targeted ablation of NPY- or leptin- responsive neurons in the ARC profoundly disturbs diurnal feeding rhythms (26, 27). Genetic deletion of NPY receptors also results in altered feeding rhythms (28). More recently, postnatal ablation of AgRP expressing neurons has been shown to impair food anticipation (29). Along with these observations, neurons in the lateral hypothalamus (LH) expressing the orexigenic neuropeptide hypocretin (HCRT or also called orexin), which are downstream to the ARC's NPY neurons, are also activated during food anticipation (30). Anorexinergic NPs appear to play a less important role in feeding rhythms. POMC is a precursor of multiple neuropeptides including the anorexigenic

alpha-melanocyte-stimulating hormones (α-MSH) which exerts its effects via melanocortin receptors (24). POMC deficient mice have shown to retain largely normal circadian activity and feeding rhythms, albeit showing altered nocturnal meal-patterns (31). On the other hand, the loss of melanocortin-3 receptor (MC3R) has been shown to significantly impair food anticipation under restricted feeding conditions (32). The dorsomedial nucleus of the hypothalamus (DMH) is an important integrating center that relays circadian inputs from the SCN to other physiological systems due to its direct connection to various brain nuclei such as the ARC, the LH, ventrolateral preoptic (VLPO) nuclei that regulate sleep/wake cycle, and the paraventricular nucleus of hypothalamus (PVN) controlling glucocorticoid secretion (33). Lesioning the DMH significantly disrupts circadian behavioral rhythms (34). The DMH has also been suggested as an important brain structure controlling FAA (35), but also (36).

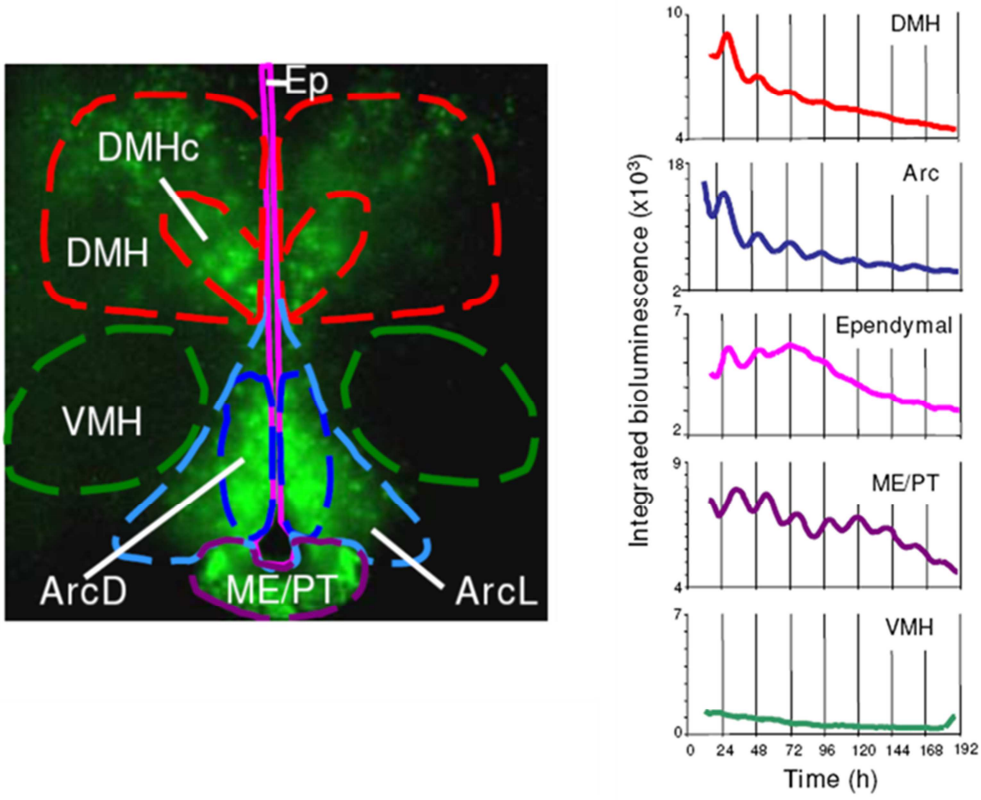

Figure 3. Self-sustaining molecular rhythms in various nuclei of the mediobasal hypothalamus organotypic slice cultures from Per2-luc mice. DMH: Dorsomedial hypothalamic nuceli, DMHc: DMH core, EP: Ependymal lining, ArcD: dorsal arcuate nucleus, ArcL: lateral arcuate nucleus, ME/PT: median eminence/pituitary, VMH: ventromedial hypothalamic nuclei. Adapted from Ref. 21.

1.4. Objectives of the current study

One of the most important features of the MBH's nuclei is the ability to perceive and integrate metabolic information carried by peripheral hormones to stabilize energy homeostasis of the body. Feeding-related hormones such as insulin, ghrelin and leptin have been shown to regulate appetite and energy expenditure via the MBH (20). Moreover, leptin and ghrelin have also been shown to regulate feeding rhythms and FAA (19). However, the physiological and molecular mechanisms conferring these effects are still elusive. I therefore hypothesize that metabolic hormones can reset molecular clocks in the MBH and thereby regulate feeding rhythms. To identify such factors, I established a cell-based approach to screen a list of candidate metabolic hormones for their ability to reset the cellular clock of hypothalamic neuronal cells. I identified an adipokines – adiponectin – as a novel mediobasal hypothalamic cellular clock modulator. In the periphery, adiponectin is known to enhance insulin sensitivity, modulating fatty acid oxidation and suppressing inflammation (37). In the CNS, it is involved in regulating food intake, energy expenditure and mood (38, 39). Importantly, circulating adiponectin levels are regulated by the metabolic status of the body. In a situation of energy excess (such as obesity) adiponectin secretion is suppressed. In contrast, during fasting, both circulating adiponectin level and the expression of its cognate receptors in the MBH are upregulated (40). Thus, I further hypothesize that adiponectin is a novel MBH circadian clock modulator which can modify the circadian feeding behaviour. In the current study, I employ *in vitro* models of MBH neurons as well as adiponectin deficient mice as an *in vivo* model to investigate the potential role of adiponectin as a modifier of the mediobasal hypothalamic circadian clock.

2. Results

2.1. Establishment of the hypothalamic circadian reporter cell line

To search for metabolic hormones capable of resetting the MBH cellular clock, I used an established SV40-immortalized mHypoE-N44 (N44 hereafter) cell line of embryonic hypothalamic neuronal origin (22). This cell line has been characterized to be GABAergic and express both *Npy* and *Agrp,* but not *Cart* and *Pomc* (22). In addition, the circadian oscillation of the molecular clockwork has been demonstrated in this cell line (22). To monitor the cellular rhythm in real-time, I engineered N44 cells to stably express a circadian reporter – *Bmal1::LUCIFERASE* (Bmal1-luc) in which the expression of luciferase is under the control of the *Bmal1* promoter – via lentiviral transduction (41). After synchronization with dexamethasone (Dex), Bmal1-Luc activity displayed a self-sustained circadian oscillation with a period length of 23.67 ± 0.1 hr (means ± SEM, n = 21) when cultured in serum-free, B-27 supplemented medium (Fig. 4 A). With this reporter cell line, I then set up a screening paradigm in which different peptide hormones were applied to the cells at roughly two opposite phases of the first circadian cycle (hr 13 and 23) separately and the peak times of the two subsequent circadian cycles after treatment were used as readout (Fig. 4 A). This anti-phasic treatment scheme was used to circumvent the potential existence of circadian dead zones, i.e. phase intervals at which the clock would be non-responsive to an otherwise active agent. I validated this experimental setting with two well established positive controls of clock-resetting reagents, forskolin (Fors; an adenylate cyclase activator) and Dex (a glucocorticoid receptor agonist) (5, 42). As expected, both of them significantly reset the cellular clock of N44 cells in a phase-dependent manner (Fig. 4 B - D). I then selected a short list of metabolic peptide hormones based on their reported binding sites and physiological effects on the MBH from the literature for our screen (Table 2). Among all candidates, only (globular) adiponectin (gAdn) showed significant effects on resetting the phase of the cellular circadian rhythm (Fig. 4 F & G). As a representative of the negative candidates, luminescence recordings from N44 Bmal1-luc cells treated with leptin are shown (Fig. 4 E). Adiponectin is an adipokine that has been shown to modulate a wide range of physiological systems in, both, the periphery and the CNS. Importantly, its functions are regulated by the metabolic state of the body (40). These screen data suggested that adiponectin may be a novel link between peripheral

energy metabolism and circadian clocks in the CNS. Thus I decided to further study the role of adiponectin in circadian rhythm regulation in detail.

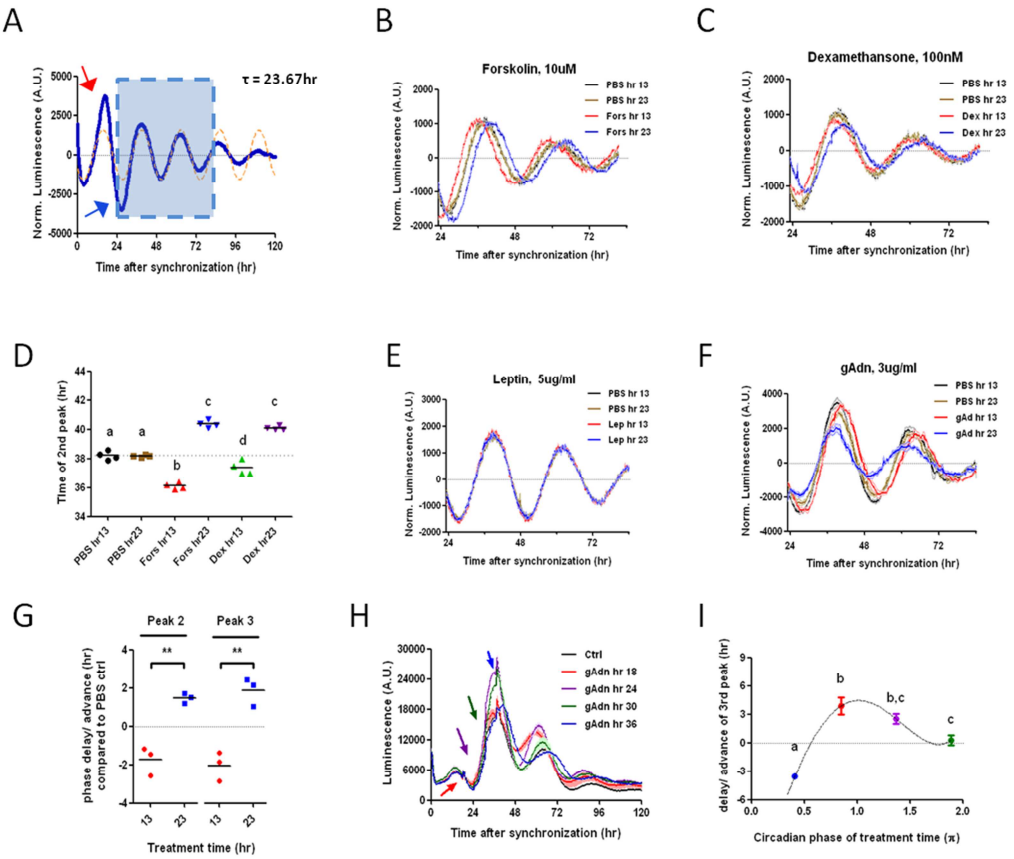

Figure 4. Adiponectin resets molecular clock in N44 cells

(A) A representative normalized bioluminescence recording of N44 Bmal1-luc cells synchronized by 2hr dexamethasone shock. The period length was calculated from 21 independent recordings across 5 passages. Two arrows (at hr13 and hr23 after synchronization) indicate the time points for the drug treatments in the peptide hormone screen. The peak times of the 2nd and 3rd circadian cycle were chosen as the readout of the screen.

(B - D) Normalized luminescence recordings from synchronized N44 Bmal1-Luc cells treated with forskolin (B) and dexamethansone (C) as a positive control of the screen, respectively. (D) Analysis of the peak time of the 2nd circadian cycle of Bmal1-luc (n=4).

(E) Leptin (5ug/ml) as a representative negative candidate of the screen (n=4).

(F and G) gAdn as a positive candidate of the screen. Normalized luminescence recordings (F) and peak time analysis (G) of N44 Bmal1-Luc cells treated with gAdn (3ug/ml; n=3).

(H and I) Phase-dependent response of gAdn-induced phase-shift in N44 Bmal1-Luc cells. (H) gAdn (5ug/ml) was treated on cells at indicated time points after synchronization. The peak time of the 3rd circadian cycle were used as readout.

(I) Phase-dependent response analysis of (H). The treatment time was adjusted into radian of the circadian Bmal1-luc rhythms. Colour code for the treatment time is consistent with (I) (n=3 per time point).

Error bars indicate means ± SEM. **p<0.01, ***p<0.001, Student's t-test. Groups denoted with different alphabets indicates statistical significance (p<0.05), One-Way ANOVA with Tukey post-test.

Table 2. Summary of the result from the screen for MBH clock-modulating metabolic peptide hormones

Drugs	Treatment 1 (at hr 13) (hr)	Treatment 2 (at hr 23) (hr)
Forskolin, 10uM	+2.21 ± 0.11 ***	-2.18 ± 0.13***
Dexamethansone, 100nM	+0.95 ± 0.24 *	-1.90 ± 0.07***
Apelin 13, 3ug/ml	-0.96 ± 0.52	-0.37 ± 0.38
gAdn, 3ug/ml	-1.74 ± 0.43 **	+1.47 ± 0.15*
TNFa, 50ng/ml	-0.44 ± 0.13	-0.17 ± 0.19
IL6, 50ng/ml	-0.50 ± 0.38	-0.77 ± 0.30
IGF1, 100ng/ml	+0.00 ± 0.18	-0.67 ± 0.06
Chemerin 9, 2nM	-0.80 ± 0.27	-0.34 ± 0.34
Chemerin 9, 1uM	+0.16 ± 0.39	-0.50 ± 0.32
Chemerin 15, 2nM	-0.54 ± 0.15	-0.30 ± 0.15
Chemerin 15, 1uM	-0.10 ± 0.18	-0.37 ± 0.32
Oxyntomodulin, 0.5uM	+0.43 ± 0.12	-0.07 ± 0.23
Leptin, 5ug/ml	+0.15 ± 0.14	+0.21 ± 0.15
Insulin, 10ug/ml	-0.10 ± 0.06	0.23 ± 0.14
Nesfatin, 3ug/ml	-0.10 ± 0.22	-0.34 ± 0.13
Visfatin, 3ug/ml	-0.07 ± 0.14	+0.03 ± 0.16
Ghrelin, 100nM	+0.00 ± 0.16	+0.07 ± 0.06
Glucagon, 100nM	-0.40 ± 0.20	-0.27 ± 0.17
Resistin, 10ng/ml	-0.25 ± 0.14	-0.05 ± 0.05
GLP-1, 100nM	-0.30 ± 0.13	+0.23 ± 0.17

+ve: phase advance; -ve: phase delay; ±SEM, *p<0.05, **p<0.01,*<0.001 vs PBS ctrl; N=3-4**

2.2. Adiponectin phase-resets molecular clocks in mediobasal hypothalamic neurons

From the screen, I had observed that adiponectin can phase-reset the Bmal1-luc rhythm in a phase-dependent manner with opposite directions as observed after forskolin or dexamethasone treatments (Fig. 4 D & G), suggesting the existence of a distinct resetting mechanism. To further characterize and confirm the phase-dependency of adiponectin's clock-resetting effect, synchronized N44 Bmal1-luc cells were treated around the course of the first circadian cycle at 6-hr intervals with constant real-time luminescence recording to allow for a more precise determination of the circadian phase at the time of treatment. Using the peak time of the subsequent circadian cycle as readout allowed me to construct a phase response curve (PRC), which confirmed the phase-dependent clock resetting effect of gAdn on hypothalamic cells (Fig. 4 H - I) distinct from the PRCs of Fors and Dex (Fig. 5).

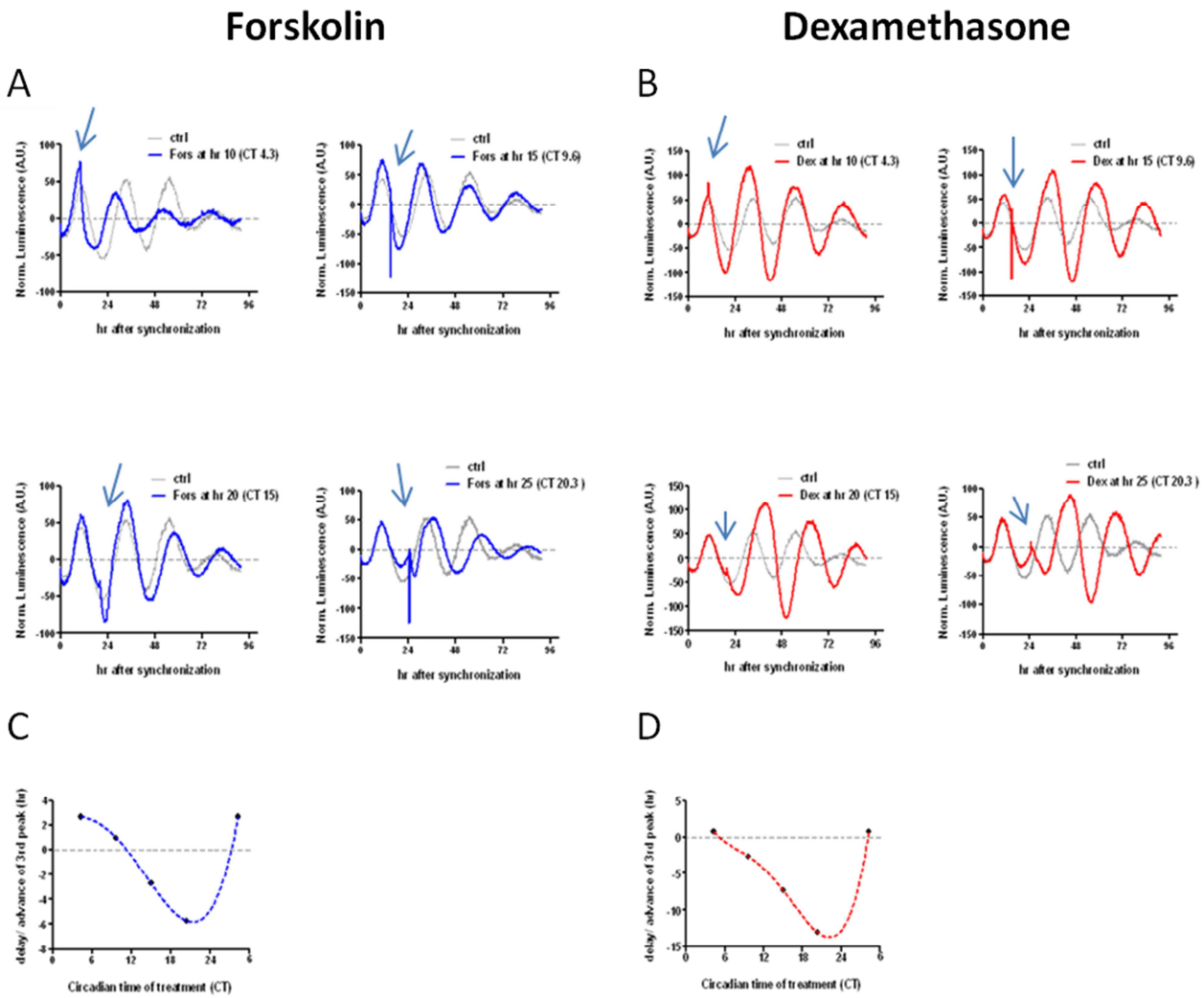

Figure 5. Resetting of molecular clock in N44 cells by forskolin and dexamethasone

(A and B) Representative normalized bioluminescence recordings of synchronized N44 Bmal1-luc cells treated with 10uM forskolin (A) and 100nM dexamethasone (B) at indicated time points. Grey curves are PBS treated controls.

(C and D) Phase-dependent response of Fors-induced (C) and Dex-induced (D) phase-shift of Bmal1-luc rhythms in N44 Bmal1-luc cells. Peak time of the 3rd circadian cycles was used as readout. Treatment time was adjusted into circadian time of Bmal1-luc rhythm with CT0 defined as the middle point of the ascending phase from the trough to the peak of the curve. The last data points of treatment were replotted.

To address the physiological relevance of the observations in immortalized N44 cells, I further tested the clock-modulating effects of gAdn on primary hypothalamic neurons and organotypic MBH slices. I isolated primary embryonic hypothalamic neurons and lentivirally transduced them to express Bmal1-luc. Upon Dex synchronization, the primary hypothalamic neurons showed a robust circadian Bmal1-luc expression rhythm

with a period length 24.45 ± 0.15 hr (means ± SEM, n = 2; Fig. 6 A). Treating the neurons at hr 68 (around the nadir) or hr 83 (around the peak) after Dex synchronization with gAdn resulted in stable phase-advances or -delays, respectively, consistent with the directions of phase-shift in N44 cells, except that the responses were more pronounced in primary neurons (Fig. 6 B & C). Multiple nuclei in the MBH show robust self-sustaining circadian rhythms (21). I therefore used the slices of the ARC/ME complex from *Per2::LUCIFERASE* (Per2-luc) reporter mice as a model to test for the phase-resetting effect of gAdn *ex vivo* (43). Preparation of these slices indeed showed robust circadian oscillations of Per2-luc rhythms over 5 days of measurement (Fig. 6 D). In line with the effects observed from N44 cells and isolated neurons, treatment with gAdn resulted in phase-shifts of the Per2-luc expression rhythm in a phase dependent manner relative to PBS treated slices (Fig 6 E - I). Together, these data suggest that adiponectin is a *bona fide* MBH molecular clock modulator.

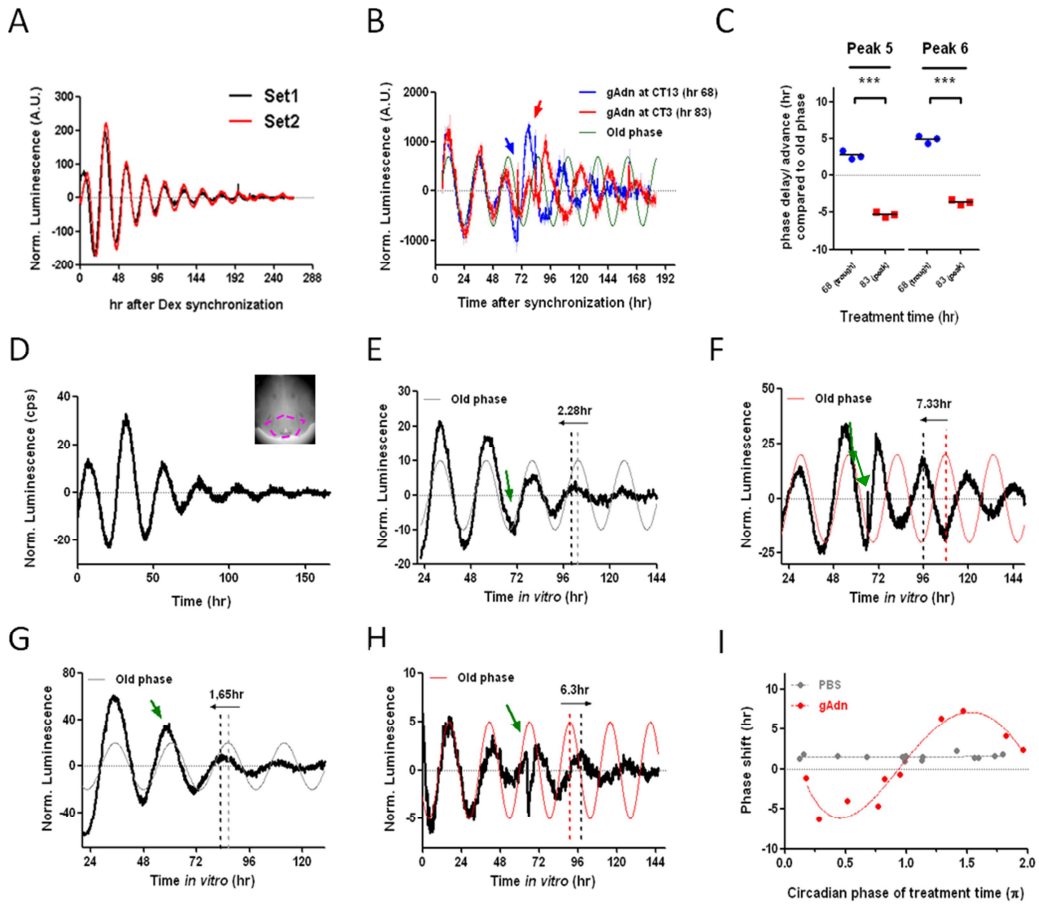

Figure 6. Adiponectin resets molecular clock in primary hypothalamic neurons and organotypic MBH slice cultures

(A) Representative normalized luminescence recordings of synchronized primary hypothalamic neurons expressing Bmal1-luc.

(B and C) gAdn resets cellular circadian rhythm in primary hypothalamic neurons expressing Bmal1-luc. (B) Normalized luminescence recordings of primary hypothalamic neurons treated with gAdn (5ug/ml) at indicated time points after synchronization. (C) Quantification of gAdn-induced phase shifts of Bmal1-luc rhythms shown in (H) (n=3).

(D) Representative normalized luminescence recording of arcuate nucleus/median eminence (ARC/ME) slices from Per2-luc mice. The highlighted region of the insert indicates the ARC/ME preparation brought for luminescence recording.

(E and F) Representative luminescence recordings of the ARC/ME slices of Per2-luc mice treated near the trough of the Per2-luc rhythm as indicated by the arrows with PBS (E) or 3ug/ml gAdn (F).

(G and H) Representative luminescence recordings of the ARC/ME slices of Per2-luc mice treated near the peak of the Per2-luc rhythm as indicated by the arrows with PBS (G) or 3ug/ml gAdn (H).

(I) Phase response curve of gAdn-induced phase shift of Per2-luc rhythms of ARC/ME slices. The treatment time was adjusted into radian of the circadian Per2-luc rhythm of slices. Note that the PBS treated slices regardless of the treatment time showed about 1-2.5 hr phase-advancement compared to the old phases which is due to the natural transition to free-running period when kept *ex vivo*. Each dot represents individual slice treated with either PBS or gAdn at indicated circadian phase.

Error bars indicate means ± SEM. ***p<0.001, Student's t-test.

2.3. Characterization of the circadian phenotype of adiponectin deficient mice

To investigate the physiological relevance of the MBH clock-modulating effects of adiponectin signaling *in vivo*, I used an established adiponectin deficient (Adipoq KO) mouse line (44, 45). To assess the circadian phenotype of Adipoq KO mice, a battery of circadian behavioral assays with running-wheels was performed. Adipoq KO mice have showed normal levels of locomotor activity compared to wild-type (WT) controls under both light-dark (LD) and constant darkness (DD) conditions (data not shown). Under LD, Adipoq KO mice displayed higher activities during the early dark phase followed by a compensatory decrease in the late dark phase (Fig. 7 A). However, Adipoq KO mice had no obvious defects on other circadian parameters of the activity rhythm that I have analyzed in LD, DD and LL (constant light ON) (Fig. 7 B – F). Interestingly, in a 6-hr phase advance experimental jet-lag paradigm, Adipoq KO mice readapted their activity rhythms faster to the new light/dark cycle than control animals (Fig. 7 G & H). These data suggest that adiponectin is not a major regulator of the master circadian clock, but it may play a modulatory role in the entrainment of behavioral rhythms to shifts in the photic cycle.

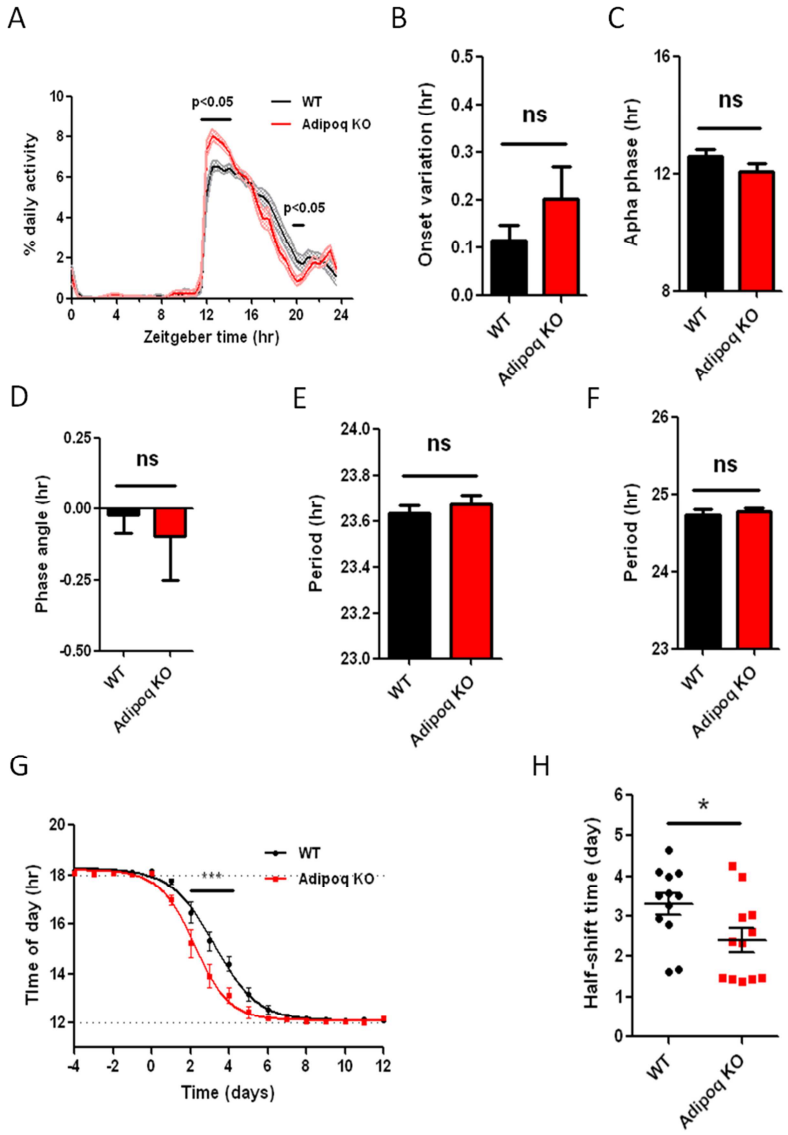

Figure 7. **Characterization of the behavioral circadian phenotypes of Adipoq KO mice assessed by running wheels**

(A) Daily activity profile integrated over 10 days in LD.

(B) Activity onset variation in LD.

(C) Alpha phase in LD.

(D) Phase angle with respect to light-OFF in LD.

(E) Average period over 10 days in DD.

(F) Average period over 10 days in LL.

(G) Activity onset under a 6 hr phase-advance experimental jet-lag paradigm.

(H) Quantification of the half-shift time of the sigmoidal regressions in (G).

Error bars indicate means ± SEM, n=12 per group, *p<0.05, ***p<0.001, ns=not significant, Mann-Whitney test.

2.4. The role of the molecular clock in MBH functions

As aforementioned, the molecular clocks residing in the MBH have been shown to regulate the local physiology *in vitro*, thus it appears that MBH clocks may contribute to the regulation of daily feeding patterns *in vivo* (46). Animals lacking components of the molecular clockwork show disrupted daily feeding behaviors (46; Table 1). To gain insights into how the circadian clock impinges on MBH functions, I analyzed the circadian regulation of mRNA levels of appetite-regulating neuropeptides (NP) in the MBH of WT and clock-deficient *Bmal1* knockout (Bmal1 KO) mice. Loss of *Bmal1* abolished the circadian expression rhythm of a well-established clock output gene, D site of albumin promoter (albumin D-box) binding protein (*Dbp*), along with drastic downregulation of its overall expression in the MBH (genotype effect, $F=340.1$, $p<0.0001$; Fig. 8 A & B). In the Bmal1 KO MBH, the 24-hr expression profiles of *Npy* ($F=11.96$, $p=0.0032$), *Cart* ($F=50.39$, $p<0.0001$) and *Pomc* ($F=51.14$, $p<0.0001$) were significantly downregulated (Fig. 8 C - F), suggesting that the circadian clock is an important regulator of appetite-regulating NP expression *in vivo*. Adiponectin is known to regulate a variety of physiological systems via its two cognate receptors, AdipoR1 and R2, which have been shown to have a broad range of tissue distribution in both the periphery and in the CNS (39, 40). To test the role of the circadian clock in the expression of AdipoRs in the MBH, I analyzed their mRNA levels in WT and Bmal1 KO mice. Consistent with the reported 24-hr expression profile of these two receptors in the periphery (47), I found that in WT mice *Adipor2* expression showed a significant circadian rhythm ($P<0.05$, cosinor analysis) while *Adipor1* did not (Fig. 8 G & H). The loss of *Bmal1* was reported to abolish the expression rhythms of *Adipor2* and led to general downregulation of both *Adipor1* and *-r2* in the periphery. In the MBH, I observed that *Bmal1* deficiency also abolished the circadian rhythm of *Adipor2,* but accentuated the overall expression of both *Adipor1* ($F=22.09$, $p=0.0002$) and *-r2* ($F=18.29$, $p=0.0006$), suggesting the existence of a tissue-specific regulatory mechanism for AdipoRs expression by the circadian clock.

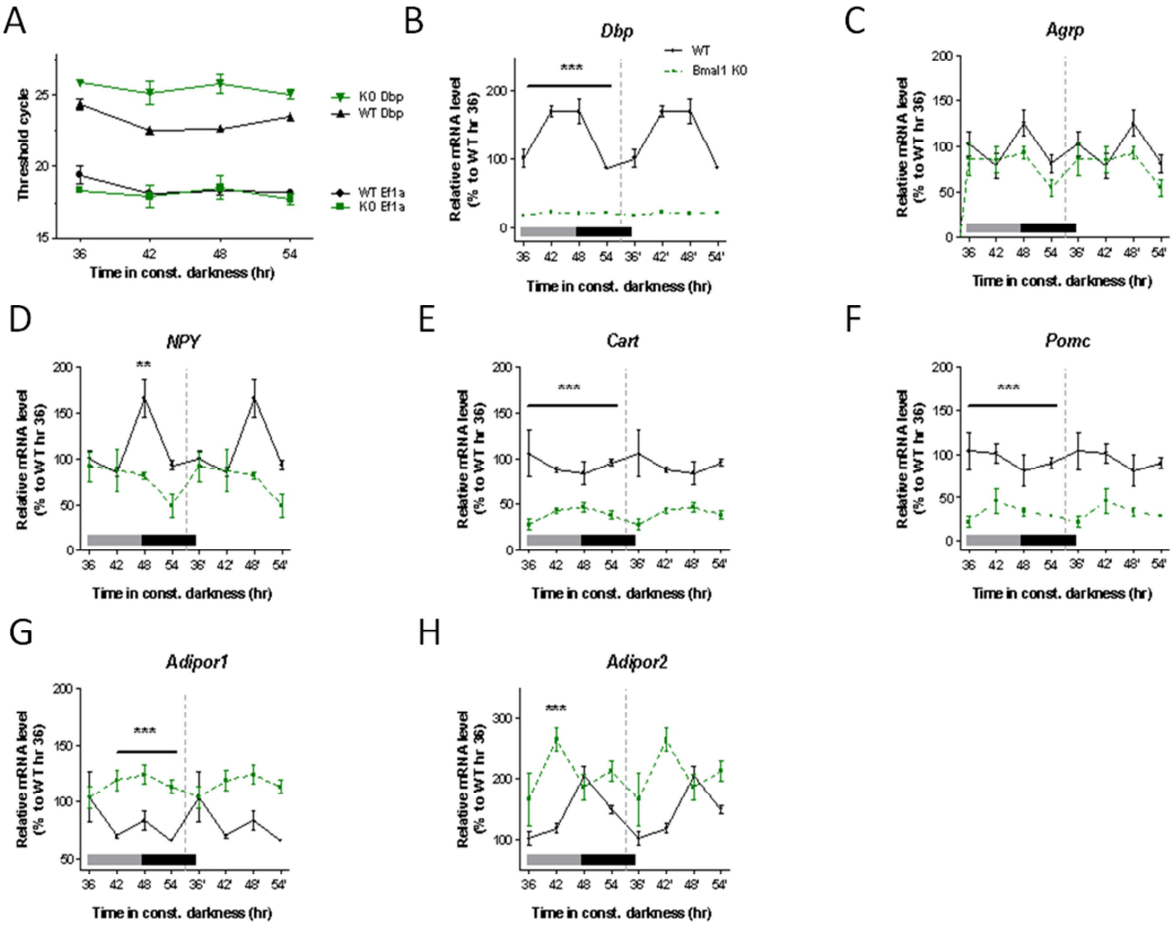

Figure 8. Characterization of appetite-regulating neuropeptides and adiponectin receptors 24 hr expression profile in the MBH of WT and Bmal1 KO mice

qPCR analysis of the MBH of WT and Bmal1 KO mice released into constant darkness for indicated timespan.

(A) Threshold cycles of *Dbp* and housekeeping *Ef1α* genes in qPCR analysis.

(B - H) Double-plotted 24-hr mRNA expression profile of selected genes.

Error bars indicate means ± SEM, n=3 per time point, **p<0.01, ***p<0.001, Two-Way ANOVA with Bonferroni post-test.

2.5. Adiponectin deficient mice have altered feeding rhythms

Adipoq KO mice consumed comparable amounts of food per day as WT, however, they consumed significantly more food during the daytime (i.e. the light phase) in LD conditions and the subjective daytime (i.e. the normal rest phase) in DD. Further analysis revealed that Adipoq KO mice had a significantly

dampened feeding rhythm in both LD (Fig. 9 A - C) and DD conditions (Fig. 9 D - F). To investigate if the loss of adiponectin could affect clock gene and appetite-regulating NP gene expression in the MBH, I analyzed the 24-hr expression profiles of corresponding transcripts in the MBH of Adipoq KO and WT mice. The circadian expression rhythms of *Bmal1*, *Per2* and *Dbp* were significantly dampened in Adipoq KO mice (Fig. 9 G - I). Also, the diurnal expression oscillations of orexigenic NPs - *AgRP*, *Npy* and *Hcrt* - were markedly blunted, particularly during the subjective nighttime, in KO mice (Fig. 9 J - L). No significant effect was observed for anorexigenic NP transcripts - *Cart* and *Pomc* (Fig. 9 M & N).

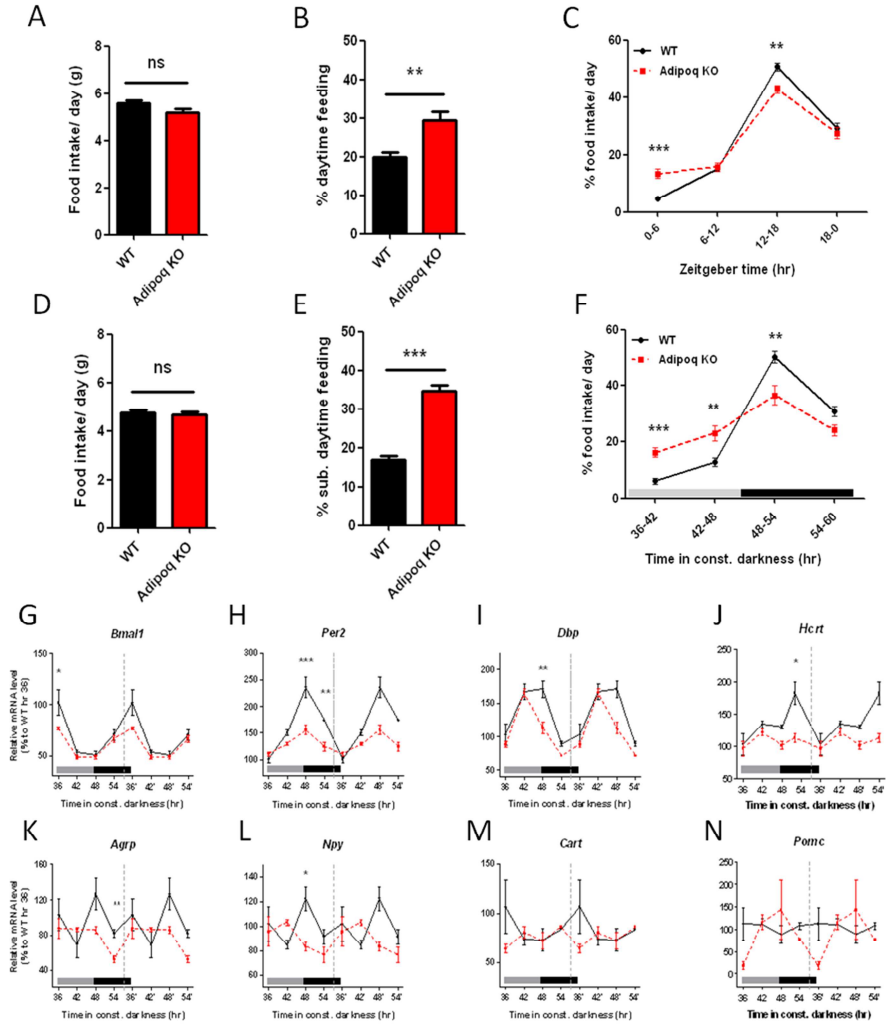

Figure 9. Adiponectin deficient mice exhibit dampened feeding rhythm and disrupted diurnal expression profile of clock genes and appetite-regulating neuropeptide genes in the MBH

(A - F) Altered feeding rhythm in Adipoq KO mice under LD (A-C) and DD (D-F) conditions. (A and D) The daily food consumption under LD and DD conditions. (B and E) Percentage of daily food consumption during the light phase (LD) and subjective daytime (DD). (C and F) 24-hr food intake profile of WT and Adipoq KO mice under LD (C) and DD (F) conditions. Data shown are an average of 2 measurements of the same cohort of mice separated by about a week (n=12).

(G - N) Double-plotted 24 hr mRNA expression profiles of selected clock genes and appetite-regulating neuropeptide genes in the MBH of WT and Adipoq KO mice released into DD for indicated time span (n=3 per time point).

Error bars indicate means ± SEM. *p<0.05, **p<0.01, ***p<0.001, Mann Whitney test for pairwise comparison and Two-Way ANOVA with Bonferroni post-test for profile data.

2.6. Tissue-specific effect of adiponectin on the circadian clocks *in vivo*

To understand if loss of adiponectin also affects clock regulation in other tissues, I also analyzed the 24-hr expression profiles of clock genes in the prefrontal cortex (PFC), the liver and femur skeletal muscles in WT and Adipoq KO mice (Fig. 10). In the PFC, I observed a general dampening of the expression rhythms similar to those of the MBH, albeit the timing of effects was different. Only minor changes in clock gene expression were observed in the liver and muscles, suggesting that the effect of adiponectin on the cellular clock may be tissue specific and apparently clocks residing in the CNS are more affected.

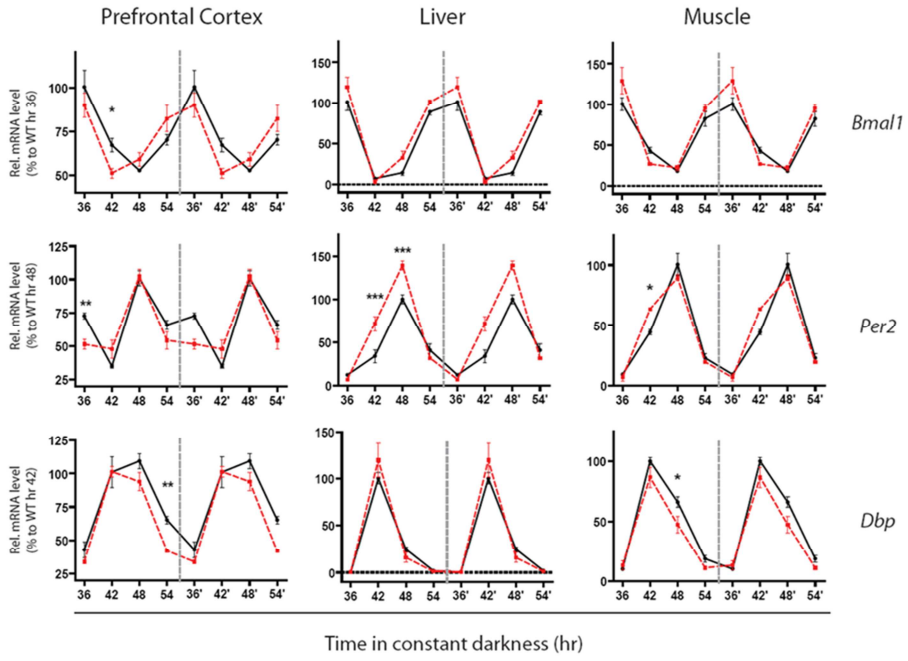

Figure 10. Tissue specific effects of adiponectin on *Bmal1* expression *in vivo*

Circadian expression profiles of selected clock genes in different tissues in WT and Adipoq KO mice released into DD for indicated time span (n=3 per time point). Error bars indicate means ± SEM. *p<0.05, **p<0.01, Two-Way ANOVA with Bonferroni post-test

2.7. *Npy* is a direct target of the molecular clock

In both Bmal1 KO and Adipoq KO mice I observed a blunted diurnal expression profile of *Npy*, together with dampened MBH clock gene oscillations. Given the pivotal role of NPY neurons in appetite regulation, these data indicate that altered *Npy* expression may play a major role in the dampened feeding phenotype of

Adipoq KO mice. In line with this claim, BMAL1 protein has been shown to rhythmically bind to the E-box elements of the *Npy* promoter in hypothalamic cells (22), suggesting that *Npy* is likely a direct target of BMAL1. To further interrogate this hypothesis, I used a short hairpin ribonucleic acid (shRNA) knock-down approach to demonstrate that reduction of *Bmal1* expression in N44 cells resulted in *Npy* downregulation (Fig. 11 A & B), indicating a positive relationship between *Bmal1* and *Npy* expression. Moreover, using a *Npy* promoter end-point luciferase assay, I demonstrated that overexpressing BMAL1 and CLOCK activates the *Npy* promoter in a dose-dependent manner (Fig. 11 C). CLOCK/BMAL1's transactivating effect was abolished by co-expressing CRY1 (Fig. 11 D). Together, these data strongly indicate that NPY is a direct target of the molecular clockwork in the hypothalamus.

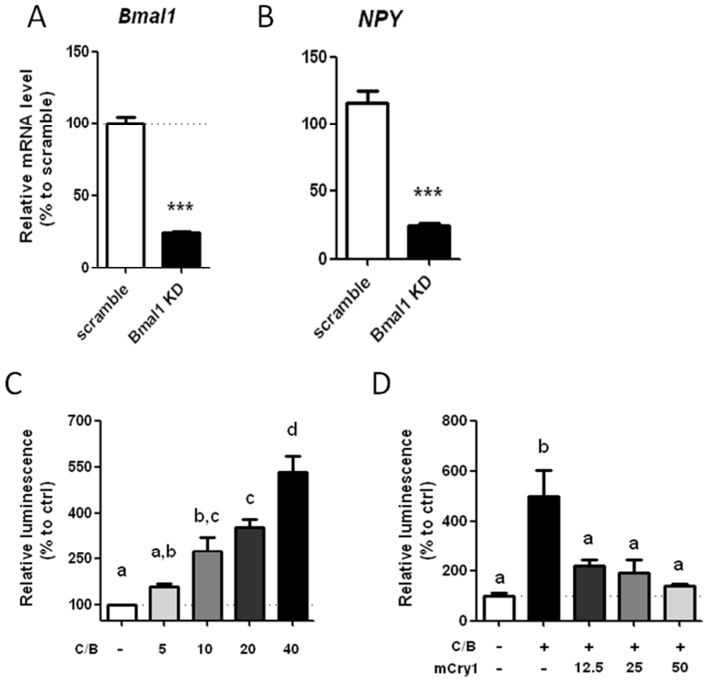

Figure 11. *Npy* is a direct target of the molecular clock

(A and B) qPCR analysis of *Bmal1* (A) and *NPY* (B) expression N44 cells with shRNA knockdown of *Bmal1* (n=3).

(C and D) *NPY* promoter end-point luciferase reporter assay in HEK293T cells. (C) *NPY* promoter activity was activated by overexpressing CLOCK and BMAL1 in a dose- dependent manner (n=6). (D) The BMAL1/CLOCK's activating effect on NPY promoter was abolished by CRY1 overexpression (n=6).

Error bars indicate means ± SEM. ***p<0.001, Student's t-test. Groups denoted with different letters indicates statistical significance (p<0.05), One-Way ANOVA with Tukey post-test.

2.8. Adiponectin regulates food anticipatory circadian rhythms

When food availability is restricted to a particular time window of the day, circadian rhythms of animals will be entrained by the feeding schedule. At the level of behavior this is characterized by the development of FAA. The molecular clockwork in the central nervous system has been shown to play a role in regulating the FAA rhythm (48). In addition, metabolic hormones such as ghrelin and leptin can modulate FAA (19). To test if adiponectin is also involved in this behavioral re-adaptation, I challenged WT and Adipoq KO mice with a time-restricted feeding (RF) regime in which food availability was gradually confined to a 4-hr time window during the late rest phase (ZT 7-11). Locomotor activity within the 3-hr time window (ZT 4-7) preceeding feeding time was defined as FAA (Fig. 12 A). Of note, under this paradigm, I did not observe overt differences in well-being between WT and Adipoq KO mice as also reflected in body weight regulation and total activity levels (Fig. 12 B & C). However, Adipoq KO mice showed a significantly delayed development of FAA (Day 3-5) compared to WT, but caught up with regard to total FAA from Day 6 on (Fig 12 D – F & J). To discern if the effects of adiponectin on food anticipatory behavioral rhythms affect the food entrainable oscillator (FEO) I resumed the *ad libitum* feeding on Day 10 until ZT11 on Day 11 followed by food deprivation until ZT11 on Day 12. The *ad libitum* feeding largely abolished the FAA of WT and Adipoq KO mice on Day 11 (Fig. 12G, H & J). The subsequent removal of food resurrected the FAA in both WT and Adipoq KO mice on Day 12, but the FAA of Adipoq KO mice was significantly reduced compared to that of WT animals (Fig. 12 I & J). This data indicate that adiponectin directly impinges on the FEO system. In parallel to FAA I also measured food intake during Day 0-10 of the RF paradigm. Adipoq KO ate less during the early phase of the RF (Day 2 - 5), but then caught up later, similar to the dynamics observed for FAA (Fig. 12 K). Notably, on Day 2 when the food access was limited to the daytime, Adipoq KO mice consumed more food than WT, which is in line with what I observed under unchallenged conditions (Fig. 9 B). Together, I demonstrated that adiponectin contributes to the robustness of the FEO and promotes behavioral re-adaptation under a timed RF regime.

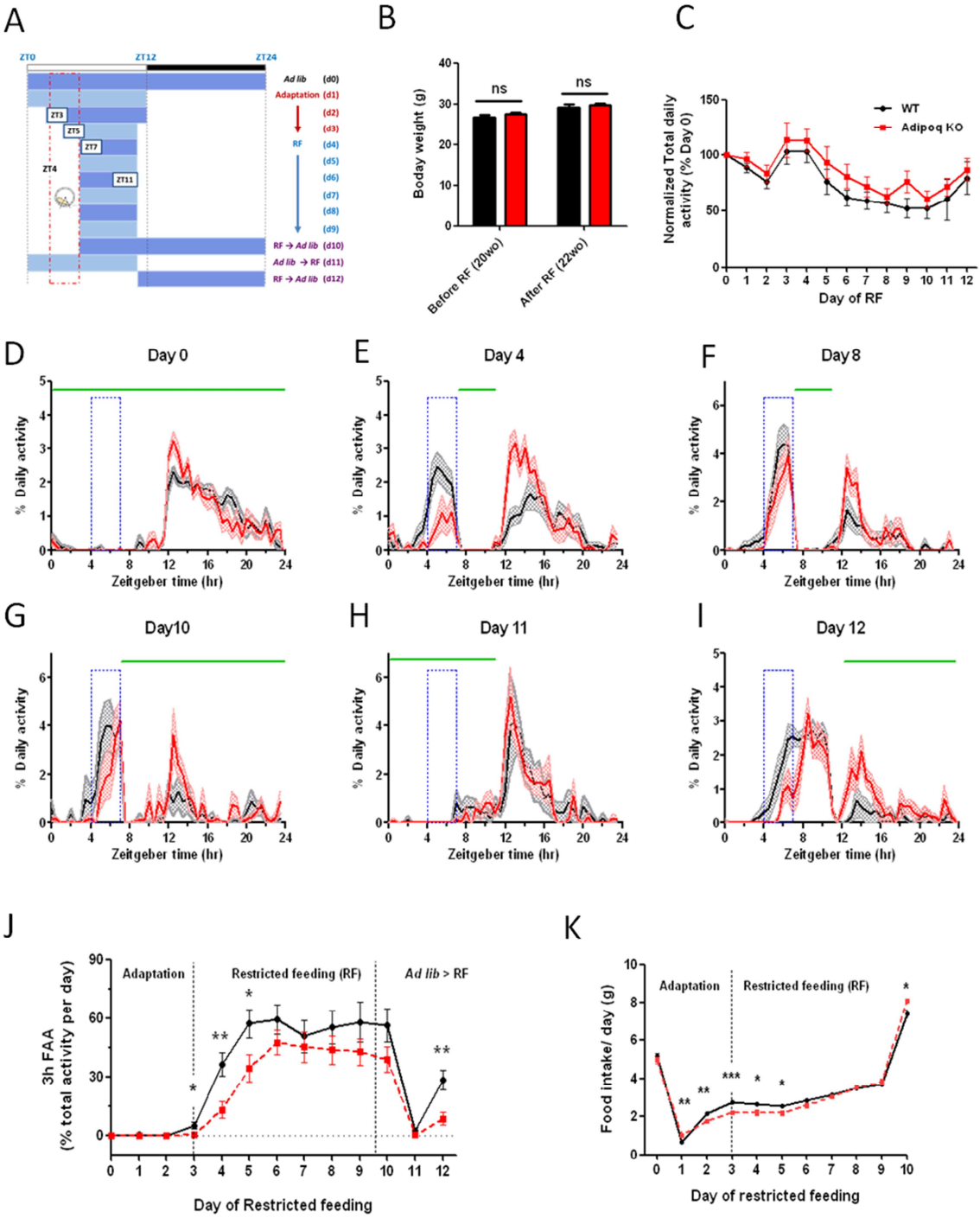

Figure 12. Adiponectin regulates food anticipatory circadian activity rhythm

(A) The time-restricted feeding (RF) regime used to induce FAA. The blue shade areas indicate the food-available time window. The rectangle with red broken border indicates the 3hr time window of the FAA.

(B) Body weight measured before and after the RF regime (n=12).

(C) Normalized total daily activity over the course of RF regime (n=12 on Day 1-10; n=6 on day 11-12, also for (D-K)).

(D - I) 24-hr activity profiles of individual day during the course of RF regime. The green bars overhead indicate feeding time; the blue rectangular windows indicate the 3hr FAA measured

(J) Quantification of the 3hr FAA over the course of RF regime

(K) Total daily food consumption over the course of RF regime.

Error bars indicate means ± SEM. *p<0.05, **p<0.01, ***p<0.001, Mann Whitney test.

2.9. Adiponectin upregulates *Bmal1* expression in the mediobasal basal hypothalamic neurons

In the luminescence recordings of my phase-resetting experiments, I consistently observed that gAdn treatment resulted in an acute and long-lasting upregulation of the Bmal1-luc signal in the raw luminescence recordings independent of the phase of treatment (Fig. 4 H). *In vivo*, I observed that adiponectin deficiency led to a dampening of the molecular clock oscillation in the MBH (Fig. 9 G - I). Together these findings suggested a positive effect of adiponectin on *Bmal1* transcription. To confirm this, I compared gAdn treatment effects with that of other clock resetting agents on unsynchronized N44 Bmal1-luc cells. I found that gAdn treatment induced Bmal1-luc activity robustly over the course of 24 hr; in contrast, Fors and Dex did change the timing of luminescence peaks, but they rather led to a decrease of Bmal1-luc raw activity (Fig. 13 A - B). These data were consistent with the conclusion from the phase-resetting experiments that the mechanism of adiponectin clock resetting may differ from that of Fors and Dex, which both exert effects mainly via *Per* – but not *Bmal1* – gene induction (5). gAdn treatment on N44 cells stably expressing luciferase under control of the constitutive mouse phosphoglycerate kinase 1 (*Pgk*) promoter (Pgk-luc) did not cause discernable changes in luciferase activity, supporting the specificity adiponectin's effects on Bmal1-luc expression (Fig. 13 C). Using qPCR I confirmed the upregulating effect of gAdn on endogenous *Bmal1* expression at the mRNA level (Fig. 13 D) and by Western blot at the protein level (Fig. 13 F). Notably, *Per2* was not acutely upregulated by gAdn (Fig. 13 E). Adiponectin is known to exist in different forms – in a globular form, as trimer, hexamer and high molecular weight (HMW) oligomers, all of which are formed via posttranslational processings of adiponectin peptides and display distinct affinities to AdipoR1 and R2 receptors (49, 50). Whilst the bacteria-expressed recombinant gAdn used so far represents a specific agonist of AdipoRs, treating unsynchronized N44 cells with full-length adiponectin (fAdn) expressed from mammalian cells (and comprising a mixture of different isoforms of adiponectin in a physiological ratio similarly resulted in a dose-dependent increase of Bmal1-luc activity, albeit with a lower efficacy compared

to gAdn alone (Fig. 13 G & H) (38, 50). To further confirm the physiological relevance of our observations, I performed gAdn treatment on synchronized Bmal1-luc expressing primary hypothalamic neurons around the peak time of the Bmal1-luc rhythm. Comparable to what I had observed in N44 cells, gAdn treatment of neurons acutely stimulated Bmal1-Luc activity (Fig 13 I & J). These cell-based data are thus in line with the *in vivo* observations that adiponectin has an enhancing effect on *Bmal1* expression. To investigate if the increase of circulating adiponectin could impinge on the MBH clock *in vivo*, with the help of my colleague Dr. Christiane Koch, we intravenously (i.v.) administered fAdn (1µg/g) to Adipoq KO mice at ZT6 (in the middle of the descending phase of the MBH *Bmal1* rhythm) via the tail vein. *Bmal1* expression in the MBH of fAdn treated mice was significantly enhanced compared to PBS treated controls (Fig. 13K). To investigate if modulating endogenous central adiponectin bioavialability could impinge on *Bmal1* expression in the MBH, I – again with the help of Dr. Koch – performed intracerebroventricular (i.c.v.) injections to centrally deliver anti-adiponectin antibodies (α-Adn) with the aim to antagonize central adiponectin signaling (39) on awake WT mice at ZT 21-22 (a few hours before the peak of MBH *Bmal1* rhythms) under dim red illumination. α-Adn treatment significantly reduced *Bmal1* expression in the MBH compared to unimmunized immunoglobulin G (IgG) controls (Fig. 13 L). In sum, *in vitro* and *in vivo* data provide compelling evidence that adiponectin signaling is a positive regulator of *Bmal1* expression in the MBH.

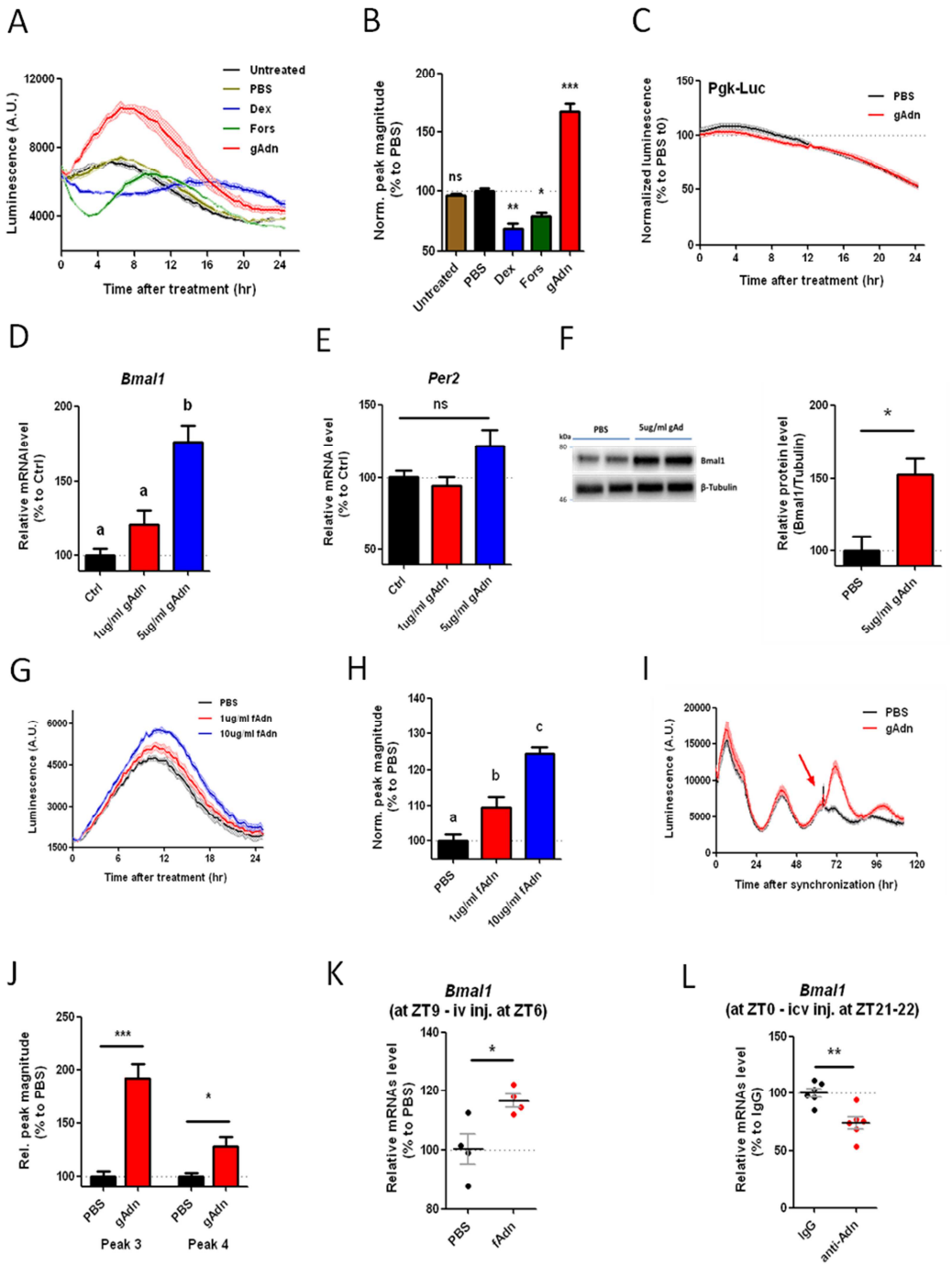

Figure 13. Adiponectin induces *Bmal1* transcription

(A and B) gAdn (5ug/ml) and others clock-resetting agents treatment on unsynchronized N44 Bmal1-luc cells. (A) Raw bioluminescence recordings of the cells treated with drugs as indicated. (B) Quantification of the normalized peak luminescence magnitudes of recordings in (A) (n=3).

(C) Normalized bioluminescence recordings of unsynchronized N44 cells stably expressing Pgk-luc treated with gAd (5ug/ml) (n=5).

(D and E) qPCR analysis of endogenous *Bmal1* (F) and *Per2* (G) expression in unsynchronized N44 cells treated with gAdn for 3 hr.

(F) Immunoblot analysis of BMAL11 protein level in N44 cells 6 hr after gAdn treatment (5ug/ml). β-tubulin was used as the loading control (n=4).

(G and H) Full-length mammalian cells-expressed adiponectin (fAdn) treatment on unsynchronized N44 Bmal1-luc cells. (G) Raw bioluminescence recordings of the cells treated with different doses of fAdn as indicated. (H) Quantification of the normalized peak luminescence magnitudes of recordings in (G) (n=3).

(I and J) gAdn treatment on synchronized primary hypothalamic neurons expressing Bmal1-luc. (I) Raw luminescence recordings of Bmal1-luc expressing primary hypothalamic neurons treated with PBS or gAdn (5ug/ml) at the time as indicated by the arrow. (J) Quantification of the normalized peak magnitudes of the recordings shown in (I) (n=3).

(K) qPCR analysis of *Bmal1* expression in the MBH of Adipoq KO mice at ZT9 after i.v. injection with PBS or fAdn (1ug/g) at ZT6.

(L) qPCR analysis of *Bmal1* expression in the MBH of WT mice at ZT0 after i.c.v. administration with control IgG or anti-adiponectin antibody (0.6ug) at ZT21-22.

Error bars indicate means ± SEM. *p<0.05, **p<0.01, ***p<0.001, ns = no significant difference. Groups denoted with different alphabets indicates statistical significance (p<0.05). One-Way ANOVA with Bonferroni post-test for multi-groups analysis compared to the control group in (B). Student's t-test in (F) and (J). Mann Whitney test in (K) and (L). One-Way ANOVA with Tukey post-test in (D) and (E).

2.10. Dose-dependency of adiponectin-induced phase resetting and *Bmal1* induction

The paradigm used for the peptide screen with synchronized N44 Bmal1-luc cells was proven to be a robust experimental setting which allows for simultaneously assessing the phase-resetting and *Bmal1* induction effects of adiponectin (Fig. 4 A). To better determine phase- and dose-dependencies of adiponectin treatment, I treated synchronized N44 Bmal1-luc cells with gAdn at various doses and at roughly two opposite circadian phases – hr 13 (near the peak; Fig. 14 A) and hr 23 (near the trough; Fig. 14 B). Consistent to the data shown in Figure 4, the direction of the phase shift was opposite when the treatments were performed at opposite circadian phases. In contrast, the dose-dependency of the phase-resetting effect (i.e., the absolute phase shift) of gAdn did not depend on treatment phase (Fig 14 B & E). Similarly, gAdn could also upregulate Bmal1-luc in a dose-dependent manner regardless of the circadian phase of the treatment (Fig. 14 C & F). These data shed light on the mechanistic nature of the gAdn-induced clock-modulating effects.

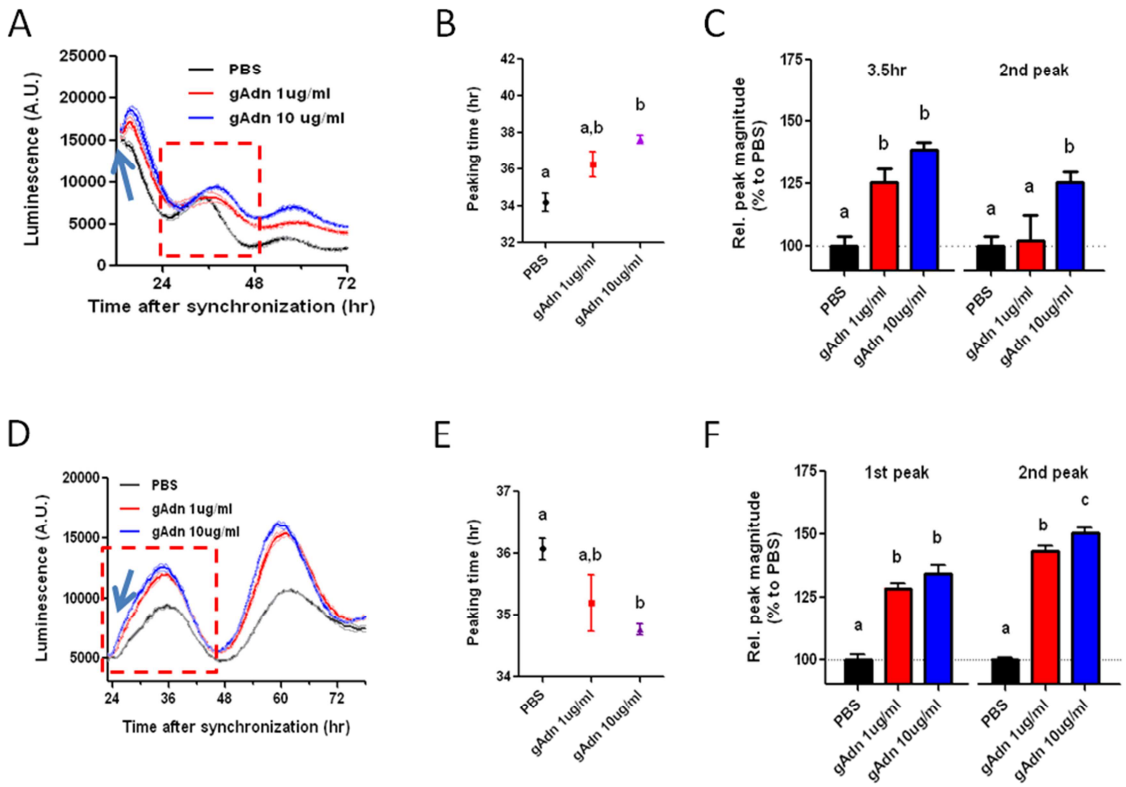

Figure 14. Dose-dependency of adiponectin-induced phase resetting and Bmal1 induction

(A - C) Dose-dependent effects of adiponectin on synchronized N44 Bmal1-luc cells treated near the peak (hr 13). (A) Raw luminescence recordings of synchronized N44 Bmal1-luc cells treated with various doses of gAdn at hr13. (B) Analysis of the peak time of the 2nd circadian cycle (rectangle with red broken-line) of recordings as shown in (A). (C) Analysis of the peak magnitude of the 2nd and 3rd circadian cycle of recordings as shown in (A).

(D - F) Dose-dependent effects of adiponectin on synchronized N44 Bmal1-luc cells treated near the trough (hr 23). (D) Raw luminescence recordings of synchronized N44 Bmal1-luc cells treated with various doses of gAdn at hr23. (E) Analysis of the peak time of the 2nd circadian cycle (rectangle with red broken-line) of recordings as shown in (D). (F) Analysis of the peak magnitude of the 2nd and 3rd circadian cycle of recordings as shown in (D).

Error bars indicate means ± SEM. Groups denoted with different alphabets indicate statistical significance ($p<0.05$), One-Way ANOVA with Tukey post-test, ns=not significant.

2.11. Tissue-specific effects of adiponectin on the circadian clocks *in vitro*

In vivo, adiponectin's clock-modulating effects were strongly tissue-specific. To delineate if this specificity occurs at the cellular level (i.e. is cell-type specific) or at the systemic level (such as influenced by systemic signals), I tested the acute *Bmal1* inducing effect of gAdn in different cell-lines engineered to stably express Bmal1-luc. Under unsynchronized conditions, gAdn treatment also acutely stimulated Bmal1-luc activity in another mediobasal hypothalamic cell line, mHypoE N41 (Fig 15 B). In contrast, gAdn treatment failed to elicit discernable changes in Bmal1-luc expression in cell lines of fibroblast origin (Fig 15 C & D). Interestingly, this tissue specificity cannot be explained by the absence of particular adiponectin receptors including T-cadherin, which presumably is a decoy receptor of adiponectin signaling in cardiac tissues (Fig. 15 E) (40). Together, this experiment revealed that the acute *Bmal1* inducing effect of adiponectin appears to be cell-type specific, probably due to the specific wiring of the downstream signaling cascades of adiponectin in different cell types (37, 40, 49).

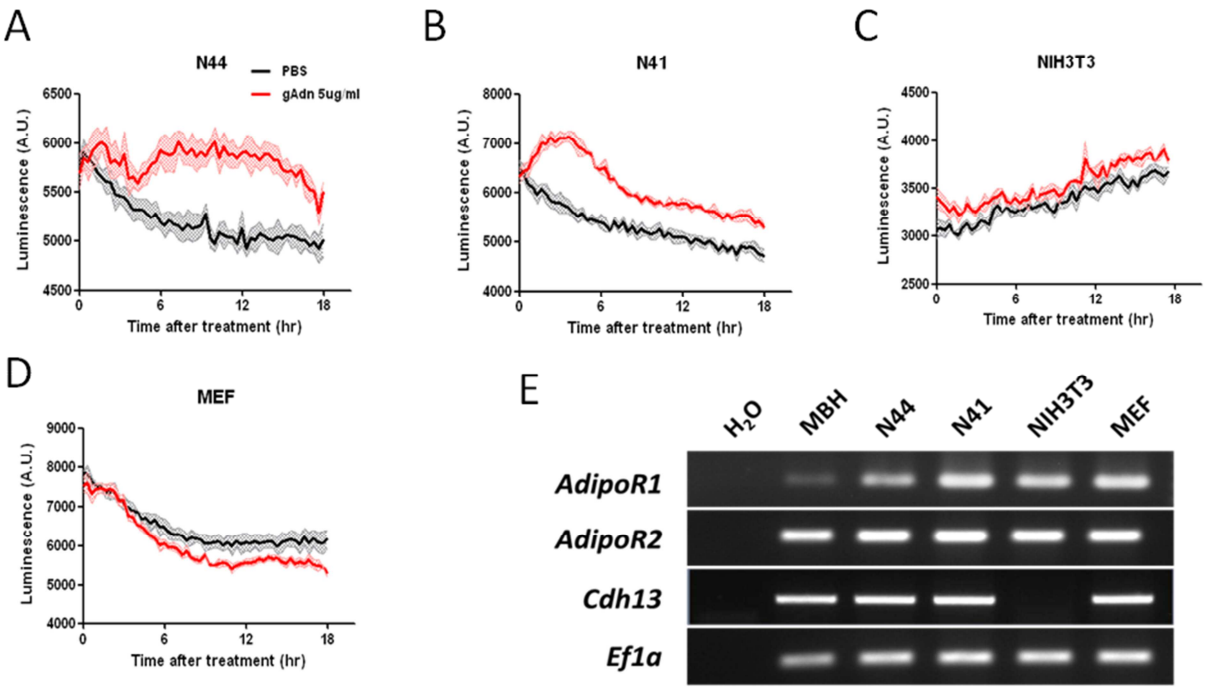

Figure 15. Tissue specific effects of adiponectin on *Bmal1* expression *in vitro*

(A - D) Raw luminescence recordings of different unsynchronized cell lines stably expressing Bmal1-luc treated with gAdn (n=4).

(E) RT-PCR analysis of mRNA expression of adiponectin receptors in cell lines used in (B-E).

Error bars indicate means ± SEM.

2.12. Differential roles of adiponectin receptors on in clock resetting

In an early phase of this study, I noticed that the phase-resetting effect of gAdn treatment in N44 cells was observed only when cells were cultured in serum-free medium (Fig. 16 A - C). One potential explanation to this phenomenon is that adiponectin signaling is already saturated by the abundant presence of adiponectin molecules in serum-containing medium. If this would be the case, then a loss-of-function approach that knocks down individual components of the adiponectin signaling cascade in N44 cells maintained in serum-containing medium would be feasible to allow us to gain insight into the molecular mechanism of adiponectin clock resetting effects. Using a shRNA approach I knocked down (KD) *Adipor1* and *-r2* individually to less than one third of the original level in N44 cells. While knocking down *Adipor2* had no effect on the mRNA level of *Adipor1*, knocking down *Adipor1* led to a simultaneous downregulation of *Adipor2* (Fig. 16 D & E). This effect did not depend on a specific shRNA, but may actually reflect an Adipor1-dependent regulation of Adipor2 expression, as transduction with a second AdipoR1-targeting shRNA gave a similar result (Fig. 17 C & D). Knocking down individual adiponectin receptors intriguingly resulted in differential effects on *Bmal1* expression: AdipoR1 KD led to downregulation of *Bmal1* while AdipoR2 KD had the opposite effect (Fig. 16 F). These observations were confirmed with a second set of shRNAs targeting distinct sequences of both *Adipor1* and *-r2* transcripts (Fig 17 C-H). Interestingly, when both *Adipor1* and *-r2* were knocked down simultaneously (R1R2 dKD), it appeared that the *Bmal1* downregulating effect of AdipoR1 KD was dominating over the upregulating effect of the AdipoR2 KD (Fig. 16 E and Fig. 17 A & B). I speculated that REV-ERBα, a transcriptional repressor of *Bmal1*, may be involved in the differential effect of the two adiponectin receptors on *Bmal1* expression. Knocking down either *Adipor1* or *-r2* individually reduced the expression of *Nr1d1* (encoding gene of REV-ERB α) (Fig. 17 B). While the downregulation of *Nr1d1* in AdipoR1 KD cells is likely secondary to the downregulation of *Bmal1,* the reduction of *Nr1d1* in AdipoR2 KD cells could explain the observed *Bmal1* upregulation in these cells (Fig. 17 B). To understand the role of adiponectin receptors in cellular circadian rhythms, luciferase activity in

synchronized N44 Bmal1-luc cells cultured in serum-containing medium with individual AdipoR knockdown was recorded. Consistent with previous data, AdipoR1 KD led to lower overall luminescence magnitudes while AdipoR2 KD had the opposite effect. I also analyzed the timing of the first peak which is related to the response of the cellular clock to the synchronizing signal and the average period over 4 circadian cycles. AdipoR1 KD resulted in a phase advance of the first peak together with period lengthening during subsequent cycles while AdipoR2 KD had no significant effects on these two parameters (Fig. 16 H - J). Together, these data indicate that AdipoR1 may mediate the clock-modulating effects of adiponectin in hypothalamic neurons. To further test this, I treated AdipoR1 KD and scramble shRNA transduced N44 Bmal1-luc cells cultured in serum free medium at hr 23 after synchronization with gAdn to analyze phase-resetting and *Bmal1* induction. AdipoR1 knockdown significantly reduced gAdn induced *Bmal1* upregulation and phase advances (Fig 16 K - N). Together, these data lead us to conclude that AdipoR1 is the major receptor that mediates the clock-modulating effects of adiponectin in MBH neurons.

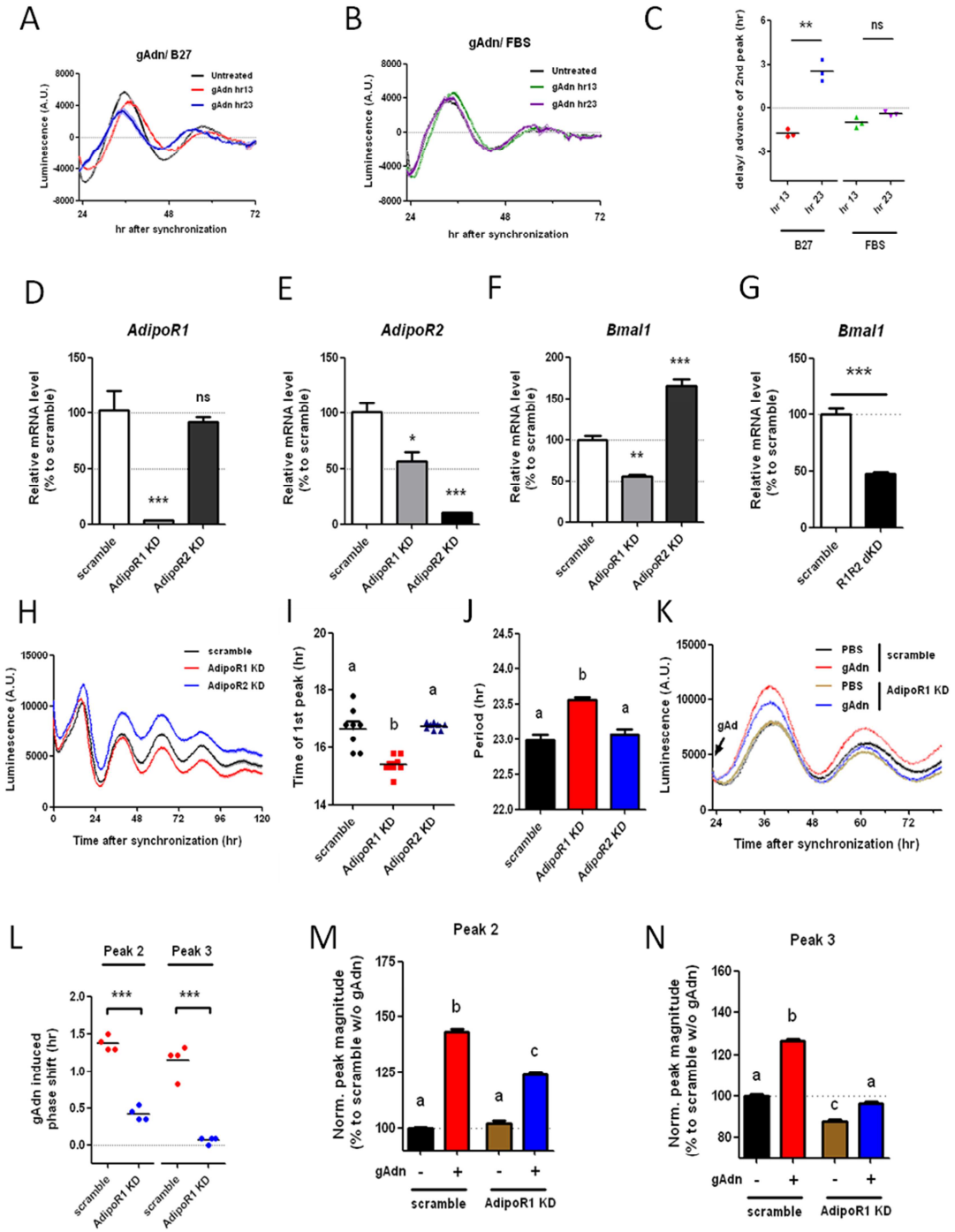

Figure 16. AdipoR1 mediates the clock modulating effects of adiponectin.

(A - C) Presence of serum abolishes the phase-shifting effect of gAdn in N44 cells. Normalized luminescence recordings of N44 cells cultured in B27 supplemented media (A) or in FBS containing media (B) treated with gAdn (3ug/ml) at indicated time points. (C) Quantification of the peak time of the 2nd circadian cycle in (A) and (B) (n=3).

(D - F) qPCR analysis of *AdipoR1*, *AdipoR2* and *Bmal1* expression in unsynchronized N44 cells with shRNA knockdown of *AdipoR1* or *AdipoR2* in serum-containing medium.

(G) qPCR analysis of *Bmal1* expression in unsynchronized N44 cells with double knockdown of *AdipoR1* and *AdipoR2* in serum-containing medium.

(H - J) Differential effects of shRNA knockdown of *AdipoR1* and *AdipoR2* on Bmal1-luc rhythm in synchronized N44 Bmal1-luc cells in serum-containing medium. (H) Raw luminescence recordings; (I) Peak time analysis of the first circadian cycle; (J) The average period length of the Bmal1-luc rhythms over 4 days of recording (n=8).

(K - N) Effects of AdipoR1 knockdown on the phase-shifting and *Bmal1* upregulating effect of gAdn treatment (5ug/ml, treated at near the trough (hr23) of the 1st circadian cycle) in synchronized N44 Bmal1-luc cells (n=4). (K) Raw luminescence recordings; Quantification of the gAdn induced phase-shift (L) and the normalized peak magnitudes of the 2nd (M) and 3rd (N) circadian cycles in control and AdipoR1 KD cells.

Error bars indicate means ± SEM. *p<0.05, **p<0.01, ***p<0.001, ns= no significant difference. Groups denoted with different alphabets indicates statistical significance (p<0.05). Student's t-test for pairwise comparison in (C, G and L). One-Way ANOVA with Bonferroni post-test for multi-groups analysis compared to the control group in (D-F). One-Way ANOVA with Tukey post-test in (I,J,M, & N).

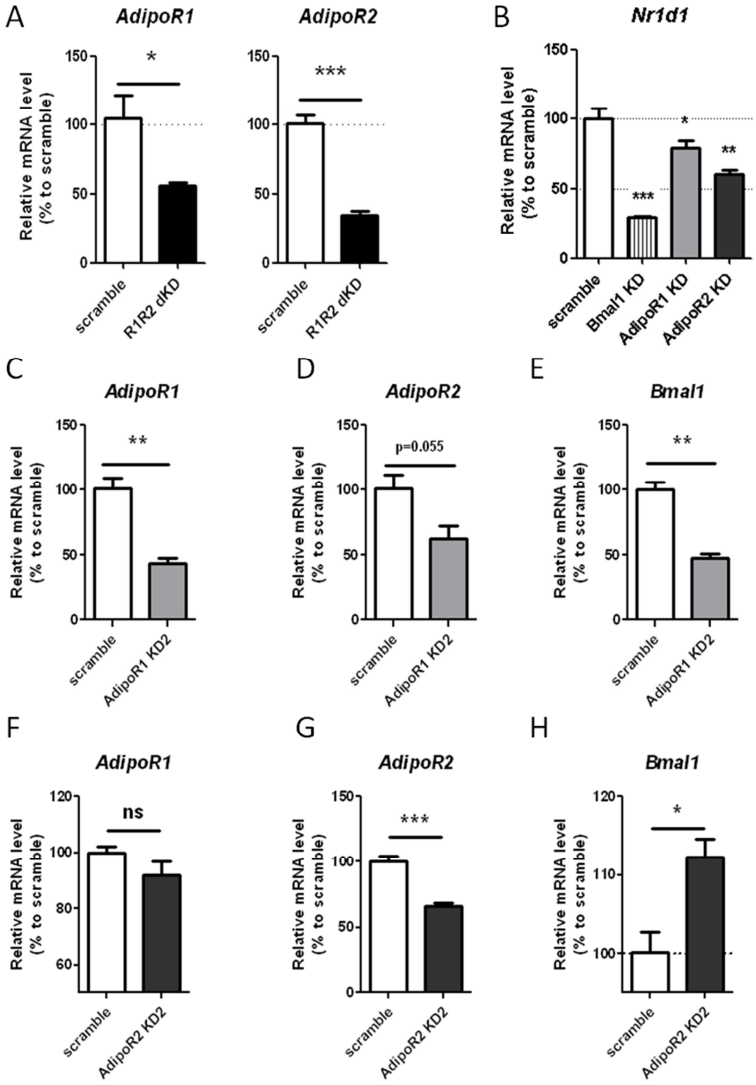

Figure 17. Supplementary data for the effects of shRNA knockdown of AdipoRs on the molecular clockwork in N44 cells.

(A) qPCR analysis of *AdipoR1 and R2* expression in N44 cells with AdipoR1 and R2 double-knockdown (n=4).

(B) qPCR analysis of *Nr1d1* expression in N44 cells with various shRNA knockdowns as indicated (n=3).

(C - E) qPCR analysis of selected genes in N44 cells with the 2nd AdipoR1 knockdown shRNA target sequence (AdipoR1 KD2) (n=4).

(F - H) qPCR analysis of selected genes in N44 cells with the 2nd AdipoR2 knockdown shRNA target sequence (AdipoR2 KD2) (n=4).

Error bars indicate means ± SEM. *p<0.05, **p<0.01, ***p<0.001, Student's t-test for pairwise comparisons and One-Way ANOVA with Bonferroni post-test for multi-groups analysis compared to control in (B).

2.13. PGC1α mediates the clock-modulating effect of adiponectin in MBH neurons

Recently, PGC1α has been identified as a crucial mediator of AdipoR1 controlled metabolic effects in skeletal muscles (51). Interestingly, PGC1α has also been identified as a modulator of the molecular clock acting via

induction of *Bmal1* expression (52). This mechanism highly resembles what I had observed for adiponectin. Therefore, I speculated that PGC1α may play a crucial role in mediating adiponectin's clock-modulating effects. To test this, I investigated if alterations of adiponectin signaling could modify the expression of *Pgc1α* in hypothalamic neurons. qPCR analysis of N44 cells cultured in serum-containing medium revealed that AdipoR1 KD resulted in dramatic reduction in *Pgc1α* expression while AdipoR2 KD had no significant effect (Fig 18 A). These observations were consistent with previous reported findings on skeletal muscles (51). Conversely, gAdn treatment of N44 cells cultured in serum free medium resulted in an upregulation of *Pgc1α* expression (Fig. 18 B) and enhanced the occupancy of an retinoic acid-related orphan receptor response element (RORE) in the *Bmal1* promoter by PGC1α, but not in the 3' untranslated region (3'-UTR) of the *Bmal1* gene (Fig. 18 C). Thus, the adiponectin-AdipoR1-PGC1α signaling cascade is conserved in MBH neurons similar to what has been shown in skeletal muscle (51). *In vivo*, I analyzed PGC1α mRNA and protein levels in the MBH of WT and Adipoq KO mice. In WT mice, I did not observe significant circadian oscillations of *Pgc1α* mRNA expression in the MBH (Fig. 18 D), but significant differences were observed in protein levels during the subjecting day and night (Fig. 18 E). Adiponectin deficiency resulted in significant downregulation of *Pgc1α* mRNA at early subjective day and night (Fig. 18 D) and of PGC1α protein during the subjective night time (Fig. 18 E). Thus, it can be concluded that adiponectin is a positive regulator of PGC1α expression in the MBH *in vitro* and *in vivo*. To characterize the role of PGC1α in regulating the molecular clockwork in MBH neurons, I investigated the cellular rhythms of synchronized N44 Bmal1-luc cells after *Pgc1α* knockdown (PGC1α KD) cultured in serum-containing medium. PGC1α KD cells displayed cellular circadian phenotypes similar to those seen in AdipoR1 KD cells - dampened Bmal1-luc magnitudes, advanced phasing and lengthened period (Fig 18 F - H), indicating that the clock-modulating functions of PGC1α and AdipoR1 use the same pathway. In synchronized N44 Bmal1-luc cells in serum-free medium, PGC1α KD diminished the phase-resetting and *Bmal1*-inducing effects of gAdn treatment at hr 23 after synchronization, similar to what was observed in AdipoR1 KD cells (Fig. 18 I - L). It has been shown that RAR-related orphan receptor alpha (RORα) activity is necessary for the circadian effects of PGC1α (52). To test if RORα is needed for adiponectin's circadian effects, N44 cells were pretreated with a recently established RORα antagonist,

VPR66 prior receiving gAdn treatment (53). I observed that VPR66 pretreatment abolished the Bmal1 inducing effect of gAdn (Fig. 19), further confirming the involvement of PGC1a-RORα signaling in adiponectin mediated clock resetting in MBH neurons.

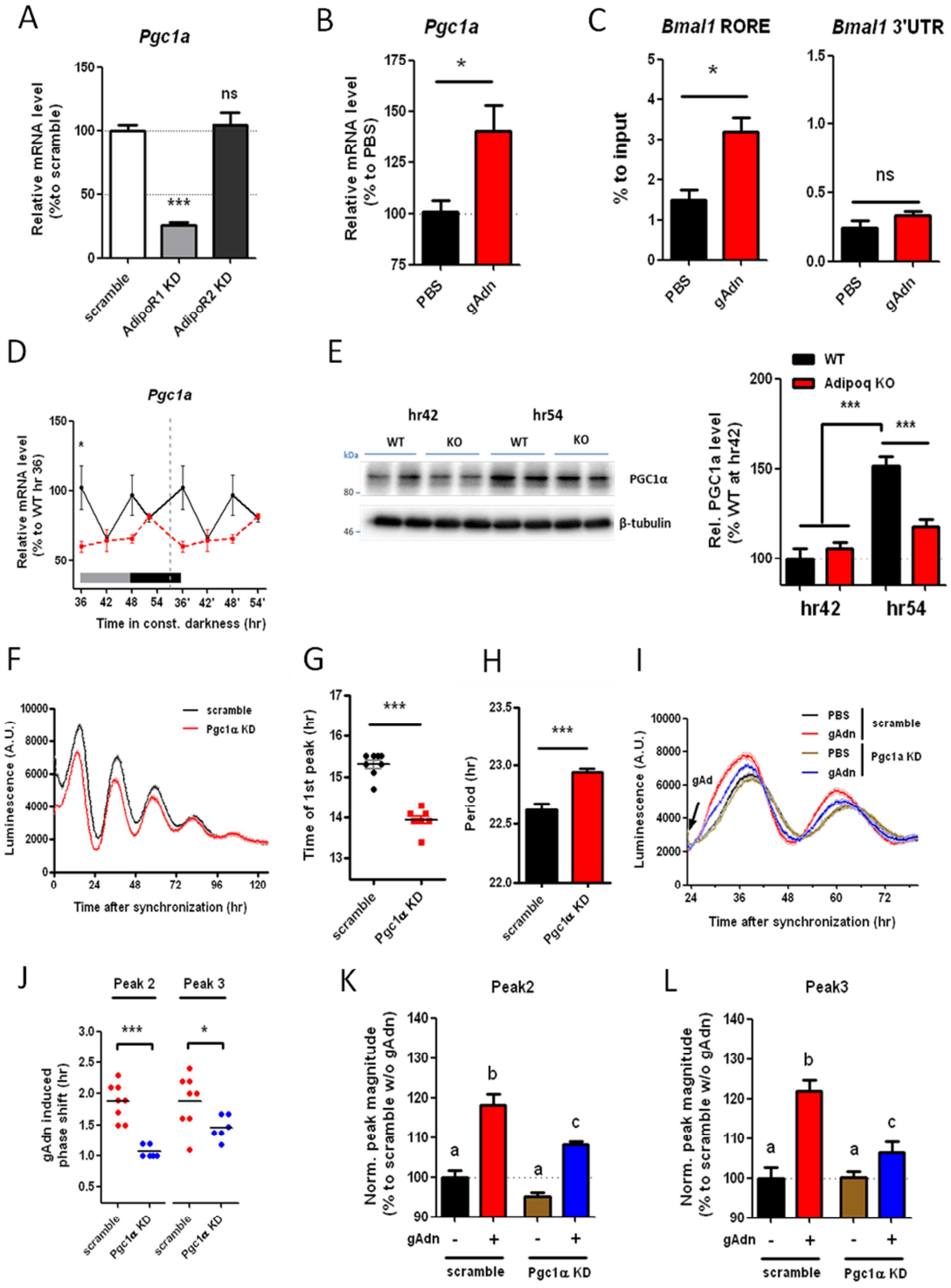

Figure 18. PGC1α mediates clock modulating effect of Adiponectin via AdipoR1.

(A) qPCR analysis of *Pgc1α* expression in unsynchronized N44 cells with shRNA knockdown of *AdipoR1* or *AdipoR2* in serum-containing medium (n=3).

(B) qPCR analysis of *Pgc1a* expression in unsynchronized N44 cells after 2hr gAdn treatment (5ug/ml) (n=4).

(C) PGC1α occupancy at the RORE sequence of the promoter and at the 3'UTR of the *Bmal1* gene in N44 cells 2 hr after gAdn treatment (5ug/ml) (n=3).

(D) Double-plotted 24-hr mRNA expression profile of *Pgc1a* in the MBH of mice released into DD for the indicated time span (n=3 per time point).

(E) Immunoblot analysis of PGC1α protein level in the MBH from mice released into DD for the indicated time span (n=4).

(F - H) Effects of shRNA knockdown of *Pgc1α* on the Bmal1-Luc rhythms in synchronized N44 cells in serum-containing medium. (F) Raw luminescence recordings; (G) Quantification of the timing of the first peak; (H) The average period of the cellular rhythm over 4 days of recording. (n=8)

(I - L) Effects of PGC1α knockdown on the phase-shifting and *Bmal1* upregulating effect of gAdn treatment (5ug/ml, treated at near the trough (hr 23) of the 1st circadian cycle) in synchronized N44 Bmal1-luc cells (n=4). (I) Raw luminescence recordings; Quantification of the gAdn induced phase-shift (J) and the normalized peak magnitudes of the 2nd (K) and 3rd (L) circadian cycles in control and PGC1α knockdowned cells.

Error bars indicate means ± SEM. *p<0.05, **p<0.01, ***p<0.001, ns= no significant difference. Groups denoted with different alphabets indicates statistical significance (p<0.05). Student's t-test for pairwise comparison. One-Way ANOVA with Bonferroni post-test for multi-groups analysis compared to the control group in (A). One-Way ANOVA with Tukey post-test in (E) and (J-L)..

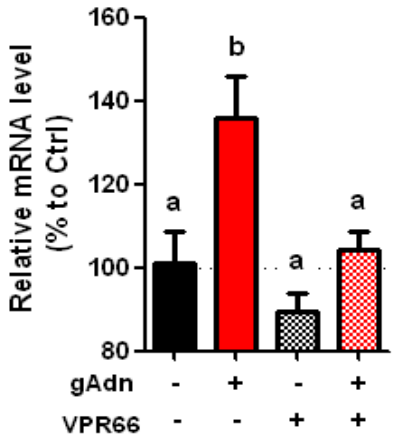

Figure 19. Adiponectin's *Bmal1* induction effect is dependent of RORα activity

qPCR analysis of *Bmal1* expression in unsynchronized N44 cells pretreated with an antagonist of RORα - VPR66 (5uM) 30 min before gAdn (5ug/ml) treatment for 3 hrs (n=4). Groups denoted with different alphabets indicates statistical significance (p<0.05). One-Way ANOVA with Tukey post-test

In the MBH, AMPK has been described as a mediator of central adiponectin signaling downstream of AdipoR1 (38). AMPK activates PGC1α activity directly via phosphorylation and indirectly via a SIRT1-dependent pathway (54, 55). Interestingly, AMPK was also shown to directly modify the molecular

clock by destabilizing CRY protein via phosphorylation (56). In N44 cells, gAdn treatment resulted in a transient phosphorylation of AMPK at Thr172 on the α-subunit which is known to stimulate the kinase activity of AMPK (Fig. 20 A & B) (57). Pre-treatment with an AMPK inhibitor, compound C, on the N44 Bmal1-luc cells significantly attenuated, but not fully abolished, gAdn induced Bmal1-luc activity (Fig. 20 C & D), suggesting that AMPK also participates in adiponectin's clock modulating effect in MBH neurons. Together, conclude that the AdipoR1-AMPK-PGC1α-Bmal1 signaling cascade mediates adiponectin's circadian effects in mediobasal hypothalamic neurons.

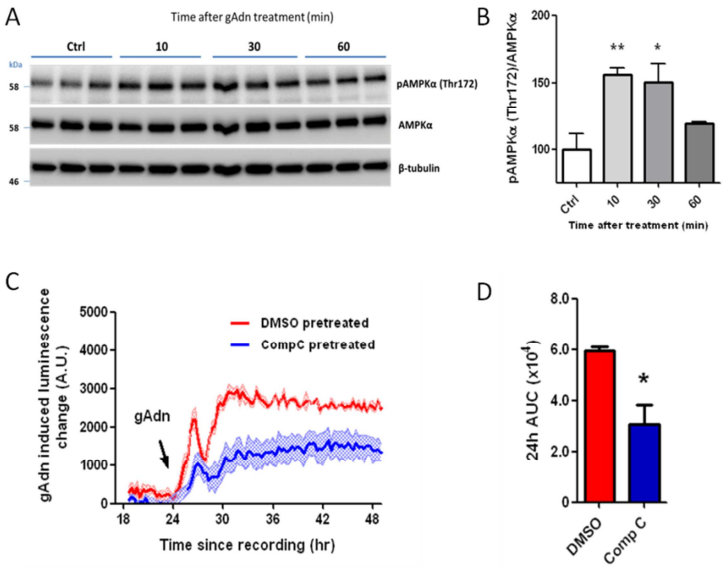

Figure 20. AMPK is involved in adiponectin induced Bmal1 upregulation.

(A and B) (A) Time-course immunoblot analysis of the phosphorylation of AMPK at Thr172 in N44 cells treated with gAdn (5ug/ml) (A). (B) Quantification of (A).

(C and D) (C) Normalized luminescence recordings of unsynchronized N44 Bmal1-Luc cells pretreated with an AMPK inhibitor - compound C (5uM) 2 hrs before gAdn treatment (5ug/ml). (D) Quantification of the 24-hr area under the curve after gAdn treatment as shown in (C).

Error bars indicate means ± SEM. *p<0.05, **p<0.01, ***p<0.001, One-Way ANOVA with Bonferroni post-test compared to control in (B) and Student's t-test in (D).

2.14. The role of central adiponectin signaling in regulating food anticipatory circadian rhythms

As mentioned, it has been shown that neuronal *Bmal1* expression plays a role in regulating circadian FAA rhythms (48). *Bmal1* expression in the DMH has been shown to contribute the FAA (58), but also (59). To

characterize the mechanism of the FAA modulating effects of adiponectin, I analyzed the 24-hr profiles of plasma adiponectin and the mRNA levels of selected adiponectin signaling components, clock genes and appetite-regulating NP genes in the MBH of WT mice that were either fed *ad libitum* or submitted to the RF regime for 10 days as depicted in Figure 12A, except that food was also removed after ZT11 on Day 10. I observed that there was a modest but significant diurnal rhythm in plasma adiponectin ($p<0.001$, cosinor analysis) under *ad libitum* feeding conditions with peak-to-trough change of 27.62 ± 8.39 %. On the other hand, the RF regime significantly reinforced this diurnal rhythm with a peak-to-trough change of 56.82 ± 6.31 % (means ± SEM, n = 3; Fig. 21 A). Interestingly, the diurnal mRNA expression of adiponectin in adipose tissues was not significantly modified by RF, suggesting the existence of post-transcriptional mechanism mediating the influence of RF on diurnal blood oscillation (Fig. 21 B). In the MBH, the RF regime significantly enhanced the amplitude in the diurnal variation of transcripts of adiponectin signaling - *Adipor1*, *Adipor2* and *Pgc1a* (Fig. 21 C - E) and of clock gene rhythms - *Bmal1*, *Per2*, *Dbp* (Fig. 21 F - H). Moreover, the RF regime dramatically reorganized the 24-hr expression profiles of appetite-regulating NP genes in the MBH (Fig. 21 I - L). Expression of *AgRP* was upregulated throughout the day and expression of *Npy* was upregulated during daytime (Fig. 21 I & J). On the other hand, RF differentially regulated the expression of anorexigenic NPs – with *Cart* expression being upregulated during the early morning (Fig. 21 K) while there was a trend for downregulation in *Pomc* during the day ($F=4.16$, $p=0.0582$) (Fig. 21 L). These data together with the impaired food anticipation phenotype in Adipoq KO mice suggest that reinforced central adiponectin signaling in the MBH may play a role in promoting the behavioral re-adaptation of the circadian system to temporally restricted feeding schedules.

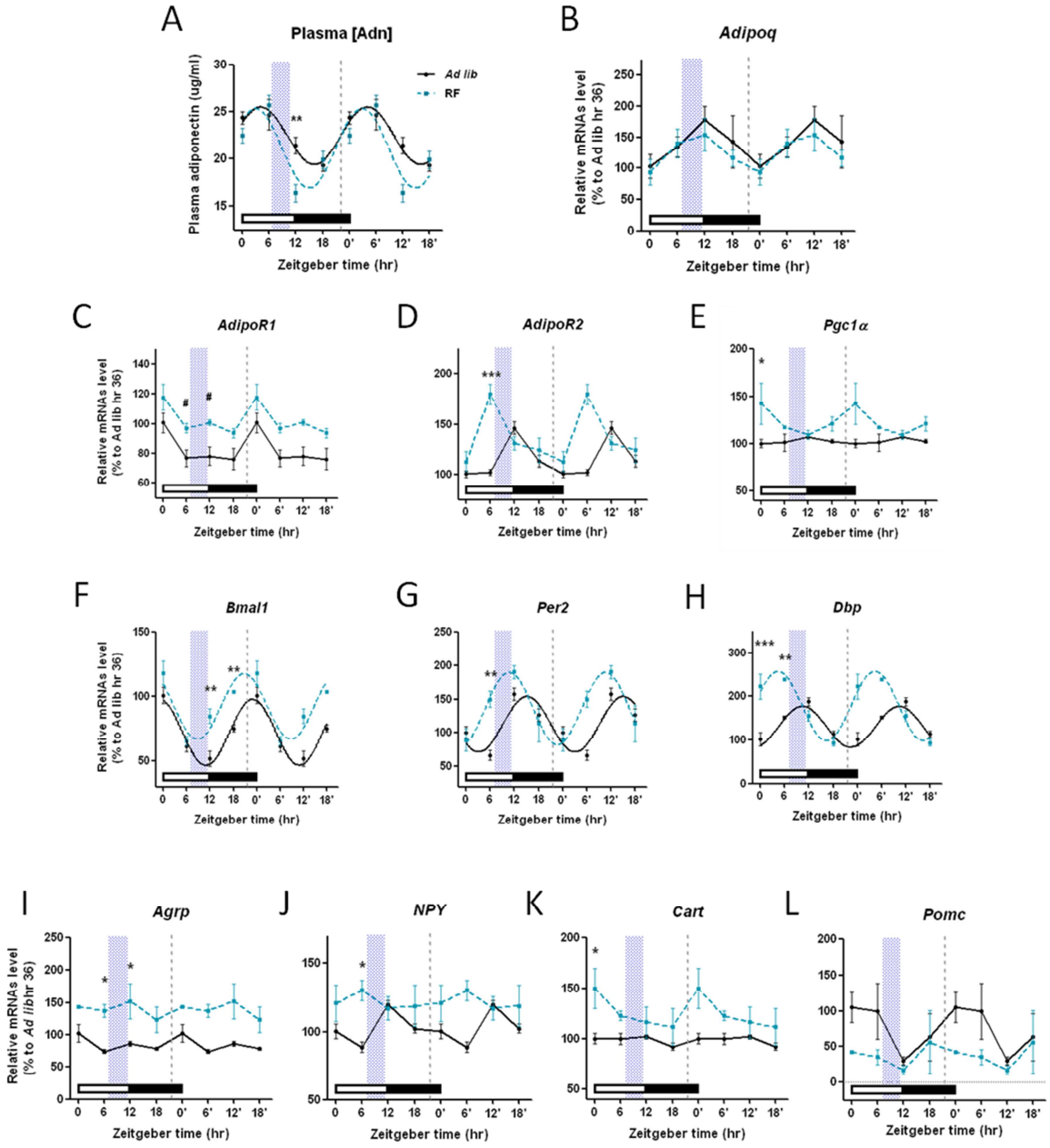

Figure 21. Regulation of the adiponectin signaling components in the MBH under time-restricted feeding regime

(A) Double-plotted 24-hr plasma adiponectin profile of WT mice under Ad libitum feeding and after 10 days in time-restricted feeding conditions as described in Fig. 3A under LD condition (n=3 per time point).

(B) Double-plotted 24-hr mRNA expression profile of *Adipoq* gene in adipose tissues (n=3 per time point).

(C - L) Double-plotted 24-hr mRNA expression level of selected adiponectin signaling components (C-E), clock genes (F-H) and appetite regulating NP genes (I-L) in the MBH of WT mice under *Ad libitum* feeding and after 10 days in RF (n=3 per time point).

Error bars indicate means ± SEM, *p<0.05, **p<0.01, ***p<0.001, Two-Way ANOVA with Bonferroni post-test for profile data.

To further strengthen the proposed role of central adiponectin signaling in food anticipatory circadian rhythms, and exclude that the observed phenotypes of Adipoq KO mice may result from general metabolic alterations as a consequence of non-circadian effects of adiponectin deficiency, in a collaboration with Dr Koch, we tested if acute pharmacological blockade of central adiponectin signaling in WT would similarly affect FAA. I submitted 2 groups of WT mice received i.c.v. administration of either DMSO vehicle or the RORα antagonist-VPR66 at ZT0 on Day 4 and 5 under the RF protocol. VPR66 treated animals showed a delay in the development of FAA mirrored by reduced food intake from Day 5 to 7 (Fig 22 A - F, H & I), similar to what was observed in Adipoq KO mice. Notably, I did not observe any significant reduction of total daily activity in VPR66 treated mice, suggesting that the general well-being was not compromised by VPR66 treatment (Fig. 22 G). Thus, these data further support the role of central adiponectin signaling in the entrainment food anticipatory circadian rhythms.

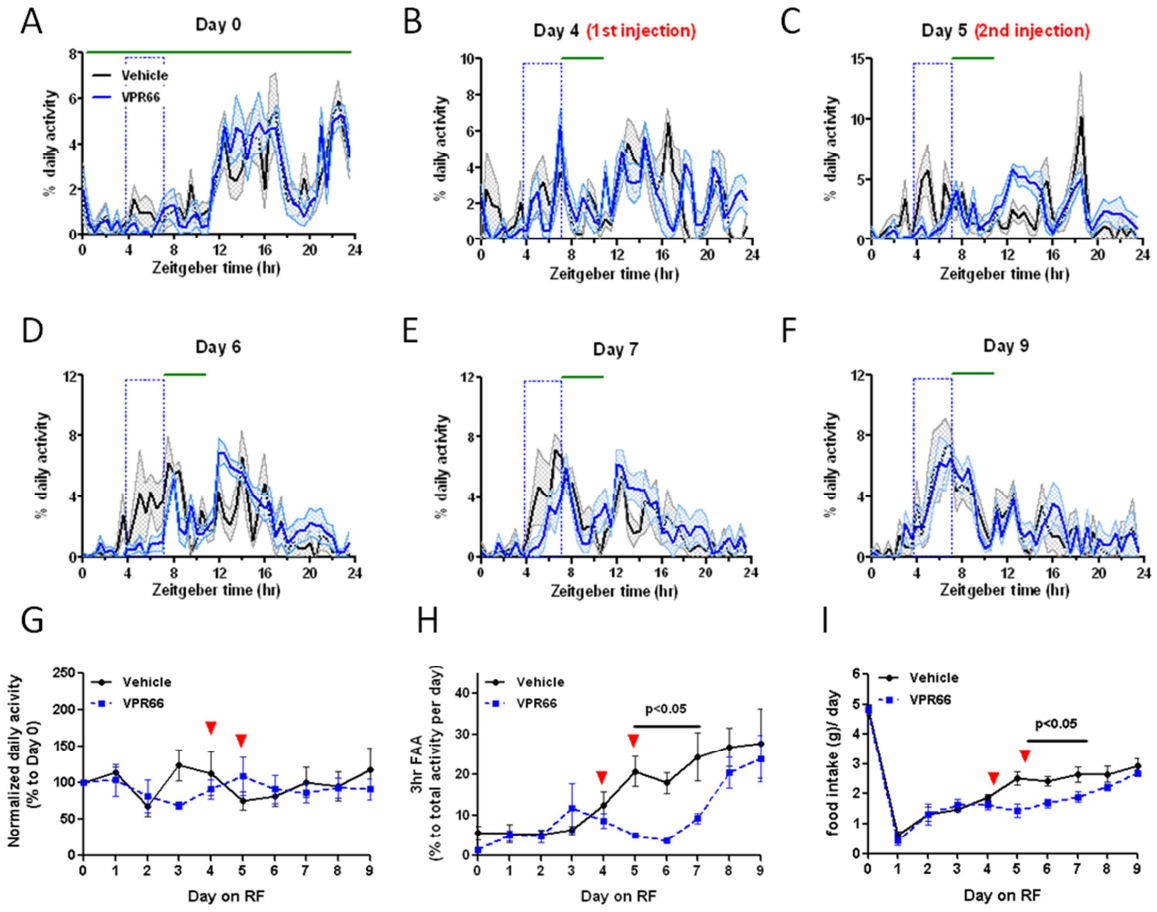

Figure 22. Central adiponectin signaling contributes to food anticipatory circadian activity rhythm.

i.c.v. administration of VPR66 (RORa inhibitor, 5uM) at ZT0 on day 4 and 5 (red arrows) under the RF regime impairs the development of FAA (assessed by infrared detectors).

(A-F) 24-hr activity profiles on individual days during the course of RF regime. The overhead green bars indicate feeding time; the blue rectangular windows indicate FAA measured. On day 4 (E) and 5 (F) mice were i.c.v. administered with 2ul VPR66 or DMSO vehicle control at ZT0.

(G) Normalized total daily activity over the course of RF regime.

(H) Quantification of the 3hr FAA over the course of RF regime.

(I) Daily food consumption over the course of RF regime.

Error bars indicate means ± SEM. *p<0.05, **p<0.01, ***p<0.001, Mann Whitney test for (G-I).

2.15. Role of adiponectin in the clock-modulating effects of high fat diet

Diet-induced obesity has been shown to down-regulate adiponectin (60). Feeding mice with a high fat diet (HFD) has been shown to disrupt circadian rhythms at both the behavioral and the molecular levels (11, 61). To investigate if adiponectin interacts with HFD-induced circadian modulations I compared circadian rhythms in WT and Adipoq KO mice fed with normal diet (ND) and HFD. HFD has been shown to acutely modulate diurnal feeding rhythms even before the development of obesity itself (11). Consistently, I also observed that HFD rapidly induced daytime feeding in WT mice compared to week 0 (i.e., fed with ND in all groups) (diet x time variation, $F=9.949$, $p<0.0001$), but failed to do so in Adipoq KO mice which already showed an accentuated daytime feeding phenotype under ND conditions (diet x time on diet, $F=0.2183$, $p=0.9267$) (Fig. 23 A). HFD did not significantly alter the total activity (Fig. 23 B) and daily energy intake (as compensated by reduced food intake) of both WT and Adipoq KO mice (Fig. 23 C & D). Consistent with a previous report, HFD led to a higher body weight gain in Adipoq KO mice compared to WT despite their energy intake was comparable (Fig. 23 C), while on ND both groups showed no significant difference (Fig. 23 E) (45). Further, HFD lengthened the free-running period of locomotor activity in DD in WT and Adipoq KO mice, indicating that adiponectin plays no role in this effect (Fig. 23 F) (11). Finally, plasma adiponectin levels were significantly reduced in WT mice after 16 weeks of HFD (Fig. 23 H). These data suggest that the loss adiponectin is unlikely to be the cause of the HFD-induced modulation of circadian system. The absence of an effect of HFD on daytime feeding in Adipoq KO mice suggests that both HFD and adiponectin deficiency converge to the same mechanism to modulate diurnal feeding patterns which may potentially be mediated by altered regulation of the appetite-regulating NP circuitry in the MBH.

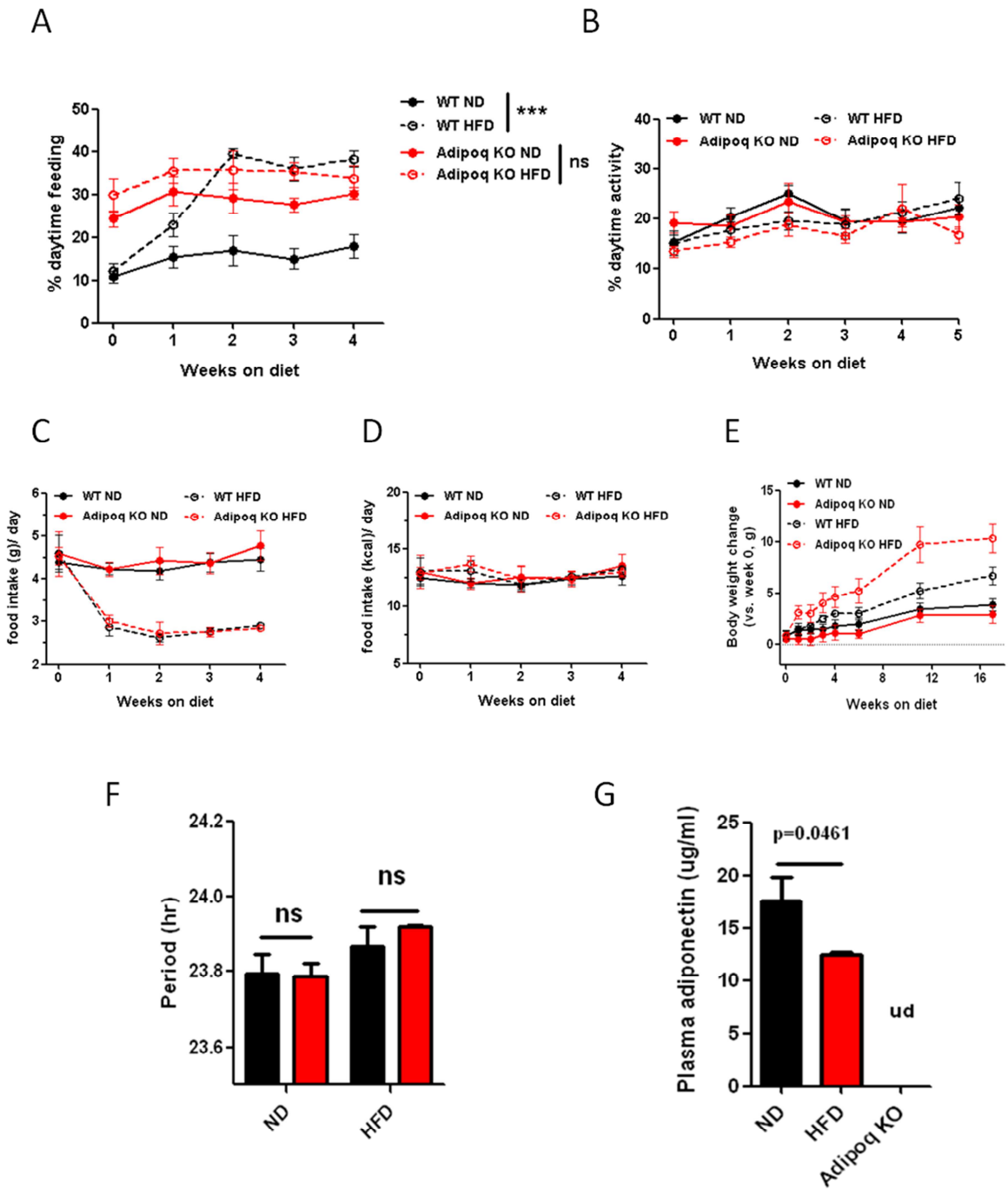

Figure 23. Role of adiponectin in the clock-modulating effects of high fat diet

(A) Normalized daily food intake during the light phase in WT and Adipoq KO mice fed with either normal diet (ND) or high fat diet (HFD) over 5 weeks of treatment. Week 0 refers to the week before HFD treatment in which all animals were fed with ND. The measurements were done once per week.

(B) Percentage of daily activity during the light phase.

(C and D) Food mass (C) and energy (D) intake per day.

(E) Body weight change since the start of experiment.

(F) Free-running period integrated from 10 days in DD on week 18-19 on diets.

(G) Plasma adiponectin level in WT ND, HFD fed groups and Adipoq ND fed group after 20 weeks on diets measured at ZT0.

Error bars indicate means ± SEM, (n=6 per group throughout the experiment). Diet x time variation, ***p<0.001, Two-Way ANOVA in (A); *p<0.05, ***p<0.001, ns=not significant, ud= undetected, Mann-Whitney test in (F&G).

3. Discussion

In this study, I identified adiponectin as a novel modulator of circadian clocks hypothalamic neurons of the MBH. *In vitro*, I found that adiponectin treatment reset the MBH clock in a phase-dependent manner and induced *Bmal1* upregulation. These effects were at least in part mediated via an AdipoR1-PGC1α dependent pathway. Adipoq KO mice, while having a largely normal circadian activity rhythm, exhibited dampened feeding rhythms accompanied by dampened clock gene circadian expression rhythms and altered appetite-regulating NP diurnal expression profiles in the MBH. Under scheduled RF, Adipoq KO mice showed delayed development of FAA and an impaired FEO. In WT mice scheduled RF reinforced the diurnal oscillation of circulating adiponectin, upregulated the expression of adiponectin signaling components and clock gene oscillations in the MBH which may be involved in promoting the reorganization of diurnal expression of appetite-regulating NPs and, thus, the re-adaptation to the altered feeding rhythm. The importance of central adiponectin signaling for food anticipatory rhythm generation was further confirmed by i.c.v. administration of RORα antagonists which phenocopied the Adipoq KO's FAA and food intake defects (Fig. 24).

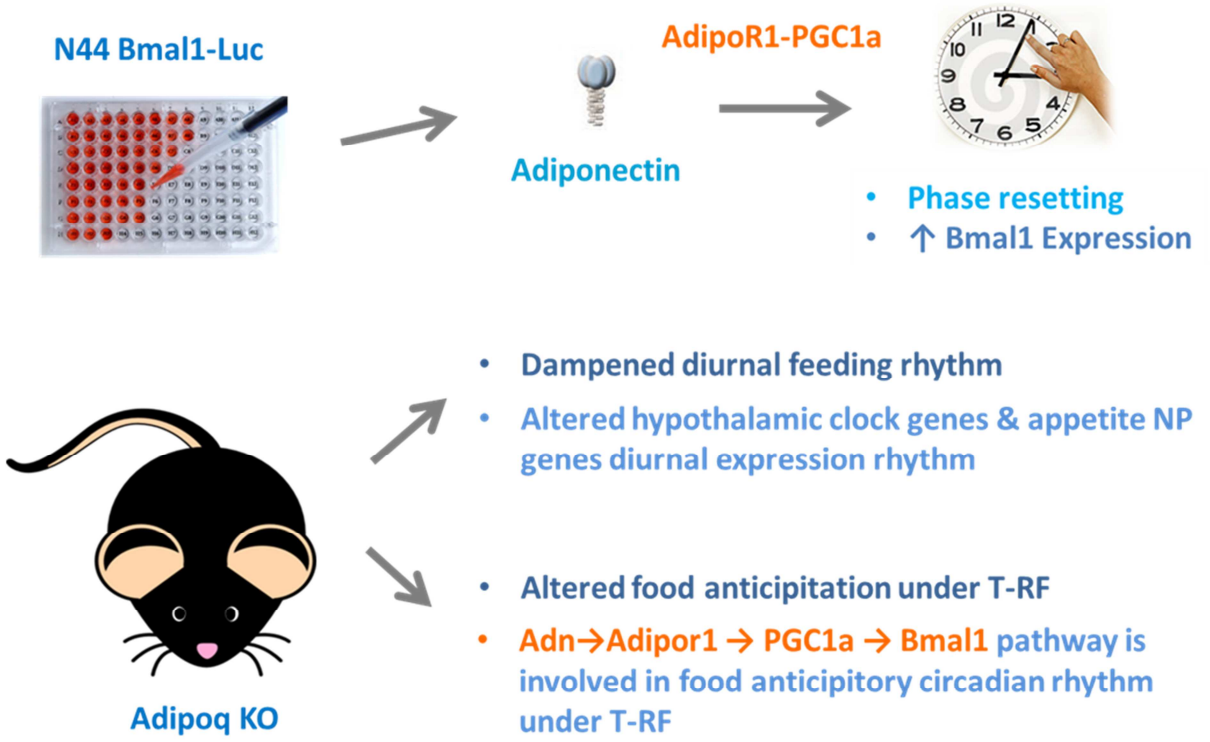

Figure 24. Summary of the findings in the current study

In this study, I identified adiponectin as a novel circadian modulator of mediobasal hypothalamic neurons which can phase-reset the clocks and transcriptionally activate *Bmal1* expression. These effects are at least in part mediated by an AdipoR1-PGC1α dependent pathway. *In vivo*, I demonstrated that adiponectin deficient mice had dampened diurnal feeding rhythms and impaired food anticipatory circadian rhythms. These phenotypes were in line with the clock-modulating effects of adiponectin and the regulatory role of molecular clockwork on appetite-regulating neuropeptides expression.

3.1. Molecular mechanism of the circadian effects of adiponectin

I demonstrated in hypothalamic cells and *in vivo* that the AdipoR1-PGC1α-*Bmal1* axis plays a crucial role in the clock-modulating effects of adiponectin. However, it is highly surprising that while the components of this signaling pathway are believed to be ubiquitously expressed, I observed a strong tissue specificity of the circadian effects of adiponectin both *in vitro* and *in vivo* (Fig. 10 & 15). One possibility is that the antagonistic effect of AdipoR2 on *Bmal1* expression may counterbalance AdipoR1's effects in certain cell types. In N44 cells the loss of AdipoR2 led to a sharp downregulation of *Nr1d1* which encodes for REV-ERB α - a

transcriptional repressor of *Bmal1* (Fig. 17 B). REV-ERB α inhibits *Bmal1* transcription via binding to the RORE in *Bmal1* promoter (62-64). It has been shown that REV-ERB α can antagonize the transactivating effect of PGC1α-RORα complexes for *Bmal1* induction (52). One potential mediator that links AdipoR2 to REV-ERB α is peroxisome proliferator-activated receptor alpha (PPARα). On one hand PPARα has been shown to transcriptionally upregulate *Nr1d1* expression (65), on the other hand AdipoR2 has been shown to activate PPARα activity in the liver (66). The AdipoR2 effects on the circadian clock and its physiological relevance in different tissues remain to be further investigated. Another explanation may lie in the involvement of AMPK activation. It has been shown in peripheral tissues and fibroblasts that persistent activation of AMPK leads to an upregulation of CRY activity and subsequent dampening of the molecular clock due to increased negative feedback via REV-ERB α (56). In this study, I demonstrated that gAdn treatment led to an acute, but short-lasting induction of AMPK activation in hypothalamic neurons (Fig. 20). Activation kinetics of AMPK appear different in this context from what has been observed in peripheral tissues (66-68). Thus, it is possible that while acute AMPK activation may transiently activate PGC1α activity and *Bmal1* expression on one hand, extended AMPK activation in certain tissues may finally lead to a dampening of the circadian clock. Obviously, as adiponectin is known to activate multiple signaling pathways (40, 49), other potential mechanisms that may locally modify the adiponectin signaling to the molecular clock may exist depending on the cellular contexts which need to be further investigated.

3.2. The role of adiponectin in photic entrainment of circadian rhythms

The lack of overt abnormalities in the circadian activity rhythm of Adipoq KO mice suggests that the SCN pacemaker may not be a direct target of adiponectin. The accelerated re-adaptation after an experimental jetlag paradigm on the other hand implies that SCN input or downstream neural substrates may be subject to adiponectin's influences (33). Brain regions such as the intergeniculate leaflet (IGL) and raphe nucleus have been shown to modulate the function of the SCN and also express AdipoR1 (ISH data from Allen Brain Atlas). Therefore, it is possible that adiponectin may locally modify the physiological functions of these

neural circuits. Further studies are needed to elucidate the mechanism of adiponectin's influences on the photic entrainment of circadian system.

3.3. The role of adiponectin in feeding rhythm regulation

The dampened feeding rhythms under both LD and DD conditions suggest that adiponectin plays a crucial role in regulating the circadian gating of appetite regulation (Fig. 9 A - F). In agreement with the *in vitro* data, the loss of adiponectin led to dampened clock gene rhythms in the MBH *in vivo* (Fig. 9 G - I). Consistent with previous findings, I provided further evidence from Bmal1 KO mice and *in vitro* data to support that MBH clocks can directly regulate NP expression (Fig. 8 & 11). However, owing to the complexity of the hypothalamic NP circuitry, the role of MBH clocks in specific subtypes of neurons *in vivo* needs to be further elucidated. From our data, I believe that the orexigenic NPs are more sensitive to adiponectin compared to anorexingenic NPs. It has been shown that central adiponectin applications acutely promote food intake (38). Therefore, the feeding phenotype observed in Adipoq KO mice may comprise of both clock-dependent and clock-independent pathways. In WT mice, I observed a significant diurnal oscillation of plasma adiponectin under *ad libitum* conditions which was further reinforced under RF conditions (Fig. 21 A). This diurnal oscillation appears to be heavily regulated by general metabolic state as under the scheduled RF protocol plasma adiponectin dramatically fell off after (re-)feeding (Fig. 21 A), consistent with the described role of adiponectin as a hunger hormone (38). Under *ad libitum* feeding conditions, circulating adiponectin gradually increased in the second half of the night and reached its acrophase during the first half of the day. This is in phase with the MBH *Bmal1* expression rhythm which peaks during the early morning (Fig. 9 G), suggesting that the late evening rise of circulating adiponectin may be important for the morning peak of MBH *Bmal1* expression. This inference is further supported by the observation that i.c.v. administration of anti-adiponectin antibodies at late subjective night diminished *Bmal1* expression in the MBH in the subjective morning (Fig. 13 L).

3.4. The role of adiponectin in food anticipation regulation

In vitro, I observed that adiponectin could robustly phase-reset MBH neuronal clocks. The lack of obvious alterations of the phasing of the MBH clock gene rhythms in Adipoq KO mice under unchallenged conditions apparently suggests that adiponectin may not be a potent regulator of the phasing of the MBH clock when food is ubiquitously available. In contrast, the strong diurnal oscillation of plasma adiponectin under RF conditions indicates that adiponectin may become important in the regulation of physiological systems and behavioral neural circuits when food availability becomes scarce. The potential role of adiponectin in the kinetics of food entrainment of clock genes rhythms in non-hypothalamic tissues should be further investigated. At the same time, how adiponectin regulated MBH clock gene rhythms are translated into FAA and food intake behaviors is similarly worth studying (Fig. 12).

The ability to anticipate physiological needs in sync with predictable availability of valuable resources provides organisms evolutionary advantages. Light is the major Zeitgeber to the mammalian circadian system that entrains behavioral rhythms to the day-night succession. However, when the daily food access time window is shifted suddenly due to seasonal (i.e., photoperiod) change or other factors such as unexpected changes in ecological landscapes (e.g., wildfire), an anticipatory timekeeping mechanism that can reorganize and optimize the physiology for foraging according to the new food available time window may therefore increase the evolutionary success of an organism and is selected by evolution (15). In the current study, I provide strong evidence that central adiponectin signaling is involved in promoting the behavioral re-adaptation under RF. It has been shown previously that the appetite-regulating system plays a major role in the regulation of FAA. Peripheral metabolic hormones such as anorexigenic leptin and orexigenic ghrelin are an integral part of the food anticipatory system (19). Leptin deficient ob/ob mice and Zuker rats show increased FAA under RF which can be suppressed by administration of recombinant leptin (69, 70). In contrast, under RF ghrelin receptor deficient mice have attenuated FAA (71, 72), despite intriguingly that ghrelin deficient mice have been reported to have a normal FAA (73). More recently, as aforementioned, orexigenic AgRP/NPY neurons in the ARC have been suggested as crucial elements of the food anticipation circuitry (29). These observations strongly point out that the interaction of metabolic

hormones and MBH, particularly the orexigenic neural circuits, is a key regulator of the food anticipatory system. Adipoq KO mice displayed a delayed development of FAA and RF-mediated entrainment of food intake. Critically, these phenotypes were mimicked by central administration of a RORα antagonist (Fig. 22), which excluded the potential secondary off-target peripheral influences downstream to the loss of adiponectin in Adipoq KO mice such as alteration of immune function or lipid homeostasis (37). In the i.c.v. VPR66 administration experiment, I noticed that VPR66 did not acutely inhibit FAA and food intake on the day of injection, but did do so with a day of delay, suggesting that it does not act directly, but by incorporation of external signals into MBH clock feedback loops. This also argues against the involvement of an acute hypothalamic orexigenic effect of adiponectin mediated via AMPK signaling (38). Rather VPR66 may antagonize the adiponectin-mediated resetting of clock gene expression rhythms in the MBH which mediates adaptation of appetite systems and behavior to the RF schedule through reprogramming of diurnal NP expression. Multiple lines of evidence support this interpretation. First, as mentioned, the orexigenic neuronal network in the MBH has been shown to be important for FAA circadian rhythms (46). Second, RF has been shown to reinforced and phase-reset the clock gene oscillations in the ARC and DMH (74, 75). Finally, extra-SCN neural *Bmal1* expression has been shown to contribute to the robustness of the FEO (48). These data are in line with our observation that adiponectin acts as a positive regulator of *Bmal1* expression and that the CLOCK/BMAL1 dimer is an important regulator of appetite-regulating NP expression (76). However, further studies with genetically and anatomically defined abrogation of central adiponectin signaling in specific neuronal subtypes will be needed to identify the responsible neural circuitries involved in the regulation of adiponectin-regulated FAA rhythms.

3.5. The role of adiponectin in high fat diet induced circadian disturbances

HFD has been shown to promote daytime feeding independent of the development of obesity (11). In this study, I also observed similar effects of HFD in WT animals, but failed to further induce daytime feeding in Adipoq KO mice (Fig. 23 A). One explanation may be a convergence of the physiological routes of adiponectin deficiency and HFD to induce daytime feeding (i.e. ceiling effect in Adipoq KO mice) – which may likely occur at the level of appetite regulating hypothalamic NPs - HFD treatment dramatically suppresses the orexigenic *AgRP* and *Npy* overall diurnal variations but upregulated the overall diurnal expression of anorexigenic *Cart* and *Pomc* (11). In our hands WT mice fed with HFD did not reach the morbid obese state in which the circulating adiponectin dramatically has been reported to drop to less than one fifth of ND fed controls according to some other papers (60), thus precluding assessment of the potential effects of the pathophysiological loss of adiponectin induced by morbid obesity in the circadian functions (i.e., in contrast to the complete prenatal loss of adiponectin in Adipoq KO mice). Under basal conditions, Adipoq mice have been reported to have only minor, if any, effects on various metabolic parameters (44, 45). However, under HFD challenge, Adipoq KO mice show significant reduction in energy expenditure, impairment of systemic glucose and lipid homeostasis and obesity compared to wild-type control animals (45). In line with this previous report, I observed that Adipoq KO mice in our hand were also susceptible to HFD-induced weight gain despite of comparable energy intake. This suggests an altered energy turnover in Adipoq KO mice under a HFD challenge. Mistimed high fat feeding during the normal rest phase has been shown to promote energy incorporation and bodyweight gain (12). Given that Adipoq KO mice also showed an increased daytime feeding phenotype, it is tempting to postulate that a part of the body weight gain may be due to mistimed feeding. However, further experiments with carefully designed feeding time windows are clearly needed to dissect the relative contribution of metabolic functions of adiponectin and feeding rhythm disruption to the body weight gain in Adipoq KO mice upon HFD challenge.

While this thesis project was under way adiponectin has been independently reported as a circadian clock modulator in peripheral organs (77). However, owing to the different approaches chosen in that study compared to ours (ectopic overexpression of adiponectin, use of heterogenic mouse models and

physiological systems of interest), a direct comparison between both studies proves difficult. Nevertheless, Hashinaga T. et al also came to the conclusion that adiponectin signaling may play an important role in maintaining proper circadian rhythms of clock gene expression in certain physiological systems under metabolic challenge (77). They reasoned that a part of the circadian effects of adiponectin may be mediated by suppressing the expression of a pro-inflammatory cytokine - tumor necrosis factor alpha (TNFα) which has been shown to modulate the circadian clock system upon inflammatory challenges (78). More recently, the expression of adiponectin in adipose tissues has been shown to be subject to molecular clock regulation via PGC1a and PPARγ (79), thus further strengthening the claim that adiponectin is an integral part of the circadian metabolic circuitry.

3.6. Clinical implications

Together, the present study reveals that adiponectin is a metabolic feedback signal to the mediobasal hypothalamic circadian clock where it regulates feeding rhythms. Disturbance of adiponectin signaling such as in obesity may therefore involve in alter feeding rhythms as seen in patients suffering from night eating syndrome (NES). NES is characterized by a lack of appetite in the morning, but consuming a half or more of daily food intake in the evening and at night associated with various sleep abnormalities (80). Obesity has a strong positive correlation with NES (81). NES patients have increased hunger at night accompanied with blunted nocturnal rise of leptin (82) and a phase advance of ghrelin (83). Mistimed intake of high energy food, on the other hand, has been shown to further promote metabolic disorders, thus forming a viscous cycle (4, 12, 13). The positive role of adiponectin in FAA suggests that people suffering from adiponectin deficiency might have impairment in adapting their feeding rhythms when food availability time window is changed. Though direct evidence that link metabolic syndromes and appetite regulation after a shift of Zeitgeber cycle is still missing, a recent epidemiological study supports this inference showing that that body mass index (BMI) has a positive correlation with social jetlag though the blood hormones has not been investigated in this study (84). Occupations that require frequent shiftwork may represent a condition when food intake becomes uncoupled from the SCN. It can be expected that people with adiponectin deficiency

will suffer from more severe loss of appetite following the shift and require more time to recover under such conflicting Zeitgeber conditions. Thus, together with other metabolic humoral factors reported in literature, I conclude that proper metabolic feedback signals to the circadian clock system either directly or indirectly are important for the robustness of the circadian clock and therefore contribute to the general well-being (Fig. 25).

Our study also sheds light on the therapeutic potential of adiponectin signaling in inhibiting circadian disruption associated with metabolic disorders. Recently, a small molecule agonist of AdipoRs (for both R1 and R2) has been developed, thus providing an opportunity to test this idea in preclinical animal models of circadian disruption (85). Timed administration of adiponectin or AdipoRs' agonists may help to normalize the altered circadian appetite rhythms in individuals suffering from obesity or NES and circadian misalignments such as shiftworkers. Development of individual receptor-specific agonists/antagonists on the other hand will be helpful to further delineate the molecular mechanism of the circadian effects of adiponectin signaling.

3.7. Concluding remarks

This study identified adiponectin as a novel modulator of the MBH clocks and is involved in regulating feeding rhythms and food anticipatory circadian rhythms which at least partly mediated by a mechanism dependent of the AdipoR1-PGC1α/RORα-Bmal1 pathway.

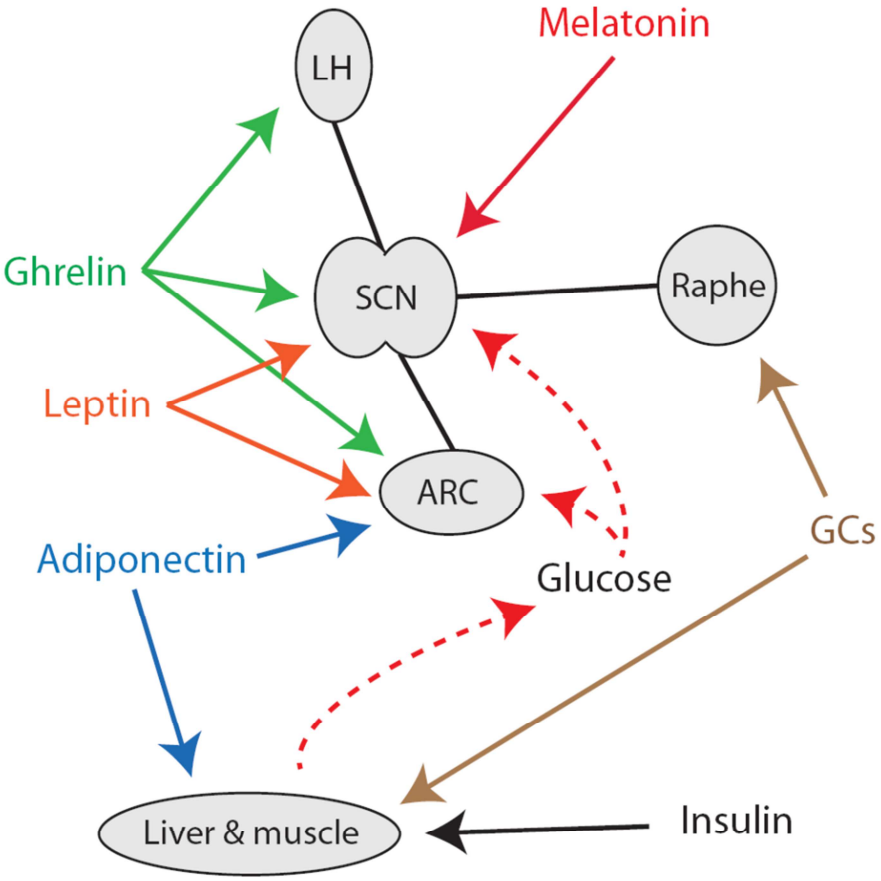

Figure 25. Overview of established endocrine feedback to the circadian clock.

While the circadian system are known to regulate the diurnal hormone abundance in the bloodstream, a number of metabolic peptide hormones such as insulin, leptin and ghrelin have been shown to modify the molecular rhythms directly on central and peripheral clock function. In the CNS, leptin and ghrelin have been shown to directly modulate the SCN clock and may also presumably affect the circadian clock in mediobasal hypothalamic structures such as arcuate nucleus (ARC) or lateral hypothalamus (LH). In this study, I identify adiponectin as a new player to modulate the MBH's clocks and may also impinge on other central and peripheral clocks as well. Melatonin and glucocorticoid (GC) are two well established feedback hormones that modify the circadian clocks of a board spectrum of central and peripheral tissues. Their productions are strongly influenced by the circadian clock. While melatonin can directly act on the SCN, the SCN is insensitive to GC. However, GC can also modulate the SCN clock indirectly via other brain structures such as the raphe nuclei of the brainstem. The central clocks may also be affected by metabolic hormones indirectly via changes in blood metabolite levels such as glucose or free fatty acid. Adapted from Ref. 9.

4. Materials and Methods

Unless otherwise stated, standard chemicals were purchased from Roth (Karlsruhe, Germany), tissue culture products were obtained from Gibco (Life Technologies GmbH, Darmstadt, Germany).

4.1. Animals and circadian behavioral experiments

Adiponectin knockout mice (JAX stock #008195) and *PER2::LUCIFERASE* animals (Per2-luc; JAX stock #006852) were purchased from the Jackson's Laboratory (Maine, USA) and maintained at the animal facilities in Göttingen or Lübeck. The use of *Bmal1* KO mice has been described previously (86). All mice used were kept on C57BL6J genetic background. For all experiments, unless stated otherwise, male mice were individually housed under 12-hour light, 12-hour dark conditions (LD; ~100 lux) with *ad libitum* access to chow pellets (normal diet (3.4% kJ fat, Ssniff #V1536) or high fat diet (45% kJ fat, Ssniff #EF D12451) and water. For experiments in constant darkness (DD), mice were first entrained under LD conditions for at least a week and then released into DD for the indicated timespans. Behavioral experiments were performed on animals aged 10-12 weeks at the beginning of the experiment. Molecular analyses were performed on 16-24 weeks old animals. All animal experiments (including MEF cells and primary hypothalamic neuron isolations) were done after ethical assessment and licensed by the Office of Consumer Protection and Food Safety of the State of Lower Saxony and the Ministry of Agriculture of the State of Schleswig-Holstein and in accordance with the German Law of Animal Welfare (TierSchG).

4.2. Plasmid construction

pLKO-WPRE-GFP was modified from the original pLKO.1-TRC (Addgene plasmid #10878) backbone in which the puromycin resistance open reading frame (ORF) was replaced with a GFP-WPRE sequence from pLenti-CMV-GFP-Zeo (637-7) (Addgene plasmid #17449) with Kpn I and BamH I restriction sites. Nucleotide sequences for generating shRNAs are listed in the Table 3 and were cloned into pLKO-WPRE-GFP using Age I and EcoR I restriction sites.

Npy-luc plasmid was generated by cloning a ~2 kb 5'-regulatory fragment of the murine *Npy* gene into pGL4 vector (Promega, Mannheim, Germany) with Kpn I and Bgl II restriction sites. This fragment has previously been identified to contain multiple E-boxes (22). The integrity of the recombinant plasmids was confirmed by sequencing.

4.3. Lentivirus production and transduction

The Bmal1-luc encoding pBluF-puro plasmid was a kind gift from Prof. Steven Brown, University of Zurich, Switzerland. To produce Bmal1-luc lentiviral particles, a 10-cm dish of HEK293T cells was cotransfected with 10 µg psPAX2 (Addgene plasmid #12260, Prof. Didier Trono, EPFL, Switzerland), 5 µg pMD2.G (Addgene plasmid #12259, Prof. Didier Trono, EPFL, Switzerland), and 15 µg pBluF-puro using Xfect transfection reagent (Clontech, Saint-Germain-en-Laye, France). Cells were rinsed with PBS on the second day and restored with fresh culture medium. Viral containing medium was harvested at 36 hr after transfection and stored at 4 °C. Cells were restored with fresh medium, and further medium collections were performed on the next day. Two collections were pooled and concentrated using LentiX concentrator reagent (Clontech) according to the manufacturer's protocol. Virus titers were determined by transducing HEK293T cells with a serial dilution of GFP encoding lentiviral particles (produced as described above but with pWPI (Addgene plasmid #12254, Prof. Didier Trono, EPFL, Switzerland) instead of pBluF-puro). At 72 hr after transduction, GFP-positive cells were counted under a fluorescence microscope. The same serial dilution was also subjected to quantitative real-time PCR (qPCR) analysis using a primer pair amplifying the viral WPRE element (forward: 5'-GGCACTGACAATTCCGTGGT-3'; reverse: 5'-AGGGACGTAGCAGAAGGACG-3') to determine viral genome abundance. qPCR was also done for Bmal1-luc virus containing medium, and the titer was estimated by comparison with pWPI abundance in relation to the GFP calibration curve. To transduce hypothalamic cells, cells at 50 % confluence were loaded with ~0.85 × 10^8 infection units (IFUs) per 1 ml medium in the presence of 8 µg/mL polybrene. Experiments on cells were carried out 72 hr after transduction.

4.4. Cell culture and circadian luminescence recording

mHypoE-N44, N41 (Cedarlanelabs, NC, USA), HEK293T, NIH3T3 (ATCC, LGC Standards GmbH, Wessel, Germany), and MEF cells were maintained in DMEM with 2mM glutamine supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin at 37 °C with 5 % CO_2. Cells stably expressing Bmal1-luc reporter via lentiviral transduction were generated polyclonally with puromycin selection. For circadian luminescence measurements, cells seeded in 96-well plates were synchronized by 100 nM dexamethasone treatment for 2 hours. After that, medium was replaced with recording medium (DMEM without phenol red supplemented with 2 mM Glutamax, 3 mM sodium carbonate, 10 mM HEPES, 2 % B-27 supplement, 1x penicillin/streptomycin and 0.5 mM D-luciferin). The plates were then sealed with transparent films and luminescence was recorded at 34 °C using the Berthold TriStar LB 941 plate reader (Berthold Technologies, Wildbach, Germany).

4.5. Primary hypothalamic neuronal culture

Hypothalami of E16 embryos were dissected and isolated using the papain dissociation system (Worthington) according to the manufacturer's protocol. $3.25x10^5/cm^2$ viable cells in plating medium (neural basal medium supplemented with 2 mM Glutamax, 2 % B-27, 10 % FBS and 1 % pen/strep) were seeded to vessels double-coated with poly-D-lysine and laminin. On the next day, the plating medium was replaced with feeding medium (same as plating medium, but without FBS) and transduced with Bmal1-luc lentivirus. 24 hr later, half the volume of the old medium was refreshed with fresh feeding medium containing 5µM cytosine arabinoside. Half the volume of the old medium was subsequently refreshed every 3 days. On day *in vitro* (DIV) 9, neurons were subjected to bioluminescence experiments in feeding medium supplemented with D-luciferin.

4.6. ARC/ME slice cultures

Luminescence was measured from cultured ARC/ME slices of heterozygous Per2-luc mice as described previously (21). Briefly, brains were isolated and harvested in ice-cold Hank's balanced salt solution (HBSS). 300 µm thick ARC/ME coronal slices (-1.80 mm and -2.10 mm relative to bregma) were prepared using a vibratome (Thermo Scientific, MA, USA). The slices were immediately placed onto a culture plate insert (Merck Millipore, Darmstadt, Germany) in 35-mm petri dishes filled with 1 ml recording medium (same as the one used for cell culture). Luminescence was measured in a LumiCycle (Actimetrics Inc, IL, USA) at 32.5 °C.

4.7. Quantitative real-time polymerase chain reaction

Isolated tissues were harvested and kept in RNAlater solution (Ambion, Life Technologies GmbH) according to the manufacturer's protocol. Total RNA of tissues and cell cultures was extracted using TRIzol reagent (Invitrogen, Life Technologies GmbH). cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) with random hexamer primers. qPCR was performed using GoTaq qPCR Master Mix (Promega GmbH, Mannheim, Germany) on a CFX96 thermocycler (Bio-Rad, Munich, Germany). Relative gene expression was quantified using a ΔΔ threshold cycle (Ct) method with adjustments of the amplification efficiencies of individual primer pairs; *Ef1α* was used as the reference gene (except for the epididymal fat pads for which *β-actin* was used as reference). Primer sequences are listed in Table 3.

4.8. Western blot

To detect BMAL1 protein in N44 cells, cells were lysed in a lysis buffer (1 % triton X-100, 2 % sodium dodecyl sulfate (SDS), 1 % sodium deoxycholate, 1 % NP40 and 1x cOmplete protease inhibitor cocktail (Roche, Grenzach-Wyhlen, Germany) in Tris-buffer saline (TBS) and subjected to sonication with the Branson 450 sonifier (Thermo Scientific; amplitude: 50, duty: 30%, duration: 30 sec) and subsequent Western blot analysis according to standard protocols of SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot (87) using anti-BMAL1 antibody (dilution 1:1000; Novus Biologicals, Cambridge, UK). To detect the

phosphorylation of the AMPK alpha subunit, N44 cells were harvested in a lysis buffer with phosphatase inhibitors (1 % triton X-100, 2 % SDS, 1 % sodium deoxycholate, 1 % NP40 and 1x cOmplete protease inhibitor cocktail, 1 mM dithiothreitol (DTT), 50 mM sodium fluoride (NaF), 1 mM sodium orthovandate ($NaVO_5$), 5 mM sodium pyrophosphate ($NaPP_i$) in TBS) and subjected to sonication and subsequent Western blot analysis with anti-phospho-AMPKα (Thr172) (dilution 1:1000) and anti-AMPKα (dilution 1:1000) antibodies (Cell Signaling Technology, MA, USA). To detect PGC1α in the MBH, tissues were dissected and frozen immediately on dry ice and stored at -80 ^{o}C until use. To extract protein, the MBH was homologized in RIPA buffer (150 mM sodium chloride ($NaCl_2$), 10 mM Tris, 0.1 % SDS, 0.1 % triton X-100, 25 mM sodium deoxycholate, 5 mM ethylenediaminetetraacetic acid (EDTA) and sodium deoxycholate) and boiled for 5 minutes. The lysates were then subjected to Western blot analysis using anti-PGC1α antibody (H300) (dilution 1:500; Santa Cruz Biotechnology, Heidelberg, Germany). In all cases, the protein concentration was determined with BCA protein assay kit (Thermo Scientific); Endogenous β-tubulin level determined with anti-β-tubulin (dilution 1:1000; Cell Signaling Technology) was used as loading reference. Densitometry analyses of bands intensity were performed with Quantity One software (Bio-Rad).

4.9. Chromatin immunoprecipitation

2 hr after adiponectin treatment, N44 cells were fixed with 1 % formaldehyde at 37 ^{o}C for 10 minutes. Cells were then lysed with SDS lysis buffer (1 % SDS, 19 mM EDTA, 50 mM EDTA, 50 mM Tris, pH8.1 with 1x cOmplete protease inhibitor cocktail) and subjected to sonication using Branson 450 sonicator (amplitude: 50, duty: 30%, duration: 30 sec, 6 repeats with 1 min intervals between each sonication) which yielded a majority of DNA fragments with sizes of 200-1000 bp. Precleared samples were incubated overnight at 4 °C with 10 µg anti-PGC1α antibody (H300). The samples were incubated with Protein-G agarose beads (Thermo Scientific) for 1 h at 4 °C followed by intensive washings. Afterward, immune complexes were eluted with elution buffer (1 % SDS, 0.1 M sodium hydrogen carbonate ($NaHCO_3$). The eluates were then treated with 40 µg/ml proteinase K and further subjected to phenol-chloroform-isoamyl alcohol purification. Purified

samples were then subjected to qPCR analysis with primer pairs flanking the RORE and the 3'-UTR of *Bmal1* and values were normalized to the pre-IP inputs. Primer sequences are listed in Table 3.

4.10. *NPY* promoter end-point luciferase assays

HEK293T cells were plated into 96-well plates coated with poly-D-lysine and transfected with the following expression plasmids: 10 ng *NPY-luc* and 2 ng *CMV-Renilla luciferase* in various combinations of the following clock gene constructs: HA-Clock, HA-Bmal1, CRY1 and pcDNA3.1 (mock transfection) using Lipofectamine LTX transfection reagent (Life Technologies). 48 hr after transfection, luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega) and the Berthold TriStar plate reader.

4.11. Intracerebroventricular administration

Intracerebroventricular (i.c.v.) cannulae were implanted to mice at 8-10 weeks of age under isoflurane anesthesia (CP-Pharma, Burgdorf, Germany) and carprofen analgesia (5 mg/kg; Rimadyl, Pfizer Deutschland GmbH, Berlin, Germany). A stainless steel guide cannula (internal cannula 2.8 mm with 1 mm projection 33 gauge, C315I-5/Spc; Plastics One, VA, USA) was stereotaxically implanted into the lateral cerebral ventricle (0.9 lateral and 0.1 posterior to bregma, 2.2 ventral to the surface of the skull). The cannula was fixed with dental cement on two small steel screws (screw 00-96x1/96). After surgery animals were single-caged and allowed to recover for 1-2 weeks with handling everyday. Substances for i.c.v. administration were dissolved in artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 25 mM $NaHCO_3$, 1.25 mM sodium phosphate monobasic monohydrate (NaH_2PO_4), 20 mM D-glucose, 5 mM potassium chloride (KCl). 2 µl in total volume were injected into manually restrained awake mice.

4.12. ELISA analysis of plasma adiponectin

Blood was collected from decapitated animals into EDTA-containing tubes. Plasma concentrations of adiponectin were determined with an ELISA detection kit for mouse adiponectin (Adipogen Inc, CA, USA) according to the manufacturer's protocol using the Epoch microplate spectrophotometer (Biotek Instruments, VT, USA).

4.13. Statistical analysis

To access the circadian parameters of luminescence recording rhythms, all traces were subjected to the subtraction of their own 24-hr moving average as baselines. To construct phase response curves, the baseline-subtracted data were further fitted with a standard sine wave regression. The intersection of the ascending cross-section of the sine wave with the x-axis was defined as 0 or 2π in radian and the peak as 0.5 π. To compare the phase before and after drug treatments, phase shifts were determined by comparing extrapolated peak times from sine wave fits of at least two consecutive circadian cycles before and after the treatment. All measurements of circadian parameters were performed using the Lumicycle analysis software (Actimetrics). Diurnal variation of 24-hr profile data was tested using sine-cosine fitting with CircWave software version 3.3 (University of Groningen, NL). General statistical analysis was performed using GraphPad Prism 5.0 (GraphPad, CA, USA). All data are represented as means ± standard error mean (SEM). In general, Student's t test was used for pairwise comparison in cell-based experiments and Mann Withney test for pairwise comparison in animal experiments. Multi-groups analyses were performed with One-Way ANOVA with either Tukey or Bonferroni post-test. 24-hr profile experiments were analyses with Two-Way ANOVA with Bonferroni post-test for comparison at specific time point.

Table 3. Sequence information of the oligos used in current study.

Target	shRNA Sequences Sense		Reference/Source
scramble	CCTAAGGTTAAGTCGCCCTCG		Addgene plasmid 8455
AdipoR1 KD1	GTACGTCCAGGCTTCAAATAA		TRCN0000249147
AdipoR1 KD2	AGATGGAGGAGTTCGTGTATA		TRCN0000257836
AdipoR2 KD1	GCAGGAATTTCGTTTCATGAT		TRCN0000175771
AdipoR2 KD2	CTTCTGATGTGATCGTATTTA		TRCN0000277168
Bmal1 KD1	GACGAACTGAAACACCTAATT		TRCN0000282305
PGC1α KD	TAACTATGCAGACCTAGATAC		TRCN0000219080, (ref)
Target	**qPCR Forward**	**Reverse**	**Reference/Source**
Eef1α	CACATCCCAGGCTGACTGT	TCGGTGGAATCCATTTTGTT	
Bmal1	TGACCCTCATGGAAGGTTAGAA	CAGCCATCCTTAGCACGGT	
Per2	GCCAAGTTTGTGGAGTTCCTG	CTTGCACCTTGACCAGGTAGG	
Dbp	AATGACCTTTGAACCTGATCCCGCT	GCTCCAGTACTTCTCATCCTTCTGT	
Nr1d1	AGCTCAACTCCCTGGCACTTAC	CTTCTCGGAATGCATGTTGTTC	
NPY	CTCCGCTCTGCGACACTAC	GGAAGGGTCTTCAAGCCTTGT	
AGRP	ATGCTGACTGCAATGTTGCTG	CAGACTTAGACCTGGGAACTCT	
CART	CCCGAGCCCTGGACATCT	GCTTCGATCTGCAACATAGCG	
POMC	ATGCCGAGATTCTGCTACAGT	TCCAGCGAGAGGTCGAGTTT	
HCRT	GTCGCCAGAAGACGTGTTC	GGTGGTAGTTACGGTCGGAC	
AdipoR1	AATGGGGCTCCTTCTGGTAAC	GCAGACCTTATACACGAACTCC	
AdipoR2	CCTTTCGGGCCTGTTTTAAGA	GAGTGGCAGTACACCGTGTG	
Adipoq	GCAGGCATCCCAGGACATC	GCGATACACATAAGCGGCTTCT	
PGC1α	AACCAGTACAACAATGAGCCTG	AATGAGGGCAATCCGTCTTCA	
Cdh13	ATGGCAGAACTCGTGATTGTC	CGCTATCGACTACCTTGCCG	
β-actin	GATGACCCAGATCATGTTTGAG	GCTGTGGTGGTGAAGCTGTA	
Target	**CHIP-qPCR Forward**	**Reverse**	
Bmal1 RORE	TTGGGCACAGCGATTGGTGG	TCCGGCGCGGGTAAACAGG	
Bmal1 3'UTR	CTGTGTGCATCGGACAGTC	CGAAGCCACCATCTGAAAC	
Target	**Cloning**		**Reference/Source**
NPY promoter	GTCCAGGAGGTGATGAACCTATGTTCTTTATGG	GCGCCCCTGTCCCAGTTGATCCTGGC	
EGFP-WPRE	ATGGTGAGCAAGGGCG	CAGGCGGGGAGGCGGC	Addgene plasmid 17449

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Curriculum Vitae

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Higher Education

1. **Doctoral candidate**, Genes and Development Program, Goettingen Graduate School for Neurosciences and Molecular Biosciences, Georg-August University Goettingen, Germany and Max Planck Institute for Biophysical Chemistry, 2010 - Present

2. **Master of Science (MSc)/ Doctor of Philosophy (PhD)** student in Neuroscience Program (Successful fulfilment of coursework and examination), International Max Planck Research School, Georg-August University Goettingen, Germany, 2009 - 2010

3. **Master of Philosophy (MPhil) in Biochemistry**, The Hong Kong University of Science and Technology (HKUST), Hong Kong, 2006 - 2009

4. **Bachelor of Science (BSc) in Biology** with Honorous, The Chinese University of Hong Kong (CUHK), Hong Kong, 2003 - 2006

Publications (in reversed chronological order)

Research articles:

1. **Tsang A.H.**, Koch C.E., Leliavski A. and Oster H. (2014) Adiponectin regulates mediobasal hypothalamic circadian clock, feeding rhythms and adaptation to restricted feeding. (Manuscript under preparation)

2. Pagel R., Bär F., Schröder T., Langenstrassen P., Michaels M.A., Kalies K., König P., Grassl G.A., Meyer M., **Tsang A.H.**, Büning J., Lehnert H., Fellermann K., Oster H. and Sina C. (2014) Circadian disruption impairs mucosal barrier function and promotes intestinal. (Manuscript under preparation)

3. Landgraf D.*, **Tsang A.H.**, Leliavski A., Koch C.E., Barclay J.L., Drucker D.J. and Oster H. (2014) Oxyntomodulin regulates resetting of the liver circadian clock by food. (Manuscript under preparation)

4. Husse J., Leliavski A., **Tsang A.H.**, Oster H., Eichele G. (2014) The light-dark cycle controls peripheral rhythmicity in mice with a genetically ablated suprachiasmatic nucleus clock. *FASEB J* fj.14-256594. (Cover article of the issue)

5. Barclay J.L., Shostak. A., Leliavski A., **Tsang A.H.**, Jöhren O., Müller-Fielitz H., Landgraf D., Naujokat N., van der Horst G.T. and Oster H. (2013) High fat diet-induced hyperinsulinemia and tissue-specific insulin resistance in Cry deficient mice. *Am J Physiol Endocrinol Metab 304(10):E1053-63* (Highlighted with an editorial commentary by Oren Froy.)

6. **Tsang A.H.***, Sánchez-Moreno C.*, Bode B., Rossner M.J., Garaulet M., and Oster H. (2012) Tissue-specific interaction of Per1/2 and Dec2 in the regulation of cellular circadian rhythms. *J Biol Rhythms* 27(6):478-89 * Equal contributions

7. Zhou R., **Tsang A.H.**, Lau E.S. and Ge W. (2012) Putitary adenylate cyclase-activating polypeptide (PACAP) and its receptors in the zebrafish ovary: evidence for potential dual roles of PACAP in controlling final oocyte maturation. *Biol Reprod* 85(3):615-25 (Cover article of the issue)

8. Zhang P., Yu P.C., **Tsang A.H.**, Chen Y., Fu A.K., Fu W.Y., Chung K.K., and Ip N.Y. (2010). S-nitrosylation of cyclin-dependent kinase 5 (CDK5) regulates its kinase activity and dendrite growth during neuronal development. *J Neurosci* 30(43):14366

9. **Tsang A.H.**, Lee Y.I., Ko H.S., Savitt J. M., Pletnikova O. Troncoso J.C., Dawson V.L., Dawson T.M. and Chung K.K. (2009). S-nitrosylation of XIAP compromises neuronal survival in Parkinson's disease. *Proc Natl Acad Sci U S A* 106: 49005 (Recommended in F1000Prime by Richard Silverman; F1000Prime.com/1162958)

Review articles and book chapters:

1. **Tsang A.H.**, Kolbe I., Seemann J., and Oster H. (2014) Interaction of circadian and stress systems in the regulation of adipose physiology. *Horm Mol Biol Clin Investig* 19(2):103-115

2. **Tsang A.H.**, Barclay J.L., and Oster H. (2013) Interactions between endocrine and circadian systems. *J Mol Endo* 52(1):R1-R16.

3. **Tsang A.H.**, Husse J., and Oster H. (2013) The role of sleep in the regulation of energy homeostasis and the development of the metabolic syndrome. In: *Chronobiology and Obesity (Garaulet M. and Ordovás J.M., ed), Springer* pp.89-109

4. Barclay J.L., **Tsang A.H.**, and Oster H. (2012) Interaction of central and peripheral clocks in physiological regulation. *Prog Brain Res.* 199:163-81

5. **Tsang A.H.** and Chung K.K. (2009). Oxidative stress and nitrosative stress in Parkinson's disease. *Biochim Biophys Acta - Molecular Basis of Disease* 1792(7):64350

Academic Awards

1. Attendance to the 63rd Lindau 63rd Nobel Laureate Meeting 2013 - sponsored by Bayer-Lindau Fellowship, Bayer Science and Education Foundation, 2013

2. GGNB Excellence Stipend, Goettingen Graduate School for Neurosciences and Molecular Biosciences, Georg-August University Goettingen, 2011 - Present

3. Stipend of the Excellence Foundation for the Promotion of the Max Planck Society, International Max Planck Research School, 2009 - 2010

4. University Grant Council (UGC) Research Travel Grant, The Hong Kong University of Science and Technology, 2008

5. Research Postgraduate Studentship, The Hong Kong University of Science and Technology, 2006 - 2009

6. Certificate of Academic Merit, Shaw College, The Chinese University of Hong Kong, 2005 - 2006

7. Dean's Honours List (annual), The Chinese University of Hong Kong, 2005 - 2006

8. Dean's Honours List (annual), The Chinese University of Hong Kong, 2004 - 2005

9. The Department/ Program Scholarship, Shaw College, The Chinese University of Hong Kong 2004 - 2005

Research Experience

1. **Doctoral thesis research student** under supervision of Prof. Henrik Oster, Circadian Rhythm Group, Max Planck Institute for Biophysical Chemistry (and later Chronophysiology Research Group, Universität zu Lübeck) 12/2010 — present (Thesis title: Metabolic resetting of circadian clocks in central nervous system)

2. **Visiting Scholar** with Prof. Kenny Chung, Department of Biochemistry, the Hong Kong University of Science and Technology 02/2009 — 07/2009

3. **Master thesis research student** under supervision of Prof. Kenny Chung, Department of Biochemistry, the Hong Kong University of Science and Technology 06/2006 — 01/2009 (Thesis title: S-nitrosylation of XIAP: Implication for the pathogenesis of Parkinson's disease)

4. **Bachelor thesis research student** under supervision of Prof. Wei Ge, Department of Biology, the Chinese University of Hong Kong 06/2005 — 06/2006 (Thesis title: Dual role of PACAP in zebrafish oocyte maturation)

5. **Laboratory Helper** in Prof. Siu Wai CHIU's laboratory, Department of Biology, the Chinese University of Hong Kong 04/2004 — 10/2005

Scientific Activities

1. Participation and poster presentation, The 63rd Lindau Nobel Laureate Meeting 2013 (Chemistry), Lindau, Germany. 30 Jun – 21 Jul 2013

2. Participation and poster presentation, the Chronobiology Summer School 2012, Berlin, Germany. 16 – 21 Sep 2012

3. Poster presentation, the Society of Neuroscience (SFN) annual meeting 2008, Washington DC, USA. 15 – 19 Nov 2008

4. Poster presentation, the Gordon Research Conference "Molecular and Cellular Neurobiology" 2008, HKUST, Hong Kong SAR, China, 08 – 13 Jun 2008

Professional skills with hand-on experience

Cell line and primary cell culture;

Electrophysiology (patch clamp technique);

Ex vivo brain-slice culture;

Fluorescence assisted cell sorting (FACS) analysis;

Fluorescent/Luminescence microscopy;

Lenti-/ Adeno-associated viral vector mediated gene transfer;

Mouse behavioral experiment;

Mouse handling and genetics;

Mouse stereotaxic surgery;

Molecular cloning;

Recombinant protein purification and Fast protein liquid chromatography (FPLC);

General techniques in biochemistry and molecular biology

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Selected publications related to the current study

Review | A H TSANG and others | *Endocrine circadian rhythms* | 52:1 | R1–R16

Interactions between endocrine and circadian systems

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Abstract

In most species, endogenous circadian clocks regulate 24-h rhythms of behavior and physiology. Clock disruption has been associated with decreased cognitive performance and increased propensity to develop obesity, diabetes, and cancer. Many hormonal factors show robust diurnal secretion rhythms, some of which are involved in mediating clock output from the brain to peripheral tissues. In this review, we describe the mechanisms of clock–hormone interaction in mammals, the contribution of different tissue oscillators to hormonal regulation, and how changes in circadian timing impinge on endocrine signalling and downstream processes. We further summarize recent findings suggesting that hormonal signals may feed back on circadian regulation and how this crosstalk interferes with physiological and metabolic homeostasis.

Key Words
- circadian clocks
- cortisol
- endocrine rhythm
- melatonin
- adipokines

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Introduction

We live in an environment shaped by various geophysical rhythms. Arguably, one of the most prominent of these rhythms is the succession of day and night. The profound environmental changes brought about by the rotation of the Earth around its axis have promoted the development of endogenous timekeepers that enable an organism to reliably predict the time of day and adjust behavior and physiology accordingly. Not surprisingly, large aspects of our endocrine system are tightly connected to the circadian (from Latin *circa diem* – about a day) clock. With recent advances in molecular life sciences and medicine, we now realize that this interaction is not only unilateral but also includes endocrine feedback on circadian clock function. This review recapitulates some of the research leading to the picture we have today of the circadian clock system in mammals and provides an overview about the most prominent connection points between circadian and endocrine regulation.

The master circadian pacemaker

In the 1970s, we witnessed a significant breakthrough in the field of chronobiology – the identification of the anatomical entity underlining the mammalian circadian rhythm. It was discovered that information about the external light–dark cycle was passed via the retino-hypothalamic tract (RHT) to not only sensory input integrating centers in the thalamus, but also to the hypothalamic suprachiasmatic nucleus (SCN), hinting at the existence of a novel photic input processing hub in the brain (Sousa-Pinto & Castro-Correia 1970, Hendrickson *et al*. 1972, Moore & Lenn 1972). The SCN is a bilaterally paired structure with high cell body density located adjacent to the third ventricle and directly atop the optic chiasm. It comprises about 50 000 neurons in humans and about 20 000 neurons in rodents. A series of electrical lesion studies provided unequivocal evidence for the

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critical role of SCN in the generation of mammalian circadian rhythms. Animals with ablated SCN become behaviorally and physiologically arrhythmic (Moore & Eichler 1972, Stephan & Zucker 1972). Critically, transplanting isolated SCN tissue into SCN-lesioned animals restores circadian rhythmicity (Ralph *et al.* 1990), and the restored behavioral rhythm of recipients is determined by the donor's intrinsic period, indicating that the SCN is indeed the master pacemaker generating circadian timing information in animals (Ralph *et al.* 1990). Brain slice explants of the SCN, but not of other tested brain areas including the cerebral cortex and arcuate nucleus, display robust circadian oscillations in firing rate *in vitro*, suggesting that the rhythmicity of the SCN is autonomous and self-sustaining (Green & Gillette 1982, Groos & Hendriks 1982, Shibata *et al.* 1982).

The molecular clockwork

The *Period* (or *Per*) gene was the first discovered clock gene (Konopka & Benzer 1971), which is conserved from fruit flies to humans. Mutations of *Per* in flies alters the circadian patterns of pupae eclosion and locomotor activity (Konopka & Benzer 1971). Since then, many more clock genes have been identified in different organisms (Zhang & Kay 2010). In the past decades, our knowledge of the molecular clockwork has been significantly expanded. The current model suggests that the central mechanism of the mammalian molecular clock is composed of a set of clock genes intertwined with a delayed interlocking transcriptional–translational feedback loop (TTL), coupled to several auxiliary mechanisms reinforcing robustness and stability (Zhang & Kay 2010). The positive limb of this TTL comprises two basic helix–loop–helix transcription factors, circadian locomotor output cycles kaput (CLOCK), and brain and muscle aryl hydrocarbon receptor nuclear translocator such as BMAL1 or ARNTL. Both form heterodimers via their PER-ARNT-SIM (PAS) domains and activate E-box-element-containing genes by recruiting transcriptional co-activators, chromatin-modifying proteins, and RNA polymerase II. In certain tissues such as the forebrain or the vasculature, CLOCK is functionally replaced by its homolog neuronal PAS domain protein 2 (NPAS2; McNamara *et al.* 2001, Reick *et al.* 2001). *Period* (*Per1–3*) and Cryptochome (*Cry1/2*) constitute the negative limb of the TTL. CLOCK–BMAL1 complexes activate the transcription of *Per* and *Cry* genes during the subjective day. PERs and CRYs translocate into the nucleus and form inhibitory complexes. With progress of the circadian cycle, PER/CRY complexes accumulate and so does their inhibitory effect on CLOCK–BMAL1 activity, shutting down *Per* and *Cry* transcription during the night (Lee *et al.* 2001). The progressive degradation of PER/CRY complexes throughout the night toward the morning releases the inhibition on CLOCK–BMAL1 transcriptional activity and thereby, completes the negative feedback loop of the circadian clock (Fig. 1).

Additional auxiliary TTLs enhance the stability of the core clock TTL and translate time-of-day information into physiological signals via transcriptional control of clock target genes (Zhang & Kay 2010). Such loops include the nuclear receptors REV–ERBα and REV–ERBβ (NR1D1 and NR1D2) and RORα (NR1F1) which regulate *Bmal1* expression via a retinoid orphan receptor responsive elements (Preitner *et al.* 2002, Ueda *et al.* 2002, Sato *et al.* 2004, Akashi & Takumi 2005, Liu *et al.* 2008), as well as the PAR basic leucine zipper proteins D-box albumin-binding protein and E4 promoter-binding protein (E4BP; NFIL3) (Cowell 2002, Ripperger & Schibler 2006) which feed-back on the expression of *Per* genes via *D-box* promoter elements (Ripperger *et al.* 2000).

Extra-SCN oscillators

The functional molecular clockwork exists not only in SCN neurons, but (almost) every single cell in the brain and periphery is capable of oscillating in a circadian manner. Molecular clock rhythms have been shown even in cultured cells, such as immortalized fibroblast cells which display robust oscillations of clock gene expression

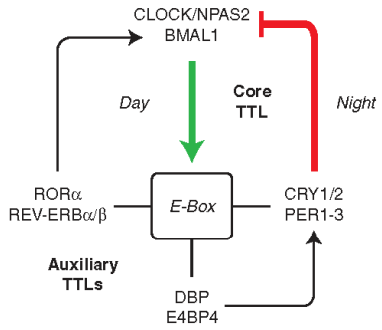

Figure 1
The molecular mammalian circadian clockwork. The transcription factors Clock/Npas2 and Bmal1 activate E-box-controlled genes including *PER1–3* and *CRY1/2* during the day. PER and CRY proteins inhibit CLOCK/BMAL1 activity during the night. Auxiliary loops stabilize this 24-h rhythm of transcriptional activation by modulating gene expression of Bmal1 and Per. For details see text.

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after a brief stimulation with high concentrations of serum (Balsalobre *et al.* 1998). Using single cell imaging techniques, Nagoshi *et al.* (2004) showed that each fibroblast cell possesses a sustained circadian clock, although at the population level the rhythm dampens quickly as a consequence of a gradual desynchronization between individual cells with different endogenous periods. Application of synchronizing agents such as serum, forskolin, glucocorticoids (GCs), or phorbol esters re-synchronizes individual cells, yielding a transiently phase-coherent population (Nagoshi *et al.* 2004). These data suggest that the cellular clocks in extra-SCN tissues are actually self-sustained and autonomous in nature, but fail to maintain coherence at the population level, in contrast to the SCN (see below). Similarly, tissue explants from a wide array of peripheral organs including heart, lung, kidney, liver, spleen, pancreas, stomach, cornea, thyroid gland, and adrenal gland show clock gene expression rhythms, but the overall rhythm dampens quickly due to the gradual loss of coherence between individual cells (Yamazaki *et al.* 2000, Yoo *et al.* 2004). Similar results have been obtained from tissue explants from various brain regions (Abe *et al.* 2002, Guilding & Piggins 2007).

SCN communication

In order to achieve a biologically relevant circadian rhythm, it is of utmost importance that individual cells of a specific tissue are synchronized to the external environment. The circadian oscillation of an SCN neuron is coupled to its neighbouring cells in an action-potential-dependent manner (Welsh *et al.* 1995). This intercellular coupling property bestows the superior robustness and resilience of the SCN circadian rhythm. For example, the SCN explant cultures exhibit robust and persistent circadian oscillations in electrophysiological activity and clock gene expression for an extended period of time, while rhythms in slices from most other brain regions and peripheral tissues dampen after a couple of days (Guilding & Piggins 2007, Guilding *et al.* 2009). SCN explant rhythms are also more resistant to clock gene mutations (Liu *et al.* 2007) and temperature fluctuations (Abraham *et al.* 2010, Buhr *et al.* 2010). One major function of the SCN is to synchronize internal biological processes to external time cues. The SCN receives photic information from both classical photoreceptors – cone and rod cells – as well as melanopsin-containing retinal ganglion cells via the RHT (Hankins *et al.* 2008). In turn, the SCN innovates other regions of the brain, in particular the hypothalamus.

The paraventricular hypothalamic nucleus (PVN) is one of the major loci relaying circadian information from the SCN to the rest of the body (Saeb-Parsy *et al.* 2000). The PVN is an important integrating center for energy homeostasis, projecting parvocellular neurons to the median eminence to control the release of hormones such as adrenocorticotrophin (ACTH) and thyroid-stimulating hormone in the anterior pituitary. The PVN also innervates the sympathetic limb of the autonomous nervous system, thereby allowing the SCN to regulate the sympathetic tone of the body over the course of the day (Buijs *et al.* 2003). Further projections of the SCN to the dorsomedial hypothalamic nucleus (Luiten *et al.* 1987), the nucleus accumbens (Phillipson & Griffiths 1985) and the paraventricular thalamic nucleus (Watts & Swanson 1987, Watts *et al.* 1987) have been described. These connections enable the SCN to exert influence on a plethora of physiological processes such as the reward system, feeding-fasting cycles, cognitive function, locomotor activity, and body temperature (Dibner *et al.* 2010). In addition to direct neural connections, the SCN secretes diffusible factors, which can function as timing cues. Membrane-encapsulated foetal SCN tissue grafts, which allow only low-molecular-weight particles to diffuse, can restore the rhythmicity of locomotor activity in SCN-lesioned hamsters in the absence of axonal outgrowth (Silver *et al.* 1996). Transforming growth factor-α (Kramer *et al.* 2001, Li *et al.* 2002), prokineticin 2 (PK2; Cheng *et al.* 2002), and cardiotropin-like cytokine (Kraves & Weitz 2006) have been implicated as SCN-secreted peptides capable of regulating behavioral rhythmicity. Given the physical proximity of the SCN to the third ventricle, these diffusible factors may help propagate the time-of-day information to more remote brain regions via the cerebrospinal ventricular system.

Endocrine rhythms: clock vs behavioral regulation

It has been long been appreciated that the circulating levels of a number of hormones vary over the 24-h cycle (Andrews & Folk 1964). Such a diurnal rhythm of a hormone or metabolite can either be a manifestation of circadian clock control or a direct or indirect response to an environmental rhythm such as the light–dark cycle. Two methodologies have been developed to track down the relative contribution of the endogenous circadian clock to diurnal hormonal rhythms in humans, namely constant routine (CR) and forced desynchrony (FD) protocols. The CR protocol aims to minimize the effects of external time cues

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and behavioral variables by equally distributing such variables across the circadian cycle. Depending on individual experimental goals, it routinely demands constant wakefulness, limited physical activity, equally distributed isocaloric snacks or constant glucose infusion and constant dim light condition (Mills *et al.* 1978). The FD protocol employs a strategy which schedules a behavioral cycle beyond the entrainable range of the circadian clock (i.e. significantly longer or shorter than 24 h) in a constant dim light environment, resulting in the free running of the endogenous circadian clock. This leads to an even distribution of certain behavioral variables in question across different phase of the circadian cycle (Kleitman 1970).

The clarification of the relative contribution of endogenous and exogenous input to the diurnal rhythm of a physiological system is of particular relevance for understanding the influence of our modern 24/7 lifestyle on the well-being of individuals. Owing to social constraints many rhythmic behaviors such as sleep/wake and food intake/fasting cycles often no longer align with their endogenous pattern controlled by the circadian clock (Scheer *et al.* 2009, Beccuti & Pannain 2011). Shift workers are an obvious example. Several epidemiological studies indicate that shift workers are predisposed for metabolic disorders and even cancer (Ohayon *et al.* 2002, Akerstedt 2003). Thus, better knowledge for the mechanistic link between circadian misalignment and hormonal deregulation may help with the development of novel medical regimes to prevent or intervene in the metabolic consequences of shift work.

GCs and melatonin represent two well-studied hormonal systems that are subject to direct and dominant regulation by the circadian clock. The circulating levels of both display robust diurnal patterns (Migeon *et al.* 1956, Ralph *et al.* 1971). Using the CR and FD experimental protocols, the secretion rhythms of cortisol (el-Hajj Fuleihan *et al.* 1997, Wehr *et al.* 2001, Aeschbach *et al.* 2003, Scheer *et al.* 2010) and melatonin (Dijk *et al.* 1999, Wehr *et al.* 2001, Cain *et al.* 2010, Gooley *et al.* 2011) have been shown to be under direct regulation by the circadian clock. Not surprisingly, both hormones also act as major hormonal output pathways that propagate the time signal from the SCN to various other tissues. In the following paragraphs, we will discuss the interaction between the central clock and these two endocrine systems.

SCN–adrenal interaction

The adrenal gland is an endocrine organ composed of two anatomically distinct structures – the cortex and medulla. The cortical part produces multiple corticosteroid hormones, while the medulla produces epinephrine and norepinephrine. The adrenal cortex is further organized into three functionally distinct subregions: the outermost zona glomerulosa producing mineralocorticoids, the middle zona fasciculata producing GCs (mainly cortisol in humans, corticosterone in rodents) and the innermost androgen-producing zona reticulata. A diurnal rhythm of the excretion of urinary ketosteroids was reported in the mid 20th century (Pincus *et al.* 1954). In the 1970s, along with the identification of the SCN as the master circadian pacemaker, the circadian secretion of corticosteroids was established as a robust hormonal output of the SCN clock (Moore & Eichler 1972, Liu *et al.* 2008). Only during the last decade, however, has the anatomical and molecular basis underlying the circadian production of corticosteroids been unveiled. Cholesterol is the precursor for the biosynthesis of steroid hormones. LDL – bound cholesterol – is imported into adrenocortical cells via LDL receptors. Cholesterol is then transported into mitochondria via steroidogenic acute regulatory protein (STAR). This import constitutes the rate-limiting step of steroidogenesis (Miller & Bose 2011). Inside the mitochondria, the side chain of cholesterol is first removed by cytochrome P450scc to become pregnenolone, which is then subjected to a series of enzyme-regulated reactions to become GC (Miller & Bose 2011). GC secretion is highly stress responsive. Together with epinephrine, GCs boost energy production and prepare the body for foraging and fight-or-flight situations. GCs exert effects on a wide array of physiological systems. In times of high energy demand, GCs help maintain blood glucose levels by promoting gluconeogenesis in liver and lipolysis in adipose tissues (Kwon & Hermayer 2013). GCs also play an important role in modulating immune (Silverman & Sternberg 2012) and cognitive functions (Sandi 2011). The majority of the effects of GCs are mediated by its ubiquitously expressed cognate nuclear receptors, glucocorticoid receptors (GRs) (Silverman & Sternberg 2012). Interestingly, despite the widespread expression pattern of GR within the brain, the SCN is devoid of GR (Okamura 2007).

The secretion of GC is the end product of hypothalamic–pituitary–adrenal (HPA) axis activation. Pituitary-released ACTH activates adrenocortical steroidogenesis through the melanocortin 2 receptor (MC2R), via a cAMP–PKA-dependent pathway which transcriptionally stimulates steroidogenic genes such as *STAR* and *CYP11A1* (Miller & Bose 2011).

Blood levels of GCs display a robust circadian rhythm, overlaid by less regular ultradian pulses with a period of

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90–120 min. The circadian rise of GCs is phase-locked to the time of awakening, peaking at few hours before the onset of the active phase, i.e. the early morning for diurnal animals such as humans and the evening for nocturnal animals such as mice (Moore & Eichler 1972, Gomez-Abellan *et al.* 2012). This GC rise promotes arousal and boosts performance during the early active phase. Importantly, GC rhythms persist under constant environmental conditions, suggesting that they are driven by the endogenous circadian clock. Surgical ablation of the SCN completely abolishes the circadian rhythm of GC in blood, indicating that the SCN is the origin of GC rhythmicity (Moore & Eichler 1972, Stephan & Zucker 1972). HPA axis activity upstream of the adrenal is also rhythmic (Watts *et al.* 2004, Henley *et al.* 2009), which led to the hypothesis that circadian regulation of GC release may be an indirect response to SCN-induced corticotrophin-releasing hormone (CRH) expression. However, this view has been challenged by several observations. First, the timing of *CRH* expression in the hypothalamus of pro-opiomelanocortin (*POMC*; precursor peptide of ACTH) in the anterior pituitary and the plasma GC surge are not organized in the expected sequential manner (Watts *et al.* 2004, Girotti *et al.* 2009). Also, implantation of ACTH pellets can restore the rhythmicity of GC in hypophysectomized rats, while denervation of the adrenal gland abolishes the daily GC rhythm, suggesting that ACTH rhythmicity *per se* is dispensable for the blood GC rhythm (Ottenweller *et al.* 1978, Ottenweller & Meier 1982). Conversely, stimulation of adrenal sympathetic nerves results in potentiated GC responses which can be abolished by hypophysectomy (Edwards & Jones 1993), suggesting a permissive function of pituitary-derived ACTH and a more direct role of sympathetic innervation in the regulation of the circadian GC rhythm. Indeed, it has been shown in viral tracing experiments that the adrenal is connected to the SCN via the spinal cord and the PVN (Buijs *et al.* 1999). In a more recent study, it has been shown that light signals are transmitted to the adrenal cortex via the SCN, inducing an up-regulation of *PER1* expression and secretion of GC independent of ACTH (Ishida *et al.* 2005).

Well before the discovery of clock genes or peripheral clocks, it was shown that adrenal glands when isolated and cultured *in vitro* display a robust circadian rhythm of metabolism and steroid secretion (Andrews & Folk 1964). In line with this, we now know that about 5% of the whole genome – including all canonical clock genes – show rhythmic expression in the mouse adrenal gland (Oster *et al.* 2006*a*). By transplanting adrenal glands from arrhythmic *PER2/CRY1* double mutant mice to WT adrenalectomized mice, and *vice versa*, we have provided evidence that a local adrenocortical clock imposes a circadian gating mechanism altering ACTH sensitivity during the course of the day (Oster *et al.* 2006*b*). This observation was further supported in a study that used a knock down of *BMAL1* in the adrenal cortex (Son *et al.* 2008). Taken together, this illustrates that while the SCN is indispensable for the circadian rhythm of GC secretion, the adrenal clock provides an additional level of control to modulate the proficiency of GC production across the circadian cycle and further clocks along the HPA axis may be involved (Fig. 2).

SCN–pineal interaction

Unlike mice and humans, many non-mammalian vertebrates can perceive photic information by extra-retinal photoreceptors (Menaker *et al.* 1997, Foster & Soni 1998), e.g. in the pineal. The pineal gland is an ancient organ that exists in most vertebrates (Menaker *et al.* 1997). In mammals, it is buried deep beneath the skull and lies within the furrow of the two hemispheres. In consequence, its photoreceptive function is lost. However, in most cases its physiology is still strongly influenced by light. A major function of the pineal is its secretion of the hormone melatonin derived from the amino acid tryptophan (Barrett & Bolborea 2012). In mammals, melatonin exerts its effects via binding to its two widely expressed cognate receptors – MT1 and MT2. The melatonin receptors belong to the $G\alpha_i/q_i$-protein-coupled receptor superfamily (Barrett & Bolborea 2012). Owing to the widespread expression of melatonin receptors, melatonin has been reported to modulate several physiological systems such as immune function (Srinivasan *et al.* 2011), metabolism (Nduhirabandi *et al.* 2012), and higher brain functions (Srinivasan *et al.* 2012). In birds and reptiles, the pineal–melatonin system is an essential part of the circadian clockwork (Gaston & Menaker 1968, Tosini & Menaker 1998). In contrast, no overt circadian disruption is observed in pinealectomized mammals (Quay 1970, 1972), but melatonin may play an important regulatory role in distributing the time signal of the SCN (see below).

The daily pattern of melatonin secretion profile has a robust profile – being low during the day; rising and peaking during the night. In contrast to the GC rhythm which is anti-phasic in nocturnal and diurnal animals, high melatonin is always confined to the dark phase. SCN lesions abolish melatonin rhythms (Klein & Moore 1979, Reppert *et al.* 1981). The SCN connects to the

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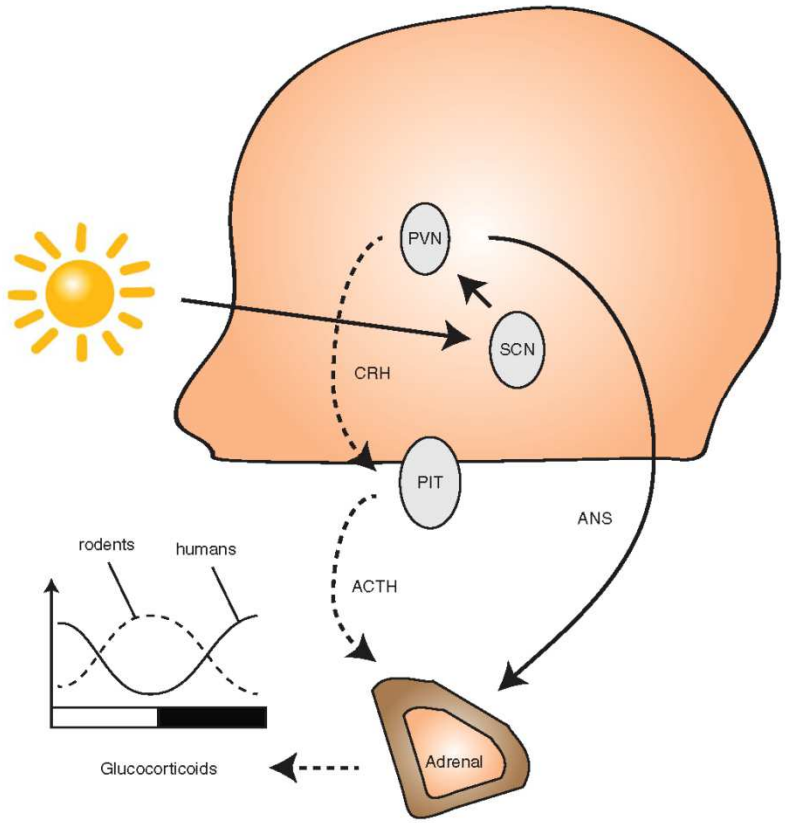

Figure 2

Interaction of central and peripheral clocks in the regulation of GC secretion. The SCN innervates the PVN from where rhythmic CRH release triggers secretion of ACTH from the pituitary (PIT). At the same time autonomic innervation (ANS) of the adrenal resets adrenocortical clocks regulating sensitivity of the steroidogenic machinery to ACTH. Synchrony between HPA axis activity and adrenal ACTH gating results in high amplitude and robust circadian GC rhythms. GC rhythms are phase-shifted between nocturnal and diurnal species indicating differential interpretation of SCN signals at downstream targets.

pineal gland via a multi-synaptic autonomic pathway which sequentially involves the PVN and then the pre-ganglionic neurons of the intermediolateral cell column of the spinal cord and finally the noradrenergic sympathetic neurons of the superior cervical ganglion (Drijfhout *et al.* 1996, Moore 1996, Larsen *et al.* 1998, Teclemariam-Mesbah *et al.* 1999; Fig. 3). The SCN releases GABA to inhibit the sympathetic input to the pineal gland during the daytime while this inhibition is released during the night (Kalsbeek *et al.* 2000). In addition, the SCN sends a constant glutamatergic stimulatory input to the pineal gland which is overwhelmed by the inhibitory mechanism during the night (Perreau-Lenz *et al.* 2004).

The role of clock genes in regulating pineal gland rhythmicity has received little attention, mainly due to the fact that many of the mouse genetic models used to study the function of the molecular clock are maintained on genetic backgrounds carrying mutations in two key enzymes of melatonin synthesis, arylalkylamine *N*-acetyltransferase (AANAT) and hydroxyindole-*O*-methyltransferase (HIOMT), resulting in melatonin deficiency (Goto *et al.* 1989, Roseboom *et al.* 1998, Vivien-Roels *et al.* 1998). *Clock*-Δ19 mutants (Vitaterna *et al.* 1994) were

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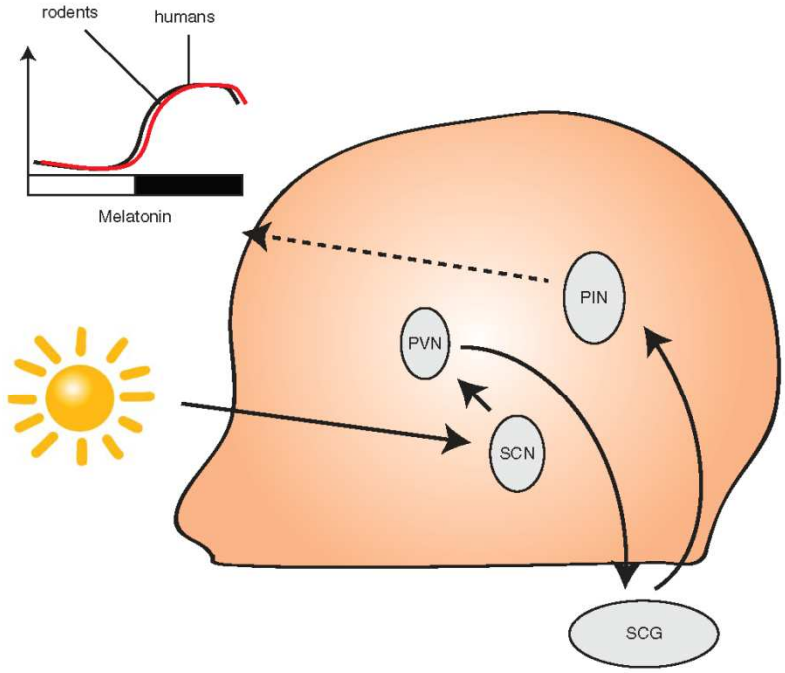

Figure 3
Melatonin release from the pineal is driven by the SCN pacemaker. The SCN innervates the PVN from where autonomous fibres descend into the spinal cord and out via the superior cervical ganglia (SCG) to reach the pineal gland (PIN). Clock genes are expressed in the pineal, but a functional contribution of a potential pineal clock to melatonin production has not been demonstrated. Unlike GCs, melatonin secretion is always confined to the dark phase, regardless of the activity profile of the animal.

back-crossed into a melatonin-proficient strain, showing that the *Clock*-Δ19 mutation leads to phase delays and dampening of the melatonin rhythm in constant darkness conditions while GC rhythms were completely abolished (Kennaway *et al.* 2003, 2006). More recently, it has been demonstrated that the melatonin biosynthesis pathway can genetically suppress the circadian perturbations of *Clock*-Δ19 mutation (Shimomura *et al.* 2010), suggesting a role of melatonin in contributing to the robustness of the SCN clock (see below). *PER1* deficiency has been shown to enhance *Aanat* transcription, enzymatic activity and hence melatonin secretion (Chen & Baler 2000, Christ *et al.* 2010). In *CRY1/2* double-deficient mice on a melatonin-proficient genetic background not only is the melatonin rhythm blunted under light–dark conditions, but also photic suppression of melatonin is abolished (Yamanaka *et al.* 2010). Together, these data suggest that clock genes impinge on pineal melatonin rhythmicity. However, owing to the lack of suitable genetic models to study the tissue-specific function of clock genes in melatonin-proficient strains, the physiological role of the molecular clock in the pineal itself remains largely unclear.

Hormonal feedback to the circadian clock

The stabilizing role of melatonin in SCN regulation mentioned above suggests that hormonal rhythms – we have discussed circadian regulation of GC and melatonin secretion – are not merely an output of the central clock. They can also feedback to the various levels of the circadian system and thereby intervene the circadian rhythm of physiology and behavior of animals (Fig. 4). In the following section, we will use these and some other hormones as examples to illustrate the crosstalk within the clock–hormones circuitry in mammals.

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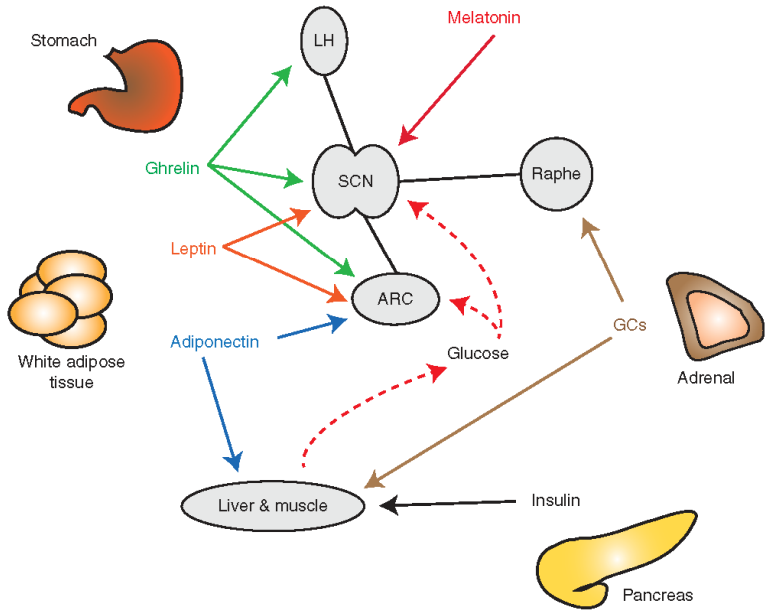

Figure 4
Endocrine feedback to the circadian clock. Various hormones can directly or indirectly feedback on central and peripheral clock function. In the brain endocrine targets with connections to the SCN include the orexinergic neurons of the lateral hypothalamus (LH), the arcuate nucleus (ARC), and the raphe nuclei of the brainstem. Other endocrine effects may be mediated via peripheral tissues and clocks such as the liver and muscle. For details see text.

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Cortisol

Exposure to jetlag or sleep perturbations (such as sleep restriction or shift work) results in a transient mismatch between the internal circadian time and the external light–dark cycle. Symptoms of jetlag include decreased alertness, motor coordination and cognitive performance, sleep disturbances, gastrointestinal disruption, and loss of appetite (Waterhouse *et al.* 2005). Sleep restriction and daytime sleep – hallmarks of a night shift work schedule – are associated with increased BMI and risk of metabolic syndrome, and alterations in circulating endocrine parameters such as insulin, glucose, and GCs (Wu *et al.* 2008, Rehman *et al.* 2010, Baron *et al.* 2011). Cortisol rhythms are also affected by jet travel, even when only three or fewer time zones are crossed (Doane *et al.* 2010), as well as by relatively subtle advances in sleep timing (Dijk *et al.* 2012).

GC steroids secreted from the adrenal gland are integral regulators of energy metabolism as well as the response to immune challenge and stress. GC disruption is associated with a variety of disorders. Cushing's disease is characterized by excess cortisol, with symptoms including hypertension, hyperglycemia, sleep disorders, depression, and weight gain (Carroll & Findling 2010). Addison's disease, characterized by a lack of cortisol, is accompanied by symptoms of weight loss, elevated sensitivity to stress, hypotension, mood disorders, and hypoglycemia (Mitchell & Pearce 2012).

GCs have been shown to directly affect circadian clock gene expression in a number of tissues, such as white adipose tissue, liver, and kidney (Gomez-Abellan *et al.* 2012, Pezük *et al.* 2012). Adrenalectomy shortens re-entrainment in the SCN, lung, and kidney following phase shifts, suggesting that GCs may serve to stabilize the phase of peripheral clocks against external noise (Pezük *et al.* 2012). In the case of jetlag-induced circadian desynchrony, it was shown that manipulation of the GC rhythm could speed up or slow down activity adaptation to the new light–dark schedule, depending on the intervention time (Kiessling *et al.* 2010). This study

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highlights the exciting therapeutic potential of GCs in the treatment of jetlag and other desynchrony disorders.

Melatonin

The best-studied physiological effect of melatonin is its modulatory function on sleep/wake cycle regulation in humans. Application of exogenous melatonin has been shown to decrease the latency to sleep, increase total sleep time, and promote sleep maintenance (Sack *et al.* 1997, Sharkey *et al.* 2001). In contrast, blocking the nocturnal release of melatonin by suppressing the sympathetic innervation to the pineal results in increased total wake time (Van Den Heuvel *et al.* 1997). Moreover, exogenous melatonin can influence sleep macro architecture (Dijk *et al.* 1995, 1997). Because of its sleep-promoting effect, melatonin treatment is frequently used to ameliorate the symptoms of jetlag or to improve sleep quality during the daytime in night-shift workers (Aeschbach *et al.* 2009).

Beyond its effect on sleep, melatonin has been shown to directly signal to the SCN. In contrast to GRs (see above), high densities of MT1 and MT2 receptors in the SCN have been demonstrated (Gillette & McArthur 1996). In rodents, timed daily administration of high concentrations of exogenous melatonin can entrain the free-running endogenous rhythm under constant darkness conditions (Armstrong *et al.* 1986, Redman & Armstrong 1988). Similarly, timed application of melatonin can entrain blind human subjects (Arendt & Broadway 1987, Sack *et al.* 2000). *In vitro*, melatonin application to cultured SCN explants affects amplitude and phase of the circadian rhythm of neuronal firing (Liu *et al.* 1997, Shimomura *et al.* 2010). The acute inhibitory effect of melatonin on neuronal activity seems to be mediated by MT1 receptor (Liu *et al.* 1997), while the phase-resetting effect relies on MT2 receptor signalling (Hunt *et al.* 2001). It is worthy of mention that melatonin is also capable of modulating the production of adrenal GCs. In humans and monkeys, acute melatonin administration suppresses the production of cortisol (Torres-Farfan *et al.* 2003, Campino *et al.* 2011). More recently, it has been demonstrated using foetal rats that timed melatonin application can entrain adrenal gland rhythms (Torres-Farfan *et al.* 2011). Thus, together melatonin and GC rhythms appear to stabilize circadian phase and precision for different physiological systems.

Ghrelin and insulin

The timing of food intake is an important entrainment signal for peripheral clocks, best characterized in, but not limited to, the liver and adipose clocks (Stephan 2002). Anticipatory behavior just before scheduled feeding (food anticipatory activity (FAA)) is seen in animals with restricted access to food. This is characterized by increased activity and changes to body temperature, GC rhythms and hepatic P450 enzymatic function (Krieger *et al.* 1977, Hirao *et al.* 2006), which function to prepare the body for the anticipated food intake. When food access is confined to the normal rest period, these processes can uncouple peripheral oscillators from the central clock that stays locked to the light regimen. Ghrelin is secreted in anticipation of feeding, regardless of the light–dark cycle, from gastric oxyntic gland cells which possess a functional clock (LeSauter *et al.* 2009). Ghrelin stimulates appetite via its actions on the hypothalamic orexigenic peptides, neuropeptide Y and orexin, and on mesolimbic reward centres (Abizaid *et al.* 2006, Toshinai *et al.* 2006). In shift workers, the post-prandial ghrelin slump is attenuated, perhaps contributing to overeating (Schiavo-Cardozo *et al.* 2012). Ghrelin administration increases FAA; however, studies on rodents lacking functional ghrelin signalling are contradictory. Mice lacking ghrelin receptors are reported to have dampened FAA (LeSauter *et al.* 2009), whilst mice lacking preproghrelin show intact FAA responses during restricted feeding (RF; Szentirmai *et al.* 2010). Ghrelin can feed back onto the circadian clock by directly affecting clock gene expression in the SCN (Yannielli *et al.* 2007). *In vivo* studies show that ghrelin treatment increases food intake, but only shifts behavioral rhythms under fasted conditions (Yannielli *et al.* 2007).

Insulin represents another potential food-inducible clock synchronizer. Insulin secretion from pancreatic beta cells is clock-gated, and disruption of the positive arm of the clock – CLOCK or BMAL1 – results in hypoinsulinemia (Marcheva *et al.* 2010, Sadacca *et al.* 2011), while disruption of the clock's negative regulators – PERs and CRYs – is associated with hyperinsulinemia (Zhao *et al.* 2012, Barclay *et al.* 2013). Insulin sensitivity is reduced in shift workers, and accompanied by increased beta cell activity, suggesting a pre-diabetic state (Esquirol *et al.* 2012). But insulin can also feed back to the clock. Tahara *et al.* (2011) used daytime RF in mice to demonstrate insulin-dependent alterations of clock gene rhythms in the liver, and a similar response was seen in primarily cultured rat hepatocytes (Yamajuku *et al.* 2012). It would be remiss to discuss the effects of insulin on the clock without discussing the effects of glucose as a direct function of insulin signalling. Glucose can directly affect circadian gene expression in fibroblasts and the SCN (Hirota *et al.* 2002, Iwanaga *et al.* 2005). In the absence of

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insulin signalling, for example in diabetic rats, circadian clock phase is shifted in the heart, suggesting that high glucose levels can directly impinge on clock regulation (Young *et al.* 2002). Under RF conditions, sucrose (but not lipid) induces phase shifts and FAA (Stephan & Davidson 1998).

The concept of food-inducible factors acting as powerful entertainers of the clock system is ratified by a number of studies which employ RF to rescue clock gene rhythms as well as physiological rhythms under desynchronous conditions. In a rat model of night work, restricting food intake to the normal activity phase restores glucose rhythms and prevents weight gain (Salgado-Delgado *et al.* 2010). In a study on a mouse model of shift work, restoring normal food intake rhythms concurrently restores clock gene rhythmicity in the liver, as well as triglyceride, glycerol and GC rhythms, and gluconeogenesis (Barclay *et al.* 2012). While these data suggest a direct link between peripheral clock regulation and energy homeostasis, the phase relationship between clock gene expression and the transcriptional activity of metabolism-associated genes is variable, suggesting an interplay between local and systemic factors (Reznick *et al.* 2013).

Leptin and adiponectin

It is widely established that clock disruption results in metabolic perturbations, and ultimately obesity (reviewed in Froy (2010)). Conversely, high fat diet (HFD) can dampen clock gene rhythmicity in the liver and fat, and well as affecting behavioral rhythms (Kohsaka *et al.* 2007). HFD results in loss of diurnal feeding patterns in rodents, and subsequent alteration to GC, insulin, and glucose rhythms (Kohsaka *et al.* 2007). A study by Kaneko *et al.* (2009) showed altered clock gene expression the brainstem of mice fed with a HFD, as well as in genetically obese mice such as *ob/ob* (lacking the leptin gene) and KK-A(y) mice (a spontaneous diabetic mouse model). However, arguably the most dramatic effects of HFD and obesity are the effects seen on circulating adipokines such as leptin and adiponectin.

Leptin is secreted from white adipose tissue in response to glucose stimulation, and signals via appetite centres in the hypothalamus to promote satiety and prevent excess energy consumption. Circulating leptin shows a diurnal rhythm, peaking in the night in humans. In the models of obesity, leptin resistance can occur and in the absence of leptin's anorexigenic effects, this is accompanied by overeating (reviewed in Gautron & Elmquist (2011)). In humans, acute HFD feeding results in lower 24-h leptin (Havel *et al.* 1999), whereas hyperleptinemia and changes in leptin rhythmicity are observed in obese subjects in accordance with increased fat mass (Considine *et al.* 1996, Rosenbaum *et al.* 1996, van Dielen *et al.* 2002). Despite having no direct effect on locomotor activity, leptin can induce *PER* expression in the SCN of female mice and potentiate the phase-shifting effects of light in these animals (Mendoza *et al.* 2011). *Ex vivo*, leptin stimulation can reset the phase of the SCN clock (Prosser & Bergeron 2003).

Adiponectin possesses insulin-sensitizing and anti-inflammatory properties (reviewed in Harwood (2012)). Circulating adiponectin levels inversely correlate with obesity and leptin levels, and weight loss results in increased adiponectin (Hu *et al.* 1996, Yang *et al.* 2001, Matsubara *et al.* 2002). Adiponectin secretion shows both ultradian and circadian rhythms, with a nadir in the early hours of the morning in healthy adults (Gavrila *et al.* 2003, Scheer *et al.* 2010). In rodents, adiponectin peaks in the end of the light phase (inactive phase) and its rhythm is shifted under HFD (Ríos-Lugo *et al.* 2010). Bullen and colleagues showed decreased adiponectin levels relative to fat mass following HFD in rodents (Barnea *et al.* 2006, Bullen *et al.* 2007). To assess the effect of adiponectin on the circadian clock, Hashinaga and colleagues used KK-Ta mice, a polygenic model of metabolic syndrome with hypoadiponectinemia. These mice have a shorter activity period under constant conditions and dampened circadian locomotor rhythms with increased light-phase activity relative to controls. Clock gene rhythms are phase-advanced in the liver and skeletal muscle in these mice. The introduction of the human adiponectin transgene into the liver of these mice restores locomotor rhythmicity, as well as hepatic clock gene phase (Hashinaga *et al.* 2013). These studies strongly indicate that leptin, adiponectin, and maybe other adipokines may have direct effects on molecular clock function.

Summary and outlook

In summary, many components of the endocrine system show strong circadian rhythmicity in both rodents and humans. Some of these hormones, such as melatonin and cortisol, are involved in disseminating the SCN timing signal to other parts of the body. Endocrine rhythms respond to factors that compromise the clock function, such as HFD, obesity, jetlag, and sleep disruption. In turn, the endocrine system feeds back on central and peripheral clocks to adapt circadian rhythms to altered physiological state. Given the profound effects endocrine and circadian

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systems have on general well-being and the development of various disorders, this mutual interaction might provide new targets for pharmacological interventions at the systemic level. Recent studies have shown that resetting of GC signalling can affect clock resetting during jetlag (Kiessling *et al.* 2010) and with the recent discovery of drugs directly impinging on clock function (Hirota *et al.* 2010, Solt *et al.* 2012) it may be possible to rescue endocrine regulation under desynchrony conditions such as shift work.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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Tissue-Specific Interaction of *Per1/2* and *Dec2* in the Regulation of Fibroblast Circadian Rhythms

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Abstract In mammals, the molecular circadian clockwork is comprised of interlocked transcriptional-translational feedback loops (TTLs). Three *Period* (*Per1-3*) and 2 *Dec* (*Dec1/2*) genes interact in regulating the activity of the transcriptional activators CLOCK/NPAS2 and BMAL1. While deletion of *Per1* and *Per2* in mice results in behavioral arrhythmicity, *Dec* deletion has less dramatic effects on activity rhythms, affecting primarily phase of entrainment and free-running period. In intact animals, clock gene mutant phenotypes are often masked due to intercellular coupling mechanisms that stabilize cellular rhythms. Therefore, to study *Per/Dec* genetic interaction at the cellular level, we isolated fibroblasts from different tissues of *Per1*, *Per2*, and *Dec2* single and double mutant mice. We show that in the cellular TTL, *Per*s and *Dec2* act in a principally synergistic way, but tissue-specific differences in this interaction are seen. A rescue of rhythmicity in *Per2* mutant cells after additional deletion of *Dec2* was observed, indicating that in the absence of *Per2*, DEC2 destabilizes TTL function. Rhythm power in *Per1/Dec2* and *Per2/Dec2* double mutants was strongly reduced, suggesting that interaction of *Dec2* with both *Per* genes is important for stabilizing clock period. Contrary to what was observed for behavior, nonsynergistic effects of *Dec2* and *Per1/2* mutations were observed on cellular clock phase regulation that do not correlate with period effects. Our data reveal cell type-specific interactions of *Per1/2* and *Dec2* in the regulation of period, phase, and rhythm sustainment, emphasizing the differential organization of the mammalian clock machinery in different tissues.

Key words *Per1*, *Per2*, *Dec2*, circadian clock, cellular rhythms, fibroblasts, luciferase

In most organisms, endogenous timekeeping systems have evolved that organize physiological and behavioral adaptation to the 24-h day (Brown et al., 2012). In mammals, these so-called circadian clocks are based on cellular oscillators driven by a set of clock genes organized in a system of interlocked transcriptional-translational feedback loops (TTLs) (Oster, 2006; Takahashi et al., 2008). Positive TTL

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elements include the transcription factors CLOCK, NPAS2, and BMAL1 (ARNTL) that drive expression of negative elements such as *Period* (*Per1-3*) and *Cryptochrome* (*Cry1/2*) genes via E-box regulatory promoter elements during the day (Bunger et al., 2000; Gekakis et al., 1998). In the night, PER/CRY protein complexes translocate into the nucleus where they interfere with CLOCK/NPAS2/BMAL1, thereby inhibiting their own transcription (Griffin et al., 1999; Kume et al., 1999; Sun et al., 1997). Toward morning, successive degradation of PER/CRY proteins releases the inhibitory pressure on CLOCK/NPAS2/BMAL1, and a new round of E-box-mediated transcription resumes (Lee et al., 2001; Yoshitane et al., 2009). The periodicity of this feedback is believed to be regulated by posttranslational mechanisms and accessory loops that stabilize the necessary delay between production and inhibitory action of negative TTL elements. One such loop involves the transcriptional modulator DEC2 (SHARP-1, BHLH-42) (Honma et al., 2002). *Dec2* transcription is controlled by CLOCK/NPAS2/BMAL1 (Hamaguchi et al., 2004; Noshiro et al., 2005), and DEC2 proteins can—depending on time and tissue—act as either activators or inhibitors of E-box transcription (Bode et al., 2011b; Rossner et al., 2008). Very similar characteristics have been observed for the PER proteins (Preitner et al., 2002; Zheng et al., 2001). In previous experiments we have shown that *Per* and *Dec* genes interact closely and in a time-of-day dependent way in regulating activity rhythms and clock gene expression in the mammalian circadian pacemaker of the suprachiasmatic nucleus (SCN) (Bode et al., 2011a, 2011b).

Unlike other tissues, the SCN is characterized by tightly coupled neurons generating a highly stabilized rhythm at the tissue level (Aton et al., 2005; Liu et al., 2007; Liu and Reppert, 2000; Maywood et al., 2006; Yamaguchi et al., 2003). Previous studies have shown that by this coupling, SCN cells become highly resistant to the destabilizing effects of clock gene mutations (Jakubcakova et al., 2007; Liu et al., 2007; Pando et al., 2002; Robles et al., 2010). Moreover, the organization of the clock machinery and, thus, the phenotype of specific clock gene mutations can differ between different tissues (Cermakian et al., 2001; DeBruyne et al., 2007b; Jakubcakova et al., 2007; Nakashima et al., 2008; Reick et al., 2001; Robles et al., 2010).

To analyze the interactivity of PER and DEC feedback in molecular clock regulation at the cellular level and in different tissues, we here compared molecular rhythms of primary fibroblasts from ear and lung tissue preparations of *Per1*, *Per2*, and *Dec2* single and double mutant mice. Our data suggest a close, cell type–specific interaction of *Per1/2* and *Dec2* in the regulation of period, phasing, and sustainment of molecular circadian rhythms, emphasizing the multiform organization of the mammalian clock machinery in different tissues.

MATERIALS AND METHODS

Primary Cell Cultures

Mouse ear and lung fibroblasts were obtained from adult male and female *Per1* mice (*Per1*^{tm1Brd}) (Zheng et al., 2001), *Per2* mice (*Per2*^{tm1Brd}) (Zheng et al., 1999), *Dec2* mutant mice (*Bhlhe41*^{tm1Mrjo}) (Rossner et al., 2008), and *PER2::LUC* mice (*Per2*^{tm1Jt}) (Yoo et al., 2004) on a C57B/6J background. Six or 7 independent preparations were done per genotype spread over several days, and always 2 or more genotypes were processed in parallel to minimize unspecific effects due to experimental variations. Tissues were extracted, rinsed with ice-cold HBSS, and subjected to mincing and subsequent enzymatic digestion. Ear tissues (2 ears per preparation) were digested with 100 µg/mL Liberase TM (Roche Applied Science, Mannheim, Germany) in the presence of 20% FBS at 37 °C and 5% CO_2 overnight. Lung tissues (2 lung wings per preparation) were digested with 100 µg/mL Liberase TM in the presence of 1% BSA at 37 °C with constant agitation for 1 h. Dissociated cells were rinsed with PBS and plated onto 60-mm cell culture dishes containing culture medium (DMEM with 20% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM stable glutamine, 2.5 µg/mL amphotericine B) with medium change every second day. Upon confluence, fibroblast cells were trypsinized, and 5×10^5 cells were seeded to a 35-mm cell culture dish containing culture medium as described above, but without amphotericine B. Viral transduction was conducted on the next day with ~1.68×10^8 infection units (IFUs) per dish in the presence of 8 µg/mL polybrene. One day after transduction, cells were rinsed with PBS and restored with fresh culture medium.

Bioluminescence Recording

Cells were synchronized by 2 h of incubation with 100 nM dexamethasone. After that, cells were changed to recording medium (same as above, but without phenol-red, plus 20 mM HEPES, 1 mM pyruvate,

100 µM D-luciferin; Biosynth AG, Staad, Switzerland). Dishes were sealed with silicon grease and cover glasses. Luminescence was measured using a LumiCycle luminometer (Actimetrics, Evanston, IL) at 32.5 °C. To assess dexamethasone sensitivity, ear- and lung-derived fibroblasts of *PER2::LUC* mice were isolated and plated into a 96-well plates with recording medium. Forty-eight hours after plating, plates were sealed with transparent film, and luminescence was recorded at 34 °C using a Berthold TriStar LB 941 (Berthold Technologies, Wildbach, Germany). Luminescence was measured over 1 h to determine reference baselines. After that, cells were treated with various concentrations of dexamethasone, and recording was resumed for additional 24 hours. Sensitivity was determined by dividing the 24-h peak value of each trace by the respective pretreatment baseline.

Lentivirus Production

Bmal1:luc encoding *pBluF-puro* plasmid was a kind gift from Prof. Steven Brown, University of Zurich, Switzerland. To produce *Bmal1:luc* lentiviral particles, each 10-cm dish of HEK293T cells was cotransfected with 10 µg *psPAX2* (Addgene plasmid #12260, Prof. Didier Trono, EPFL, Switzerland), 5 µg *pMD2.G* (Addgene plasmid #12259, Prof. Didier Trono, EPFL, Switzerland), and 15 µg *pBluF-puro* using Xfect transfection reagent (Clontech, Saint-Germain-en-Laye, France). Cells were rinsed with PBS on the second day and restored with fresh culture medium. Viral containing medium was harvested at 36 h after transfection and stored at 4 °C. Cells were restored with fresh medium, and further medium collections were performed on the next day. Two collections were pooled and concentrated using LentiX concentrator reagent (Clontech) according to the manufacturer's protocol. Virus titers were determined by transducing HEK 293T cells with a serial dilution of GFP-encoding lentiviral particles (produced as described above but with *pWPI* (Addgene plasmid #12254, Prof. Didier Trono, EPFL, Switzerland) instead of *pBluF-puro*). At 72 h after transduction, GFP-positive cells were counted under a fluorescence microscope. The same serial dilution was also subjected to real-time quantitative PCR (qPCR) analysis using a primer pair amplifying the viral *WPRE* element (forward: 5′-GGCACTGACAATTCCGTGGT-3′; reverse: 5′-AGGGACGTAGCAGAAGGACG-3′) to determine viral genome abundance. qPCR was also done for *Bmal1-luc* virus-containing medium, and the titer was estimated by comparison with the *pWPI* abundance versus GFP calibration curve.

Gene Reporter Assays

Dbp:luc plasmid was a kind gift from Prof. Ueli Schibler, University of Geneva, Switzerland. HEK 293T cells were plated into 96-well plates coated with poly-D-lysine and transfected with the following expression plasmids: 10 ng *Dbp-luc* and 2 ng *CMV-Renilla luciferase* with various combinations (20 ng each) of the following clock gene constructs: *HA-Clock*, *HA-Bmal1*, *HA-Dec1*, *FLAG-Dec2*, *Per1-GFP*, *Per2-V5*, and *pcDNA3.1* (mock transfection). We used Lipofectamine LTX transfection reagent (Life Technologies, Darmstadt, Germany). Forty-eight hours after transfection, luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega, Mannheim, Germany) using the Berthold TriStar.

Statistics

For statistical comparisons, 6 or 7 independent fibroblast preparations for each tissue and genotype were used. Rhythmicity and period length were assessed by χ^2 periodogram analysis over the first 3 days on 24-h moving average baseline-subtracted luminescence recordings. Dampening times (D) were determined by fitting a dampened sine wave to baseline-subtracted luminescence recordings over 5 days using a Levenberg-Marquardt algorithm on the following equation: $Y(t) = \text{Amplitude} * \sin(2 * \pi * \text{Frequency} * t + \text{Phase}) * e^{-(t/D)} + \text{Offset}$. Dampening times are expressed relative to rhythm period length (1 CT = (2 * π * Frequency)/24 h). To determine phase, the first peak of the baseline-subtracted luminescence curve was measured relative to the time of synchronization. All measurements were done using the Lumicycle analysis software (Actimetrics, Evanston, IL). Group statistics were done with GraphPad Prism 5 (GraphPad, La Jolla, CA).

RESULTS

Rhythmic Bmal1:luc Expression in Per/Dec-Deficient Fibroblasts

We isolated and cultured primary fibroblasts of ear and lung tissues from adult wild-type (WT) and homozygous *Per1* (*P1*), *Per2* (*P2*), *Dec2* (*D2*), *Per1/Dec2* (*P1D2*), and *Per2/Dec2* (*P2D2*) single and double

mutant mice. We chose 2 similar cell types from 2 different organs as this would allow us to compare tissue-specific effects under comparable culture conditions. At 60% confluence, cells were infected with *Bmal1:luc* lentivirus. Transduction efficiency was determined on parallel infections using a GFP-expressing lentivirus. More than 95% of cells were expressing the transgene in all tested preparations (Suppl. Fig. S1). Upon confluency, cells were synchronized by dexamethasone treatment and transferred into a luminometer for luciferase activity measurements. Cells of all genotypes and tissues showed at least 2 full cycles of circadian rhythmicity, with the exception of *P2* (both tissues) and *P1D2* (lung). Representative luminescence recordings and corresponding normalizations are shown in Figure 1A for ear and Figure 1B for lung preparations. To exclude that a difference in sensitivity to dexamethasone treatment might underlie the observed differences in rhythmicity in the 2 cell types, we stimulated *PER2::LUC* fibroblasts from both tissues with increasing amounts of dexamethasone and monitored the direct luciferase response over the next 24 hours. Luciferase activity was dose-dependently and similarly induced by dexamethasone in both tissues (Suppl. Fig. S2).

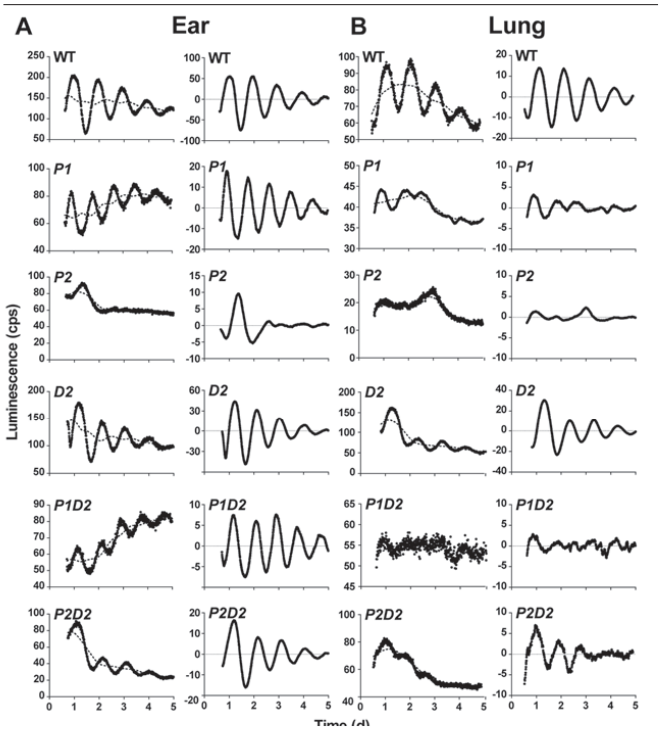

Figure 1. Cellular circadian rhythms in ear- and lung-derived *Per/Dec2* single and double mutant fibroblasts. (A, B) Representative luminescence recordings from wild-type (WT), *Per1* (*P1*), *Per2* (*P2*), *Dec2* (*D2*) single, and *Per1/Dec2* (*P1D2*) and *Per2/Dec2* (*P2D2*) double mutant fibroblasts derived from adult mouse ear (A) and lung tissue (B). Left panels show raw data (black circles) and 24-h moving average baselines (dotted lines). Right panels show the same curves after baseline subtraction.

Tissue-Specific Period Regulation in Per/Dec-Deficient Fibroblasts

Period length was shortened in *P1* relative to WT cells from both tissues (Fig. 2A, B). *D2* cellular rhythms were significantly shortened in ear-derived (Fig. 2A) but not in lung-derived fibroblasts (Fig. 2B). *P1D2* ear fibroblasts showed short periods comparable to those seen in *P1* single mutant cells (Fig. 2A), indicating that period length is affected less by *Dec2* than by *Per1* in this tissue. In contrast in lung cells, arrhythmicity was observed after simultaneous mutation of *Per1* and *Dec2* (Fig. 2B). Surprisingly, circadian rhythmicity was rescued in *Per2* mutant fibroblast preparations after additional deletion of *Dec2* (*P2D2*). While *P2D2* ear fibroblasts showed short periods comparable to those of *D2* cells (Fig. 2A), in lung-derived *P2D2* cells periods were shorter than those of either WT or *D2* single mutant fibroblasts (Fig. 2B). Comparable results were obtained when sine wave fits were used instead of periodogram analyses (Suppl. Fig. S3).

Tissue-Specific Regulation of Dampening Time and Rhythm Strength in Per/Dec-Deficient Fibroblasts

The restoration of circadian molecular rhythmicity in *P2D2* double mutant cells was very reminiscent of the rescue in behavioral locomotor rhythmicity observed in *P2D2* double mutant animals (Bode et al., 2011a). To further assess interactive effects of *Per1*, *Per2*, and *Dec2* on cellular rhythmicity, we quantified *Bmal1:luc* activity rhythm sustainment by determining

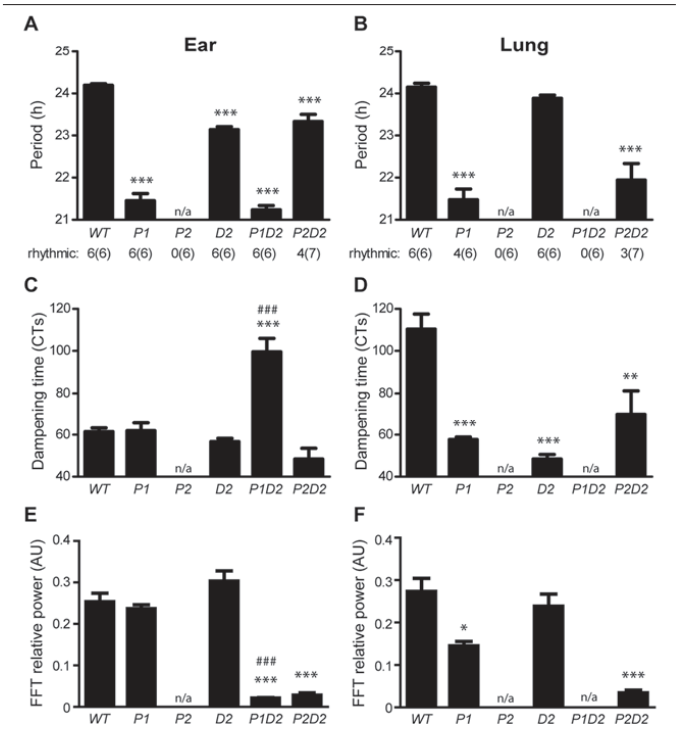

Figure 2. Period length and rhythm dampening in ear- and lung-derived *Per/Dec2* single and double mutant fibroblasts. (A, B) Period length as determined by χ^2 periodogram analysis on baseline-subtracted recordings from ear-derived (A) and lung-derived cells (B). Values below depict numbers of rhythmic and total (in parentheses) preparations. (C, D) Dampening times as determined by dampened sine wave fitting on baseline-subtracted recordings from ear-derived (C) and lung-derived cells (D). Dampening times were normalized to internal period to allow a better comparison of rhythm stability between cells with different internal periods. (E, F) Fast Fourier transformation (FFT) power analyses of luciferase activity during the first 3 days of the recordings from ear-derived (E) and lung-derived cells (F). All data are presented as means + SEM (n = 6-7); *$p < 0.05$, ***/###$p < 0.001$, 1-way ANOVA with Bonferroni posttest vs. WT (*) or P1 (#); n/a = no rhythm detectable.

dampening times and rhythm stability after fitting a dampened sine wave to baseline-subtracted luminescence recordings. For ear tissue-derived fibroblasts, dampening times were unaltered in *P1*, *D2*, and *P2D2* while, surprisingly, an increase in amplitude stability was observed in *P1D2* cells (Fig. 2C). In contrast, in lung-derived cells, rhythm sustainment was strongly and consistently decreased in *P1*, *D2*, and *P2D2* compared with WT (Fig. 2D). Rhythm stability values correlated well with dampening times (Fig. 2E, F), with faster dampening correlating with reduced rhythm power. Notable exceptions were seen in ear-derived P1D2 and lung-derived *D2* cells. In ear *P1D2* cells, reduced rhythm power rates suggested that the extended amplitude sustainment was compromised by low period stability, making the oscillation rather unreliable. At the same time, in *D2* lung fibroblasts, short dampening times were combined with relatively high rhythm power, indicating that early rhythm cessation may not derive from unstable period regulation per se.

Tissue-Specific Regulation of Circadian Phase in Per/Dec-Deficient Fibroblasts

One of the most prominent interactive phenotypes observed in *Per/Dec* double mutant mice was seen in the regulation of locomotor activity onset (i.e., phase angle) under entrained conditions and after release into free-run (Bode et al., 2011a, 2011b). We did not measure rhythm entrainment in our cells (e.g., by ambient temperature cycles). However, because dexamethasone synchronization represents a type 0 resetting paradigm, release into free-run can be simulated—and thus *Bmal1:luc* phase differences between genotypes estimated—in vitro by comparing the time of the first peak of luminescence in rhythmic fibroblasts after synchronization, and it has been shown in cultured fibroblasts that after synchronization, the intrinsic phase relationship among the expressions of different clock genes are well preserved and comparable to the in vivo situation (Saini et al., 2012). WT ear fibroblasts peaked at 13.4 ± 0.4 h after synchronization (Fig. 3A). In *P1* cells—and similar to what was seen for period length (Fig. 2A, B)—phasing of the first peak was advanced (11.0 ± 0.6 h), while in *D2* (17.4 ± 0.1 h), *P1D2* (17.3 ± 0.6 h), and *P2D2* (16.3 ± 0.7 h), cells peak phase was delayed relative to WT fibroblasts (Fig. 3A). In lung-derived cells, the situation was similar with WT cells peaking at 11.2 ± 1.2 h after synchronization, *P1* cells showing highest luciferase activity significantly earlier at 2.9 ± 0.1 h, and *D2* cells showing a delayed

peak (17.6 ± 0.2 h) compared with WT cells. Unlike what was seen in ear-derived cells, phasing in *P2D2* lung cells (9.5 ± 1.0 h) was not delayed relative to WT fibroblasts (Fig. 3B).

Correlation between Period Length and Circadian Phase

Previous data from animals and humans suggest a strong correlation between period length and phasing of behavioral and molecular rhythms (Brown et al., 2008; Liu et al., 2007; Meng et al., 2008; Oster et al., 2003; Ralph and Menaker, 1988; Zheng et al., 1999). In our study, we also observed some genotypes in which shortened period coincided with advanced phasing (e.g., *P1* for both tissues; compare Figs. 2 and 3). To further analyze this relationship, we plotted peak time against period length for all preparations. When both tissues were pooled, no significant correlation between the 2 measures was found (data not shown). However, when we compared genotype effects separated for tissues, phasing did not correlate with period in ear-derived fibroblasts (Fig. 4A), whereas a strong correlation ($p < 0.001$) between the 2 parameters was seen for lung (Fig. 4B). In all cases, the extent of advance in phasing was much higher than that of period shortening, suggesting that period variations may contribute but are not sufficient to explain the observed phase advances.

Interaction of PER and DEC Proteins in the Regulation of Dbp Transcription

Our cell culture data suggested that *Per1/2* and *Dec2* have tissue type-independent synergistic roles in period regulation. So far, physical interaction of PER and DEC proteins has not been demonstrated, but it is known that they can both act on CLOCK/BMAL1 activity at *E-box* promoters (Bode et al., 2011b; Honma et al., 2002; Jin et al., 1999; Rossner et al., 2008). Genetic and modeling experiments suggest that relieving *E-box* repression, for example by enhanced destabilization of PER proteins, shortens free-running period (Gallego et al., 2006; Maywood et al., 2011). We therefore hypothesized that synergistic de-repression of CLOCK/BMAL1 activity may underlie the interactive effect of *Per1/2* and *Dec2* deletions on period regulation. The opposite effect would be expected upon overexpression of PER and DEC proteins. To test this we measured luciferase activity in HEK 293T cells transfected with a *Dbp-luc* reporter, *Clock*, and *Bmal1* and various combinations of *Per1*, *Per2*, and *Dec2* expression plasmids. As expected, expression of *Clock* and *Bmal1* resulted in a prominent increase in luciferase activity (Fig. 5A). Coexpression of *Per1*, *Per2*, or *Dec2* alone led to a moderate inhibition of CLOCK/BMAL1 (although significance was not reached in a 1-way ANOVA). In contrast, cotransfection of *Per1* or *Per2* with *Dec2* produced a significant repression of CLOCK/BMAL1 activity with luciferase activity levels comparable to control conditions. A second tissue type–independent finding from our fibroblast experiments was that P2D2 mutant cells had consistently longer periods than P1D2 mutants. Assuming that this effect was also mediated by differential regulation of CLOCK/BMAL1 transactivation and following the same argument as above, this would mean that PER1 and DEC1 together (i.e.,

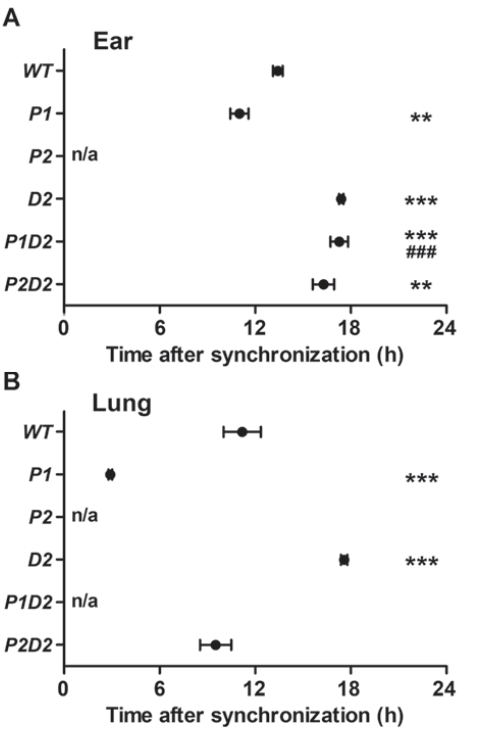

Figure 3. *Bmal1:luc* peak phase in ear- and lung-derived *Per/Dec2* single and double mutant fibroblasts. Timing of first peaks of baseline-subtracted luminescence recordings after dexamethasone synchronization in ear-derived (A) and lung-derived *Bmal1:luc*-transduced fibroblasts (B). All data are presented as means ± SEM (n = 6-7); $^{**}p < 0.01$, $^{***/\#\#\#}p < 0.001$, 1-way ANOVA with Bonferroni post-test vs. WT (*) or P1 (#); n/a = no rhythm detectable.

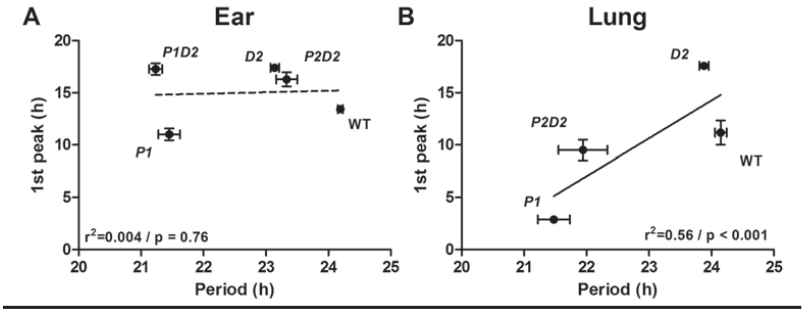

Figure 4. Correlation of phase vs. period length in ear- and lung-derived *Per/Dec2* single and double mutant fibroblasts. (A, B) Correlation of phase and period in ear-derived (A) and lung-derived synchronized fibroblasts (B) of wild-type (WT), *Per1* (*P1*), *Per2* (P2), *Per1/Dec2* (*P1D2*), and *Per2/Dec2* (*P2D2*) mutant mice. All data are presented as means ± SEM (n = 6-7). Correlation lines and goodness of fit criteria are listed in each diagram. Solid lines depict significant degrees of correlation.

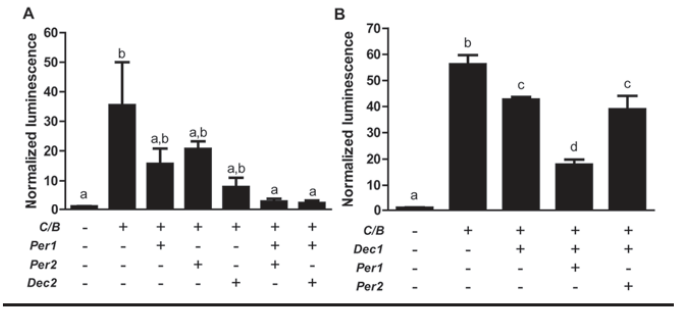

Figure 5. Interaction of *Per*s and *Dec*s in the regulation of CLOCK/BMAL1-induced *Dbp-luc* activity. (A, B) Luciferase activity in HEK 293T cells after transfection with different combinations of expression plasmids depicted below. Data are presented as means ± SEM (n = 3); letters depict significantly different groups by 1-way ANOVA with Tukey posttest.

resembling the P2D2 mutant situation) should have a stronger inhibitory effect on CLOCK/BMAL1 than PER2/DEC1 (resembling P1/D2). We tested this by cotransfecting *Dec1* with *Per1* or *Per2* in HEK 293T cells as described above. While *Dec1* alone had a moderate effect on CLOCK/BMAL1 activity, cotransfection with *Per1*, but not with *Per2*, caused further repression of *Dbp-luc* activity (Fig. 5B). Together, these data suggest that the interaction of *Per1/2* and *Dec2* in the regulation of the circadian TTL may—at least with respect to period—be mediated via *E-box* regulation.

DISCUSSION

Our data suggest a predominantly synergistic interaction between *Per1*, *Per2*, and *Dec2* in cellular clock regulation. This interaction is tissue-specific, affecting rhythmicity itself as well as rhythm sustainment, period length, and stability. In contrast, non-synergistic interactions were seen in the regulation of cellular clock phase. Luciferase promoter assays suggest that *Per/Dec* interaction is mediated by interaction at the level of *E-box* regulation.

Synergistic but Tissue-Specific Interaction of Dec2 and Per1/2

It has previously been shown that the effects of clock gene mutations on circadian rhythmicity may differ between tissues (Cermakian et al., 2001; DeBruyne et al., 2007b; Jakubcakova et al., 2007; Nakashima et al., 2008; Reick et al., 2001; Robles et al., 2010). Due to its unique intercellular coupling, the SCN pacemaker is particularly robust against genetic perturbation (Hogenesch and Herzog, 2011; Liu et al., 2007). This seems also true for *Per/Dec* mutations. When one compares the cellular data obtained from this study with previous reports on *Per/Dec* mutant behavior and SCN clock gene expression (Bode et al., 2011a, 2011b; Rossner et al., 2008; Zheng et al., 2001; Zheng et al., 1999), overall larger effects on cellular rhythmicity than on SCN-controlled locomotor behavior are observed (Table 1). For example, deletion of *Dec2* had little effect on running-wheel activity rhythm period, phase, and stability, whereas period (ear), phase (ear and lung), and sustainment (lung) were affected in *D2* fibroblasts. The absence of communication between single oscillators in cell culture, however, may not be the only reason for the observed differences. Tissue-specific alterations in the clock gene machinery itself seem to also play a role as cells from ear or lung preparations react differently to genetic perturbation. For example, *Bmal1:luc* peak phase was delayed in *P2D2* ear fibroblasts, whereas

Table 1. Comparison of behavioral and cellular phenotypes.

	Behavior			*Ear Fibroblasts*			*Lung Fibroblasts*		
Mutation	Period	Phase	Sustain[a]	Period	Phase	Sustain[b]	Period	Phase	Sustain[b]
Per1	↓	←	n.c.	↓	←	n.c.	↓	←	↓
Per2	↓	←	↓	x	x	↓[c]	x	x	↓[c]
Dec2	n.c.	n.c.	n.c.	↓	→	n.c.	n.c.	→	↓
Per1/Dec2	↓	←	n.c.	↓	→	↑	x	x	x
Per2/Dec2	↓	←	n.c.	↓	→	n.c.	↓	n.c.	↓

x = not determined; n.c. = no change; ↕ = reduced/increased; ↔ = advanced/delayed.
a. Behavioral rhythmicity in constant darkness.
b. *Bmal1:luc* activity dampening time.
c. A lack of rhythmicity was interpreted as a sustain of 0.

no change relative to WT was seen in lung-derived cells. In contrast, rhythm sustainment was unaltered—although rhythm power was strongly reduced—relative to WT cells in *P2D2* ear fibroblasts whereas lung-derived cells showed accelerated dampening in their luminescence rhythm. Along this line, a mutation of *Per1* has previously been shown to result in circadian arrhythmicity in dispersed SCN neurons and in tail tissue-derived fibroblasts (Liu et al., 2007), whereas rhythmicity was preserved in our setup (though with accelerated dampening and reduced rhythm power in lung). Together these data indicate a certain degree of variability in the composition of the clock gene machinery between tissues or, alternatively, the existence of tissue-specific modulators of clock function. A tissue-specific nature has previously been postulated for the role of *Clock* in circadian regulation (Kennaway et al., 2006). Interestingly, a mutation of *Clock* also differently affects *Dec2* expression in different tissues (Noshiro et al., 2005).

Dec2 Deletion Rescues Rhythmicity in Per2 but Not in Per1 Mutant Cells

A phenotypic rescue of clock rhythmicity after additional mutation of *Dec2* in a *Per2* mutant background was seen both at the behavioral level (Bode et al., 2011a) and in fibroblasts (Table 1), similar to what had previously been observed for circadian behavior and in vivo clock gene rhythms after deletion of *Cry2* and *Per2* (Oster et al., 2002). Together, these data suggest that *Per2* function in the circadian TTL is strongly modulated by other clock regulators and that under certain conditions, *Per2* may even be dispensable for circadian rhythm generation. This interpretation is further supported by recent studies that report normal rhythmicity and period regulation in $Per2^{ldc}$ mutant mice (Pendergast et al., 2010; Xu et al., 2007). Surprisingly, our data suggest that an additional mutation of *Dec2* in a *Per1* mutant background can stabilize cellular rhythm sustainment, while at the same time rhythm power is strongly reduced. It is generally believed that cellular oscillators in culture do not communicate with each other (Liu et al., 2007). Therefore, rhythm dampening in cell culture is mainly based on 2 factors: individual differences in endogenous cellular period lengths and rhythm destabilization at the molecular level. Imaging studies in WT fibroblast cultures show that cellular clocks are self-sustained and single cells robustly cycle over extended periods of time while overall phase coherence is rapidly lost (Leise et al., 2012; Nagoshi et al., 2004). Our data support the hypothesis that individual period variation may contribute to rapid dampening in *Per1* mutant cells and that—specifically in ear-derived fibroblasts—this effect is compensated after additional mutation of *Dec2*. The finding that in both *P1D2* and *P2D2* double mutant cells rhythm sustainment does not correlate with increased rhythm power indicates a rather high variability in day-to-day period regulation, rendering the remaining rhythm highly unreliable. *Per1* mutant mice show a high variability in locomotor activity, rhythm period, and length regulation under free-running conditions (Zheng et al., 2001), and this effect is preserved in *P1D2* double mutant animals (Bode et al., 2011b) and cells (this article), suggesting that the absence of *Per* genes may confer intrinsic instability to the clock without principally affecting rhythm sustainment per se. However, lacking single-cell recordings, we cannot distinguish between inter- and intracellular effects in our setup.

The observation that rhythm sustainment in ear-derived *P1D2* mutant cells seems improved—although with reduced power—when compared

with WT cells appears surprising but may emphasize 2 important aspects. First, circadian clocks have not evolved under free-running conditions. Although for physiological entrainment it is important that clocks are capable of predicting time under zeitgeber free conditions and as a counterbalance against external noise, for most species optimization of entrainment under natural zeitgeber cycles might have been evolutionarily much more relevant (Hut and Beersma, 2011). Second, cell culture conditions only poorly recapitulate the native environment to which a cell is exposed inside the body. Thus, interactions between the altered genetic machinery of a mutant cell and components of the culture medium may critically interfere with the regulation and stability of cellular circadian rhythms. Some components of the clock gene machinery have been shown to interact with external signaling pathways. For example, CRY proteins regulate glucocorticoid receptor signaling and cellular energy metabolism in murine fibroblasts (Lamia et al., 2011). Such implications should be taken into consideration when interpreting circadian phenotypes under in vitro conditions.

Nonsynergistic Interaction of Per1 and Dec2 in the Regulation of Cellular Clock Phase

Our analyses of clock phase reveal 2 major findings. First, loss of *Per1* has an opposite effect on *Bmal1-luc* phase regulation than loss of *Dec2*. In ear-derived *P1D2* double mutant cells, phase regulation resembles that of *D2* mutants, whereas in lung-derived cells, loss of both genes results in arrhythmicity. Second, peak phase relations between different genotypes are not always correlated to period length. Theoretical considerations suggest that entrainment phase of an oscillatory system to an external zeitgeber is influenced by internal period (Roenneberg et al., 2008). Experimental data from human and animal studies support these postulations (Brown et al., 2008; Ralph and Menaker, 1988; Toh et al., 2001; Xu et al., 2005; Zheng et al., 1999). The extent of this effect, however, is highly dependent on zeitgeber strength. Activity rhythms in rodents, for example, can be stably entrained by extremely low light intensities (Foster et al., 2007), and standard laboratory light regimens are usually far beyond this entrainment threshold, thus minimizing the observed effects of internal period on phase adjustment. In cell culture conditions, the determination of phase will further depend on the selected readout. In this project we used luciferase activity as a real-time reporter of *Bmal1* promoter activity. However, with deletion of TTL components in clock gene mutant cells, the normal phase relationship between different TTL transcripts may be lost. Thus, phase relationships between genotypes may be different if another readout (e.g., another clock gene) is chosen. Of note, such shifts in clock gene mRNA profiles were also observed in *Per/Dec* mutant SCN (Bode et al., 2011a, 2011b). Third, genetic mutations may differentially affect resetting pathways and period regulation (Jakubcakova et al., 2007). The fact that in *Per/Dec* mutant fibroblasts phase differences are much more pronounced than those observed in free-running period lengths suggests that this may represent a major factor in this context. This assumption is supported by the fact that both *Per* genes have been implicated in resetting of the TTL in both the SCN and the periphery (Dibner et al., 2010).

Per1/2 and Dec2 Interact at the Level of E-Box Regulation

Our reporter gene assays show that *Per1/2* and *Dec2* interact in regulating CLOCK/BMAL1 activity on the *Dbp* promoter. Such mode of interaction has been suggested before for mammals (Rossner et al., 2008) and for the *Per/Dec* orthologs *period* and *clockwork orange* of the *Drosophila* clock (Kadener et al., 2007). In vitro experiments on nonsynchronized cells, however, only poorly reflect the dynamics of an oscillatory system, and it has indeed been suggested that *Dec* function in E-box regulation may depend on clock phase (Rossner et al., 2008). A direct physical interaction of PER and DEC proteins has so far not been shown, but even though our transcription assay data indicate that E-box effects are sufficient to explain the period effects observed in *Per1/2-Dec2* mutant cells, other mechanisms may play a role or become even more important for the regulation of clock phase and stability or for explaining tissue specific effects of *Per/Dec* mutations.

CONCLUSION

The analysis of oscillatory behavior after combinatorial deletion of different genetic components of the biological clock, in particular in cells, may help us to better understand the complex interactions of the numerous components of the circadian clockwork (for a successful example in plants, see Locke et al.,

2005). In mammals this approach is hampered by the fact that multiple mutations frequently result in arrhythmicity, which then prevents further measurements (Cho et al., 2012; DeBruyne et al., 2007a; Oster et al., 2002). We show that in cells, *Per/Dec2* interaction is either synergistic (period and rhythm power) or nonsynergistic (phase) and highly tissue-specific. Given that in the SCN, *Dec1* and *Dec2* have partly redundant function (Honma et al., 2002; Rossner et al., 2008), it would be interesting to extend these studies to *Dec1* mutant cells. Many clock components specifically regulate biological processes outside circadian timekeeping (Bradshaw and Holzapfel, 2010; Franken et al., 2007; Rosenwasser, 2010; Sidman et al., 1962). Therefore, describing the modes of clock gene interactions in different tissues may be particularly useful to identify pharmacological targets for the tissue-specific intervention into clock-controlled as well as noncircadian physiological systems.

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CONFLICT OF INTEREST STATEMENT

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

NOTE

Supplementary online material is available on the journal's website at http://jbr.sagepub.com/supplemental.

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