Role of the nuclear receptor Rev-erb alpha in circadian food anticipation and metabolism

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Rôle du récepteur nucléaire *Rev-erbα* dans les mécanismes d'anticipation des repas et le métabolisme

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UNIVERSITÉ DE STRASBOURG

THÈSE

présentée à la Faculté des Sciences de la Vie et de la Santé de l'Université de Strasbourg pour l'obtention du grade de Docteur

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Soutenue le 29 juin 2012

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Rapporteur externe	Prof Urs Albrecht (Fribourg, Switzerland)
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A vous deux,

« Le Soleil envie la Terre comme la Terre envie le Soleil.
L'un reçoit la lumière et l'autre brille de mille feux. »
J.D.

Note to the reader

The work presented in this manuscript was carried out from 2008 to 2012 in the:

- Department of Neurobiology of Rhythms, Institute of Cellular and Integrative Neurosciences, University of Strasbourg, Strasbourg, France;

and in collaboration with other laboratories:

- Department of Ecology, Physiology, and Ethology, Hubert Curien Multidisciplinary Institute, University of Strasbourg, Strasbourg, France (short stay of three months);
- Valrose Institute of Biology, University of Nice, Nice, France (short stay of two months);
- Physiology of Reproduction and Behavior, François Rabelais University, French Institute of the Horse and Equitation, Nouzilly, France.

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Delezie J, Challet E. Interactions between metabolism and circadian clocks: reciprocal disturbances. Ann N Y Acad Sci. 2011;1243(1):30-46. *(Review)*

and in general-public journals:

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Delezie J, Challet E. La rythmicité circadienne au cœur du métabolisme. Biofutur 2011, 325, 31-34. *(French review)*

Challet E, ***Delezie J,*** Mendoza J. Circadian clocks and nutrition. Correspondances en Métabolisme Hormones, Diabètes et Nutrition 2009 May-June, Vol. XIII. N°3. *(French review)*

A few words in Shakespeare's language…

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Et un peu plus dans la langue de Voltaire…

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> Il a fallu que je fasse toute la route depuis mon p'tit bled perdu au fin fond de la [Lorraine], jusqu'à la grisaille de [Strasbourg], pour trouver l'amour de ma vie. Même en gambergeant pendant un million d'années, il me serait jamais venu à l'idée d'associer l'amour avec la capitale de l'[Europe]. Et aujourd'hui encore, ce qui a suivi ressemble à un rêve lointain. Pourtant ce rêve était bien vrai, et il a changé nos deux vies à tout jamais […]. *True Romance*

Abstract

Circadian oscillators rely on transcriptional mechanisms that regulate a wide array of biological activities. In mammals, the suprachiasmatic nucleus pacemaker is the chief conductor of the body that sets the tempo and dynamics through timekeeping signals to unify the timing of the different performers—the so-called secondary or peripheral oscillators. Each element of this biological orchestra shares common molecular mechanisms, in which clock genes such as *Per1-3*, *Cry1-2*, *Clock*, *Bmal1* and *Rev-erbα* are essential.

In this large multi-oscillatory system, one putative performer, the food-entrainable oscillator, has been shown to respond to the restriction of food availability to a specific time of the day. As a result, beyond the reach of the main conductor, the food-entrainable oscillator is able to adjust the phase and amplitude of a plethora of behavioral and physiological events controlled by secondary oscillators. This is especially highlighted by the ability of animals to predict feeding time, as evidenced by food-seeking behaviors a few hours prior to the expected food access. In addition, many circadian oscillators are specifically involved in metabolic functions for a proper energy balance. The tight relationship between the metabolic states of the body and functioning of circadian oscillators is particularly illustrated by the genetic perturbations of the molecular clockwork.

The work performed during this PhD thesis aimed at investigating the role of the transcriptional silencer *Rev-erbα* in both the circadian clockwork of the food-entrainable oscillator and metabolic regulations. Firstly, by evaluating food-anticipatory components in animals fed once a day at the same time, we showed that mice lacking *Rev-erbα* display a reduction in locomotor activity prior to food access compared to littermate controls. Accordingly, the rises in body temperature and corticosterone that anticipate mealtime are also diminished. Interestingly, daily p-ERK expression in hypothalamic regions and daily PER2 expression in the cerebellum of *Rev-erbα* KO mice are not phase-adjusted to feeding time. These results indicate that *Rev-erbα* participates in the integration of feeding signals and in food-seeking behaviors. Secondly, by investigating energy balance in fasted, normal chow or high-fat fed animals, we revealed that *Rev-erbα* KO mice exhibit greater reliance on lipid fuels as energy substrates, contributing to a mild hyperglycemic state. We also found that *Lipoprotein lipase (Lpl)* expression, important for fatty acid uptake and utilization, is strongly up-regulated in peripheral tissues of *Rev-erbα* KO mice, predisposing mice to obesity. In this regard, we uncovered a new molecular pathway that ties clock-driven *Lpl* expression to energy homeostasis. These findings highlight the significance of daily *Rev-erbα* oscillations to prevent the appearance of the metabolic syndrome.

In conclusion, we provide evidence that REV-ERBα may be a part of the food-entrainable oscillator clockwork that triggers food-anticipatory components, and represents a pivotal player to link the core clock machinery to metabolic pathways.

Introduction

Les rythmes biologiques sont une propriété fondamentale du vivant. En effet, la plupart des espèces animales présentent une rythmicité journalière (*p.ex.* rythme d'activité-repos). Cette rythmicité est la conséquence d'une adaptation de ces espèces aux variations périodiques de leur environnement (*p.ex.* cycle lumière-obscurité). La rythmicité des fonctions biologiques repose sur l'existence d'un système endogène composé d'oscillateurs – ou horloges – biologiques capables d'imposer une rythmicité circadienne (*circa* : environ, *dies* : jour) à de nombreux paramètres physiologiques et comportementaux, tels que les métabolismes lipidique et glucidique, les sécrétions hormonales (*p.ex.* corticostérone), la prise alimentaire ou le cycle veille-sommeil.

Chez les mammifères, l'horloge principale est localisée dans les noyaux suprachiasmatiques (NSC) situés à la base de l'hypothalamus. Bien que ces noyaux présentent des oscillations auto-entretenues proches de 24 h, ces dernières sont ajustées principalement par le cycle d'alternance jour/nuit. Plus exactement, l'activité des NSC est synchronisée (*c.-à-d.* remise à l'heure) par la lumière du jour, qui leur est transmise directement par la rétine via le tractus rétino-hypothalamique. A partir des NSC, l'information va être relayée vers différentes structures centrales (*p.ex.* l'hypothalamus) ou périphériques (*p.ex.* le foie) dont les activités seront à leur tour synchronisées sur 24 h par l'intermédiaire de voies neuronales et neuroendocrines. D'autres oscillateurs, dits secondaires, sont localisés dans différentes parties du cerveau et dans les organes périphériques (Fig. 1).

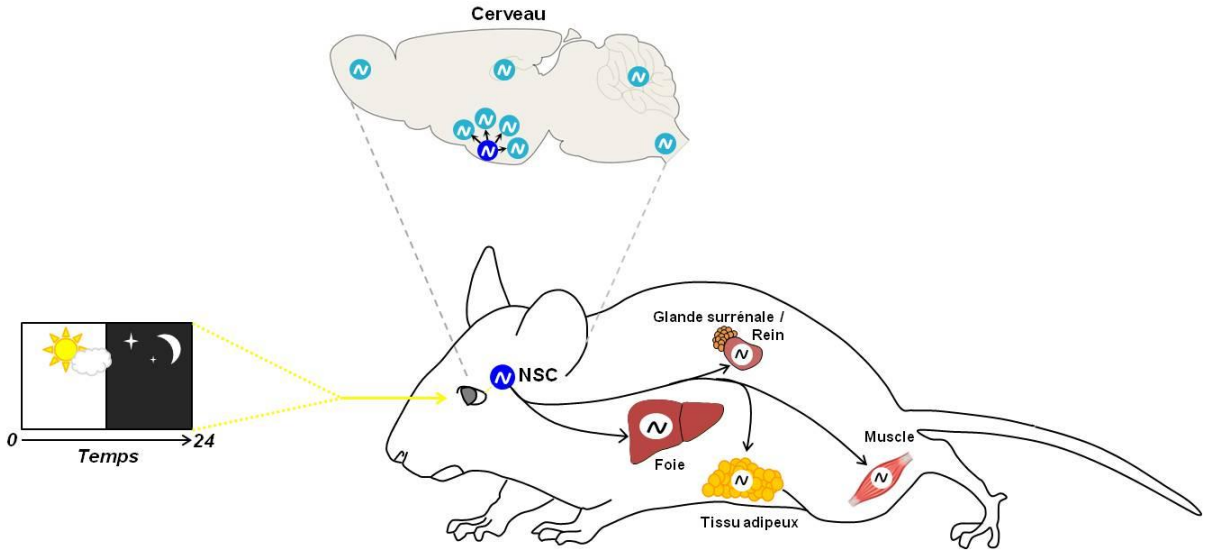


Figure 1. Les oscillateurs centraux et périphériques

L'activité cellulaire des noyaux suprachiasmatiques (NSC), situés à la base de l'hypothalamus, est synchronisée sur 24 h par l'alternance rythmique du jour et de la nuit. Ce message rythmique est ensuite distribué par les NSC à différents oscillateurs centraux (*c.-à-d.* dans le cerveau) ou périphériques (*p.ex.* le foie).

Sur le plan moléculaire, la genèse des rythmes circadiens est basée sur la coexpression de gènes spécifiques appelés « gènes d'horloges ». Parmi ces gènes, *Per1-3, Cry1-2, Bmal1, Clock, Rorα* et *Rev-erbα* constituent le cœur de la machinerie moléculaire des oscillateurs. La régulation des boucles moléculaires de rétroaction qui génèrent les oscillations circadiennes peut être décrite comme suit : le point de départ est une première boucle positive qui introduit deux facteurs de transcription, CLOCK et BMAL1. Ces facteurs dimérisent et vont transloquer dans le noyau, puis se fixer à l'ADN et initier, via une séquence E-box, la transcription des gènes *Per1-3, Cry1-2, Rorα* et *Rev-erbα*. La seconde boucle dite négative, est formée par les protéines PER et CRY qui, lorsqu'elles s'accumulent dans le cytosol, s'associent et vont transloquer à leur tour dans le noyau, au sein duquel le complexe PER:CRY inhibe sa propre transcription en interagissant avec le dimère CLOCK:BMAL1. Une boucle supplémentaire fait intervenir les récepteurs nucléaires RORα et REV-ERBα, qui ont respectivement un effet activateur et inhibiteur sur la transcription de BMAL1 via une séquence RORE. Cette boucle additionnelle représente ainsi le lien entre lesdites positive et négative et permet d'assurer la précision des oscillations circadiennes. Le fonctionnement de ces boucles conduit par conséquent à un rythme d'expression de différents ARNm et protéines qui varient sur 24 h (Fig. 2).

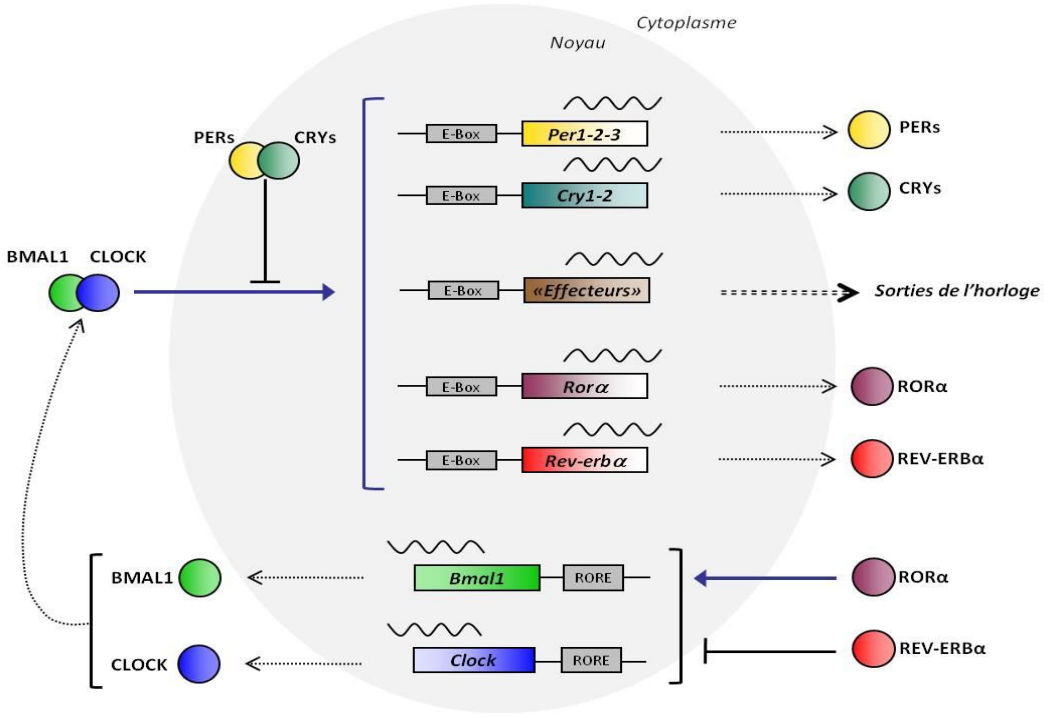


Figure 2. Modèle cellulaire simplifié de la rythmicité circadienne

Les oscillations moléculaires des gènes d'horloges se déroulent sur 24 h grâce à un jeu réciproque d'activation (flèches bleues) et d'inhibition (flèches noires) de la transcription.

Comme introduit plus haut, les NSC, en tant que chef d'orchestre, communiquent avec l'ensemble de l'organisme pour ajuster de nombreuses fonctions biologiques sur 24 h. La destruction des NSC chez le rongeur induit une arythmie comportementale parallèle à une abolition du rythme de prise alimentaire, de température ou de corticostérone. Les NSC ont des connexions directes avec différents noyaux situés en particulier dans l'hypothalamus et qui contrôlent le cycle veille-sommeil ou encore la prise alimentaire (*p.ex.* le noyau dorsomédian). Les NSC peuvent aussi recevoir des informations du noyau ventromédian, de l'aire hypothalamique latérale ou du noyau arqué, qui jouent des rôles clés dans le métabolisme énergétique. Ce réseau de communication sous le contrôle des NSC, permet dès lors l'organisation rythmique de fonctions comportementales et métaboliques.

Les gènes d'horloges susnommés ne sont pas seulement exprimés dans les NSC mais sont potentiellement présents dans tous les tissus, toutes les cellules de l'organisme. Leur expression a été particulièrement décrite dans le foie, au sein duquel ils contrôlent localement environ 10% du transcriptome. Parmi les 49

récepteurs nucléaires identifiés chez la souris, acteurs moléculaires essentiels pour la régulation du métabolisme, environ 40% d'entre eux ont une expression qui varie au cours du cycle lumière-obscurité, en particulier dans le foie et le tissu adipeux. Au sein de cette famille de récepteurs nucléaires, des données récentes suggèrent que le gène *Rev-erbα* est un acteur essentiel des rouages des horloges biologiques et du métabolisme.

En effet, des souris pour lesquelles le gène *Rev-erbα* a été invalidé, montrent un phénotype circadien altéré, en particulier une période endogène réduite, ainsi qu'une réponse à la synchronisation photique augmentée. Ceci indique clairement que la machinerie moléculaire de l'horloge principale des NSC est affectée en l'absence de *Rev-erbα*. De la même manière, ces souris présentent des modifications sur le plan métabolique, notamment un niveau élevé de cholestérol et de triglycérides. En outre, *Rev-erbα* a été impliqué dans la sécrétion biliaire et le métabolisme des lipides en contrôlant l'expression du gène *Srebp1c*.

Objectifs et résultats – partie 1

La première partie de mon travail de thèse a été de définir le rôle joué par le récepteur nucléaire *Rev-erbα* dans les mécanismes de synchronisation par la nourriture d'une horloge circadienne putative, non encore localisée, appelée « horloge alimentaire ». Cette dernière – si tant est que ce soit une structure unique – semble permettre aux animaux d'exploiter les régularités temporelles de la disponibilité en nourriture dans leur environnement, même en l'absence de l'horloge principale des NSC. Ainsi, différentes espèces sont en mesure de rechercher des ressources alimentaires au moment le plus propice de la journée. En laboratoire, des rongeurs placés en condition de restriction alimentaire temporelle (*c.-à-d.* un accès à la nourriture limité à quelques heures par jour et à heure fixe) montrent une augmentation d'activité locomotrice quelques heures avant l'heure du repas, en parallèle à une augmentation de température et de sécrétions hormonales. Ces sorties physiologiques et comportementales seraient donc le reflet d'un mécanisme adaptatif lors de périodes de raréfaction des ressources alimentaires. Il est fait l'hypothèse que le mécanisme moléculaire à l'origine de cette horloge permettant la

synchronisation alimentaire (*c.-à-d.* à l'heure des repas) serait similaire à celui des NSC.

Afin d'explorer davantage les rouages moléculaires de cette horloge alimentaire, nous avons étudié des souris déficientes (ou knockout ; KO) pour le gène d'horloge *Rev-erbα*. De manière intéressante, en condition de lumière-obscurité 12 h - 12 h, l'activité locomotrice ainsi que l'augmentation de température en anticipation de l'heure du repas observées chez des souris témoins, étaient diminuées chez des souris KO pour *Rev-erbα* (Fig. 3). Ce résultat a également été observé chez des souris KO placés en condition d'obscurité totale, ou porteuses de lésions des NSC. En parallèle à une diminution de l'activité locomotrice et de la température corporelle, le pic de libération de corticostérone en anticipation de l'heure du repas présent chez les souris sauvages, n'était pas observé chez les souris KO pour *Rev-erbα* ; indiquant qu'une sortie physiologique additionnelle contrôlée par l'horloge alimentaire était altérée chez ces dernières.

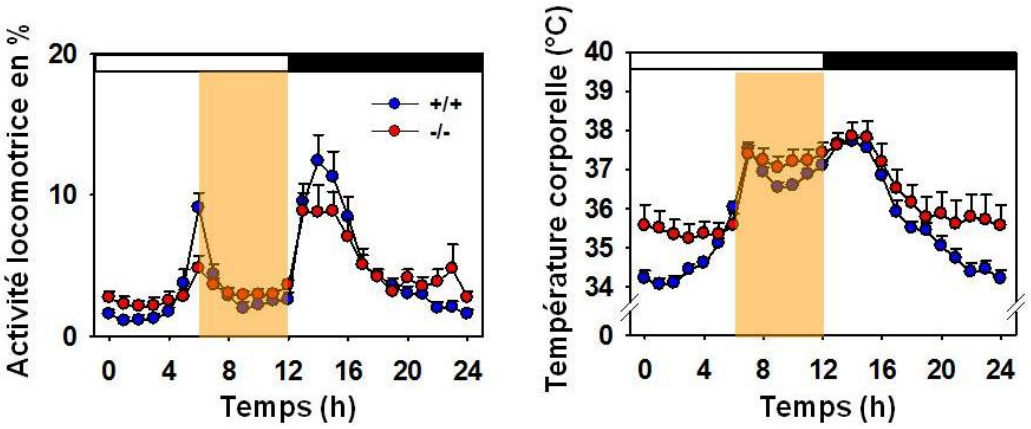


Figure 3. Activité locomotrice de roue (à gauche) et température corporelle (à droite) en condition de restriction alimentaire

Quelques heures précédant l'accès à la nourriture (rectangle orange), les souris témoins (en bleu) présentent une forte augmentation de l'activité locomotrice, en parallèle à une élévation de leur température corporelle. Ces composantes d'anticipation sont diminuées chez les souris KO (en rouge).

Toutefois, la synchronisation de l'oscillateur hépatique à l'heure du repas était conservée chez les souris KO (Fig. 4). De la même manière, les oscillations de la protéine d'horloge PER2 dans les structures hypothalamiques était modifiée sous l'effet de la restriction temporelle dans les deux génotypes. Autrement dit, la synchronisation des oscillateurs hypothalamiques et de l'oscillateur hépatique à

l'heure des repas, était préservée en l'absence du gène d'horloge *Rev-erbα*. Cependant, ni PER2, ni p-ERK (un marqueur d'activation cellulaire) ne répondaient clairement à la restriction dans le cervelet des souris KO.

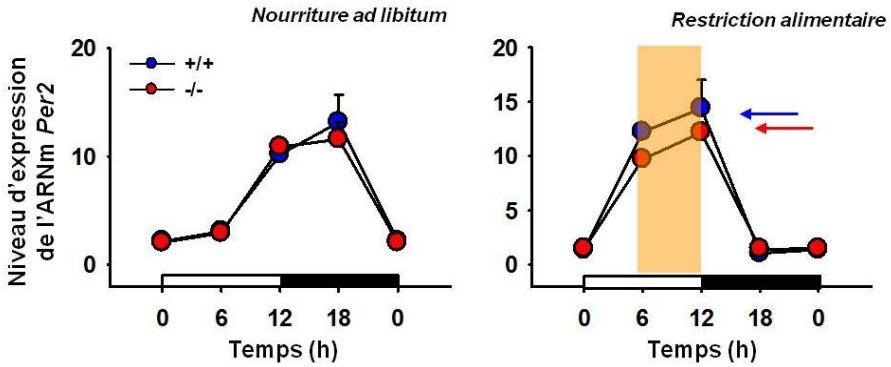


Figure 4. Expression de l'ARNm *Per2* dans le foie

La restriction alimentaire temporelle induit un changement de phase du rythme de *Per2* dans les deux génotypes.

L'ensemble de ces résultats suggèrent un rôle essentiel de *Rev-erbα* dans le fonctionnement de cette horloge alimentaire qui pourrait, parmi les régions cérébrales étudiées, faire intervenir le cervelet dans la modulation de l'expression de l'activité anticipatrice. Néanmoins, du fait de la participation de *Rev-erbα* aux régulations du métabolisme, nous avons évalué des paramètres métaboliques comme le niveau de glycémie ou de glycogène hépatique. De manière inattendue, nous avons découvert que les souris KO avaient des niveaux plus élevés de glucose et de glycogène hépatique en comparaison des souris témoins, indépendamment de la condition de nourrissage (Fig. 5). De plus, en période de jeûne induite par la restriction temporelle, les KO montraient des valeurs de température corporelle nettement supérieures à celles de souris témoins (Fig. 3). Ces observations rendaient compte de changements non négligeables dans l'homéostasie énergétique des souris déficientes en *Rev-erbα* qui pourraient contribuer à l'altération des sorties comportementales et physiologiques de l'horloge alimentaire.

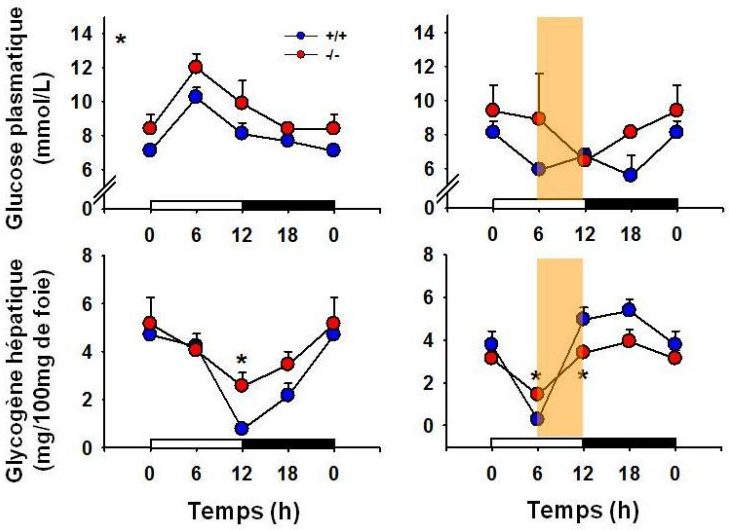


Figure 5. Rythme de la glycémie (haut) et du glycogène hépatique (bas)

En période de nourrissage *ad libitum* (gauche) ainsi qu'en période de restriction alimentaire (droit), les niveaux de glucose plasmatique et de glycogène hépatique sont plus élevés chez les souris KO (en rouge) en comparaison aux souris contrôles (en bleu).

Objectifs et résultats – partie 2

La seconde partie de mon travail de thèse a dès lors consisté à étudier le phénotype métabolique des souris KO pour *Rev-erbα*. Par une association originale d'approches *in vivo* et *in vitro* pour l'exploration des métabolismes glucidique et lipidique, nous avons pu mettre en évidence que le gène *Rev-erbα* joue un rôle essentiel dans la balance énergétique. De manière remarquable, les souris KO ont une plus grande adiposité et sont hyperglycémiques, même si elles mangent la même quantité de nourriture standard (ou normocalorique), au même moment de la journée et avec une activité physique similaire aux souris témoins. En outre, l'hyperglycémie a également été observée après un jeûne de 24 h (Fig. 6), et n'est pas la conséquence d'un défaut de sécrétion d'insuline ou d'une insulino-résistance – comme le confirment notamment les résultats du clamp euglycémique hyperinsulinémique (Fig. 6).

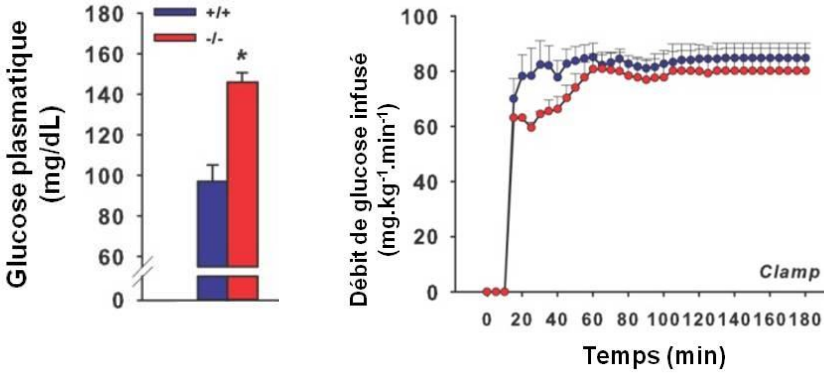


Figure 6. Concentration de glucose plasmatique après une période de jeûne de 24 h (à gauche) et clamp euglycémique hyperinsulinémique (à droite)

Les souris KO (en rouge) montrent une hyperglycémie même après une période de jeûne.

La différence majeure entre les souris sauvages et les souris déficientes en *Rev-erbα* réside dans la manière dont ces dernières métabolisent les nutriments : en effet, nos données de calorimétrie indirecte ont révélés que les KO synthétisent plus de lipides pendant la phase de prise alimentaire (*c.-à-d.* phase nocturne) contribuant à une surchage lipidique, et utilisent moins de glucides pendant la phase de repos (Fig. 7), contribuant au maintien d'une légère hyperglycémie sur le cycle de 24 h. De façon intéressante, cette augmentation de l'utilisation des lipides (ou des corps cétoniques) en l'absence de *Rev-erbα* est également observée lors d'une période de jeûne de 24 h.

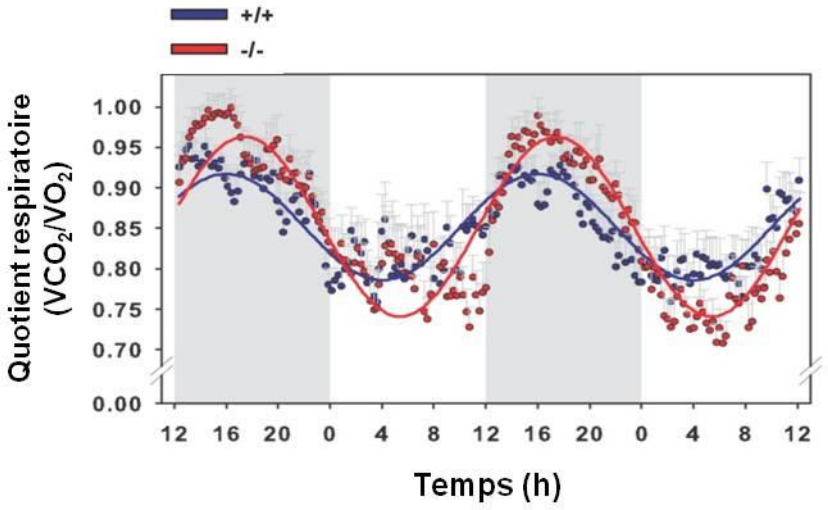


Figure 7. Calorimétrie indirecte

Les souris KO (en rouge) métabolisent davantage les glucides de nuit et les lipides de jour, en comparaison aux souris contrôles (en bleu).

En outre, nous avons montré que le phénotype « gras » est très accentué lorsque les KO sont sous nourrissage hypercalorique (*c.-à-d.* riche en graisses ; Fig. 8). Cette obésité induite par la nourriture va conduire à de multiples altérations métaboliques. Des modifications de la quantité et du rythme d'expression de la glycémie, de l'insuline, de la leptine (Fig. 8) et des acides gras libres sont également constatées de manière importante chez les souris KO. Les oscillateurs périphériques tels que le foie et le tissu adipeux, sont également touchés par une surcharge lipidique. Notamment, l'expression hépatique de gènes de la lipogenèse est augmentée chez les souris KO.

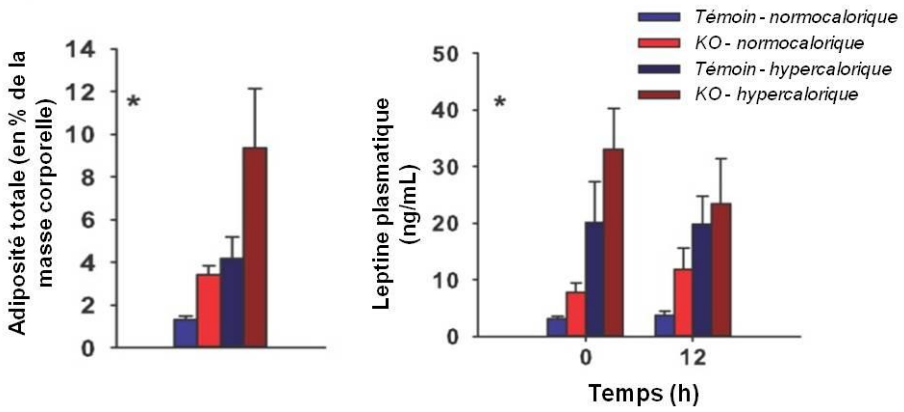


Figure 8. Adiposité totale (à gauche) et concentration de leptine (à droite)

Indépendamment de la condition de nourrissage (normo- ou hypercalorique), les souris KO ont une plus grande adiposité corrélée à de plus grandes concentrations de leptine.

Enfin, nous avons démontré que le phénotype « gras » des souris KO résultait également d'une perte de contrôle par les oscillateurs périphériques, en l'absence de REV-ERBα, de l'expression d'un gène clé du métabolisme, la *Lipoprotéine lipase* (*Lpl*), intervenant dans l'hydrolyse des triglycérides et l'entrée des acides gras libres dans les cellules. En effet, le gène de la *Lpl* était surexprimé dans différents tissus périphériques tels que le foie, le muscle et le tissu adipeux (Fig. 9). Cette surexpression notamment dans le tissu adipeux et le muscle conduit respectivement à un meilleur stockage et à une utilisation préférentielle des acides gras.

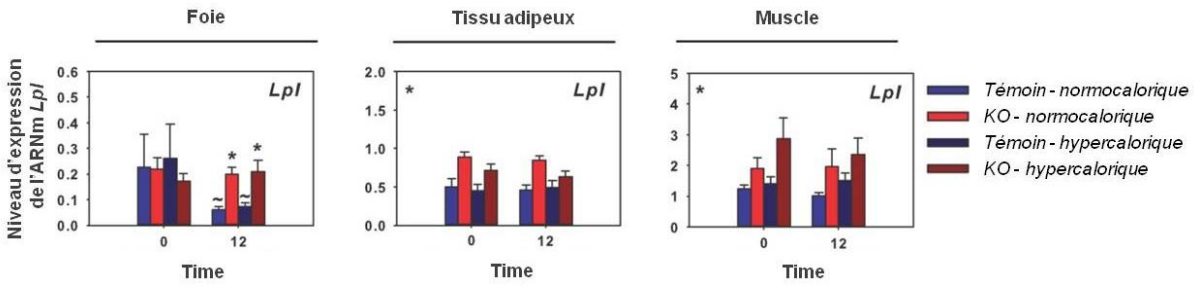


Figure 9. Expression du gène de la *Lipoprotéine lipase* dans différents tissus

L'ARNm de la *Lpl* est surexprimé à différents temps dans les organes périphériques des souris KO pour *Rev-erbα.*

De façon intéressante, nous avons pu révéler que la régulation de l'expression de l'ARNm de la *Lpl* n'impliquait pas directement le répresseur transcriptionnel REV-ERBα mais l'activateur transcriptionnel CLOCK (Fig. 10), qui en l'absence de REV-ERBα *in vivo* démontrait des niveaux d'expression d'ARNm supérieurs à ceux mesuraient chez des souris témoins.

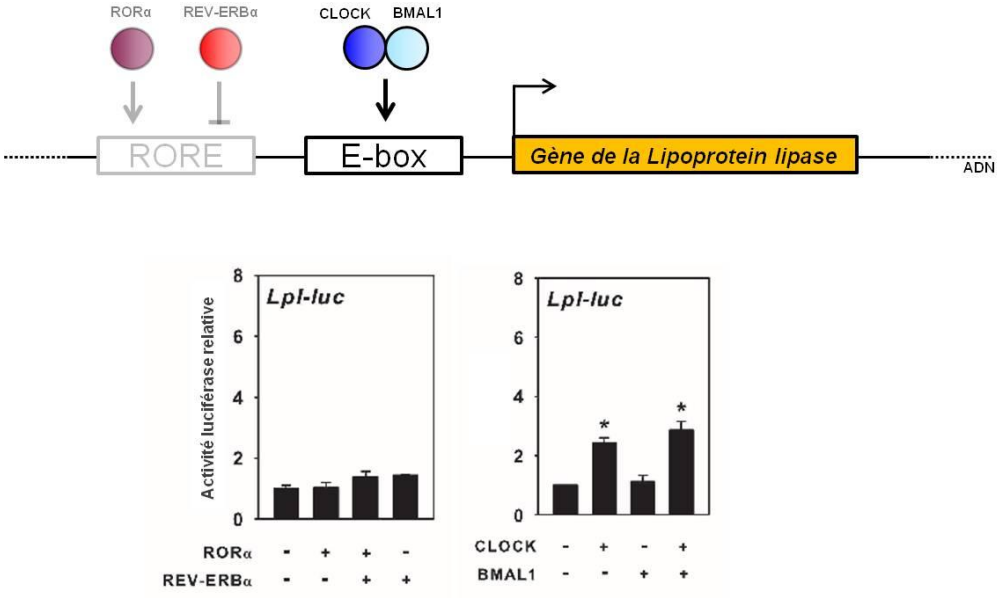


Figure 10. Dosage de l'activité luciférase

L'analyse des séquences promotrices du gène de la *Lpl* a mis en évidence une E-box fonctionnelle qui peut interagir avec le facteur CLOCK.

Conclusion

L'ensemble de nos données indique que le répresseur transcriptionnel *Rev-erbα* joue un rôle charnière dans les fonctions circadiennes ainsi que dans le métabolisme. En effet, d'un point de vue circadien, l'absence de *Rev-erbα* altère la synchronisation à l'heure des repas – démontré par une réduction des sorties comportementales et physiologiques de l'horloge alimentaire, ainsi que par l'absence d'ajustement du rythme de PER2 dans l'oscillateur cérébelleux. Sur le plan métabolique, la délétion de ce gène modifie notamment le métabolisme des lipides – démontré par une accumulation excessive de tissu adipeux, une utilisation préférentielle des acides gras, ainsi qu'une perte de contrôle de l'expression de la *Lpl*.

Néanmoins, la possibilité que la diminution de la capacité d'anticiper l'heure des repas soit essentiellement reliée aux modifications de la balance énergétique ne peut pas être exclue. Cette hypothèse qui donnerait dès lors un rôle moindre aux perturbations du cœur de la machinerie moléculaire circadienne induites par l'absence même du gène d'horloge *Rev-erbα*, est soutenue par la synchronisation de l'oscillateur hépatique à l'heure des repas, ainsi que par l'ajustement dans divers oscillateurs hypothalamiques de l'expression de la protéine d'horloge PER2 en condition de restriction alimentaire.

En conclusion, au-delà de positionner *Rev-erbα* à l'interface entre régulations circadiennes et métaboliques, ces travaux indiquent l'importance de prendre en considération les modifications du métabolisme induites par la délétion d'un gène d'horloge – notamment dans un organisme entier – pour l'étude des fonctions circadiennes telles que l'entraînement à l'heure des repas. En ce sens, l'utilisation d'outils génétiques permettant de désactiver spécifiquement l'expression d'un gène d'horloge dans un tissu donné ou à un stade précis du développement pourrait être une méthode de choix pour mieux explorer les rouages de cette mystérieuse horloge alimentaire.

α-MSH: alpha-melanocyte-stimulating hormone
Acc: acetyl-Coenzyme A carboxylase alpha
ACTH: adrenocorticotropic hormone
AgRP: agouti-related peptide
AL: *ad libitum*
AMPK: adenosine monophosphate-activated protein kinase
AMY: amygdala
ApocIII: apolipoprotein CIII
ARC: arcuate nucleus
AVP: arginin vasopressin
BAT: brown adipose tissue
Bmal: *brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like*
BNST: bed nucleus of the striata terminalis
cAMP: cyclic adenosine monophosphate
Cd36: cluster of differentiation 36
CKI: caseine kinase I
Clock: *circadian locomotor output cycles kaput*
Cpt1a: carnitine palmitoyltransferase 1A (liver)
Cpt1b: carnitine palmitoyltransferase 1B (muscle)
CRB: cerebellum
CRE: cAMP responsive element
Cry: *cryptochrome*
DMH: dorsomedial nucleus of the hypothalamus
Elovl6: ELOVL fatty acid elongase 6
FAA: food-anticipatory activity
Fas: fatty acid synthase
FAT: food-anticipatory thermogenesis
FEO: food-entrainable oscillator
G6pc: glucose-6-phosphatase, catalytic subunit
GABA: gamma aminobutyric acid
Gk: glucokinase
Glut2: glucose transporter 2
Glut4: glucose transporter 4
GRP: gastrin-releasing peptide
Gsk3b: glycogen synthase kinase 3 beta
HFD: high-fat diet
i.c.v: intracerebroventricular
i.p: intraperitoneal
ipRGC: intrinsically photosensitive retinal ganglion cell
KO: knockout
LDL: low-density lipoprotein

LH: lateral hypothalamus
Lpl : lipoprotein lipase
MAP: methamphetamine
mRNA: messenger ribonucleic acid
MyHC: myosin heavy chain
NAc: nucleus accumbens
NAD: nicotinamide adenine dinucleotide
NAMPT: nicotinamide phosphoryl-transferase
NEFA: nonesterified fatty acid
Npas: *neuronal PAS*
NPY: neuropeptide Y
NTS: nucleus of the solitary tract
PACAP: pituitary adenylate cyclase-activating polypeptide
PAS: period-arnt-sim
Pepck: phosphoenolpyruvate carboxykinase 2 (mitochondrial)
Per: period
Pgc1: peroxisome proliferator-activated receptor gamma coactivator 1
Ppara: peroxisome proliferator-activated receptor alpha
Pparg: peroxisome proliferator-activated receptor gamma
PVN: paraventricular nucleus of the hypothalamus
PVT: paraventricular nucleus of the thalamus
Rev-erb: reverse viral erythroblastis oncogene product
RF: restricted feeding
Ror: *retinoic acid-related orphan receptor*
RORE: REV-ERB/ROR Response element
RQ: respiratory quotient
SCN: suprachiasmatic nucleus of the hypothalamus
Sirt: sirtuin
Srebp1c: sterol regulatory element binding transcription factor 1
TG: triglycerides
Tr: thyroid receptor
Ucp: uncoupling protein
VIP: vasoactive intestinal peptide
VLDL: very low density lipoprotein
VMH: ventromedial nucleus of the hypothalamus
WAT: white adipose tissue
WT: wild-type
ZT: zeitgeber time

Table of content

Chapter 1 GENERAL INTRODUCTION

1. Beats, rhythms and life[1]

1.1 Introduction

Dynamic cyclic events and the steady recurrence of identical rhythm patterns are a common feature of both music and life. Various characteristic and repetitive pulses can be identified in different kinds of music, such as hip-hop, blues or classical music, just as we can observe various regular recurring events in nature. Some occur at a high frequency (e.g., human heartbeats) and others at a much slower frequency (e.g., annual migration). The idea that life is organized rhythmically, which has been originally hypothesized a long time ago (see part 1.2), paved the way for the development of a discipline of research studying biological rhythms, called chronobiology (from the greek: *chrónos*, meaning "time"; *bios*, "life" and *-logia*, "study of").

The historical emergence of this relatively new scientific research area radically changed our way of seeing living creatures, from basic organisms such as algae to more complex organic systems such as human beings. Since the last century, the study of biological rhythms is turning out to be one of the most fascinating interdisciplinary research fields in modern biology, receiving a vast amount of attention from other disciplines. I will present first the brilliant thoughts and discoveries that gave birth to chronobiology. Then, I will progressively introduce the different concepts and terminology used in our research area and show that biological rhythms can vary according to their duration and can have very complex and fascinating beats, the same as rhythmic patterns in music.

1.2 *Horologium Florae*

The observation of rhythmic events in living species comes first from description on leaf movements, which has been probably known over 2300 years ago (mentioned in Wolfgang Engelmann, 2002). Daily periodic movements of the leaves

[1] This title is freely inspired from "Beats, Rhymes and Life*",* the fourth album of the famous hip-hop groug "A Tribe Called Quest".

of the tamarind tree, *Tamarindus indica*, were initially and clearly reported by Androsthenes of Thasos, admiral under Alexander the Great, 400 years before Christ (mentioned in Bretzl, 1903). Much later, in 1729, the French astronomer Jean Jacques Ortous de Mairan observed the daily opening and closing of the leaves of a sensitive heliotrope plant (probably *Mimosa Pudica*)[2]. de Mairan then performed an experiment to test whether this biological "behavior" was simply a response to the daily appearance of the sun. To do so, he confined the plant to the dark (i.e., a constant condition of darkness). He observed that in the absence of sunlight, the leaves of this plant opened during the usual daylight hours and closed at night. Even if de Mairan did not conclude that *Mimosa Pudica* has an internal clock, his experiment was the first scientific demonstration of the *persistence* of a rhythmic event in constant darkness.

It took 30 years before further scientific investigations on this biological mechanism in plants followed de Mairan's observation. Indeed, independent researches by Duhamel Du Monceau and Zinn in 1759 (for review, see McClung 2006), showed that the rhythm of leaf movements in constant darkness was independent of fluctuations in environmental temperature. In other words, this "clock" mechanism appeared to be *endogenous*. In 1832, De Candolle gave more details on the period of this inner clock. He showed that in constant light conditions, opening and closing rhythm of the leaflets of a sensitive plant (e.g., *Mimosa*), was maintained with a period close to, but not exactly, 24 h. To make use of circadian terminology, this signifies that the endogenous period of *Mimosa*'s clock runs free (or *free-runs*[3]) with its own timing (Fig. 1). De Candolle also showed that the rhythm could be inverted by manipulating the light-dark cycle. Later on, Darwin tried to understand the selective advantage of the periodic leaves movements (mentioned in Bunning and Moser 1969).

These pioneering observations paved the way for further studies focusing on biological rhythms, and thus the establishment of the discipline of chronobiology.

[2] The botanical observations of de Mairan would have passed into obscurity, if his colleague, M. Marchand, had not published the results for him.

[3] "Free running" being a term used to describe a circadian rhythm that is not entrained to external time cues.

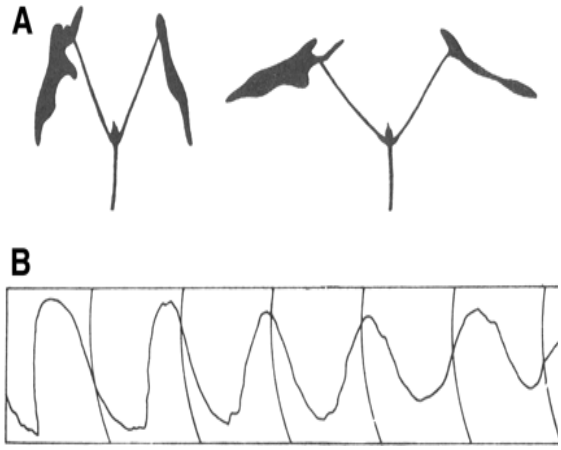


Figure 1. Leaf movements of a representative species

(A) Sleep movements of *Phaseolus coccineus*. The position of the primary leaves of a seedling at night is at the left and during the day is at the right. (B) Circadian rhythm of leaf movements of *P. coccineus* entrained to light/dark cycles and monitored in continuous light. As can be inferred from the leaf positions in (A), the peaks of the curve represent the nightime leaf position. The vertical lines indicate 24-h intervals. The period for this trace is ~27 h. From (McClung 2006)

1.3 Chronobiology, modern times

In the 20th century, chronobiology became its own discipline of research (in parallel to evolution, genetics and development). Observations of rhythmic events became numerous, particularly in animals. For example, the psychologist JS Szymanski reported in 1914 that goldfish swimming occurs with a 24-h activity rhythm, even in the absence of external cues such as light. In 1922, Curt Richter reported daily pattern of locomotor activity in rats. In 1937, researchers created an international organization for the study of biological rhythms (for review, see Koukarri)[4]. At the same time, brilliant and leading figures in chronobiology were influential in establishing many concepts and key criteria to define the biological clock.

1.3.1 Halberg, "Circadian" rhythm

In the 1950s, Franz Halberg—regarded as the father of chronobiology—observed several kinds of differences among groups of mice in the counts of circulating blood eosinophils when they were sampled at different clock hours. He also noticed that after blinding, rectal temperature in mice showed an approximately 24-h periodicity. He created the term *circadian*, which comes from the Latin *circa*, meaning "around" and *dies*, "day" (Halberg et al. 1977; Halberg et al. 2003). As a result, biological phenomena observed with a roughly 24-h period (i.e., from 20 to 28 h) were so named.

[4] Research on biological rhythms was strong and colorful in Germany and the rest of Europe in the 18th and 19th centuries. In the 20th century, in spite of the prevalent skepticism of scientific establishment in the USA, chronobiology no longer hid, became popular and conceptual advances were flourishing. In the sense, the 1960 symposium at Cold Spring Harbor Laboratory laid the groundwork for the field of chronobiology.

Circadian: relating to biologic variations or rhythms with a frequency of 1 cycle in 24 ± 4 h; circa (about, approximately) and dies (day or 24 h). Note: term describes rhythms with an about 24-h cycle length, whether they are frequency-synchronized with (acceptable) or are desynchronized or free-running from the local environmental time scale, with periods slightly yet consistently different from 24 h … (Halberg, 1977)

In spite of further extensive studies of circadian rhythms, many other important cycles were concurrently delineated and studied. This includes *infradian* rhythms, which show a period longer than 24-h and can occur at monthly, seasonal or yearly intervals (e.g., annual migration); and *ultradian* rhythms, which occur with a period shorter than 24-h (e.g., thermoregulation). It is important to mention that within each cycle, the time at which the peak of a rhythm occurs is called the "acrophase", whereas the time at which the trough of a rhythm occurs is the "bathyphase". The difference between the peak (or trough) and the mean value (mesor) of a wave is called the amplitude (Fig. 2)[5].

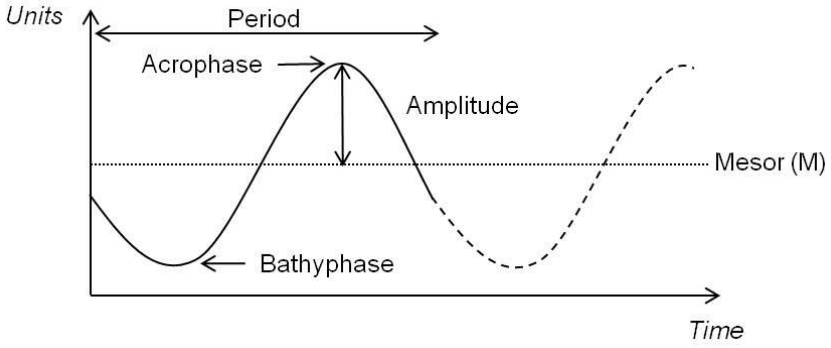


Figure 2. Terms used to describe circadian rhythms.

The period is defined as the time to complete one cycle. The mesor is the average value around which the variable measured oscillates. The amplitude of the rhythm is defined as the distance between the mesor and the acrophase (or half the peak-to-peak amplitude). Within each cycle, the acrophase and the bathyphase represent the time point where the parameter measured shows the highest or the lowest value, respectively.

Whether we measure, hour by hour, the number of dividing cells in any tissue, the volume of urine excreted, the reaction to a drug, or the accuracy and the speed with which arithmetical problems are solved, we usually find that there is a maximum value at one time of day and a minimum value at another. (Aschoff 1965)

[5] Acrophase and other parameters of a rhythm are defined by a mathematical model, usually a cosine function.

1.3.2 Aschoff, light is a Zeitgeber

Jürgen Aschoff, one of the brilliant founders of chronobiology, used for the first time in 1954 the German term *Zeitgeber* ("time giver" or "synchronizer") to define an exogenous (external) cue that triggers some sort of change in an organism's endogenous clock. Light is the most prominent entraining agent for a wide variety of species, but other, so-called non-photic *Zeitgebers* such as food, which will be discussed further, can influence internal clocks. In 1960, Aschoff observed that an increase in the intensity of light in constant (light) conditions causes a lengthening of the free-running period for a nocturnal (dark-active) organism and a shortening of the period for a diurnal (light-active) organism[6] (Aschoff 1960). He also contributed in unraveling the physiological mechanisms that regulate circadian rhythms in birds, mammals and especially in humans (Chandrashekaran 1998). Indeed, Aschoff & Wever from 1962 demonstrated that in the absence of environmental cues (e.g., light) human's endogenous clock free runs with a period slightly longer than 24 h (Aschoff 1965)—an observation also made by the French explorer and scientist, Michel Siffre (1964), when he isolated himself in a deep cave without time cues for several months.

1.3.3 Bünning, the heritability of circadian rhythms

In the early 1930s, Erwin Bünning, a great biologist and botanist, demonstrated the inheritance of circadian rhythms. In 1932, Bünning started to cross bean plants with different endogenous periods and demonstrated that the next generation had periods of intermediate duration, thus refuting the idea that daily rhythmicity is *learned,* as first argued earlier by Richard Semon (1904). In 1935, Bünning undeniably determined in plants the genetic origin of the "biological clock," a term he coined. However, it took several years until the existence of a genetic-basis for circadian rhythms was firmly admitted. Bünning also laid out the basis of photoperiodism. He suggested that plants display endogenous phases of light and dark sensitivity, important in the photoperiod response of flowering (mentioned in Koukkari and Sothern 2006).

[6] A phenomenon extended and called now "Aschoff's Rule".

1.3.4 Pittendrigh, temperature compensation

The biologist Colin Pittendrigh (1918-1996) "*brought to chronobiology a degree of experimental elegance and rigour which remained unsurpassed"* (Chandrashekaran 1998). Work by Pittendrigh confirmed Bünning's hypothesis that a rhythmic process underlies the photoperiodic time-measurement. He defined several key criteria (highlighted below) a biological clock must have to be so called. His scientific research on *Drosophila pseudoobscura* in the early 1950s[7], demonstrated that daily rhythm in eclosion *persists* in constant conditions and can be *entrained* by light cycles close to the flies' endogenous period; and more importantly, that the period of eclosion remains relatively constant when exposed to changes in environmental temperature. In other words, the eclosion rhythms of *Drosophila* were *temperature compensated*, unlike the rates of most chemical reactions (Pittendrigh 1954).

In 1958, Pittendrigh also developed the concept of the phase response curve (PRC), which predicts how the biological clock would be affected by a change in its light schedule (Pittendrigh 1954; Menaker 1996; McClung 2006). It was also in 1960 that Patricia DeCoursey demonstrated on a time graph a daily light sensitivity rhythm of locomotor activity in rodent by testing the effects of light pulse at different times of the day (De Coursey 1960). The PRC is now a powerful and precise tool for the study of circadian rhythms (Fig. 3-4).

[7] As an aside, it is important to mention that it was also in the 50's that Gustav Kramer and Klaus Hoffman demonstrated that starlings, which use the sun to navigate, possess an internal clock, important to compensate for the sun's movements throughout the day, and thus essential for migration.

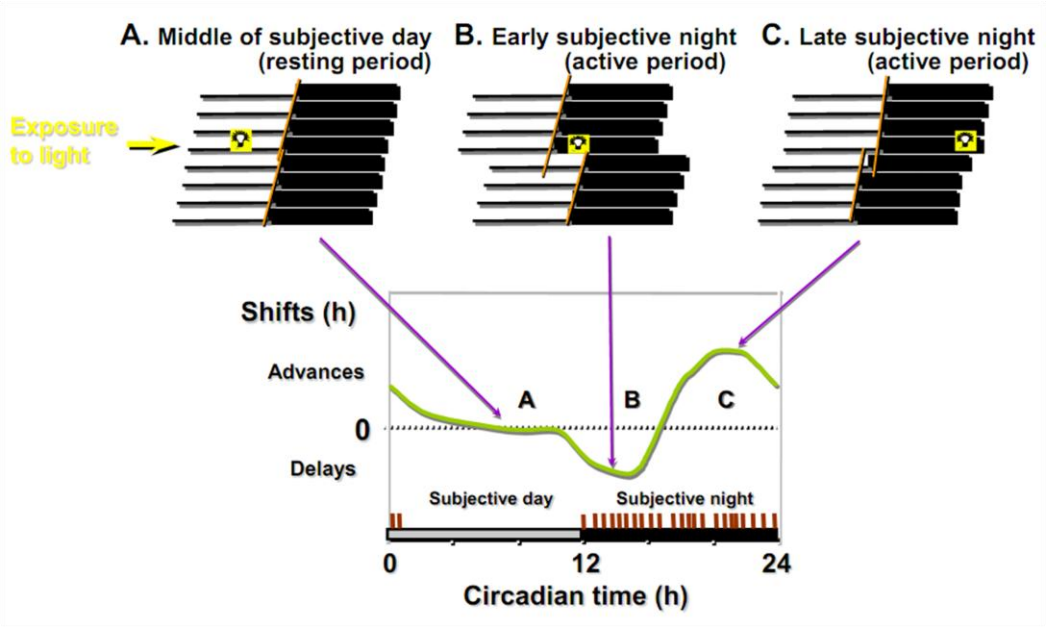


Figure 3. Light-pulse phase response-curve of a nocturnal animal

Animals are kept in constant darkness. The free-running activity can be phase shifted by light-pulses in a phase-dependent manner. (A) When light-pulse is given in the middle of the subjective day (light/rest-phase for a nocturnal animal), there is no strong effect on the onset of an animal activity. Whereas, light-pulses administrated at the beginning of the subjective night (i.e., dark/active phase for a nocturnal animal), phase-delayed the activity rhythm, while light-pulse delivered at the end of the active period caused phase-advance. Note that “subjective” day/night are defined by the segments of inactive and active period, respectively, during entrainment by a light-dark cycle.

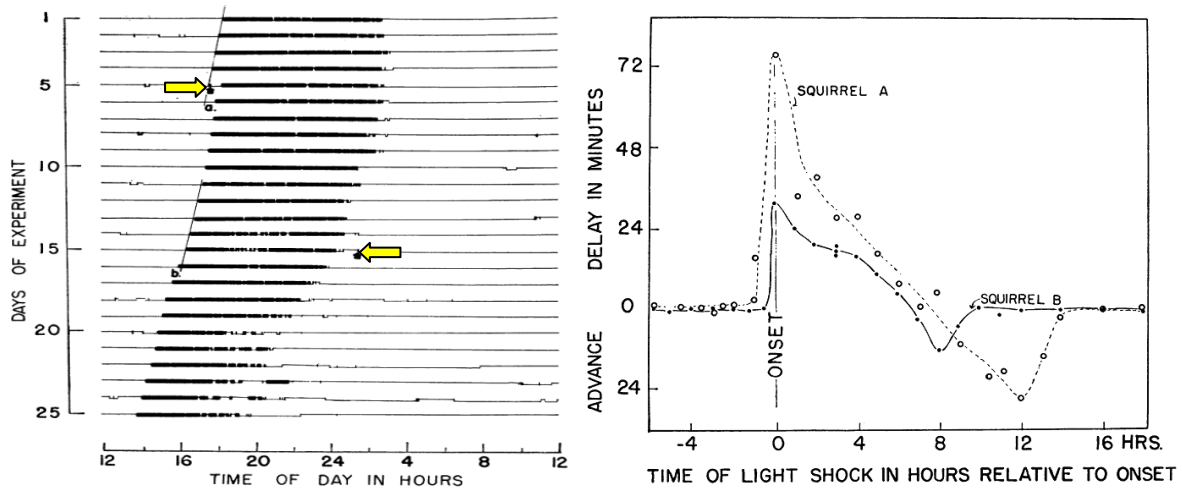


Figure 4. Light-pulse phase response-curve of a flying squirrel rodent

(*Left*) 24-h activity record, called actogram, of a flying squirrel. The first yellow arrow indicated the first light-pulse, which was given 1 min after the onset of the daily activity period and which caused a phase-delay. The second yellow arrow points to a ligh-pulse that was given 9 h after the onset of activity, which caused a phase-advance of the locomotor activity the following day. (*Right*) Light-reponse curves for two flying squirrels, A and B. Adapted from (De Coursey 1960).

1.4 Take-home message

In living creatures, most essential biological functions show a rhythmic pattern close to 24 h. These endogenous daily variations, called circadian rhythms, allow organisms to anticipate and prepare for periodic changes in the environment, such as light, temperature, food availability and other periodic phenomena. Circadian rhythms are generated by biological clocks, which are a temperature compensated system. Circadian rhythms are mostly synchronized to the daily environmental light-dark cycle—ultimately determined by the Earth's rotation. When isolated from periodic environmental time cues or *Zeitgebers*, the endogenous rhythm persists (i.e., free-runs), with a period slightly longer or shorter than 24 h (Fig. 5).

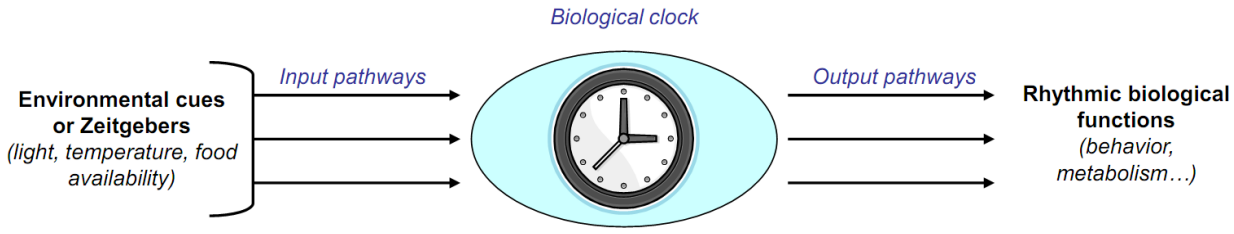


Figure 5. Input and ouput pathways of a biological clock

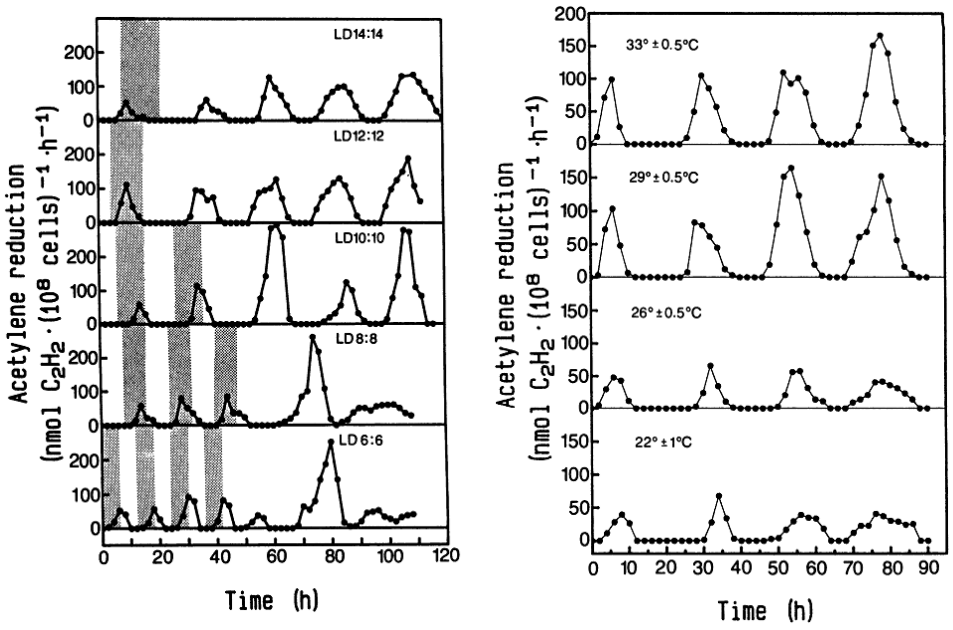
Different *Zeitgebers* can entrain the internal biological clock in order to regulate circadian rhythms to a precise 24-hour period.

2. One clock to rule them all

In the recent past, scientific investigations were conducted in bacteria, fungi, plants, birds and mammals, as well as molecules and cells. The fact that circadian rhythms have been observed in a wide range of organisms, suggests that temporal organization result from an evolutionary process, which started when life consisted only of single cells.

2.1 The tree of life

As aforementioned, circadian rhythms—thus the presence of a biological clock—have been revealed in organisms ranging from prokaryotes to eukaryotes. As an aside, it is important to remember than until the mid 1980s, it was thought that only eukaryotes had circadian rhythms. However, several research groups in the 80's discovered that prokaryotes were also capable of expressing circadian rhythms (Johnson et al. 1996) that meet all the criteria of bona fide biological clocks. For instance, the prokaryote, *Synechococcus*[8], has been shown to possess a circadian clock regulating cell division[9] that is entrained by a light-dark cycle and that is temperature-compensated (Fig. 6) (Sweeney and Borgese 1989; Huang et al. 1990).



[8] *Synechococcus* is a unicellular *cyanobacterium* that is very widespread in the marine environment.

[9] Cell division cycle in cyanobacteria can be a much more rapid process than circadian cycle. However, it has been demonstrated that the circadian clock mechanism is independent of cell division cycle, while the later depends on the phase of the circadian clock such that cell division is gated to occur only in specific circadian phases.

Figure 6. Rhythms of nitrogenase activity of Synechococcus sp. RF-1

(*Left*) Rhythms of nitrogenase activity in several different light-dark (LD) cycles followed by constant light conditions (LL). The cultures were adapted to the LD conditions for one week before exposing them to LL. (*Right*) Endogenous rhythm of nitrogenase activity of Synechococcus sp. RF-1 at several different temperatures. The cultures were all initially grown at 28°C under a 12:12 LD regimen for 1 week. They were then transferred to LL at 22, 26, 29, and 33°C, respectively. The N2-fixing activity of these cultures incubated at various temperatures was assayed at 2 h interval for a period of 4 days. From (Huang et al. 1990)

Hence, the 20th century has left us with an impressive list of living organisms that exhibit circadian rhythms (Fig. 7) (for review, see Dunlap 1999; Vansteensel et al. 2008). At present, a common statement is that circadian rhythms are ubiquitous in living systems. Besides the observations of rhythmic behavioral/physiological outputs in many organisms supporting this thought, additional evidence comes from the identification of specific genes, named “clock genes”, which are the genetic basis for circadian oscillations and which can be expressed in virtually all cells.

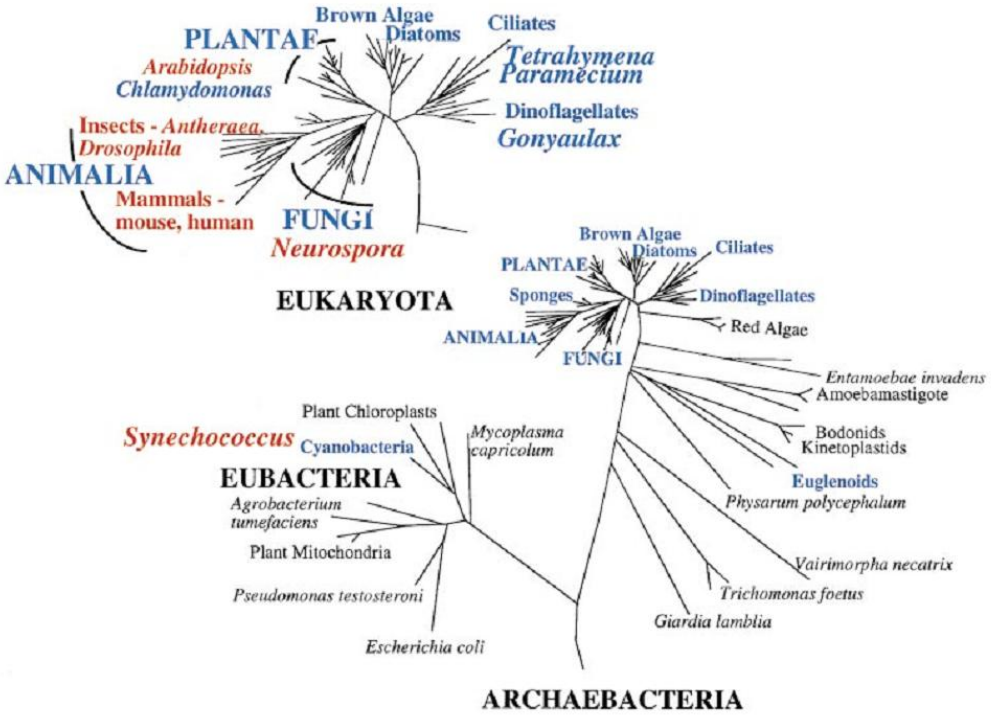


Figure 7. Circadian systems in the universal tree of life

The diagram, designed by Jay C. Dunlap, represents the range of organisms in which circadian systems/rhythms have been described. In blue are represented the phylogenic groups in which circadian rhythms have been investigated. In red are represented those species in which genetic and molecular analysis of clock mechanism has progressed significantly. Line lengths correspond to evolutionary distance between phylogenic groups. From (Dunlap 1999)

2.2 Discovery of (the) clock gene

In 1971, Konopka and Benzer isolated the first clock mutant *Drosophila melanogaster*. They showed that a gene mutation on a specific region of the X chromosome drastically alters the period length of the circadian clock regulating eclosion and locomotor activity rhythms (Konopka and Benzer 1971). The gene was thus named *Period*, and its precise location discovered later (Bargiello and Young 1984). This was the first description of a genetic component of a circadian clock. From then on, “clock” mutation was also demonstrated in *Neurospora* and the green algae *Chlamydomonas* (mentioned in Ralph and Menaker 1988).

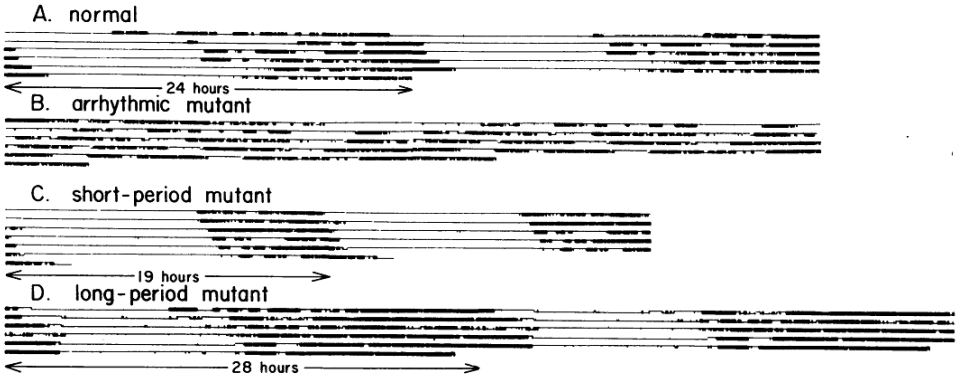


Figure 8. Individual locomotor activity rhythms of normal or mutant flies kept in constant darkness

All flies were previously exposed to a 12 h-12 h light-dark cycle. Each line represents the start of a successive interval. Vertical black bars represent the level of activity. For visual continuity, data are double-plotted, so that two days are represented horizontally. From (Konopka et al. 1971)

Later, Ralph and Menaker described for the first time a single-gene mutation that specifically affects the circadian clock of a vertebrate. This mutation, *Tau,* caused a dramatic shortening of the period (called *Tau*) of the circadian locomotor rhythm of golden hamsters (Fig. 8) (Ralph et al. 1988). In the 1990s, Takahashi and colleagues succeed in identifying the first mammalian clock gene by inducing circadian clock mutations in mice with the *N-ethyl-N-notrosourea* (ENU)-induced mutagenesis strategy. As a result, a semidominant mutation in a key gene, named *Clock*, was found to dramatically affect circadian rhythms (Fig. 9). *Clock* mutant mice displayed an abnormally long period of daily activity and eventually became arrhythmic in constant darkness (Vitaterna et al. 1994; King et al. 1997).

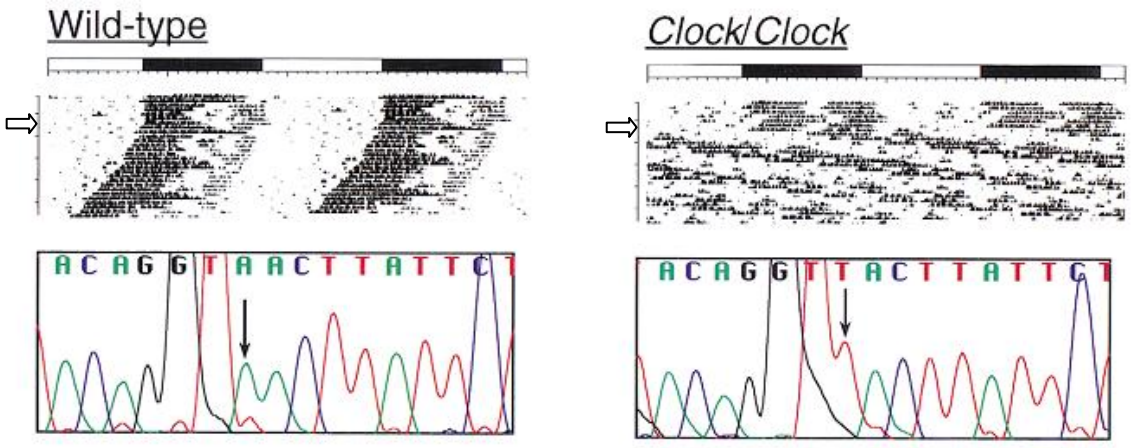


Figure 9. Effect of the *Clock* mutation in mice

Locomotor activity and DNA sequence data from wild-type and *Clock/Clock* mutant mice. On the actogram, the white and black bars represent the day and night periods, respectively. Note that activity records are double-plotted. Mice were kept in light-dark cycle for 8 days and then transferred to constant darkness (arrows). Below each actograms are represented the DNA sequence to illustrate the A→T transversion that causes the mutation. Adapted from (King et al. 1997)

All these findings demonstrated that clock genes are fundamental components of a biological clock and are necessary for normal circadian function. Considerable progress has been now made in discovering many more genetic components of the biological clock[10]. In addition, the ongoing characterization of these genes at the transcriptional and translational levels contributes to precisely characterizing their role and notably, how they interact to generate circadian oscillations (see part 2.3.2). Hereafter the focus will be especially on mammals.

2.3 Discovery of the first *pacemaker* in mammals

Since several physiological processes are controlled in a circadian way, the question of an anatomical substrate (in parallel to the identification of clock genes) at the basis of the temporal organization of dynamic events—like humans optimize their social/professional activities by referring to their wristwatch—has emerged decades ago. By referring to the results presented above, I will introduce what we currently know regarding the functioning of the circadian timing system in mammals. Is there only one main clock which drives all the circadian rhythms? Or are several

[10] The cross-species approach, in particular between fruit flies and mice, was a driving force in chronobiology to identify clock gene by homology of DNA sequence.

clocks/oscillators[11] involved? Where are they localized? Do they all contain the same "clock" genes?

2.3.1 The suprachiasmatic nucleus: a great conductor

Considering the fact that mammals showed daily rhythms of biological functions, synchronized to the 24-h light-dark cycle, it has been thought that an entrainment pathway must involved an organ that is sensitive to the periodic variations of illumination. In this context, a cerebral structure identified relatively early in the 20th century became the subject of much attention.

In 1937, Pate demonstrated that enucleation in cats induced atrophy of a specific region of the hypothalamus—*nucleus ovidens*. Much later in 1972, a direct projection from the retina to the *suprachiasmatic area* of the hypothalamus was demonstrated in the rat (Moore and Lenn 1972). This area was further described as a bilateral region containing two groups of neurons (10000-12000 total in population) dorsal to the optic chiasm and just lateral to the periventricular nucleus and third ventricle (Moore et al. 2002). Of note, these nuclei are also subdivided in a ventral (core) area and a dorsal (shell) area based on their afferents and the neurochemical nature of cells in each area (Fig. 10, 12).

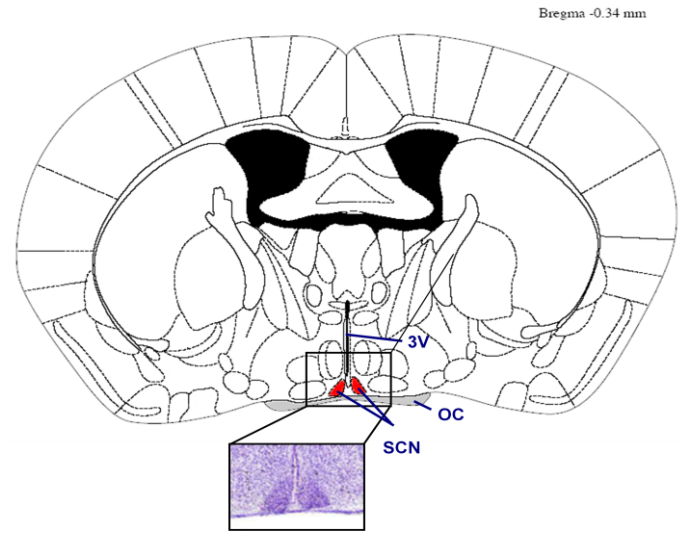


Figure 10. SCN localization in mice

Coronal section of a mouse brain showing the localization of the SCN, and below cresyl violet staining demonstrating the dense cellular packing in the ventromedial and dorsolateral part of the SCN. See text for description. OC: optic chiasma; 3V: third ventricule. Adapted from the Franklin-Paxinos atlas.

[11] I am adding the term "oscillator" to define a biological entity capable of generating circadian rhythms (e.g., of gene expression), but, amongst other things, that does not impose its rhythmicity to other entities.

Interestingly, Curt Richter at about the same time identified the anterior hypothalamus as a “biological pacemaker” involved in sleep-wake cycles (Richter 1967). Two years later, the effect of a removal of the *suprachiasmatic nucleus* (SCN) by bilateral electrolytic lesions in rats was tested by Moore and Eichler (1972) and Stephan and Zucker (1972). Surprisingly, the SCN ablation abolished the rhythm of adrenal corticosterone (Moore and Eichler 1972), as well as those of locomotor activity, food intake and drinking behavior (Stephan and Zucker 1972; Nagai et al. 1978). Further observations were then made in other mammals (Fig. 11) (Edgar et al. 1993; DeCoursey et al. 1997).

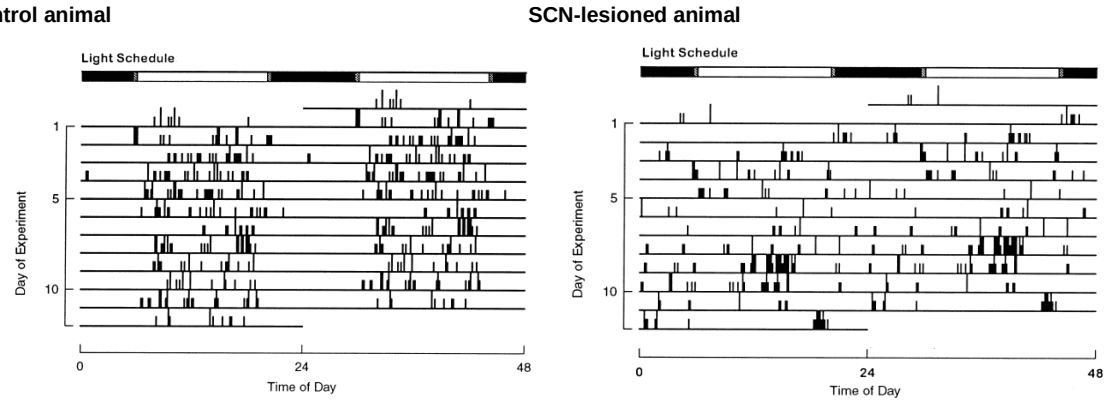


Figure 11. Effect of SCN lesions in the diurnal rodent, *Ammospermophilus leucurus*

Two actograms representing the locomotor activity of an intact SCN animal (Left) and an SCN-lesioned animal (Right). Recordings were performed using microchip transponder scanner for a period of 12 days. Each line corresponds to one day. Vertical black data line represents the number of bouts of activity per 10-min bin. Above the actograms, the schedule bars indicate the light and the dark conditions. Adapted from (DeCoursey et al. 1997)

In 1979, a brilliant work by Inouye and Kawamura tested the autonomy of the rat SCN clock. They isolated a small hypothalamic “island” that contains the SCN, thus free of all neural inputs from other brain structures. Then, they recorded neural (multiple unit) activity from two electrodes simultaneously, one in or near the SCN and the other in different neural locations. In intact animals, SCN and all brain sites tested presented a circadian rhythmicity of spontaneous neural activity, while in animals (that were also blinded) with this hypothalamic island containing the SCN, rhythmic electrical activity was lost elsewhere but persisted in the island (Inouye and Kawamura 1979). In brief, the rhythmic activity of the island did not depend on inputs from other brain areas.

A few years later, it was demonstrated that brain slices containing the SCN maintained a circadian rhythm in electrical activity (Green and Gillette 1982). It was also shown after isolation of the SCN that the peptide hormone vasopressin—which is synthesized in and secreted daily by the SCN (see Fig. 12, for the distribution of the different peptides in the SCN)—continue to be released in a circadian fashion for several days *in vitro* (Moore 1983; Earnest and Sladek 1986). Of interest, in the early 90's, a circadian rhythm in membrane conductance was revealed in isolated basal retinal neurons (Michel et al. 1993). Afterward, isolated neurons from the rat SCN were shown to express independently phased circadian firing rhythms, indicative of the presence of single-cell circadian oscillators (Welsh et al. 1995).

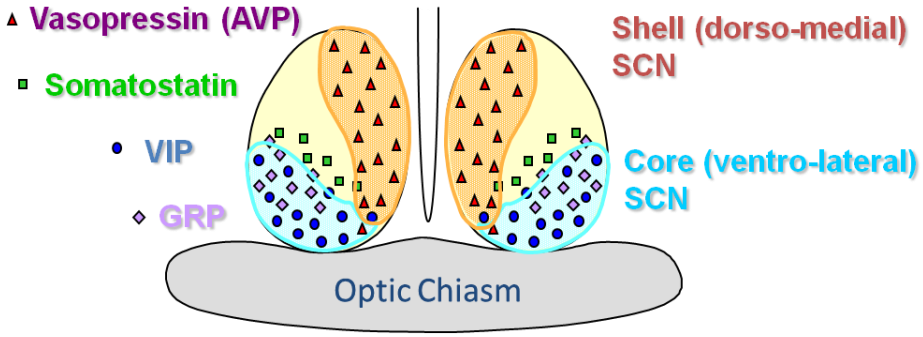


Figure 12. Drawing representing neuropeptide distribution in the SCN neurons

Many of the neurons within the core SCN express the neuropeptides VIP, GRP and the neurotransmitter GABA (not represented here). Neurons of the dorsal shell mostly express the neuropeptide AVP and GABA as well. AVP: arginine vasopressin; VIP: vasoactive intestinal polypeptide; GRP: gastrin-releasing peptide.

To further investigate a role for the SCN as a main conductor of circadian rhythmicity, Ralph and colleagues in 1990 used wild-type and *Tau* mutant hamsters (the latter having shortened endogenous periods, see part 2.2). By transplanting the SCN from a donor to a host animal bearing SCN lesions (SCN-x), they were capable of restoring a circadian rhythmicity. Interestingly, the transplant restored a free-running rhythm that was characteristic of the period of the donor. Indeed, if the donor was a *Tau* mutant hamster, the SCN-lesioned wild-type hamster behaved as a *Tau* mutant. SCN-lesioned *Tau* mutant in turn, could be converted to a wild-type hamster. In other words, the restored rhythms always exhibited the period of the donor genotype (Ralph et al. 1990).

All these findings showed that the SCN is a driving oscillator in the circadian system. The SCN will therefore be referring as a circadian *pacemaker*, i.e., a self-sustained biological oscillator that can be entrained to external cues and distribute a rhythmic message to the rest of the body.

2.3.2 Light input to the pacemaker

SCN neurons form an endogenous pacemaker sensitive to the daily light-dark cycle which appears to orchestrate a wide range of physiological and behavioral circadian rhythms in mammals. Since terminals of retinal axons were discovered in that location (Moore et al. 1972), a mediating role for the mammalian visual system in photic entrainement has been thus extensively investigated.

The eye is the sole light-sensitive tissue in mammals[12]. The SCN, especially the core region, receives input from the retina through a monosynaptic projection from retinal ganglion cells, the *retinohypothalamic tract*, which uses glutamate as a main neurotransmitter and is the main carrier of photic information[13]. The retina contains many neurons interconnected by synapses. Three types of neurons, called photoreceptors, are sensitive to light: rods that are primarily used in situations of less intense light and provide black-and-white vision; cones that function best in bright daytime light and are responsible for colour vision; and the intrinsically-photosensitive retinal ganglion cells (ipRGCs), crucial for non-image forming functions, that are presented below.

In 1999, Freedman and colleagues observed that photoentrainment was maintained in mice lacking both rods and cones, thus demonstrating that synchronization of circadian wheel-running activity to the light-dark cycle depends on an additional photoreceptor (Freedman et al. 1999). Meanwhile, a group of retinal ganglion cells were shown to contain a sensitive photopigment, called melanopsin that is excited in particular by blue-light. It was also revealed that the anatomical distribution of melanopsin-positive retinal cells was similar to the pattern of cells known to connect with the SCN (Provencio et al. 2000). Interestingly, the thus named

[12] In other vertebrates, light can be perceived by the pineal gland or deep brain photoreceptors.

[13] Two addition neural pathways connect to the SCN: an indirect retinal projection from the ventral lateral geniculate nucleus of the thalamus, the geniculo-hypothalamic tract, which uses the neuropeptide Y; and a raphe-hypothalamic pathway that connects raphe nuclei to the SCN and uses 5-HT as neurotransmitter.

ipRGCs were capable of depolarization even when all synaptic inputs from rods and cones were pharmacologically blocked, supporting Freedman's observation. In addition, the spectral sensitivity of ipRGCs and the fact that these cells respond more sluggishly to light than cones and rods, suggested that they could be the primary photoreceptors for photic entrainment of the SCN pacemaker (Berson et al. 2002). This has been further confirmed by Hattar and his colleagues, who showed that axons of ipRGCs directly target the SCN (Hattar et al. 2002).

To formally investigate the role of melanopsin in light entrainment of the SCN pacemaker, Panda and colleagues generated mice lacking the melanopsin gene. These mice entrained normally to a light-dark cycle and did not show altered endogenous period in constant darkness. However, they displayed altered light-induced phase resetting of the SCN demonstrating that melanopsin is required for normal circadian photoentrainement (Panda et al. 2002). Of interest, mice lacking ipRGCs expressed more deficits in circadian light responses (Guler et al. 2008). Moreover, mice deficient in both melanopsin and classical photoreceptors exhibited an entrainment phenotype comparable to that of enucleated mice (Panda et al. 2003). In short, partial functional redundancy may exist between ipRGCs, rods, and, to a lesser extent, cones to ensure normal photic entrainment (Fig. 13) (Paul et al. 2009; Altimus et al. 2010; Lall et al. 2010).

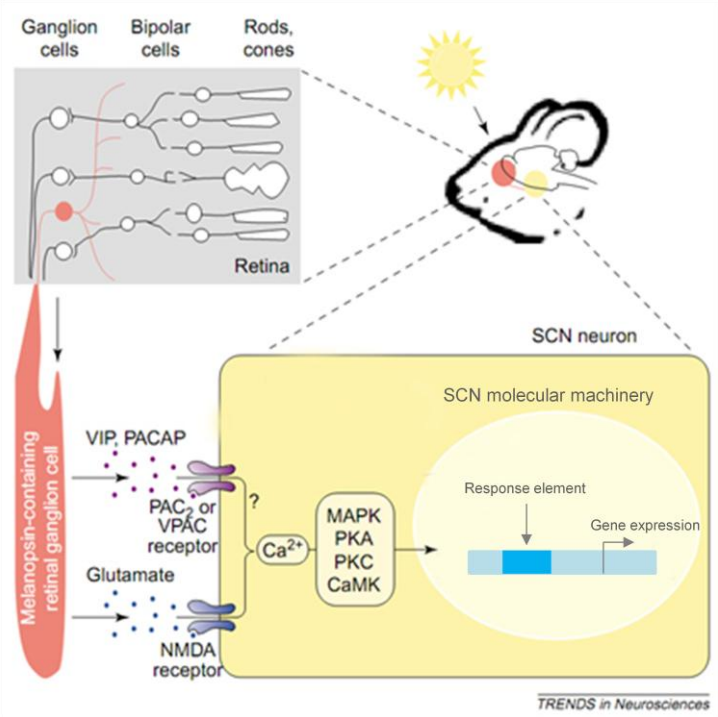


Figure 13. Schematic view of retinal circuitry and light entrainment

Photic signals are conveyed to the SCN via photoreceptor cells, especially the melanopsin-containing cells, which project directly to the SCN along the retinohypothalamic tract. Signal transduction to the molecular oscillator likely involves VIP, PACAP and glutamate releases at terminals. Changes in intracellular Ca2+ concentration lead to activation of several kinase pathways (e.g., MAPK, PKA) that in turn activate the transcription of different genes that are part of the SCN molecular architecture. Adapted from (Morse and Sassone-Corsi 2002).

Among the multiple neurotransmitters that can affect SCN cells, glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) release from the retinohypothalamic terminals play a crucial role for mediating the circadian actions of light (Morse et al. 2002; Meijer and Schwartz 2003; Hannibal 2006). SCN cells contain receptors sensitive to the peptides, NMDA, PAC2 and VPAC, leading to the activation of signaling pathways that will lead in turn to transcription-transduction and phosphorylation reactions that will influence the SCN's molecular machinery. In this context, neurons located in the ventrolateral part of the SCN have a strong capability of light-induced gene expression. The *c-Fos* gene, which has DNA-binding properties for transcriptional activation and whose transcription is regulated by phosphorylated CREB (Ginty et al. 1993), is proposed as a functional marker of the photic pathway. Indeed, bright light pulses administrated during the subjective night have been shown to induce FOS expression in the SCN (Fig. 14) (Rea 1989; Kornhauser et al. 1990).

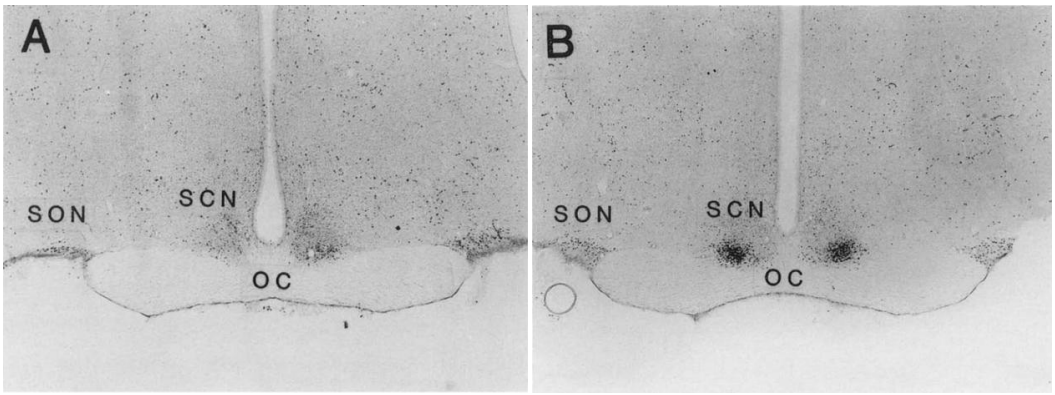


Figure 14. Photomicrographs of SCN coronal sections stained for c-*Fos* immunoreactivity.

(A) Control rat, and (B) a rat which received 15 min of bright (2200 lux) white light exposure. SON, supraoptic nucleus; OC, optic chiasm. Magnification = 30x. From (Rea 1989)

To summarize, for a proper phase-adjustment of the mammalian pacemaker to the external environment, SCN neurons receive direct input from the retina in order to synchronize cellular activities to the 24-h light-dark cycle. In this context, the ipRGCs play an important role to mediate photic entrainment and activate (through glutamate and PACAP releases) multiple protein kinases, immediate early genes such as c-*Fos* and clock genes (Fig. 15), which will be further presented in the following part.

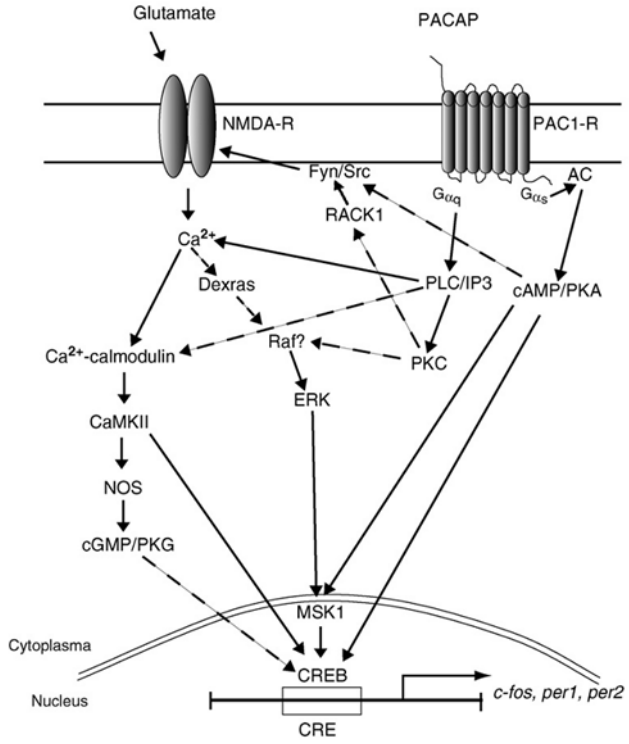


Figure 15. Signal transduction of photic messages in the mammalian SCN

Glutamate and PACAP releases following photic stimulation interacts with NMDA and PAC1 receptors, respectively. The light-induced increase of Ca2+ leads to the activation of several cytoplasmic mechanisms that in turn leads to the phosphorylation of the transcription factor CREB and to induction or change of clock gene expression. In parallel, the stimulation of the PAC1 receptor activates several signaling pathways (e.g., cAMP/PKA), resulting in the potentiation of attenuation of glutamate signaling. CaMKII: Ca^{2+}/calmodulin-dependent kinase II, IP_3: inositol 1,4,5-trisphosphate, NMDAR: *N*-methyl-d-aspartate receptor, PKC: protein kinase C, PLC: phospholipase C. From (Hannibal 2006)

2.3.3 A journey to the center of the clock

The process of SCN synchronization involves the transcriptional activation of different genes. As outlined above, light-induced cellular changes in calcium and kinase levels within the SCN (Motzkus et al. 2000; Hannibal 2006) lead to activation of immediate early genes, such as c-*Fos,* containing a cAMP responsive element (CRE) in their promoter region (Fig. 15). Interestingly, the *Period* (*Per*) clock gene, in particular its two isoforms *Per1* and *Per2* (Albrecht et al. 1997; Albrecht et al. 2001), have been shown to be light-responsive and to also possess CRE sequence in their promoter (Travnickova-Bendova et al. 2002). Thus the *Per* genes play a major role for the establishment of a new circadian phase following a change/perturbation in the light-dark schedule (Fig. 16).

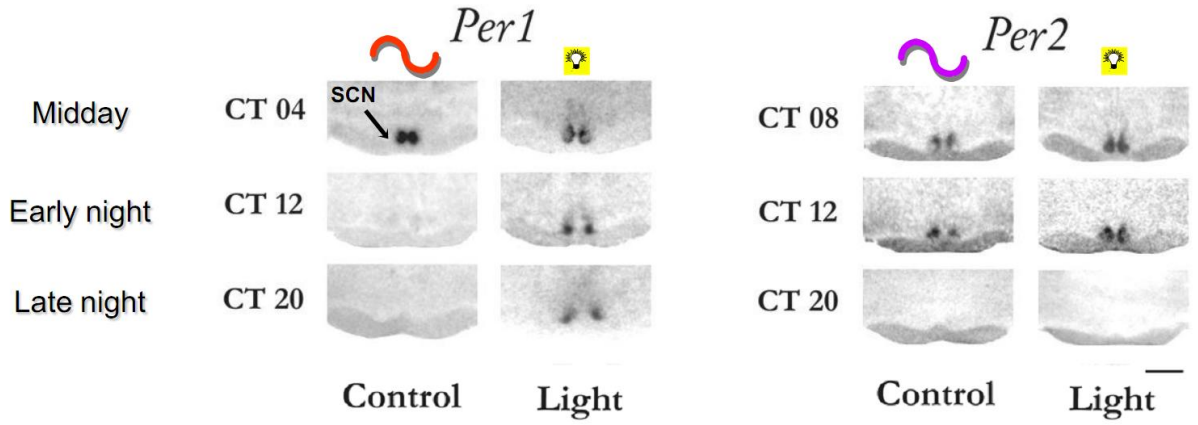


Figure 16. Photic modulation of *Per* genes in a diurnal rodent

Coronal sections of the SCN of diurnal rodents exposed to a light-pulse (100 lux, 1 h) or not (control) during the subjective day (midday or Circadian Time 04) and the early (CT12) and late night period (CT20). Scale bar = 1 mm. Adapted from (Caldelas et al. 2003)

However, in the absence of environmental light variations, biological rhythms are still observed in the circadian range, from cellular (e.g. electrical activity of SCN neurons) to behavioral levels (e.g., locomotor activity of an animal). These self-sustained activities take their origin from a clockwork that orchestrates rhythmic gene expression, and thus periodic fluctuations in cellular functions. Indeed, the mammalian molecular basis of circadian oscillations relies on clock genes/proteins, which control their own transcription/translation. Among some of the discovered factors are the essential *Period 1-3* (*Per1, Per2, Per3*), *Cryptochrome 1,2* (*Cry1, Cry2*), circadian locomotor output cycles kaput (*Clock*) or its analogue neuronal PAS domain protein 2 (*Npas2*), brain and muscle Arnt-like 1 (*Bmal1*), reverse viral erythroblastis oncogene products (*Rev-erbα* and *Rev-erbβ*) and retinoic acid-related orphan receptors (*Rorα, Rorβ, Rorγ*) (Ko and Takahashi 2006; Guilding et al. 2007).

Clock genes are co-expressed (in virtually all tissues) and their products reciprocally interact at the transcriptional/translational levels to generate circadian oscillations. At least three auto-regulatory feedback loops are interconnected (Fig. 17): one positive in which CLOCK and BMAL1 dimerize to activate the E-box[14] mediated transcription of *Per* and *Cry* genes; one negative whereby upon reaching a critical concentration, PER and CRY proteins enter into the nucleus to inhibit the transactivation mediated by CLOCK:BMAL1, therefore inhibiting their own transcription; and an interconnecting loop, in which *Rors* can activate the transcription of *Bmal1* and *Npas2*, whereas *Rev-erbs* can repress *Bmal1*, *Clock* and *Npas2*, via retinoic acid–related orphan receptor response element (RORE[15]) (Preitner et al. 2002; Guillaumond et al. 2005; Crumbley et al. 2010; Crumbley and Burris 2011). This loop ensures the fine-tuning of circadian rhythms. In addition, post-translational mechanisms such as protein phosphorylation, affect stabilization, degradation and subcellular localization of clock proteins, thus contributing to the molecular clockwork[16] (Lee et al. 2001; Ko et al. 2006).

[14] The consensus sequence for the E-box element is CANNTG (where N = ANY base).
[15] The 11pb consensus sequence for the RORE is WAWNTRGGTCA (where W = A or T, N = ANY, R = A or G).
[16] This model is continuously completed.

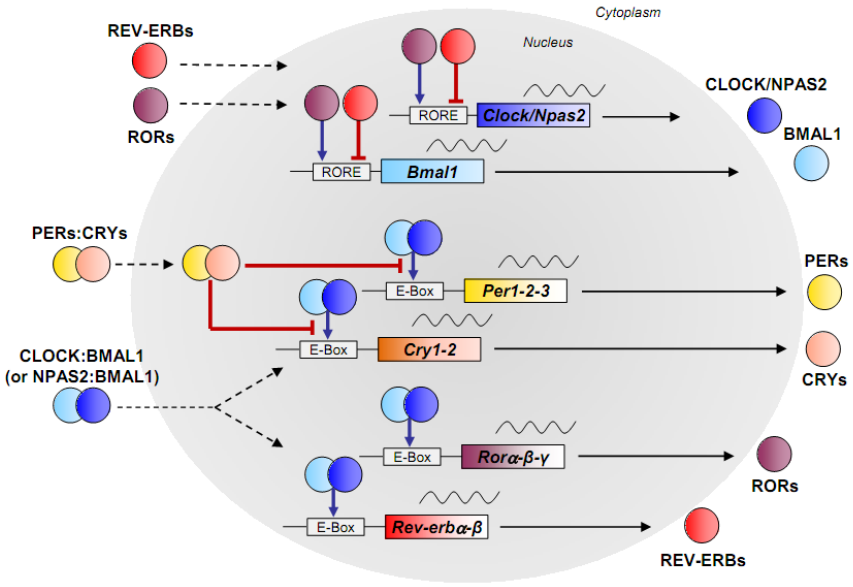


Figure 17. Simplified cellular model of the mammalian molecular circadian clockwork

Circadian rhythms are generated by transcriptional/translational feedback regulatory loops of core clock genes. CLOCK (or NPAS2) and BMAL1 can dimerize to rhythmically transactivate genes containing a specific DNA sequence (E-box) in their promoter region. As a result, the transcriptional activity of *Pers, Crys, Rors*, and *Rev-erbs* is enhanced, and their products from translational activity are cyclically released in the cytoplasm. Then, when PERs and CRYs proteins reach a critical concentration, they form heterodimers that translocate into the nucleus to repress transcriptional activity induced by CLOCK (NPAS2):BMAL1, leading to their own repression. An additional loop involves the nuclear receptors *Rev-erbs* and *Rors*, which can translocate into the nucleus to modulate *Bmal1, Clock*, and *Npas2* transcription via opposite action on a RORE sequence located in their promoter.

Overall, this highly sophisticated clock mechanism takes ~24 h to complete one full oscillation. Thus, each clock component has its acrophase at a specific time of the day[17] (Fig. 18) and mutation/deletion have profound consequences on circadian organization (see part 5.3).

[17] Intriguingly, regardless of whether an animal is diurnal or nocturnal in terms of behavior, the circadian pattern of clock gene expression in the SCN is not markedly different. Not to mention the same aspect in SCN electrical activity that occurs during the day period in both diurnal and nocturnal animals.

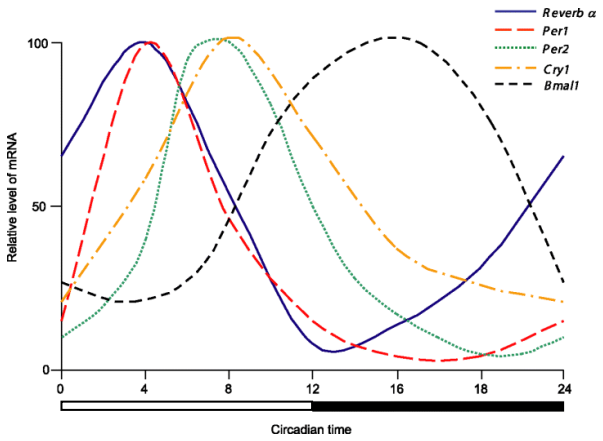


Figure 18. Circadian variations of clock genes mRNA in the SCN

Rev-erb α and *Per1* mRNA levels peak early in the subjective day, *Per2* and *Cry1* levels peak later in the subjective day, while *Bmal1* peaks in antiphase to these, in the subjective night. Expression of clock proteins are delayed by 4–6 h with regard to mRNA. Subjective night is indicated by the black bar along the time axis. Note that *Clock* (not represented) is continuously expressed. From (Guilding and Piggins 2007)

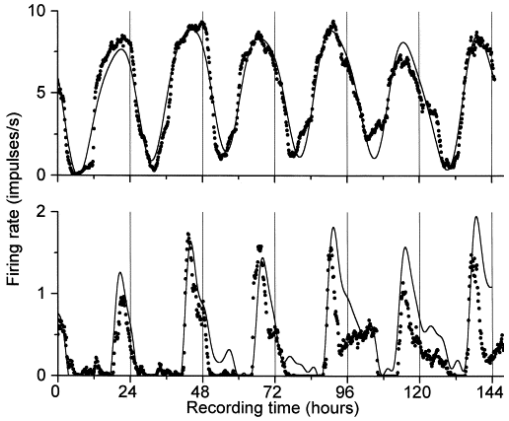


Figure 19. Circadian variations of electrical activity in SCN neurons

2 cells were recorded from separate mouse SCN explants. Both display a circadian rhythms in firing rate.

From (Herzog et al. 1997)

It is now well-established that the circadian clockwork can function within a single cell. Besides, SCN neurons are circadian pacemakers that can show rhythms in electrical activity when isolated from other neurons (Fig. 19). However, SCN neurons *in vivo* are part of a network. Hence, in order to be in an exact synchrony, SCN neurons may communicate with each other through gap-junctions (Jiang et al. 1997; Long et al. 2005), synaptic interactions, GABAergic transmission (Shirakawa et al. 2000) and neuropeptide release (Aton et al. 2005; Kim et al. 2009). As a result of synchronization, the precision of the period of the SCN pacemaker increases as a function of its cells interactions. In other words, the fluctuations in the period of electrical activity decrease as single-cells synchronize to a cellular network, which synchronize further to an entire living organism (Fig. 20). As an aside, it is important to mention that we currently have little knowledge regarding the inter-relationships within the SCN between rhythmic neural electrical activity and rhythmic gene

expression[18]. Despite this fact, it clearly appears that both ionic and molecular mechanisms are in perpetual reciprocal interaction (and can even be phased over time) to produce a functional pacemaker (for review, see Colwell 2011).

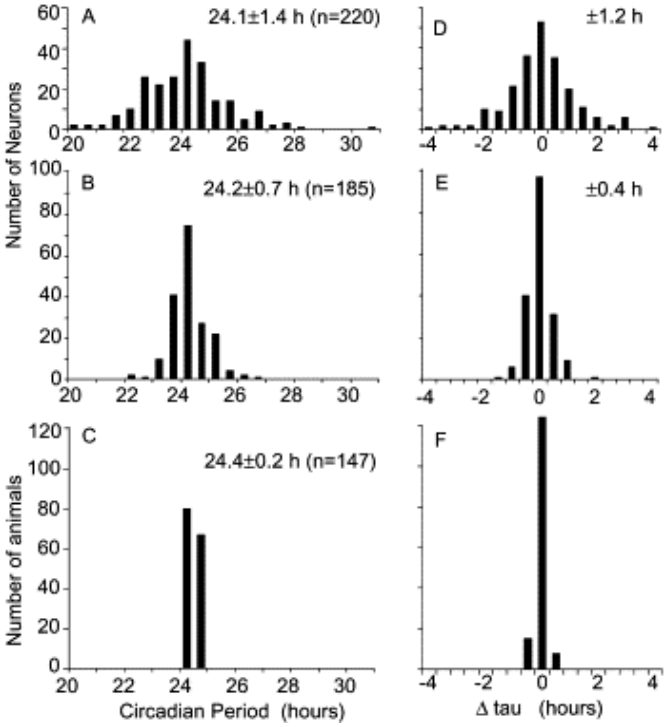


Figure 20. Distribution of circadian periods in two types of SCN culture and at the animal level

(A, D) Electrical activity in dispersed cell culture. Note the strong fluctuations in the intrinsic periods between single cells. (B) Electrical activity of single-cell recorded in SCN slice culture. Note that the periods vary less between single-cells. (C) Period of locomotor activity in individual rats, depicting slight variations from one animal to another. (D, E) Period variations within a dish and a slice. (F) Distribution range of behavioral rhythm. From (Honma et al. 2004)

In the context of synchronization, the SCN pacemaker uses many output pathways to adjust biological functions to a 24-h schedule. The daily increase in SCN neuron firing rate itself has been assumed to be an output of the circadian system (Colwell 2011). It will be show in the following part that the periodic synchronous fluctuations of thousand of neurons are interpreted outside the SCN by other brain regions and peripheral tissues at a specific time of the day.

2.4 The mammalian circadian timing system

Up to now, I focused on the SCN pacemaker to introduce the fundamental mechanisms at the basis of circadian oscillations. However, the SCN is not the sole biological entity that can exhibit self-sustained activity, rhythmic clock gene expression, and/or that can distribute a periodic message to the rest of the body. Indeed, the circadian timing system is multi-oscillatory by nature, particularly in

[18] In the context of cell-autonomous oscillations and intercellular coupling, the participation of glial cells has to be taken into consideration.

mammals. It is composed of several endogenous oscillators in anatomically discrete locations that form a highly sophisticated circadian network, regulating an amazing array of biological functions. In this context, major brain areas (e.g., hypothalamic nuclei) and peripheral organs (e.g., liver) possess their own clockwork to regulate the transcription of genes and hence local cellular events.

2.4.1 The brain oscillators

The output of the clock machinery consists of changes in neural activity and gene expression associated with neurotransmitter releases. Apart from generating self-sustained circadian clock gene expression, the interlocked positive and negative feedback loops within the SCN are essential for the rhythmic transcriptional control of circadian output genes or *clock-controlled genes (CCGs)* (Fig. 21).

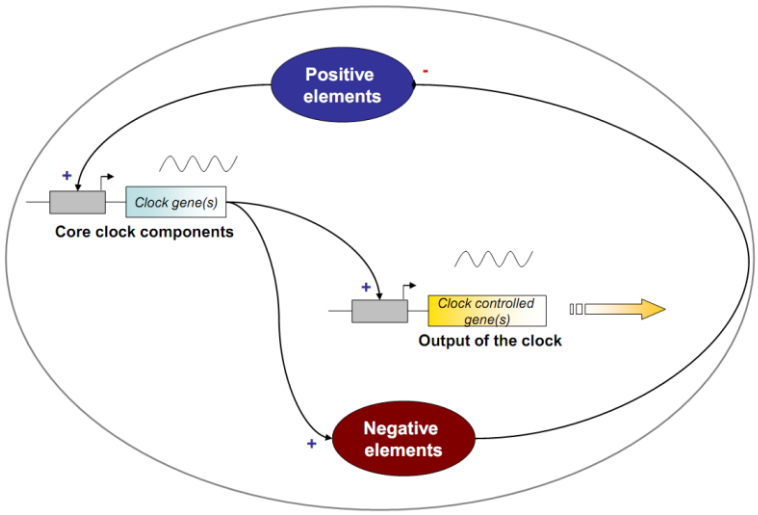


Figure 21. *Clock-controlled genes* linked to the circadian molecular mechanism.

Both positive and negative limbs reciprocally interact to govern the transcription of CCGs.

GABA, glutamate, AVP, VIP, transforming growth factor α (TGFα), prokineticin 2 (PK2), cardiotrophin-like cytokine (CLC), are strong candidates for intrinsic timekeeping signals that travel from the SCN to the rest of the brain (Guilding et al. 2007). For instance, rhythmic immunoreactivity for the AVP CCGs has been demonstrated in efferent projections of the SCN (van Esseveldt et al. 2000). Interestingly, as demonstrated by SCN grafts experiments, the humoral release of AVP is important for the restoration of circadian electrical activity in the paraventricular nucleus (PVN) of the hypothalamus in the absence of direct neural connections (Tousson and Meissl 2004). In addition, AVP release from SCN terminals has been demonstrated to be crucial to control daily variations in

corticosterone levels (Kalsbeek et al. 1992; Kalsbeek et al. 1996). Another interesting candidate, PK2, has been shown to be expressed on a circadian basis and to be modulated by light exposure (Cheng et al. 2005). Furthermore, the intracerebroventricular injection of PK2 inhibits locomotor activity (Cheng et al. 2002). Recent observations in mice lacking PK2 support its role in the circadian control of locomotor activity (Li et al. 2006). Intriguingly, transplantation experiments of isolated SCN tissue have demonstrated that SCN projections are not required for the control of circadian locomotor rhythms, suggesting that a diffusible factor such as PK2 may sustain circadian activity (Silver et al. 1996). However, graft transplantation of SCN failed to restore endocrine rhythms (Meyer-Bernstein et al. 1999), showing that the daily SCN output signaling are either accomplished through synaptic connections or rely on nearby tissues that are direct targets of local diffusible factors.

Detailed topography of the SCN efferents has been revealed by studies using anterograde and retrograde tracing techniques (Watts and Swanson 1987; Watts et al. 1987; Kalsbeek et al. 1993). Within the hypothalamus, the SCN connects with the PVN, dorsomedial nucleus (DMH), ventromedial nucleus, lateral hypothalamic area (LH), arcuate nucleus (ARC) and retrochiasmatic area. SCN efferents can also terminate in the preoptic area, the bed nucleus of the stria terminalis and the lateral septum. The paraventricular nucleus of the thalamus (PVT) is also directly innervated. SCN projections to the habenula and amygdala (AMY) are also considered (Fig. 22) (for reviews, see Kalsbeek and Buijs 2002; Saper et al. 2005; Dibner et al. 2010). Furthermore, it has been revealed that SCN efferents can indirectly control crucial neuroendocrine rhythms for the distribution of rhythmicity to the entire body, such as those of melatonin. In short, a multisynaptic pathway involving the PVN, the preganglionic sympathetic neurons in the spinal cord and the superior cervical ganglia, allow the SCN to control the rhythmic synthesis of melatonin[19] by the pineal gland (Moore and Klein 1974; Kalsbeek et al. 2006).

[19] Melatonin, secreted during the night, is a key hormone for seasonal and circadian variations in biological functions.

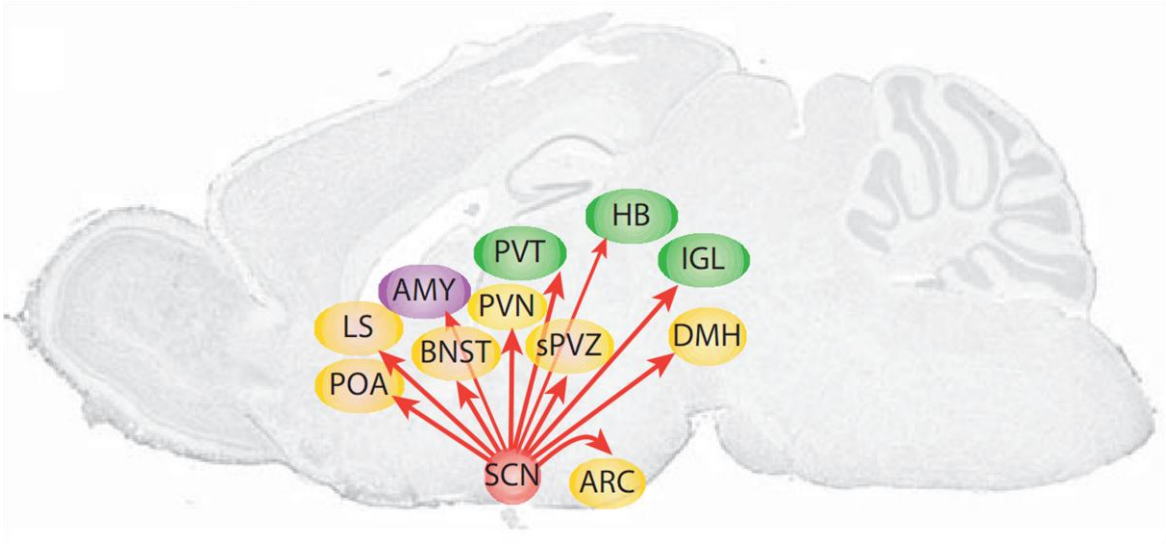


Figure 22. Direct SCN projections

Efferent pathways from the SCN (*red*) to hypothalamic (*yellow*) and thalamic (*green*) brain regions. AMY, amygdala; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; DMH, dorsomedial hypothalamus; HB, habenula; IGL, intergeniculate leaflet; LS, lateral septum; POA, preoptic area; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; SCN, suprachiasmatic nuclei; sPVZ, subparaventricular zone. From (Dibner et al. 2010)

The discovery of clock genes, amongst other things, uncovered in turn a substantial number of brain structures, outside the SCN, that exhibit circadian oscillations. The retina was the first autonomous oscillator—pacemaker—to be discovered outside the SCN. Cultured retina exhibited circadian rhythms of melatonin synthesis that can be entrained by light cycles; that was free-running in constant darkness; and that was temperature compensated[20] (Tosini and Menaker 1996; Tosini and Menaker 1998). The olfactory bulb is one additional brain structure that can also oscillate in a self-sustained manner (Granados-Fuentes et al. 2006). The habenula (Hb), especially the lateral Hb, may contain an autonomous oscillator as well (Zhao and Rusak 2005; Guilding et al. 2010). A vast number of brain locations have now been identified as extra-SCN oscillators—thus oscillators that do not meet the criteria to be classified as pacemakers. Indeed, more than 20 extra-SCN tissues, such as the hippocampus and the AMY have shown at least one rhythmic cycle of clock components observed at the mRNA or protein level (for an exhaustive list, see Guilding et al. 2007).

[20] Of note, the *Tau* mutation shortened the free-running rhythm of melatonin synthesis by 4 h, suggesting that common mechanisms may be shared between the SCN and the retina pacemakers.

Circadian rhythms have also been observed by using transgenic *Per1-luciferase* rats. Interestingly, 14 of the 27 brain areas examined expressed *Per1* in a rhythmic fashion *in vitro* (Abe et al. 2002). Among these structures, the ARC nucleus, the pineal and pituitary glands all expressed rhythms that persisted for several days *in vitro,* although rhythmicity in some regions damped more rapidly over time than others (Fig. 23).

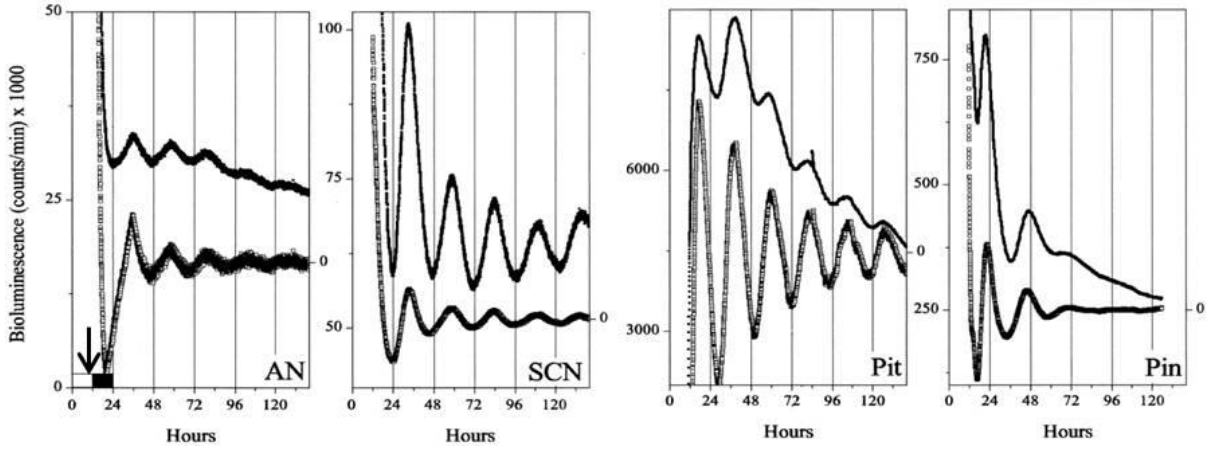


Figure 23. Real-time recording of bioluminescence from *Per1-luciferase* transgenic rats

Tissues were explanted 1 h before light-offset (see arrow and light and dark periods in the top left plot). Note that the SCN differs from other tissues in the phase and amplitude of PER1-luc circadian expression. AN: arcuate nucleus; Pit: pituitary gland; Pin: pineal gland. Adapted from (Abe et al. 2002).

All these findings indicate that the brain contains several circadian (strong or weak) oscillators that regulate multiple biological functions (Fig. 24). However, most oscillators depend on signals derived from the SCN for their cellular activities. In that respect, the SCN delivers circadian time information to the whole body through neuronal (e.g., autonomous nervous system) and humoral routes to fine-tune its control of many brain and peripheral tissues.

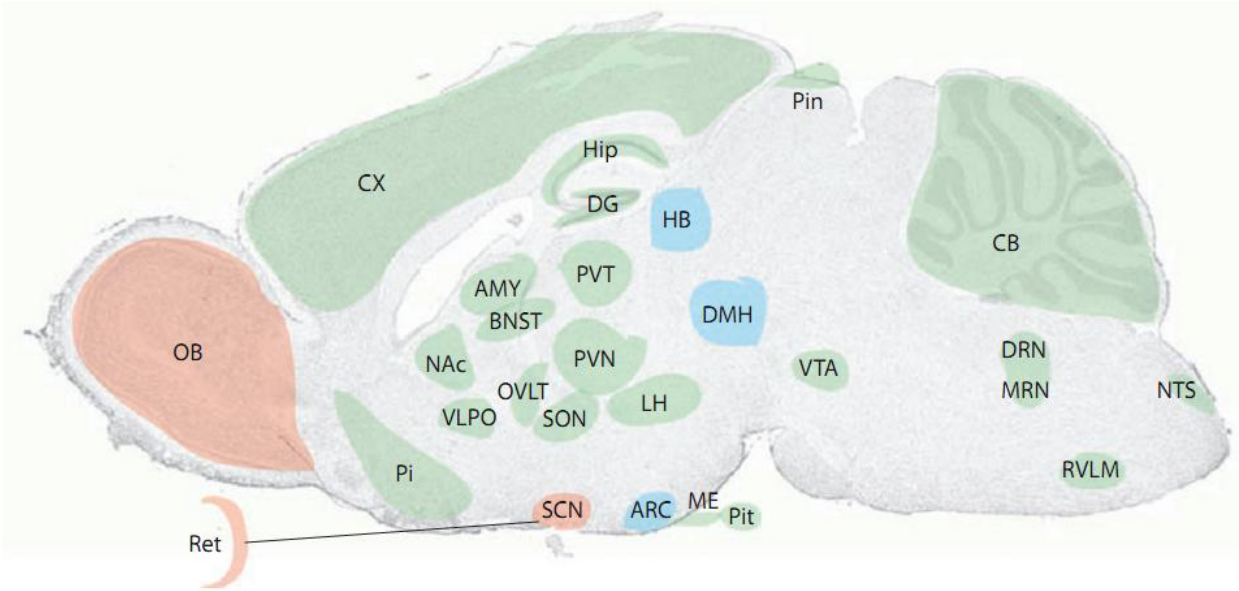


Figure 24. Potential circadian oscillators in the mammalian brain

Self-sustained circadian oscillators are shown shaded in red, semiautonomous oscillators in blue, and slave oscillators in green. AMY, amygdala; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; CB, cerebellum; CX, cortex; DG, dentate gyrus; DMH, dorsomedial hypothalamus; DRN, dorsal raphe nucleus; HB, habenula; Hip, hippocampus; LH, lateral hypothalamus; ME, median eminence; MRN, median raphe nucleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OVLT, vascular organ of the lamina terminalis; Pi, piriform cortex; Pin, pineal gland; Pit, pituitary gland; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; Ret, retina; RVLM, rostral ventrolateral medulla; SCN, suprachiasmatic nuclei; SON, supraoptic nucleus; VLPO, ventrolateral preoptic area; VTA, ventral tegmental area. From (Dibner et al. 2010).

2.4.2 The peripheral oscillators

As aforementioned, several hypothalamic nuclei receive direct neuronal connections from the SCN (Watts et al. 1987; Dibner et al. 2010). Interestingly, these nuclei project in turn to peripheral tissues, such as the liver (la Fleur et al. 2000; Shibata 2004), pancreas (Buijs et al. 2001) and adipose tissue (Kreier et al. 2002) via autonomic output pathways.

Indeed, the role of the autonomic nervous system is not only illustrated by the sympathetic innervations that permit the SCN to control melatonin release, but also by the multisynaptic pathway that connects the SCN to the adrenal gland. Autonomic projections of the SCN via the PVN to the intermedio-lateral column of the spinal cord to the adrenal contribute to the daily release of the adrenocorticotropic hormone (ACTH) (Buijs et al. 1999). This network is highlighted by the finding that light induces (clock) gene expression and corticosterone secretion in/by the adrenal gland via the

SCN-sympathetic nervous system—since SCN lesions and transection of a sympathetic nerve route completely suppressed this effect (Ishida et al. 2005).

Virtually all peripheral tissues contain a circadian oscillator, as many express clock genes and can, in culture, show 24-h rhythms in gene expression (see part 4.1.2 for more detailed) (Tei et al. 1997; Damiola et al. 2000; Abe et al. 2002). As an example, the adrenal gland exhibits rhythmic clock gene expression that is disturbed in clock mutant mice (Oster et al. 2006). In addition, circadian oscillations are also observed in several peripheral tissues by using real-time visualization of circadian expression of *mPER2::LUC* reporter *in vitro* (Fig. 25) (Yoo et al. 2004) and *in vivo* (Tahara et al. 2012).

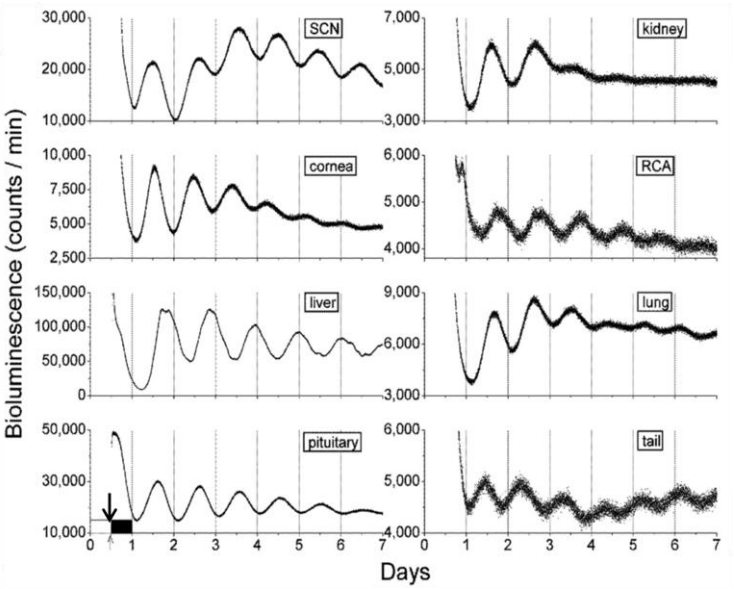


Figure 25. Circadian oscillations of PER2-luc in the SCN and peripheral tissues

Tissues were taken 1 h before light-offset (see arrow and light and dark periods in the bottom left plot). Note that all tissues show circadian oscillations for several days that dampened differently over time. From (Yoo et al. 2004)

Surprisingly, some tissue explants can sustain circadian cycles that dampen over several days, even when they were derived from SCN-lesioned animals. However, SCN lesions produced internal phase desynchrony between tissues but not between cells within a given tissue. These observations suggest that the SCN plays a role as a phase coordinator and that, organ-specific synchronizers at the cell and tissue levels may exist to ensure a coupling between cells and thus tissue oscillations for several cycles (Yoo et al. 2004). Nevertheless, this conclusion was not supported by other studies which showed that the circadian oscillations observed in tissue explants did not necessarily imply that cells were synchronized *in vivo* (Yamazaki et al. 2000; Stratmann and Schibler 2006), and that the SCN central pacemaker is essential to impose phase coherence between oscillating cells within a peripheral

organ (Guo et al. 2006). In addition, contrary to peripheral oscillators, the coupling between SCN neurons also renders them more resilient to clock gene mutations (Liu et al. 2007).

Taken together, these findings indicate that the SCN, in coordination with the light-dark cycle, is essential to synchronize cell-autonomous oscillators in the brain and peripheral organs, via direct and indirect neural projections as well as releases of humoral factors. However, light is not the unique input or *Zeitgeber* that can orchestrate rhythmic activities in an oscillator. I will show that non-photic cues such as food can influence the brain (including the SCN) as well as peripheral oscillators (Fig. 26).

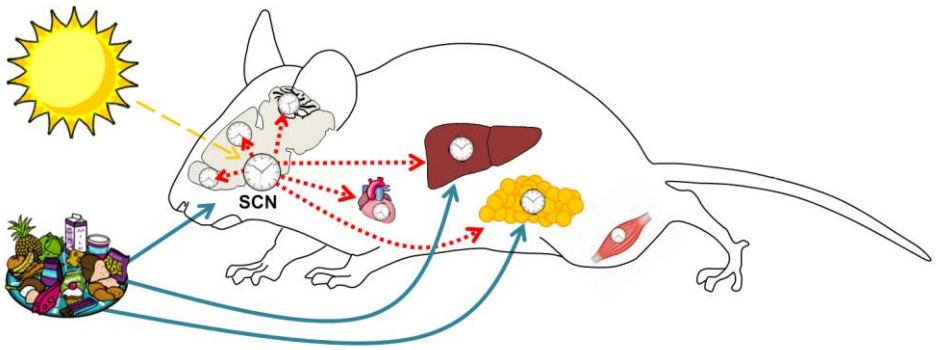


Figure 26. Organization of the circadian timing system

The master clock, located in the suprachiamatic nuclei (SCN) of the hypothalamus, adjusts the timing of many secondary clocks/oscillators in the brain and peripheral organs, in part via nervous pathways (dotted red lines). Light perceived by the retina is the most potent synchronizer of the SCN clock (dashed yellow arrow) while mealtime can synchronize peripheral clocks (blue arrows).

2.4.3 Adaptive value of circadian oscillations

Long before humans introduced the concept of time and invented a clock mechanism to better apprehend their own world and organize their diverse activities, probably the first and simplest living organisms on earth adapted to nature's motions by developing a sensitive internal measuring system to evaluate the approximate duration of external events, and the intervals between them, enabling them not to merely passively follow their environment, but on the contrary anticipate changes in an active manner and optimize their own metabolism.

In such a perspective, it is therefore not surprising to observe periodic fluctuations of life functions in a wide variety of species and to identify gene sequences in portions of multiple organisms' DNA that are at the base of a remarkable molecular clock mechanism. The likely ubiquitous nature of the latter can be viewed as the result of evolution. Indeed, organisms having a clock machinery (functional or not) probably share a common ancestry. Biological clocks could represent a vestigial mechanism, providing evidence for evolution. In that respect, chronobiology may in the future unlock some of the mysteries relating to development and evolution.

In any case, it is certain that either the inheritance of the characteristics of a well-adapted ancestral predecessor, or the fact that more than a few creatures on, in and above earth have evolved an internal time-keeping system in parallel, imparted a non-negligible survival advantage allowing them to deal with nature's fickleness, such as the daily variations of sunlight, temperature, water/food availability, as well as greater phenomena such as seasonality, severe cold conditions, etc.

Furthermore, the presence of a clock timing system not only improves the ability of a living organism to adapt—metabolically speaking—in a rhythmic environment but considerably enhances its fitness under competitive conditions (e.g., feeding, mating and predation). In this context, the benefit of a clock system is also highlighted by its ability to respond to several stimuli, even in distinctive conditions. For instance, cavefish have evolved for millions of years in the perpetual darkness of subterranean caves in Somalia and present complete eye degeneration. Nevertheless, this fascinating animal still retains a circadian molecular clock that can be entrained by feeding time (Cavallari et al. 2011).

The benefits of having a clock therefore accrue to all organisms across their diverse kingdoms.

3. The mysterious food-entrainable oscillator

3.1 Food as a Zeitgeber, first evidence

Foraging is fundamental for an animal to survive and can be influenced by several factors such as genetics and learning and memory capacities. Another element that may help animals in their search for food resources is the circadian timing system. Although light is the most prominent Zeitgeber, several non-photic stimuli, such as food, have been also shown to entrain circadian rhythms. Interestingly, feeding time can act not only on the SCN and peripheral tissues, but also on an oscillator which is not yet well-defined, called the *food-entrainable oscillator* (FEO), which helps multiple animals in their search for food.

> *"A primary task of the foraging organism is to exploit spatial and temporal regularities of food availability in the environment."* (Mistlberger 1994)

3.1.1 Honey bees

In the early 20th century, it was already known from von Buttel-Reepen that honey bees were able to learn the time of day when flowers secrete nectar (mentioned in Moore 2001). Meanwhile, the Swiss physician August Forel, when he had breakfast with his family on a terrace each morning, noticed that some forager bees from a hive located not far from his summer home, started to visit his breakfast table and even arrived, after a few days, at the time when breakfast was served. He also noticed that bees still came even if no food was put on the table, suggesting that this daily visit was not related to odor or other stimuli from food (mentioned in Renner 1960). He suggested that "*the bees remember the hours at which they had usually found sweets … they have a memory for time* (*Zeitgedächtnis*)".

In 1929, Ingeborg Beling, a student of the famous ethologist Karl von Frish, published the first study on the time-sense of bees. Beling observed that honey bees were able to associate the time of day with the presentation of a food reward. By training bees to collect a sugar solution at specific location and during specific periods of time on several consecutive days, she observed that bees continued to return to the sugar source with greatest frequency at 24-h intervals even if sugar was no longer present (Fig. 27); food presented at 19-h or 48-h intervals did not train the

bees. In addition, Beling and her colleague Otto Wahl demonstrated a few years later that bees shielded from external factors such as light, temperature and humidity could still be trained to collect nectar and pollen at different times of day[21]. However, von Frish and colleagues did not conclude that time-sense in bees relies on an endogenous clock (mentioned in Moore and Rankin 1983; Moore 2001).

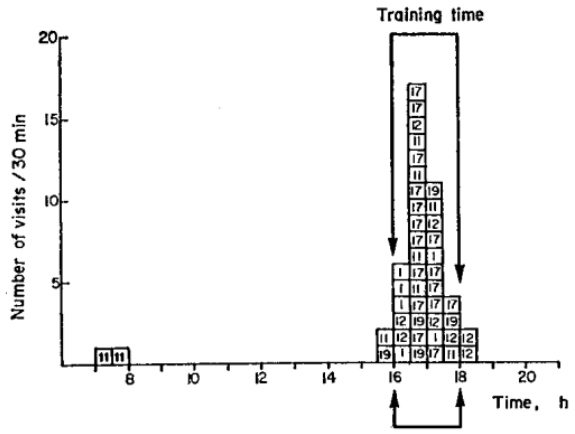


Figure 27. The time-memory of bees

The bees were trained to come to a sugar source at a fixed time (16 to 18 h) during several consecutive training days. As they visited the sugar they were marked individually. The left graph shows the results on the test day, when sugar was omitted. The bees (individually numbered, see bar graphs) continued to arrive at the feeding location at the same time of the day. From (Saunders 1977)

Evidence for the presence of an endogenous biological clock was given a few decades later by Max Renner (1955), in a series of translocation experiments. Renner trained bees in his laboratory in France to collect sugar water between 8:15 p.m. and 10:15 p.m., and then he transported the bees overnight to New York, where they were placed in similar laboratory conditions. He observed that the following day of departure, bees arrived at the feeding table at the same previous *French* fixed time, despite having been displaced (Renner 1960). Renner's studies give strong evidence that bees are oriented in time with an endogenous clock.

3.1.2 Craving rats

In the early 1920s, Curt Richter would have first observed a phenomenon of circadian food anticipation in rats (mentioned in Davidson 2006). Later, Bolles and colleagues, as well as Edmonds, showed that when food[22] was restricted to a 1-h span, rats exhibited increases in locomotor activity preceding food presentation, even thought food was presented during the light period (when rats are resting) or two times per day (Bolles and Stokes 1965; Bolles and Moot 1973; Edmonds 1977). In

[21] Bees were apparently moved 180 m below the surface of earth, in a salt mine, to exclude cosmic radiation influence.
[22] Normocaloric standard chow pellets

1979, Friedrich K. Stephan demonstrated that timely controlled food-seeking behaviors did not depend on the SCN pacemaker. He showed that bilateral SCN lesioned rats—thus arrhythmic in locomotor and feeding/drinking behaviors—that were previously fed on an *ad libitum* (AL) basis and then exposed to a restricted feeding (RF) schedule (i.e., rats received one meal every 12-h intervals) in constant darkness[23], still displayed food-anticipatory locomotor activity (Fig. 28). Stephan concluded that food-anticipatory activity (FAA) was controlled by an oscillator outside the SCN (Stephan et al. 1979; Stephan et al. 1979; Stephan 1983).

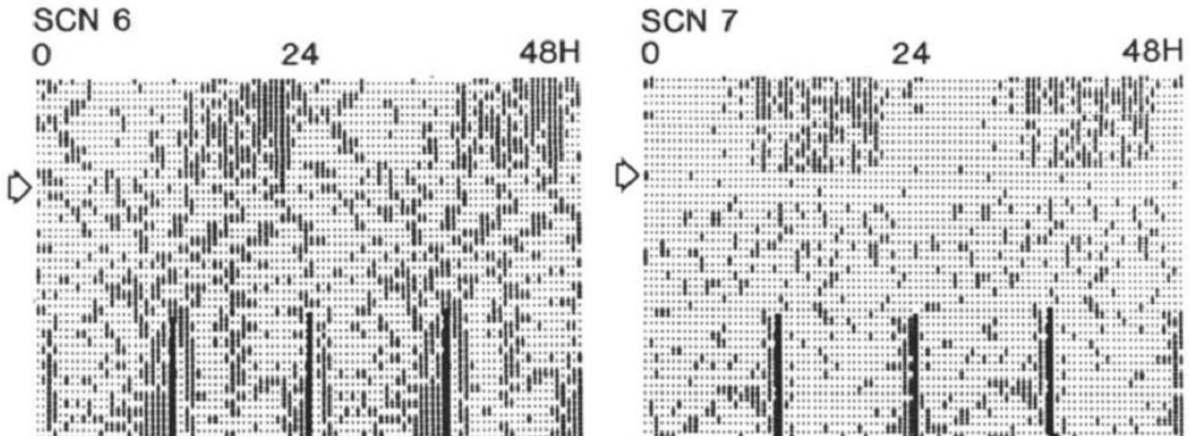


Figure 28. Food-anticipatory activity in SCN-x rats

Double-plotted actograms (48 h time scale) showing locomotor activity for 2 rats (SCN6-7) in constant darkness. The arrow indicates the time when rats were SCN lesioned. Black line indicates the beginning of 1 h restricted feeding (food was given every 12-h intervals, thus twice a day). Note the increase of activity prior to the two mealtimes. From (Stephan 1983)

3.1.3 Food anticipation in nature

Of note, other species such as fishes (Lopez-Olmeda et al. 2010), birds, mice, hamsters, rabbits[24] (Caba and Gonzalez-Mariscal 2009), golden shiners (Lagud and Reebs 2000), marsupials and primates (Boulos et al. 1989) can all show abrupt food-seeking behaviors prior to feeding time (for a review, see Mistlberger 1994). As aforementioned, even cavefish that have evolved in dark subterranean caves can show FAA in response to periodic food availability (Fig. 29) (Cavallari et al. 2011).

[23] To avoid a masking effect (i.e., inhibiting effect of light) on locomotor activity, such experiments are conducted in constant dark conditions.

[24] Rabbit pups are normally fed by nursing only once a day and show a clear locomotor anticipatory behavior to the arrival of the nursing mother (see Caba and Gonzalez-Mariscal 2009).

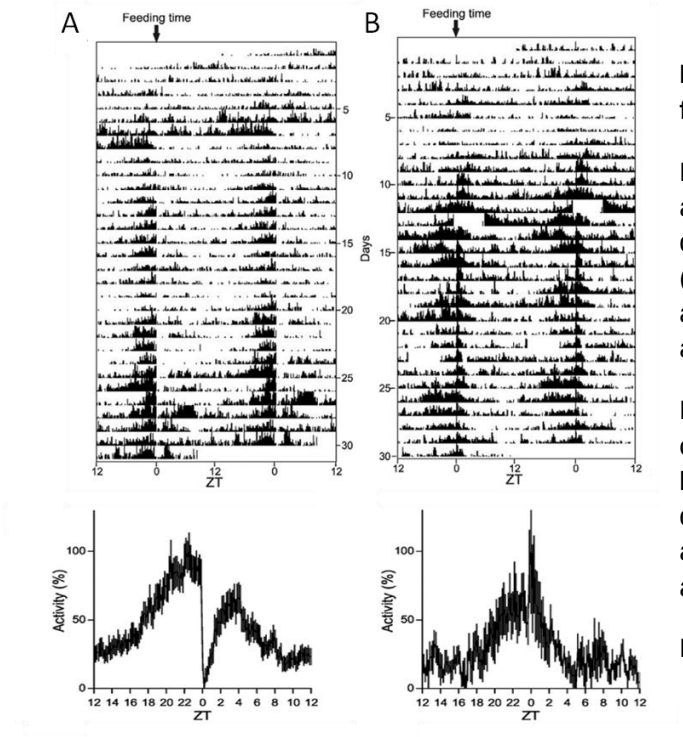


Figure 29. Food-anticipatory activity in fishes

Representative actograms of zebrafish (A) and cavefish (B) maintained under constant darkness and fed once a day at a fixed time (ZT = 0). The mean waveforms of zebrafish and cavefish are represented below the actograms.

Each point in the mean waveform has been calculated as the mean 6 SEM from 10 min binned data across all the experimental days (n = 30) shown on each actogram and all experimental aquaria (n = 5 for zebrafish and n = 3 for cavefish).

From (Cavallari et al. 2011)

3.2 The circadian characteristics of the food-entrainable oscillator

As demonstrated above, there is much evidence of an extra-SCN oscillator sensitive to feeding time that can control circadian behavioral activity. This oscillator was the object of much attention in the early 80s, particularly in rats. Numerous studies characterized the properties of the so-called FEO.

3.2.1 Transient cycles and persistence

Stephan in the early 80s observed that SCN-lesioned rats[25] exposed to a 24-h interval RF schedule exhibited after phase shifts of mealtime, transient cycles that are characteristics of photic entrainment (Fig. 30) (Stephan 1984). Meanwhile, other groups demonstrated that rats previously fed at a fixed time of the day, could show after being in AL conditions a more rapid entrainment when reexposed to RF, and could also exhibit bursts of activity associated with the former mealtime when food deprived. In other words, a previous experience of a scheduled RF facilitates the (re)appearance of FAA in response to food deprivation (Fig. 31) (Coleman et al. 1982; Stephan 1992; Mistlberger 1994). Of note, FAA can persist at its usual phase

[25] As an aside, the study of FAA is facilitated in SCN-X rats, since the circadian control of locomotor activity by the SCN pacemaker is eliminated, making it easier to assess the FEO output under restricted feeding challenge.

for a few days in some food-restricted animals that are placed back in AL conditions. However, in most cases, persistent activity is not observed following a day of AL access. On the other hand, FAA can reappear at its usual period even after repeated periods of AL access interrupted by food deprivation, or even 50 days after the last RF schedule (Clarke and Coleman 1986; Mistlberger 1994). Thus, this suggests that the FEO rapidly uncouple from (circadian) effectors that control (at least) locomotor activity, rather than dampen over time (Mistlberger 1994).

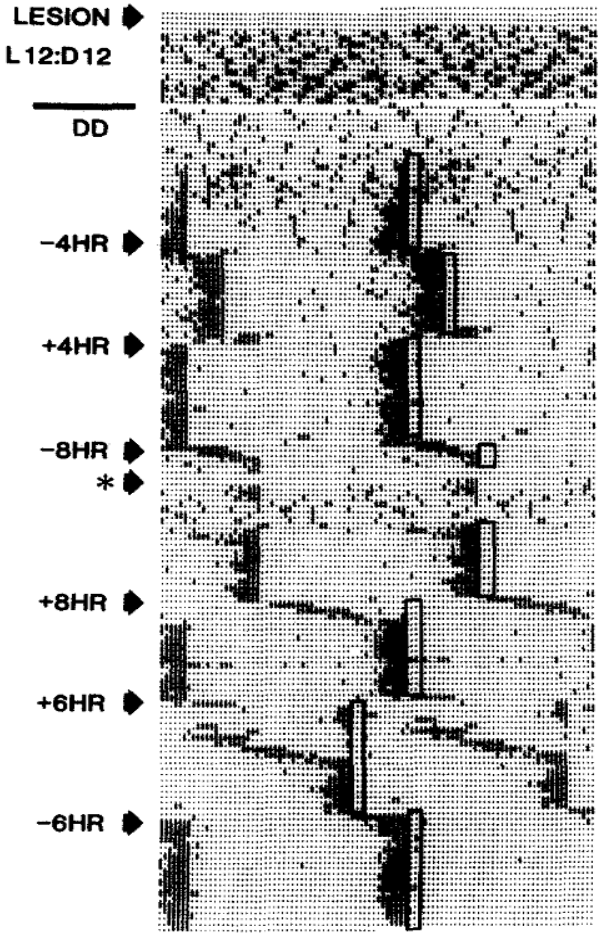


Figure 30. Food-anticipatory activity in an SCN-X rat followed phase shifts of mealtime

Double-plotted actogram of daily locomotor activity. Rectangles indicate 2 h food access during RF. DD or constant dark conditions. Phase shifts of food access are shown on the left. * indicates 2 days power failure and loss of data.

Note that strong FAA appeared ~2-3 h prior to mealtime immediately few days after RF. Following phase delays of food access (e.g., - 4 h or - 8 h), clear transient cycles in activity are observed. Following phase advances, however, this rat did not show clear transients in activity. From (Stephan 1984)

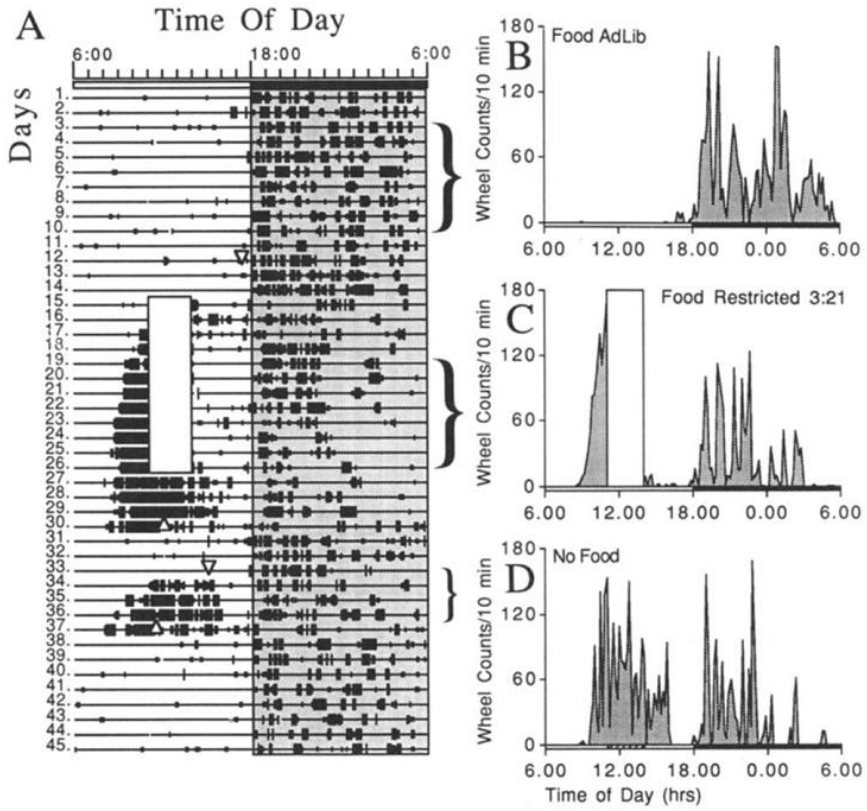


Figure 31. Food-anticipatory activity in a normal rat under light-dark conditions

(A) Single-plotted actogram of wheel-running activity of a rat during AL food and RF access. The white rectangle during the light period indicates time of food access. Triangles represent the beginning (pointing down) or end (pointing up) of total food deprivation. (B, C, D) Waveforms of wheel running during AL (7 day average), RF (7 day average), and total food deprivation (3 day average), respectively. From (Mistlberger 1994)

3.2.2 Feeding behavior and entrainment limits

Interestingly, rats both with and without SCN lesions are not capable of anticipating feedings at 18-h intervals (Stephan et al. 1979). Further experiments demonstrated that FAA is expressed only if food access is given under a circadian range. As a comparison, anticipatory wheel-running activity in SCN-ablated rats could not be reliably observed at 22-h intervals or shorter, and at 31-h intervals and longer (Fig. 32) (Boulos et al. 1980; Stephan 1981).

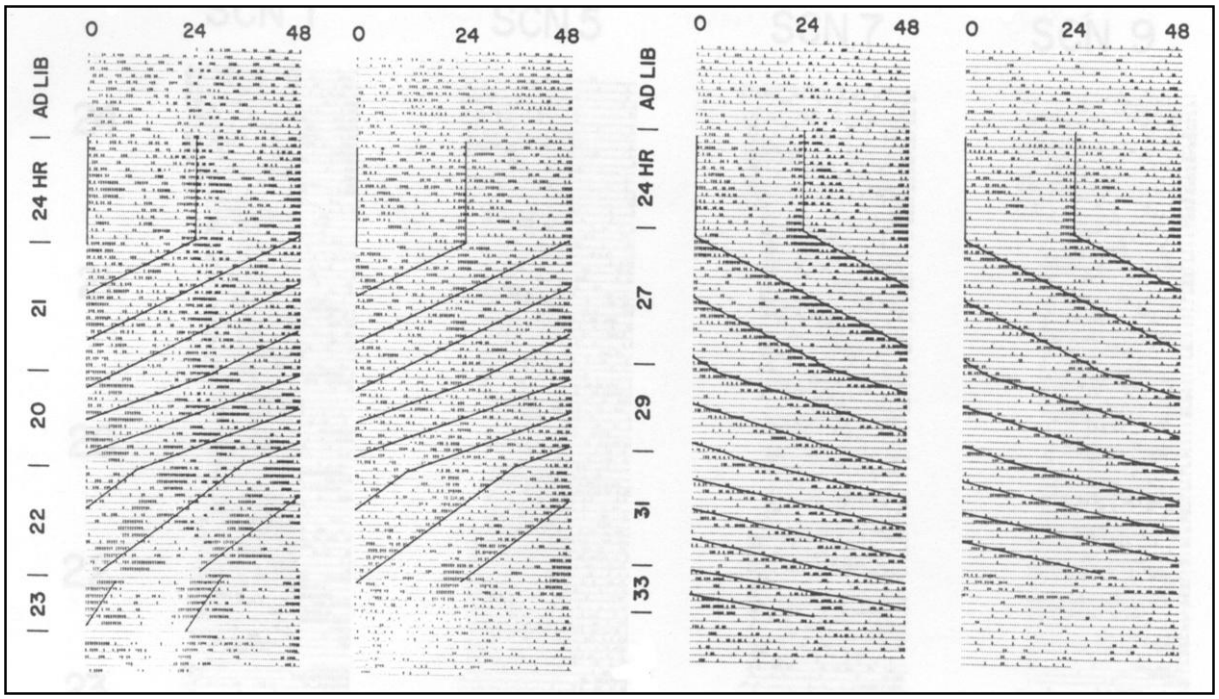


Figure 32. Limits of food entrainment in SCN-X rats

Rats bearing SCN lesions were maintained in constant darkness and exposed to RF at short interval periods (i.e., 21, 20, 22 and 23-h intervals between food access; left figures) and long interval periods (i.e., 27, 29, 31, 33-h intervals; right figures). Note that rats entrained to the 24-h feeding schedule and can show consistent entrainment to schedules between 23 and 29 h. Black lines indicate food access and number on the left indicate the period of the feeding schedule. From (Stephan 1981)

The circadian limit of entrainment and the fact that FAA onset occurs regularly with a positive phase angle (i.e., a few hours before mealtime, see Fig. 31), especially for long intervals, may suggest that the mechanism at the base of the FEO is relatively supple and have a memory of phase displacement. The latter can be also supported by the post-feeding activity that can be seen in some animals (Fig. 32, left actograms, 24-h and 21-h intervals). The study of the free-running capacity of this putative FEO is obviously limited by the deleterious effect of prolonged food deprivation. These experiments are nevertheless informative of the *circadian* nature of the FEO.

3.2.3 Conclusion on FEO's properties

The demonstration of a circadian range of entrainment, split transients and strong phase-specific persistence suggests that FAA is not simply the result of associative learning, classical conditioning or hourglass mechanism[26]. On the contrary, the above FAA characteristics are indicative of an oscillator that possesses a flexible intrinsic mechanism and that probably has the ability to retain or "remember" a circadian phase—likely associated with the memory of a specific location, as observation of food anticipation in bees supports this thought[27].

All these findings thus confirm the existence of one (or multiple) oscillator(s) that drive FAA under restricted food access and that is anatomically independent of the SCN.

3.3 Food anticipation, outputs and inputs

3.3.1 The multiple aspects of the food-entrained physiology

In addition to general and wheel-running lomocotor activities, other behavioral/physiological parameters appeared to be controlled by the FEO as well. Not surprisingly, drinking behavior can be synchronized to the periodic food access, increasing (i.e., number of licks) a few hours before mealtime or being maintained during food deprivation (Boulos et al. 1980; Clarke et al. 1986). FAA can also be observed by assessing lever pressing behavior (Boulos et al. 1980) or by evaluating food-bin approaches (Mistlberger 1994).

Concomitant to an increase of locomotor activity, a rise of body temperature can also be measured in normal or SCN-X rats prior to feeding time. In addition to this food-anticipatory thermogenesis (FAT), a diet-induced thermogenesis (DIT) is observed as well (Fig. 33) (Krieger 1974; Nelson et al. 1975; Challet et al. 1997). The anticipatory rise in temperature, regardless of its association with physical activity, can be viewed as an adaptive mechanism that prepares an organism to ingest food efficiently. Moreover, DIT is related to the cost of digestion, absorption and nutrient processing and is as well a consequence of enhanced brown-adipose tissue (BAT)

[26] Not excluding, however, the participation of memory, conditioning, etc.
[27] As starling birds give evidence of interactions between compass navigational system, memory and circadian clock systems.

thermogenesis due to excessive food consumption during a short time window. On the other hand, the decrease in body temperature before FAT can reflect the economizing strategy to save energy during food deprivation and to optimize food-seeking behaviors at a specific time of the day (Fig. 33).

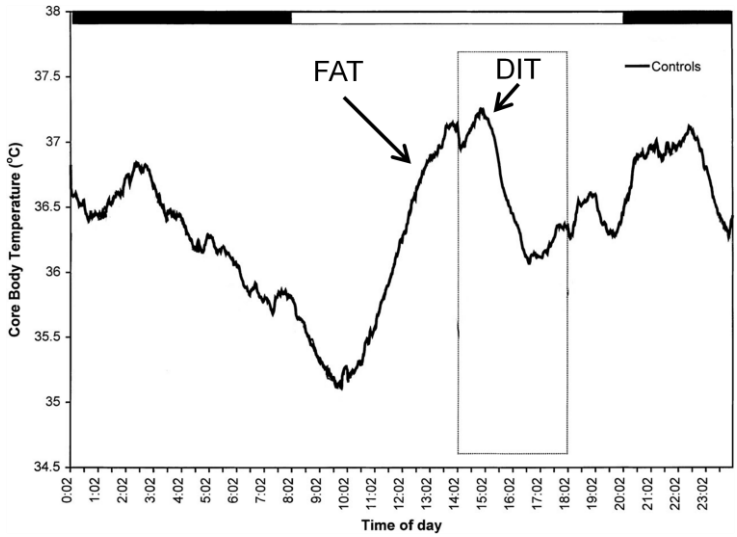


Figure 33. Rise in body temperature before mealtime in control rats

Mean core body temperature during 2 days of restricted feeding. The dashed rectangle indicates food access time. FAT: food-anticipatory thermogenesis; DIT: diet-induced thermogenesis. Adapted from (Davidson et al. 2000)

Corticosterone is another physiological parameter that shows a drastic elevation in anticipation to food arrival (Krieger 1974; Nelson et al. 1975). In nocturnal animals fed on AL basis, a regular peak of corticosterone occurs at the day-night transition and is controlled by the SCN pacemaker (Fig. 43). However, if food is restricted to a temporal window during the day, a second peak of corticosterone is observable before feeding time (Fig. 34). Of note, this RF-induced peak is not related to a possible stress induced by a food deprivation, since neither 24-h ACTH levels nor 24-h adrenal and plasma corticosterone levels were higher in food-restricted animals (Wilkinson et al. 1979). In addition, rats or mice that were stressed by tube restraint for 2 h at the same time for several days do not develop an anticipatory corticosterone peak prior to stress-time (Ottenweller et al. 1987) nor show a phase advance of circadian activity rhythm (Challet et al. 1998). Thus, these findings suggest that the anticipatory acrophase of corticosterone induced by feeding time is likely controlled by a FEO.

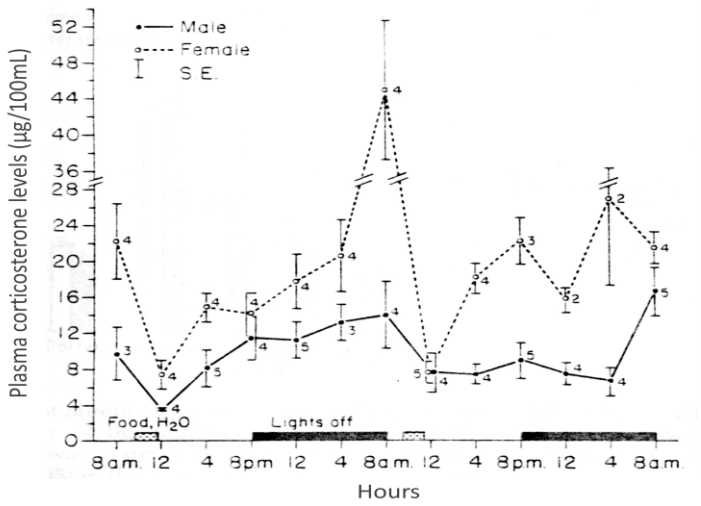


Figure 34. Rise in plasma corticosterone before mealtime in control rats

Mean plasma corticosterone levels in male and female rats under RF schedule. Food access is indicated by white rectangles along the X-axis. Note that both male and female rats showed a peak of corticosterone before food access. Adapted from (Krieger 1974)

Finally, other metabolic variables, such as leptin and ghrelin rhythms can be phase-shifted in response to restricted day feeding (Fig. 35) (Bodosi et al. 2004; Martinez-Merlos et al. 2004). Both leptin and free fatty acids are entrained by a 2-h RF schedule. In particular, leptin and free fatty acids levels increase after and before mealtime, respectively, and can persist during food deprivation. However, i.c.v. administration of leptin failed to elicit FAA, suggesting that leptin rise is likely a direct response to food intake. On the other hand, the elevation of free fatty acids before mealtime could reflect mobilization of energy substrates to sustain FAA (Martinez-Merlos et al. 2004). Parameters such as glucagon, insulin, and cholesterol, can also be affected by RF (Velasco et al. 1994; Diaz-Munoz et al. 2000; Martinez-Merlos et al. 2004). However, further investigations are needed to ensure that these metabolic actors are controlled by the FEO in the absence of a circadian control from the SCN pacemaker, and are not only a response to a low metabolic state.

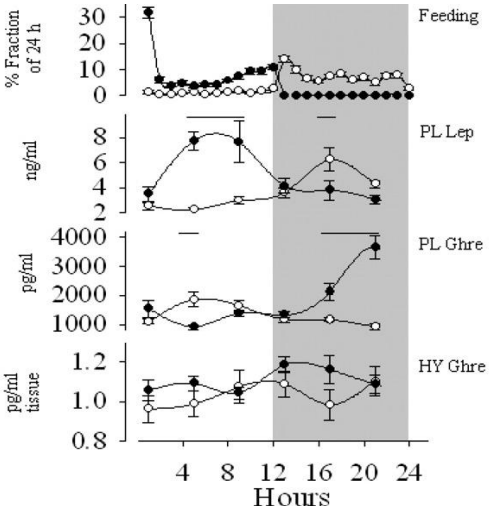


Figure 35. Effects of feeding restricted to the light cycle on the diurnal rhythms of leptin and ghrelin in rats

In rats with feeding restricted to the 12-h light period, phase-shift of plasma leptin (Pl Lep) and plasma ghrelin (Pl Ghre) can be observed. Hypothalamic ghrelin contents (HY Ghre) was not significantly affected. Filled symbols, RF rats; open symbols, free-feeding rats. Gray column, dark period. Adapted from (Bodosi et al. 2004)

3.3.2 Will run for almost anything

As outlined above, standard food provided only for a few hours per day can elicit behavioral and physiological activation, especially in rats. A temporal restriction protocol can be replaced by a hypocaloric feeding protocol, in which a limited amount of food is given at a fixed time point (i.e., usually 50% of the AL daily intake). In this procedure as well, food anticipation is observed in SCN intact rats (Challet et al. 1997). In addition, it has been mentioned that the anticipation of two mealtimes can be observed in rats (Mistlberger et al. 2012). Of note, if three meals are given for instance at 8-h intervals (thus three times per day), FAA is only observed for two out of the three meals and sometimes FAA jumps or shifts (with transient cycles) to another feeding time (Mistlberger 1994). This observation gives support to the control of FAA by the circadian system and not (only) by a memory/learning system.

Besides normocaloric food, access to salt solutions or salty food for 2 h each day failed to induce FAA (Rosenwasser et al. 1985). Similar results have been shown in rats that had access to a daily meal of protein, carbohydrate or fat, plus a free access to a complementary diet. Conversely, rats limited to two daily single-macronutrient meals (i.e., associated protein-fat, or protein-carbohydrate meals) can exhibit FAA (Mistlberger et al. 1990). Thus, the energetic content of a meal appears to be crucial to entrain the FEO. This view is partially confirmed by a study demonstrating that glucose is sufficient to affect the circadian properties (i.e., elicit transients after phase-shift of feeding time) of the FEO (Stephan and Davidson 1998). Of note, gastric distention does not seem necessary to synchronize the FEO (Stephan 1997).

Palatable but nonnutritive meals, such as saccharin, do not arouse the FEO, excluding taste as a main factor of entrainment (Mistlberger and Rusak 1987). Brief access to sucrose solution produces behavioral anticipation only after food deprivation in rats (Pecoraro et al. 2002; Waddington Lamont et al. 2007). Other attractive snacks, such as chocolate provided once a day at the same time without food deprivation, can induce FAA (Fig. 36) (Mendoza et al. 2005). However, this is not observed if the chocolate has no nutritional value, again confirming the importance of food energy content (Mistlberger et al. 1987). On the other hand, in hamsters, temporally limited daily access to chocolate can elicit FAA only if the SCN

is ablated or if the animals have limited access (i.e., 70%) to their standard food (Abe and Rusak 1992). In free-fed mice, however, a palatable chocolate snack failed to induce food anticipation, even if mice avidly consumed the snack and even if some related food-seeking behaviors can be detected (Hsu et al. 2010). Thus, the ability of the circadian system, in particular the FEO, to respond to palatable food, may depend on the motivational and metabolic states specific to each species, in addition to the nutritive aspects of the food.

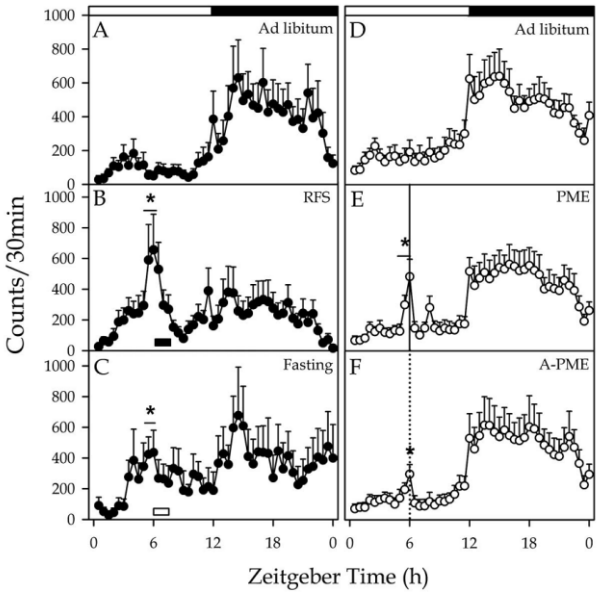


Figure 36. Daily fixed access to a palatable meal entrained locomotor activity in rats.

Mean activity waves for locomotor activity in restricted feeding scheduile (RFS; white circles) and in palatable meal entrainment (PME; black circles) groups during AL (A, D), during entrainment (B, E) and for the 3 days in food deprivation (C) or 4 days following interruption of palatable meal (F). Horizontal black bar indicates mealtime and vertical line indicates palatable mealtime (B, E). Horizontal white bar and dotted line indicate time of expected mealtime (C, F). Values represent mean±S.E. * Post hoc Tukey $P<0.05$. From (Mendoza et al. 2005)

From all these findings, we have seen that FAA can be induced by temporal restriction, hypocaloric feeding and to some extent by a daily palatable meal. Hence, the role of brain reward pathways in anticipatory behavior in rats can be highly considered. In addition, some addictive substances such as methamphetamine (MAP) have been demonstrated to act on the circadian timing system. In particular, daily injections of MAP have been shown to induce behavioral activation a few hours before the time of injection that can persist on the day of withdrawal (Shibata et al. 1994; Iijima et al. 2002). In addition, in SCN-X rats and mice, continuous MAP exposure can restore a locomotor rhythm in the circadian range (Honma et al. 1987), while in intact animals exposed to constant dark conditions, MAP lengthens the free-running period (Tataroglu et al. 2006). Of interest, the free-running activity induced by MAP can be entrained by a RF schedule in SCN-x rats. However, no FAA is detectable in this experimental design (Fig. 37) (Honma et al. 1989).

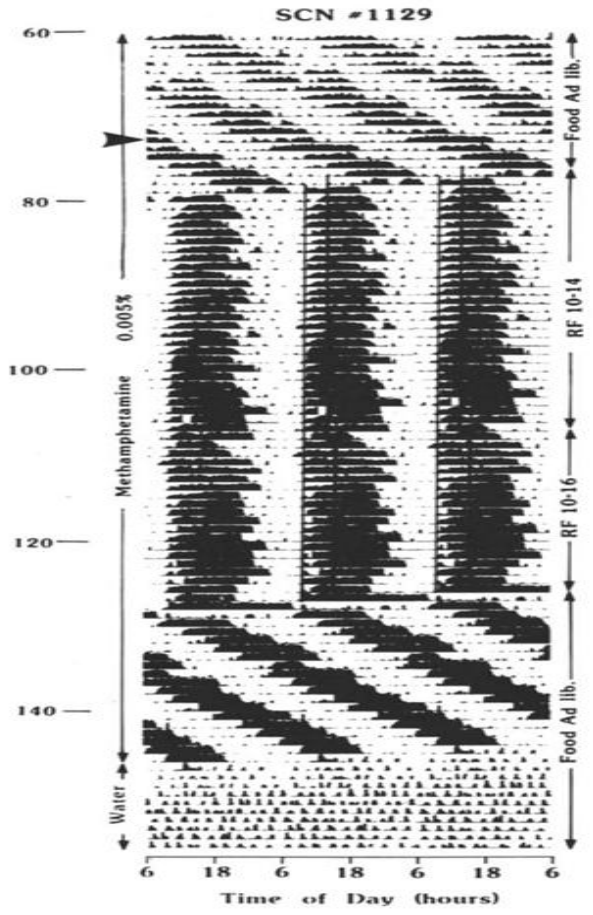


Figure 37. Methamphetamine-induced locomotor rhythm entrains to restricted daily feeding in SCN lesioned rats.

Triple plotted actographs of SCN lesioned rats before, during and after the RF schedule. Note that rats were also blinded during the procedure (black arrow heads). Parallel vertical lines in the actograph indicate (left) methamphetamine treatment and (right)

RF schedule: RF 10-14 means the food presentation from 10 to 14 h and RF 10-16 from 10 to 16 h.

Note that no FAA can be detected during the RF protocol. Note also that after termination of methamphetamine treatment, animals were *de novo* arrhythmic.

From (Honma et al. 1989)

The intriguing effect of MAP suggests that a methamphetamine-sensitive oscillator (MASCO), also independent of the SCN, can control behavioral rhythmicity. However, it is not excluded that both MAP and food can act on the same circadian system, a unique oscillator (originally the FEO), or more likely, a multiple oscillator system (at least FEO + MASCO). As an aside, this system could be tightly associated with brain regions that form reward pathways and could rely on close molecular feedback loops that generate circadian oscillations in the SCN and extra-SCN tissues as will be further discussed.

3.4 When restricted feeding affects the circadian timing system

Usually, locomotor activity, body temperature, fatty acid synthesis (Hems et al. 1975), or expression of specific genes (see below), start to increase at the beginning of the light-dark transition, provided that free-fed nocturnal animals are synchronized to a 12:12 light-dark cycle. Conversely, after having been acclimatized to a circadian feeding schedule, many animals' body functions adapt to be in phase with feeding time.

3.4.1 Peripheral tissues

In response to RF, the stomach has been shown to increase its capacity for distension (Martinez-Merlos et al. 2004). Disaccharidase and even mitotic activities showed rhythmic changes in the gastro-intestinal system with higher levels around the feeding time (Nishida et al. 1978; Scheving et al. 1983). Moreover, clock gene oscillations in peripheral tissues can be shifted by feeding time and thus be uncoupled from the circadian control of the central SCN pacemaker (Damiola et al. 2000; Stokkan et al. 2001; Feillet et al. 2006). In particular, the liver (Fig. 38), kidney, heart and pancreas, showed gradual phase resetting of rhythmic gene expression in response to daytime feeding, similar to a change in photoperiod, indicative of the involvement of a clock-dependent mechanism (Damiola et al. 2000). Moreover, the liver oscillator, important for glucose and lipid homeostasis, has been demonstrated to exhibit significant metabolic phase adjustments and increased levels of metabolic genes expression in response to food restriction (Lima et al. 1981; Baez-Ruiz et al. 2005). The fact that many physiological, metabolic and endocrine events (e.g., temperature, corticosterone changes as outlined above) are affected by a RF schedule suggests that some peripheral signals may serve to regulate, or even entrain the circadian functioning of other organs and tissues that communicate with (or represent) the FEO.

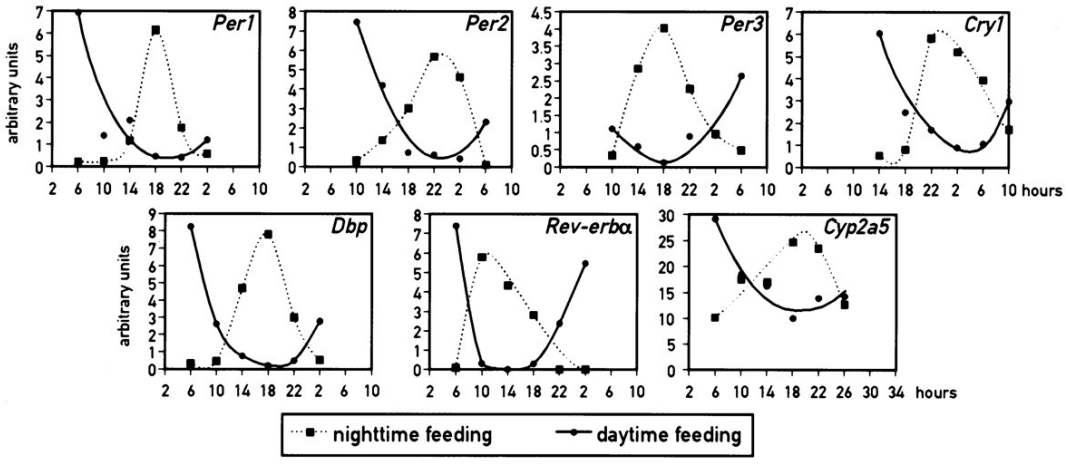


Figure 38. Circadian accumulation of clock and clock-controlled genes in the liver

Daytime feeding changes the phase of circadian gene expression in the liver. Mice, kept under a light-dark regimen (lights on 6 a.m., lights off 6 p.m.), were fed exclusively during the light phase (6 a.m. to 6 p.m.) or during the dark phase (6 p.m. to 6 a.m.). From (Damiola et al. 2000)

In this context, glucocorticoids are good candidates. Indeed, the glucocorticoid hormone analog dexamethasone has been shown to induce circadian gene expression in cultured fibroblasts and to transiently change the phase of clock gene expression in the liver, kidney, and heart (Balsalobre et al. 2000). Besides, glucocorticoids can feed back to the central nervous system to modulate brain oscillators (Sage et al. 2004; Malek et al. 2007). The nutrient-responsive adenosine monophosphate-activated protein kinase (AMPK) is another candidate to inform the core clock machinery of metabolic changes (see part 4.4.2) (Lamia et al. 2009)).

Thus, feeding schedule has strong Zeitgeber properties on peripheral tissues, as evidenced by the uncoupling of peripheral oscillators from the SCN. Interestingly, peripheral organs connect directly through nervous pathways to the brain and can release multiple metabolic signals that can affect the core clock machinery.

3.4.2 Brain nuclei

Contrary to peripheral oscillators, the SCN pacemaker is relatively impervious to the synchronizing effect of mealtime, provided that the animals are exposed to a light-dark cycle and ingest enough daily energy. Under severe food restriction (i.e., caloric restriction), however, the phase of the SCN and its synchronization to light are modified (Challet 2010). Different studies have measured c-FOS expression, as a marker of neuronal activity (Dragunow and Faull 1989) in different brain areas, to assess their responses to a scheduled feeding. In food-entrained rats, increased c-FOS immunoreactivity before and after mealtimes have been observed in the DMH, LH, perifornical area, and only after feeding in the PVN (Angeles-Castellanos et al. 2004; Gooley et al. 2006). In hypothalamic regions such as the LH, DMH and perifornical area, c-FOS expression has even been shown to persist for a few days when food was not provided at the expected time (Angeles-Castellanos et al. 2004). Several additional structures such as the BNST, lateral septum, nucleus accumbens (NAc), AMY, prefrontal cortex and PVT, showed up-regulated c-FOS expression in anticipation to food access (Fig. 39) (Angeles-Castellanos et al. 2007).

Besides, dopamine release is up-regulated in advance of a scheduled mealtime in the NAc (Hsu et al. 2010). In addition, the neuropeptide Y (NPY) and agouti-related peptide (AgRP) has been shown to increase prior to mealtime in, for

instance, the ARC nucleus, the PVN but not the DMH (Yoshihara et al. 1996; Crowley et al. 2005). PER2 rhythms are also sensitive to scheduled feeding in limbic forebrain areas (Waddington Lamont et al. 2007). Moreover, a phase-shift of *mPer1* and *mPer2* expression is observed in mice fed only for 4 h during the daytime in the cerebral cortex and hippocampus, as well as moderately in the striatum, piriform cortex and PVN, and without effect in the SCN (Wakamatsu et al. 2001). Hypocaloric feeding has also been shown to affect PER1 and PER2 expression in multiple brain nuclei (e.g., DMH and ARC) (Feillet et al. 2008). These studies support the view that the FEO is probably not a unique structure, but involves several hypothalamic and corticolimbic brain regions.

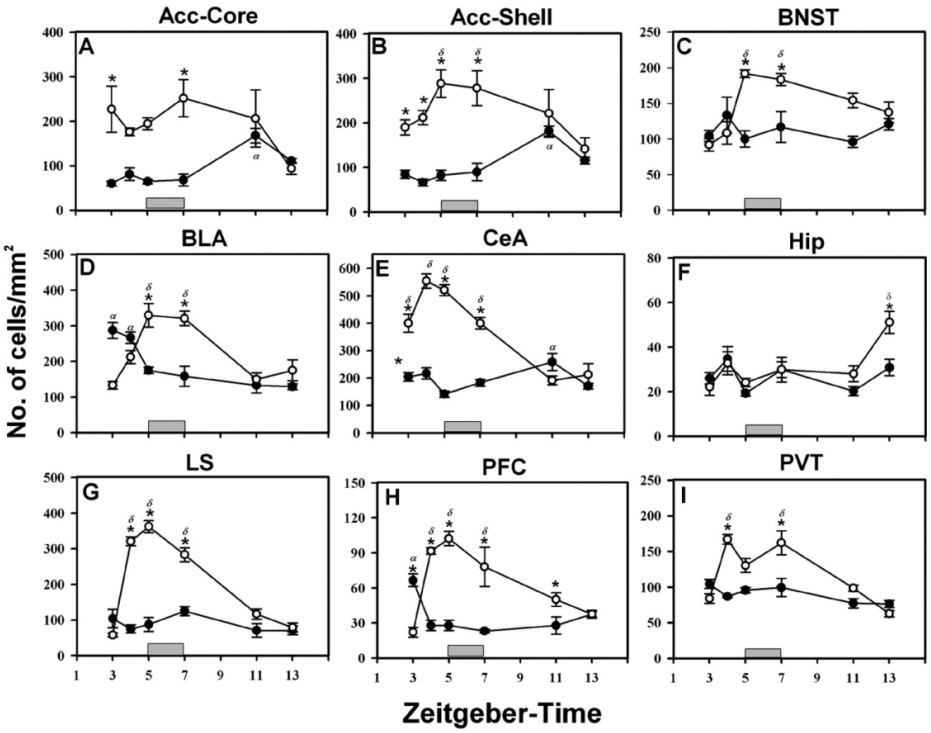


Figure 39. Temporal patterns of c-FOS immunoreactivity in corticolimbic structures
Food-entrained rats (○) and their ad libitum controls AL (●). Significant cellular activation can be observed in all structures during the time when rats are anticipating food access and after mealtime. The hippocampus (F) is the only structure that does not show cellular activation associated with FAA and feeding. The horizontal bar on the abscissa represents food access. Values are mean±S.E. * Significant difference between AL and RF group obtained with the Tukey post hoc test ($P<0.01$), α for the AL group between peak and low time points, and δ for RF. From (Angeles-Castellanos et al. 2007)

It is worth noting that subdivisions of specific brain nuclei, such as the compact and ventral parts of the DMH, do not exhibit the same degree of response to RF in mice. In particular, m*Per1* mRNA expression was strongly induced after feeding in the compact part of the DMH, while no significant rhythmicity was detected in AL

conditions (Mieda et al. 2006). In addition, the PER2 rhythm has been shown in some limbic forebrain structures to be dependent on adrenal integrity (Lamont et al. 2005), suggesting that corticosterone levels during a RF-schedule can act downstream of the SCN to synchronize the activity of structures, probably important to anticipate food access.

In addition to the synchronizing properties of RF, palatable meals also have an entraining effect on extra-SCN oscillators. Indeed, in rats with unrestricted standard food access, c-FOS and PER1 expression were shown to be in phase with a daily palatable meal in the PVT and limbic structures (Mendoza et al. 2005; Mendoza et al. 2005; Angeles-Castellanos et al. 2008). However, no effects are observed on PER2 levels in the limbic system in response to a daily access of sucrose solution in freely fed rats (Waddington Lamont et al. 2007). This was observed despite the efficiency of a sucrose solution in eliciting dopamine release in the NAc (Bassareo and Di Chiara 1999; Rada et al. 2005). Taken together, these results show that some “hedonic” feeding signals can affect gene expression. However, the effects appear rather limited since the expression pattern of additional clock components is lacking. Therefore, the accurate synchronizing properties of a palatable meal on the core clock machinery remains to be further investigated.

To summarize, the results cited above give evidence that feeding synchronization—and the negative metabolic state associated with food restriction—implies phase-adjustment of metabolic processes and gene expression in peripheral and brain tissues. As a result, many organs could likely play an essential role in food anticipation. Thus, the mammalian body is being carefully explored with a "fine-tooth comb" to delineate the FEO substrate(s) and its intrinsic core clock mechanism.

3.5 The food-entrainable oscillator, a needle in a haystack

As indicated above, the relevance of peripheral oscillators as well as brain nuclei for the generation of food-entrainable oscillations seems undeniable. Nonetheless, very little is known as of yet regarding the location and the exact molecular basis on which the FEO relies. The identification of the neural substrate and the molecular basis of the so-called FEO have indeed proved to be a real challenge these last 30 years. Different approaches have been used, but neither anatomical nor molecular approaches have really succeeded in elucidating the neural substrate or the precise molecular functioning of this mysterious oscillator.

3.5.1 Lesional and pharmacological approaches

Subdiaphragmatic vagotomy was used in one study to remove afferents from the periphery to the brain and severely impact the normal function of the gastro-intestinal tract. In this study, there was no change in the amount of FAA (Comperatore and Stephan 1990). Notably, the vagus nerve does not seem necessary for the ghrelin reponse to nutrients (Williams et al. 2003). To further investigate neural routes of communication between the periphery and the central nervous system, capsaicin-induced visceral deafferentation was performed in rats bearing SCN lesions (Davidson and Stephan 1998). However, as for vagal transection, capsaicin deafferentation did not prevent FAA, thus questioning the implication of neural pathways. The role of the liver oscillator in response to food restriction has also been investigated in rats rendered cirrhotic with carbon tetrachloride. These rats displayed, however, similar FAA and RF-entrained free fatty acids levels compared to normal rats (Escobar et al. 2002; Escobar et al. 2005). Nonetheless, abnormalities in liver functioning were not described in these studies. Of note, adrenalectomy did not block FAA either (Stephan et al. 1979). This result opposes to the highlighted role of corticosterone in the resetting of the circadian system, and can suggest that corticosterone signaling is somewhat dispensable for food entrainment. Of great interest, a study has investigated the role of the digestive system in the food-entrained oscillations that underlie FAA. The authors entrained rats to dual RF (i.e., two meals per day) and observed that FAA occurred for both meals. However, PER1-LUC rhythmicity in the digestive system (including liver, esophagus, stomach and colon) showed entrainment only to one-meal, dispelling the

hypothesis that FAA arises as a unique output of rhythms in the gastrointestinal system. Thus food anticipation is likely an output of a circadian FEO, likely located in the brain (Davidson et al. 2003).

On the other hand, brain regions can detect blood-borne feedback signals that can be indicative of perturbations in energy balance, such as diminished circulating metabolic fuels (e.g., glucose) or high food intake in a short time-period. In that context, lesion or induced-degeneration of brain structures essential for relaying circulating signal from the periphery, such as the area postrema[28] and the ARC nucleus[29], failed to affect FAA (Mistlberger and Antle 1999; Davidson et al. 2001). On the contrary, lesion of the parabrachial nuclei, which receive information from visceral and gustatory afferents and direct inputs from the area postrema and the nucleus of the solitary tract (NTS), altered food-anticipatory bin approaches and slightly attenuated FAT (Davidson et al. 2000). In one study, destruction of the ventromedial nucleus have also been found to affect FAA (Inouye 1982), but another study points to the importance of a sufficient time for recovery after lesion and the evaluation of food entrainment (Mistlberger and Rechtschaffen 1984). A lesional approach focusing on the paraventricular and lateral hypothalamic areas also failed to abolish FAA or mealtime associated food-bin approaches (Mistlberger and Rusak 1988). The pharmacological depletion of orexin neurons, essential for regulation of arousal and appetite, induced hypophagia and weight loss but did not affect anticipation of a daily meal (Mistlberger et al. 2003). Rats bearing large lesions of the AMY, hippocampus or NAc were able to anticipate meal timing (Mistlberger and Mumby 1992). Of note, specific ablation of the shell part of the NAc had a positive effect on FAA (Mendoza et al. 2005). Treatment with the dopamine antagonist haloperidol did not affected food-entrained rhythms in intact rats either (Mistlberger et al. 1992). However, 6-hydroxydopamine, a neurotoxin that selectively kills dopaminergic and noradrenergic neurons, injected into the PVN did eliminate the anticipatory rise of corticosterone (Honma et al. 1992). Lesion of the PVT appeared to change the phase of the food-anticipatory corticosterone peak (Nakahara et al. 2004), without affecting FAA (Landry et al. 2007). Finally, zinc-induced anosmia in rats did not affect anticipation

[28] A region that lacks the blood-brain barrier (BBB).

[29] The presence of a BBB is controversial for the ARC since some vessels in the ventromedial part lacks some BBB markers. At least, the close relationship of the ARC with the median eminence makes it important to integrate metabolic changes in the periphery.

during scheduled feeding, indicating that olfaction does not play an exclusive role—*Forel's assumption* (Coleman and Hay 1990).

Of great interest, the DMH has been shown to be crucial for producing circadian rhythms such as those of sleep and waking, locomotor activity, feeding and corticosteroid production. In addition, the DMH connects with several hypothalamic nuclei including the lateral area and the PVN (Chou et al. 2003; Saper et al. 2005). The DMH has thus been tested as a putative FEO. In that context, a first study demonstrated that cell-specific lesions of the DMH in rats caused a drastic reduction in the preprandial rise in locomotor activity, body temperature and wakefulness (Fig. 40). The authors additionally showed that the degree of FAA correlated with the number of remaining DMH neurons (Gooley et al. 2006).

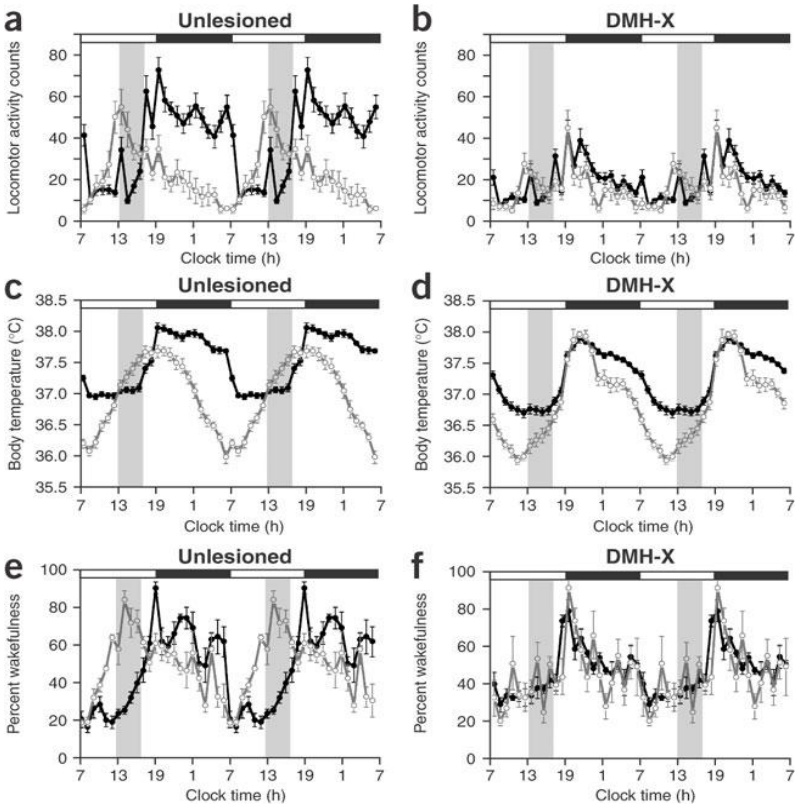


Figure 40. DMH lesions attenuate food entrainment.

After 3 weeks of restricted feeding, rats were deprived of food for 2 d so that the underlying behavioral and physiologic rhythms could be observed in the absence of the daily feeding-time cue. Black, rhythms during *ad lib* feeding; gray, rhythms during food deprivation period. (**a**–**f**) Unlesioned rats showed a marked shift induced by restricted feeding in the rhythms of locomotor activity (**a**), body temperature (**c**) and wakefulness (**e**). However, in DMH-lesioned rats, rhythms of locomotion (**b**), body temperature (**d**) and wakefulness (**f**) remained in phase with the light-dark cycle rather than with the daily meal. Data are reported as hourly mean ±s.e.m. White-black bars at top, light-dark cycle; vertical gray bars, timing of food availability during restricted feeding. Rhythms during *ad lib* feeding and food deprivation are plotted twice to emphasize daily rhythmicity. From (Gooley et al. 2006)

Nevertheless, further studies focusing on the DMH showed, by using similar and complementary methods to induce lesion and assess food anticipation, that food entrainment in DMH-x rats was normal and even persisted during a food deprivation (Landry et al. 2006; Landry et al. 2007). Clock gene expression, temperature and activity rhythms could be RF-entrained in DMH-ablated mice as well (Moriya et al. 2009). Conversely, two additional studies gave evidence that the DMH may still play a role in the control of food anticipation. The first in mice showed that after widespread destruction of the mediobasal hypothalamus, comprising the DMH and parts of the VMH and ARC, a reduction of FAA to a similar extent than the initial study of Gooley et al. 2006 was observed but without affecting the entrainment of *Per2* gene expression to mealtime in the liver (Tahara et al. 2010). The second study explored the tight connection between the SCN pacemaker and the putative DMH FEO in rats. The authors first revealed the presence of DMH GABA-projections to the SCN that can inhibit its intrinsic neuronal activity. Then they reported that DMH-lesion, which results in a diminution of FAA, leads simultaneously to increased neuronal activity in the SCN (at the level of c-FOS expression). Intriguingly, the concomitant lesion of the SCN in the same animals (i.e., previously DMH-lesioned) restored FAA. They thus proposed that in intact animals, the DMH may play a role in food entrainment by inhibiting the activity of the SCN (Acosta-Galvan et al. 2011). However, the SCN lesions appeared rather incomplete in the actograms provided in Acosta-Galvan et al. 2011, and the metabolic state of the animal, especially after deep surgery, has not been communicated. Therefore, further studies are needed to clarify the exact role of the DMH. In addition, it is important to note that, in both this last study and those cited above, further behavioral, physiological or gene expression measures, as well as their evaluation in different lighting conditions, are truly lacking for the study of food entrainment.

From all these results, it appears likely that the FEO is not confined to only one (peripheral or central) structure. In the contrary, the FEO may be a network of distributed circadian oscillators (weak or strong, see Fig. 24) that allow the integration of multiple messages and that permit sustained food-entrainable rhyhms of behavior and physiology even in the absence of one component (Fig. 41).

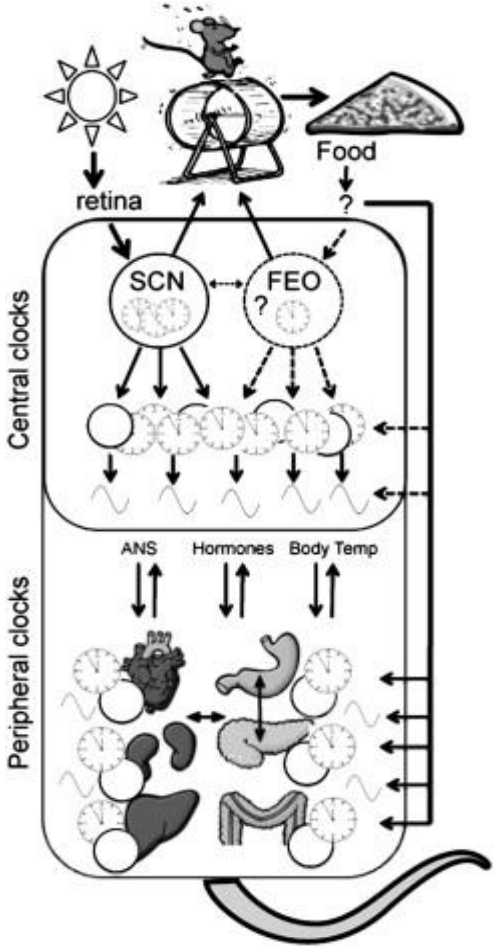


Figure 41. Cartoon of the mammalian circadian system, illustrating known (solid arrows and circles) and hypothesized (dashed arrows and circles) components and pathways

Circles without clock hands represent processes directly driven by other clocks or external stimuli. Sine waves represent overt rhythms. Light–dark cycles entrain a circadian pacemaker in the suprachiasmatic nucleus (SCN). SCN outputs via polysynaptic pathways drive daily rhythms of activity and feeding, autonomic efferents, hormones and body temperature, all of which contribute to phase control of circadian clocks and driven processes in peripheral organs. If food is temporally restricted, locomotor activity comes under control of a timing mechanism (most likely neural) with circadian properties that generates food anticipatory activity. There may be a central food-entrainable oscillator (FEO) coordinating behavioral and physiological rhythms to mealtime, or there may be a distributed system of central and peripheral FEOs, entrained in parallel by feeding related stimuli. From (Mistlberger 2011)

3.5.2 Gene deletion strategy

Genetic strategies allowing modifications of the genome, especially in mice, have been widely used to study the FEO clockwork. Clock mutants were the first models phenotyped in the context of food entrainment. In spite of having a strong circadian SCN defect (Vitaterna et al. 1994), *ClockΔ19* mutant mice exhibit higher and longer FAA than wild-type mice when challenged with RF, both in light-dark or constant dark conditions (Pitts et al. 2003). This result was also confirmed with the *Clock*-null mutant mice that also showed normal food-entrained clock gene expression to RF (Horikawa et al. 2005). It is interesting to note that both mutations did not affect the SCN functioning in the same manner and neither affected food entrainment. This could in part be explained by functional redundancy between clock genes, since NPAS2, a paralog of the CLOCK protein, can also heterodimerize with BMAL1 (Reick et al. 2001). In this context, deletion of *Npas2* has been shown to have effect on *Per2* mRNA oscillations in extra-SCN tissues (Reick et al. 2001). However, in response to temporal RF, mice knockout for *Npas2* only displayed a

slightly delayed FAA (Dudley et al. 2003). The SCN functioning is also severely affected in mice double-mutant for *Cry1* and *Cry2* (van der Horst et al. 1999). These double-mutant mice expressed delayed and unstable FAA in light-dark or constant dark conditions, and disrupted FAA was also noticed when the SCN pacemaker was lesioned (Iijima et al. 2005).

A role in food anticipation for the main clock components *Per1* and *Per2* has also been investigated. Mice with a mutated *Per1* gene ($Per1^{Brdm1}$, considered a null-mutant allele) exhibited FAA at levels close to their wild-type littermates (Feillet et al. 2006). On the contrary, $Per2^{Brdm1}$-mutant mice (that carry a deletion in the PAS domain) expressed reduced food anticipation of wheel-running and general activities as well as body temperature in response to temporal RF or hypocaloric feeding, regardless of the lighting conditions (Fig. 42). *Per2* mutation, however, did not interfere with hepatic clock gene entrainment to RF (Feillet et al. 2006). On the contrary, in $Per2^{ldc}$-mutant mice (a null-mutant allele) normal FAA both in a light–dark cycle and in constant darkness was observed. In addition, double $Per1^{ldc}$;$Per2^{ldc}$ mutant mice exhibited normal FAA (Storch and Weitz 2009). The different consequence relative to complete or partial loss-of-function mutation strategies (i.e., creating partially or completely non-functional proteins) on the circadian timing system may explain these discrepancies (for review, see Challet et al. 2009).

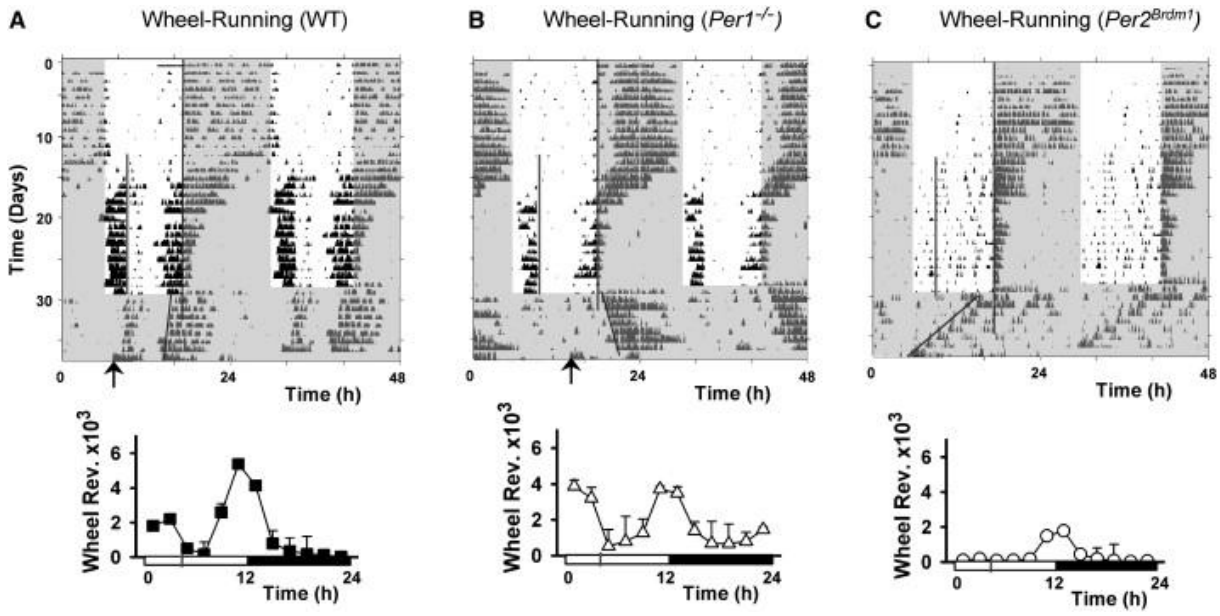


Figure 42. Daily wheel-running activity in wild-type (WT) (A), *Per1* (B), and *Per2* (C) mutant mice with hypocaloric feeding under light-dark conditions

Gray shading indicates lights off. Animals were fed ad libitum, submitted to hypocaloric feeding, and subsequently released into constant darkness with food ad libitum with the exception of the last day (arrow) when no food was accessible. The gray line indicates the time when hypocaloric food was provided. The arrow shows FAA at the expected time in both WT and *Per1* mutant mice fed ad libitum in constant darkness. The bottom graph represents the mean daily-activity profile during the last 8 days of hypocaloric feeding period (n = 6 in WT and *Per1*−/− mice and n = 4 in *Per2*^{Brdm1} mice; mean ± SEM). The gray line on the X axis indicates time of feeding. Note the lack of food-anticipatory activity in *Per2* mutant mice (C). From (Feillet et al. 2006)

As studies that explored a role for the DMH in food anticipation gave diverging conclusions, investigations on the involvement of the *Bmal1* clock gene in the FEO (and DMH) is controversial as well. *Bmal1*$^{-/-}$ mice (null-allele) are arrhythmic in constant darkness and strong alterations of their SCN clock machinery are observed (Bunger et al. 2000). In addition, when challenged with RF, *Bmal1*$^{-/-}$ mice did not show significant increase of locomotor activity and body temperature prior to mealtime (Fuller et al. 2008). By using viral vector containing the *Bmal1* gene, Fuller and colleagues were able to demonstrate that the injection of this vector in the SCN of the *Bmal1*$^{-/-}$ mice can restore light-entrainable circadian rhythms (i.e., those of locomotor activity and body temperature) without positively affecting food-entrained rhythms. Moreover, injection of the viral vector containing the *Bmal1* gene in the DMH can restore the ability of *Bmal1*$^{-/-}$ mice to entrain to RF without affecting light entrainment. The authors thus concluded that the DMH harbors a *Bmal1*-based FEO (Fuller et al. 2008). However, these results have been challenged by further studies showing that some FAA can be observed in the *Bmal1*$^{-/-}$ mice (Mistlberger et al. 2008; Pendergast et al. 2009; Storch et al. 2009). Besides, methodological and data issues have been raised regarding Fuller and colleagues' findings (Fuller et al. 2009; Mistlberger et al. 2009; Mistlberger et al. 2009). These caveats aside, it is important to mention that *Bmal1*$^{-/-}$ mice have profound developmental and metabolic defects (see part 4.3.1). To avoid these problems, a recent study has investigated food-entrainable activity in mice lacking *Bmal1* only in the nervous system. They showed that the emergence of FAA was severely delayed in these mice, supporting an essential role of the *Bmal1* clock gene (in the nervous system) for a rapid adaptation to periodic feeding (Mieda and Sakurai 2011).

Mice lacking specific feeding-related genes have also been studied. Genetically obese Zucker (*fa/fa*) rats that carry a mutation in the leptin receptor (see part 4.2.1 for a detailed description) displayed anticipatory rhythms greater than that

of control rats, that were maintained during food deprivation. This can suggest that leptin action is not an essential entrainment cue for the FEO (Mistlberger and Marchant 1999). On the other hand, mice carrying a mutation in the ghrelin receptor (GHSR knockout (KO) mice), exhibited diminished FAA and c-FOS immunoreactivity in several hypothalamic nuclei in response to RF (Blum et al. 2009; LeSauter et al. 2009). Moreover, hypothalamic c-FOS expression patterns in unrestricted wild-type (WT) mice receiving daily i.p. injections of ghrelin can mimic those of mice under restricted feeding schedules (Blum et al. 2009). These results indicate that ghrelin can play a strong role in food entrainment. The role of ghrelin is highlighted by two additional studies. The first demonstrated that injections of ghrelin in free-fed animals (that were food-deprived following the injection) can increase general locomotor activity and subsequent food intake. In addition, the authors of the first study showed that the stomach oxyntic gland cells contained a putative FEO, since ghrelin and clock gene expression (i.e., PER1 and PER2) were controlled by the time of food availability and rhythmic expression of oxyntic cell ghrelin was abolished in *Per1-Per2*$^{(Brdm1)}$ double-mutant mice (LeSauter et al. 2009). The second study gives evidence that the injection of ghrelin or an antagonist of the ghrelin receptor can increase or decrease, respectively, FAA prior to access to a palatable meal (Merkestein et al. 2012). Ghrelin thus seems to be a strong humoral signal to modulate food anticipation and motivational associated-behaviors such as feeding.

Genetic perturbations of several additional genes also led to interesting phenotypes regarding food entrainment. As outlined above, orexin is essential to regulate feeding and arousal. Orexin KO mice display reduced wakefulness and FAA but normal FAT before feeding time (Akiyama et al. 2004; Mieda et al. 2004; Kaur et al. 2008). Nevertheless, chemical destruction of hypothalamic orexin-producing neurons did not affect FAA (Mistlberger et al. 2003), thus not supporting an indispensable role for orexin. The role of the melanin-concentrating hormone (MCH, a neuropeptide that stimulates feeding) in food anticipation has been explored in mice deficient for the MCH1 receptor, with no effect (Zhou et al. 2005). On the other hand, melanocortins, crucial for the regulation of energy homeostasis, may influence food entrainment. Indeed, mice deficient for the melanocortin receptors MCR3 exhibit attenuated wakefulness and locomotor activity prior to food presentation concomitant with impaired clock gene expression in AL and RF conditions (Sutton et al. 2008).

Deletion of the orphan nuclear receptor *Ear2* in mice, essential for the development of the *locus coeruleus*, reduced FAA in response to a RF challenge. Of note, *Ear2*$^{-/-}$ mice have impaired *Per1* and *Per2* expression in the frontal cortex as well as diminished NA levels in this brain region (Warnecke et al. 2005). Investigations focusing on the role of mu-opiod receptors in adapted-reward behaviors are also informative. During or prior to the activation of anticipatory behavior to a coming reward, the mesolimbic opioid system is activated (Spruijt et al. 2001). Of interest, mu-Opioid receptor KO mice do not show evidence of ethanol self-administration, or display attenuated MAP-induced behavioral sensitization and cocaine conditioned place preference—or reward cocaine effect—compared to wild-type littermates (Roberts et al. 2000; Hall et al. 2004; Shen et al. 2010). In the context of food entrainment, mu-KO mice showed reduced locomotor activity in anticipation of food access (Kas et al. 2004). This may suggest that a functional opioid system is essential to adapt behavior to the limited temporal access of a daily reward such as food. Finally, mutation in mice of the ionotropic glutamate receptor δ2 gene (*Grid2*), selectively expressed in Purkinje cells, led to an impairment of motor coordination and to a severe reduction of food-anticipatory components (Mendoza et al. 2010).

Hence, the FEO location does not appear to be confined to a unique structure. Multiple genes involved in clock regulation pathways, metabolic and feeding pathways, or even reward pathways, have emerged concurrently as important actors to drive food entrainment.

3.6 General conclusion

The necessary effort to feed life has favoured the evolution of a food-entrainable system to track temporal regularities and anticipate predictable changes of food resources in nature. Indeed, animals that occupy different time niches can forage at the most appropriate time of the day to ensure their survival. The increased expression of anticipatory behavior and physiology during artificially (i.e., in laboratory conditions) reduced food availability can thus reflect the triggering of circadian, motivational and metabolic processes to ensure that foraging takes place in the most efficient manner.

From the studies presented above, the molecular (circadian) control of food anticipation is still elusive in mammals—both at the tissue and cellular levels—, but two working hypotheses emerge. The first is an assumption that food entrainment is dependent on at least a few clock genes (i.e., *Per2, Bmal1, Npas2* and *Cry1-2*) and thus a food-entrainable clockwork model can be shaped and served to further integrate the role of other clock-related genes yet to be tested (for a review, see Challet et al. 2009). The second hypothesis opts for the involvement of other (circadian) mechanisms for food entrainment to be determined and supporters based their hypothesis on the observations that canonical clock genes are not involved for the functioning of the FEO nor for the MASCO[30] (Mohawk et al. 2009; Storch et al. 2009).

Incidentally, most of the studies that investigate the functions of genes in food entrainment used *germline* KO mice, which make the interpretation of results a bit challenging. Indeed, on one hand, the circadian system has been demonstrated to be relatively impervious to genetic perturbations *in vitro* (Baggs et al. 2009), which is supported *in vivo* by the few strong effects of clock gene deletion on the SCN pacemaker. Thus, significant impairment of the FEO clockwork could be hard to detect. On the other hand, clock gene deletion (as well as lesion of brain nuclei) can have broad consequences, in particular, on energy homeostasis (see part 4.3.1), rendering the evaluation of circadian food entrainment more difficult.

Finally, beyond the need to also (re)consider the participation of reward and learning processes in food entrainment (Silver et al. 2011), study of the simplest organisms (e.g., bee) could probably be informative for further investigations of the food-entrainable system. In parallel, the use in the future of inducible and reversible knockout, or even, optogenetic tools for targeted neural inhibition/activation may help to find the “needle(s) in the haystack”.

[30] The close nature between the MASCO and the FEO (a sole entity or two different systems) was mentioned earlier.

4. The circadian metabolism

4.1 Circadian control of metabolic functions

In the previous part, we have seen that food restriction triggers direct consequences on the circadian system and energy balance. The following part will show that metabolic regulations are tightly regulated within the brain and peripheral tissues and that clock genes play a crucial role in this context.

4.1.1 The hypothalamus

Considerable experimental work has highlighted the important role of hypothalamic nuclei in the control of feeding and energy metabolism. The ventromedial hypothalamic nucleus, LH and ARC nucleus contain glucose-sensing neurons and receive nutritional information from blood-borne signals and neuronal messages from brainstem nuclei, including parabrachial nucleus and NTS. Among the key hormones that modulate feeding via hypothalamic activity, leptin synthesized by adipocytes acts in particular on ARC to inhibit appetite and stimulate energy expenditure (Ahima and Lazar 2008). Insulin, released from the *β* cells of the pancreas, also has anorexigenic effects on the metabolic hypothalamus (Gerozissis 2008). On the other hand, ghrelin, released by the stomach (that play a strong role in food entrainment), activates NPY/AgRP containing neurons in the ARC to increase appetite and decrease energy expenditure (Nogueiras et al. 2008).

As outlined before, an important role of the SCN is to orchestrate the internal ticking of different central and peripheral tissues. Therefore, 24-h rhythmic patterns can be observed for a plethora of metabolic substrates and hormones, such as glucose (Yamamoto et al. 1987; La Fleur et al. 1999), non-esterified fatty acids (NEFA), insulin (Yamamoto et al. 1987) and leptin levels (Kalsbeek et al. 2001), and are abolished in the absence of the SCN (Fig. 43). In addition, expression in the SCN of receptors to metabolic hormones (i.e., insulin, ghrelin and leptin) raises the possibility that peripheral hormonal signals can feedback to the SCN (Unger et al. 1989; Hakansson et al. 1998; Zigman et al. 2006).

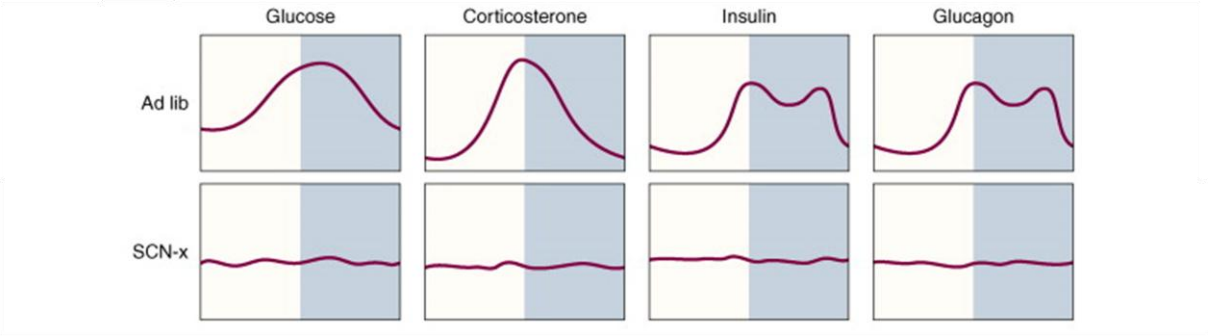


Figure 43. Daily rhythms in basal plasma glucose concentrations and a number of glucoregulatory hormones during different experimental conditions

Plasma glucose, corticosterone, insulin and glucagon concentrations across the 24-h light/dark cycle show clear day/night rhythms in intact rats under conditions of *ad libitum* (Ad lib) feeding but are abolished by an SCN lesion (SCN-x). Adapted from (Kalsbeek et al. 2010)

4.1.2 Circadian transcriptome

Microarray analysis of the mouse liver transcriptome showed that 9% of more than 2000 genes studied oscillate in a circadian manner and may be under the control of the SCN (Akhtar et al. 2002). Thus, the SCN modulates both behavior and metabolism by altering the phase of peripheral oscillators provided that food is freely available. Recent genome-wide transcriptome analyses performed in the SCN, liver, adrenal gland of mice, revealed that around 10% of transcripts are regulated in a circadian manner (Ueda et al. 2002; Oishi et al. 2003; Lowrey and Takahashi 2004; Oishi et al. 2005; Miller et al. 2007; Hughes et al. 2009). Of note, among these transcripts are found key regulators of glucose and lipid metabolism, and components of the xenobiotic detoxification pathway. Of the 49 nuclear receptors, that are key actors of metabolic regulations (see below), approximately 40% were cyclic in the liver or white adipose tissue (WAT) (Yang et al. 2006). Liver posttranscriptional and translational mechanisms contribute as well to circadian coordination (Reddy et al. 2006).

Therefore, the whole circadian system participates in the daily variation of metabolism. Several biological approaches and genetic models have given further insight into the maintenance of metabolic homeostasis and the crosstalk between the circadian system and metabolism, as will be further discussed below.

4.2 Metabolic diseases are associated with circadian disturbances

4.2.1 Genetic obesity and diabetes

Monogenic causes of obesity and diabetes are relatively rare, especially in humans. For instance, early-onset obesity has been associated with a mutation in the leptin receptor in humans (Clement et al. 1998). In rodents, however, genetic syndromes of obesity and diabetes mellitus offer models of choice to analyze circadian disturbances associated with metabolic physiopathology.

In obese Zucker rats, which carry a mutation (i.e., *fa*) in the leptin receptor gene, daily rhythms of locomotor activity, body temperature and feeding are phase-advanced (i.e., the active phase starts in the afternoon in contrast to the nocturnal onset in *fa/?* littermates) and their day-night amplitude is generally reduced (Fukagawa et al. 1992; Murakami et al. 1995; Mistlberger et al. 1998). The molecular clockwork in peripheral tissues is consistently disrupted in genetically obese, diabetic or not, mice. More precisely, in obese *ob/ob* and KK mice as well as in obese and diabetic KK-A^Y mice, the amplitude of daily profiles of clock gene expression in the liver or white adipose tissue is generally reduced, if not barely sizeable (Ando et al. 2005; Ando et al. 2011). The results are more contrasted in obese and diabetic *db/db* mice because in their liver, expression of clock genes can be either decreased (i.e., *Per2*), up-regulated (i.e., *Per1*) or phase-advanced (i.e., *Bmal1*) (Kudo et al. 2004). Of interest, in *ob/ob* mice, alterations in peripheral clocks occur earlier than metabolic symptoms, such as morbid obesity and hyperinsulinemia, thus suggesting that metabolic disturbances are not the main cause of altered clockwork (Ando et al. 2011). Within the central nervous system of *ob/ob* or KK-A^Y mice, clock gene oscillations have been studied in the NTS. The most salient changes concern *Bmal1* and *Rev-erbα* whose daily expression is up- and down-regulated, respectively (Kaneko et al. 2009). Moreover, the molecular clockwork of the SCN is not markedly affected in leptin-deficient, obese (i.e., *ob/ob*) mice (Ando et al. 2011). Unexpectedly in view of the severe dampening of overt rhythmicity in *db/db* mice, the amplitude of SCN molecular oscillations is significantly increased in these mice compared to *db/+* littermates (Kudo et al. 2004). Finally, it is worth mentioning that *ob/ob* mice show increased photic resetting of the master clock (Sans-Fuentes et al. 2010).

4.2.2 Diet-induced obesity

In nature, species are not often confronted to food abundance. On the contrary, food can be rare for most animals and foraging is a key for survival (thus contributing to energy expenditure). Consequently, obese animals in the wild are uncommon, except for some species in which overweighting is important to support long periods of fasting (e.g., penguins, bears), and those species living in proximity to humans and consuming greasy food that is discarded by people. Most laboratory-based research on obesity is carried out in rodents (West and York 1998). Control animals usually have an *ad libitum* access to a normocaloric chow diet. Despite this free access, most rodents regulate their consumption and the time when they eat and do not become obese.

Obesity can be induced by feeding rodents on an *ad libitum* basis with a high-fat diet (HFD, more than 50% of metabolizable energy derived from fat). Short-term high-fat feeding reduces circadian variations of leptin levels in rats (Cha et al. 2000), and humans (Havel et al. 1999), thus suggesting that dampened circulating leptin could contribute to the development of obesity. In mice, high-fat feeding attenuates the daily pattern of food intake, with a higher consumption during the day and a concomitant decrease during the active period, before significant mass gain. Moreover, changes in the concentration and temporal pattern of expression of glucose, insulin, leptin and NEFA are also observed (Fig. 44) (Kohsaka et al. 2007).

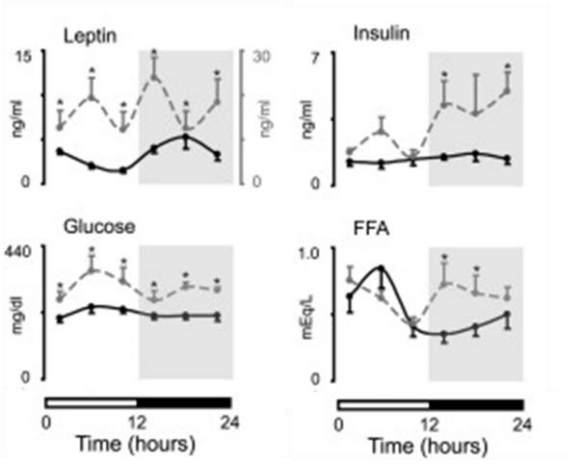


Figure 44. High-Fat Diet Alters Diurnal Patterns of Metabolic Markers.

Mice were maintained on a 12:12 LD cycle and fed either RC (black lines) or HF diet (gray dotted lines) ad libitum for 6 weeks (n = 6–8 per group per time point).(A) Diurnal variation in serum leptin, glucose, insulin, free fatty acid (FFA), and corticosterone levels. Adapted from (Kohsaka et al. 2007)

High-fat feeding also leads to difficulties in maintaining wakefulness during the active period and increases non-rapid eye movement sleep in mice (Jenkins et al. 2006), as well as postprandial sleepiness in humans (Wells et al. 1997), suggesting that metabolic state affects neural structures regulating sleep. Equally interesting, HFD seems to have a direct effect on the main circadian clock. In mice, HFD lengthens the free-running period (Kohsaka et al. 2007) and disrupts photic synchronization of the SCN to light, as shown by slower re-entrainment to shifted light-dark cycle and reduction in light-induced phase-shifts (Mendoza et al. 2008). There is also a clear change in neuropeptide expression in the mediobasal hypothalamus, despite no major modification of the core clock machinery in that region (Kohsaka et al. 2007). In the brainstem, more precisely in the NTS, mice fed with HFD display altered daily patterns of clock gene expression (Kaneko et al. 2009). Taken together, these results suggest that central dysfunctions may contribute to the development of obesity.

Oscillations of clock genes have been found in human WAT (Garaulet et al. 2011), but no difference has been detected in the characteristics of these oscillations between lean, overweight and diabetic individuals (Otway et al. 2011). By contrast, the circadian timing of peripheral tissues is markedly modified by fat overload in rodents. HFD alters the diurnal variation in glucose tolerance and insulin sensitivity by influencing clock functioning (Rudic et al. 2004). In the liver, changes are seen in the level as well as the rhythmic pattern of major components of lipid homeostasis and adiponectin metabolic pathway, with parallel modifications in clock gene expression (Kohsaka et al. 2007; Barnea et al. 2009). In HFD-fed animals, expression of metabolic actors is asynchronous in liver and adipose tissue, suggesting the importance of temporal coordination among metabolic tissues. Furthermore, HFD also attenuates the amplitude of *Clock*, *Bmal1* and *Per2* in adipose tissue (Kohsaka et al. 2007). Hence, altered circadian clock function within adipose tissue may promote excess fat storage (especially intra-abdominal fat).

In summary, impairment of clock gene oscillations and metabolic pathways may explain the altered coordination of metabolic functions and clock-controlled output signaling, contributing to obesity and associated disorders (e.g., diabetes, sleep disturbances). Recent evidence also demonstrates that disruption of the circadian timing system solely, has various consequences on metabolism.

4.3 Circadian disruption is associated with metabolic dysfunctions

4.3.1 Altered endogenous clockwork

As for the study of the FEO, one of the first clock genes studied was *Clock*. Homozygous C57BL/6J *Clock* mutant mice are hyperphagic, show a dampened feeding pattern, with increased food intake during the rest period, and attenuated energy expenditure at night, thus contributing to fat excess. *Clock* mutant mice display severe metabolic alterations, including hypercholesteronemia, hypertriglyceridemia, hepatic steatosis and hyperglycemia. In addition, *Clock* mutation leads to day-night changes in the expression of hypothalamic neuropeptides, like CART (Cocaine and amphetamine regulated transcript) and orexin mRNA, which play a role in central circuits regulating feeding and arousal (Fig. 45) (Turek et al. 2005). Furthermore, *Clock* mutation induces profound changes in glucose homeostasis, such as increased insulin sensitivity and altered gluconeogenesis (Rudic et al. 2004). Of interest, in the liver, *Clock* has been shown to directly drive the expression of *glycogen synthase 2* via E-boxes (Doi et al. 2010).

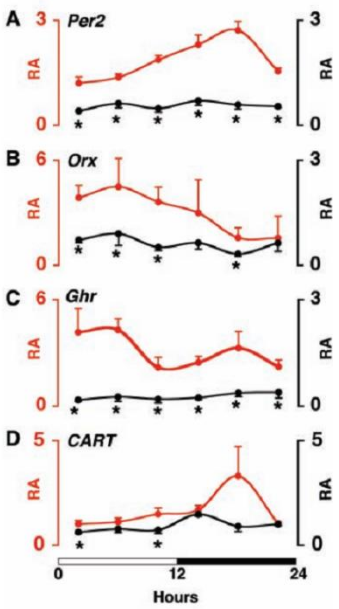


Figure 45. *Clock* mutation alters the diurnal rhythms of clock and metabolic genes.

Clock mutant mice display altered circadian patterns and abundances of *Per2* mRNA (A) and mRNAs encoding hypothalamic peptides involved in energy balance. 12:12 LD cycle is indicated by bar at the bottom. Values for WT (redline) and *Clock* mutant (black line) mice are displayed as relative abundance (RA; mean +/- SEM). Brains were collected at 4-h intervals across the 24-h cycle. Orx: orexin; Ghr: ghrelin; CART: cocaine- and amphetamine-regulated transcript. From (Turek et al. 2005)

In *Clock* mutant mice generated on a CBA/6CaH background (which synthesize melatonin contrary to the C57BL/6J strain), impairments in glucose tolerance, insulin secretion, liver *Pepck* mRNA expression and increased insulin sensitivity are also observed, although these mice do not become spontaneously obese unlike C57BL/6J *Clock* mutants (Kennaway et al. 2007). Moreover, *Clock*

(Jcl:ICR) mutant mice display levels of triglycerides and NEFA lower than control mice, and cholesterol and glucose levels do not differ. Due to reduced lipid absorption and hepatic lipogenesis, these mice challenged with HFD show reduced body mass elevation, leptin and insulin levels (Oishi et al. 2006; Kudo et al. 2007). In spite of these discrepancies, due notably to strain-related differences, mutation of the *Clock* gene clearly has an impact on lipid metabolism, since *Clock* has been shown to participate in liver cholesterol accumulation (Kudo et al. 2008) and to amplify obesity in *ob/ob* mice (Oishi et al. 2006).

Bmal1, a close partner of *Clock*, has been shown to participate in adipocyte differentiation and lipogenesis (Shimba et al. 2005). Sensitivity to exogenous insulin is increased by deletion of *Bmal1*, while gluconeogenesis is suppressed (Rudic et al. 2004). Liver-specific deletion of *Bmal1* in mice nicely confirmed its strong involvement in glucose metabolism. *L-Bmal1*$^{-/-}$ mice have impaired expression of clock-related genes involved in hepatic glucose regulation such as *glucose transporter 2*, *glucokinase* or *pyruvate kinase*. *L-Bmal1*$^{-/-}$ mice were hypoglycemic during the resting period, were more glucose tolerant, have normal insulin sensitivity and production, and normal total body fat content compared to wild-type mice (Lamia et al. 2008). Altogether, these results demonstrate that *Clock* and *Bmal1* modulate hepatic function to regulate glucose and fatty acid homeostasis.

Recent elegant studies have explored the role of *Clock* and *Bmal1* in the pancreatic islets, demonstrating that the pancreas harbors a functional circadian oscillator (Fig. 46) (Marcheva et al. 2010; Sadacca et al. 2011). They showed that pancreas-specific *Bmal1* mutant mice have higher blood glucose levels during the whole 24 h, impaired glucose tolerance and reduced insulin secretion. Moreover, pancreatic islets of these mutant mice have altered development and produced less insulin, suggesting that intact clock functioning in the pancreas is essential for normal insulin secretion (Marcheva et al. 2010). This study establishes clearly that circadian components can regulate local metabolism.

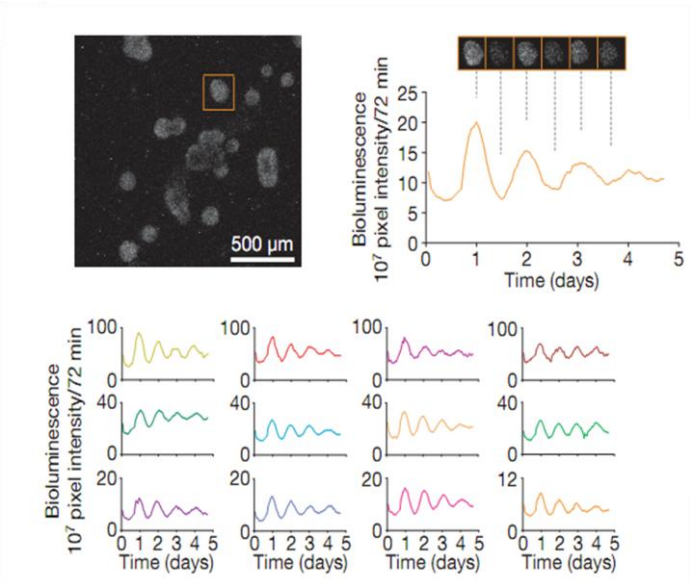


Figure 46. Cell autonomous oscillator in pancreas

Islets from *Per2Luc* mice were imaged, and the orange trace at the right represents the bioluminescence rhythm collected from the islet in the orange square (left). Traces from other islets are shown below. Adapted from (Marcheva et al. 2010)

Mutation of the core clock gene *Per2* can also lead to abnormal conditions. In *Per2*$^{-/-}$ (*Per2tm1Brd*) mice, daily corticosterone rhythm is markedly attenuated. Of interest, when fed with HFD, these mice eat more during the rest period compared to wild-type mice and develop bigger obesity (Yang et al. 2009). Moreover, the rhythmic pattern in *Per2*$^{-/-}$ mice of alpha-melanocyte-stimulating hormone (α-MSH), a powerful appetite suppressing peptide, is disrupted and peripheral injection of α-MSH induces weight loss (Yang et al. 2009). In mice in which *Per2* is fully ablated, lipid metabolism was altered with decreases in both plasma NEFA and total TG, and TG contents in the WAT (Grimaldi et al. 2010). In *Per2*$^{-/-}$ mice, and to a lesser extent in *Per1*$^{-/-}$ mice, glucose tolerance was increased (i.e., improved) compared to wild-type animals (Dallmann et al. 2006). By contrast, in *Per1*$^{-/-}$;*Per2*$^{-/-}$ double-mutant mice (129/sv background) glucose tolerance and insulin sensitivity were both attenuated (Lamia et al. 2008). Triple mutant mice for *Per1-Per2-Per3* gain more body mass on HFD than wild-type mice, similar to *Per3* single mutant mice, thus indicating that the *Per3* mutation alone accounts for the obese phenotype (Dallmann and Weaver 2010).

Along with core clock components, secondary actors of circadian oscillations take part in metabolic functions. *Rorα*, activator of *Bmal1* transcription, has been shown to participate in the regulation of *Apolipoprotein C-III* (*Apo C-III*) gene involved in triglyceride metabolism (Raspe et al. 2001), and lipid homeostasis in skeletal muscle (Lau et al. 2004). *Rev-erbα*, repressor of *Bmal1*, is involved in adipocyte differenciation (Fontaine et al. 2003; Wang and Lazar 2008) and rhythmic bile and

lipid homeostasis (Le Martelot et al. 2009). *Rev-erbα*$^{-/-}$ mice displayed elevated serum Apo C-III and triglycerides levels (Raspe et al. 2002). Moreover, although the deletion of *Rev-erbα* alone has only a minor effect on hepatic glucose regulation (Le Martelot et al. 2009), the alterations are more pronounced with concomitant mutation of *Per2* (Schmutz et al. 2010). Indeed, the expression of key enzymes of glucose metabolism, such as glucose-6-phosphatase or glucokinase, was more affected in the liver of *Rev-erbα*$^{-/-}$*/Per2* mutant mice.

4.3.2 Altered rhythmic environment

There are two main external causes of disruption in circadian rhythmicity. The first one concerns chronic changes in timing of light-dark cycles, such as shift-work or chronic jet-lag. The second way relates to behavioral activity (e.g., physical activity, feeding) occurring on a regular basis during the usual resting period, such as during night-work. As detailed below, both situations have deleterious consequences on circadian organization and metabolic health.

Housing mice to light-dark cycles too short (i.e., 20 h) for enabling daily synchronization of their master clock leads to larger body mass gain and increased insulin/glucose ratio, indicative in the fasted state of insulin resistance (Karatsoreos et al. 2011). In human subjects exposed to controlled 28-h sleep-wake cycles under dim light (so-called ‘forced desynchronization’ protocol), circadian misalignment impairs glucose tolerance and reduces sensitivity to insulin (Scheer et al. 2009). Additionally, constant exposure of mice to bright or dim light leads to increased body mass and reduced glucose tolerance compared to mice housed under a regular light-dark cycle (Fig. 47) (Fonken et al. 2010). Repeated weekly shifts of 24-h light-dark cycle in rats fed with regular chow diet lead to circadian desynchronization and trigger higher body mass gain (Tsai et al. 2005).

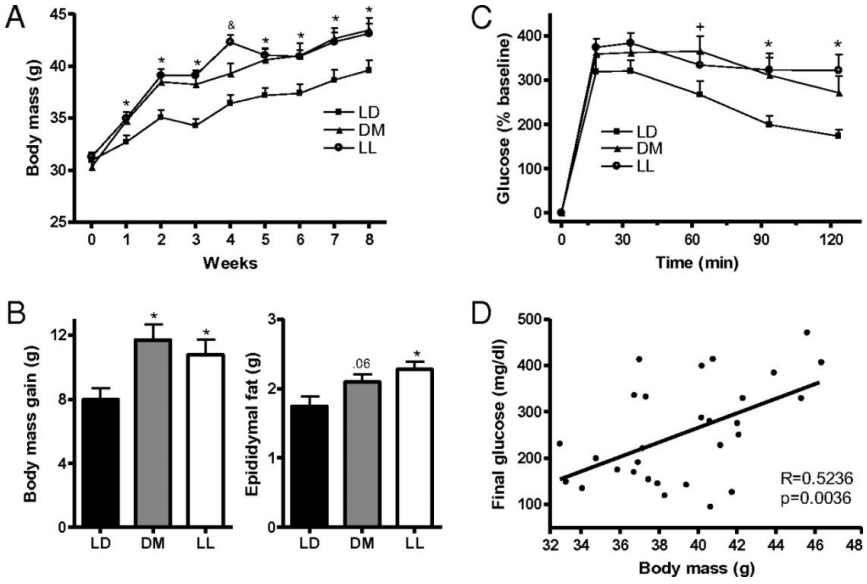


Figure 47. Body mass, fat pad mass, and glucose tolerance were altered in mice exposed to bright or dim light at night.

(A) Weekly body mass for mice throughout the study (*$P \leq 0.05$ when ligh-dark (LD) differs from both ligh-light (LL) and dim-light (DM) groups; $^{\&}P \leq 0.05$ between all groups). Mice exposed to light at night had elevated body mass beginning 1 wk after placement in experimental light conditions and continuing throughout the remainder of the study. (*B*) Body mass gain and epididymal fat pad mass differed among groups at the conclusion of the study, suggesting increases in body mass may be caused by changes in body fat composition. (*C*) Mice exposed to either DM or LL had reduced glucose tolerance, and DM and LL mice failed to recover blood glucose as rapidly as LD mice (*$P \leq 0.05$ when LD differs from both LL and DM groups; $^{+}P \leq 0.05$ when glucose level is higher in the DM group than in the LD group). (*D*) Body mass at the time of the GTT correlated positively with final blood glucose levels. (Fonken et al. 2010)

Being nocturnal animals, laboratory rats and mice exposed to a light-dark cycle consume most of their daily food during the night period. Access to food restricted to a few hours during the light phase usually leads to mild body mass loss or no change at all (Castillo et al. 2004; Feillet et al. 2006; Sutton et al. 2008). In more rare cases, food-restricted rats with chow diet available only for a few hours can increase their body mass (Martinez-Merlos et al. 2004). A clear contribution of circadian timing of food intake in body mass gain has been shown in Zucker rats (Fig. 48) (Mistlberger et al. 1998). As mentioned above, *fa/fa* rats ingest a large proportion of food during the usual resting phase (i.e., light phase) in nocturnal rats. This study was the first to demonstrate that by limiting food access to the normal period of activity and feeding (i.e., nighttime in nocturnal rats), Zucker rats gained less body mass compared to free-fed animals, in spite of similar amounts of food intake between both groups (Mistlberger et al. 1998). More recent observations confirm

nicely the metabolic consequences of unusual timing of feeding. Mice fed ad libitum with HFD display a rapid increase in daytime feeding that takes place weeks before the obese phenotype is detectable (Kohsaka et al. 2007). Furthermore, when HFD was restricted to the light phase, mice gained more body mass than those on the same diet but with access limited to the dark phase (Fig. 48) (Arble et al. 2009).

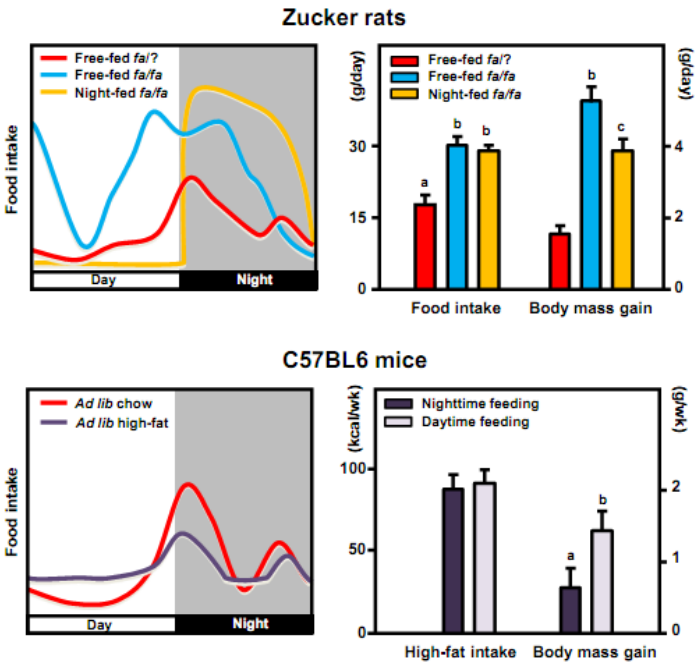


Figure 48. Importance of feeding time in genetic or diet-induced obesity

In Zucker rats (upper panels), obese individuals (i.e., *fa/fa*; blue lines and bars) eat more during daytime than control littermates (*fa/?*; red lines and bars). When food access is limited to nighttime (orange lines and bars), *fa/fa* rats gain less body mass in spite of similar whole energy intake compared to free-fed individuals (modified with permission from (Mistlberger et al. 1998)). In C57BL6 mice (lower panels), free access to a high-fat diet is associated with an increase in daytime feeding (purple line), while chow feeding is mostly nocturnal (red line). If high-fat feeding is restricted to daytime or nighttime hours (light and dark purple bars, respectively), body mass gain is larger in the former, despite comparable energy intake (drawn from data in refs. Martinez-Merlos et al. 2004; Kohsaka et al. 2007).

Another paradigm used to awaken rodents during their resting period (i.e., daytime) is forced activity that leads to internal desynchronization (Salgado-Delgado et al. 2008). Keeping food intake to the normal feeding period (i.e., night) in spite of diurnal forced activity prevents body mass and metabolic changes (Salgado-Delgado et al. 2010). Considering that exposure to bright light at night is an aggravating factor for the occurrence of pathologies in human night-workers (Arendt 2010), it may be clinically relevant to develop shift-work models in day-active animals.

4.4 Transcriptional networks connecting molecular clockwork and metabolic pathways

From genetic strategies allowing modifications of the genome in mice, it has been discussed above that most, if not all, clock gene deletions lead to a broad range of metabolic diseases. In addition, the effects of unhealthy food and incorrect timing of food intake on metabolic regulatory centers and the core clock machinery have been introduced. In this last section, an overview of the emergent understanding of the communication within the circadian clock circuitry will be given. The different molecular pathways by which clock-related nuclear receptors are involved in metabolism and the effects of nutrient "sensors" on core clock components are illustrated below (Fig. 49).

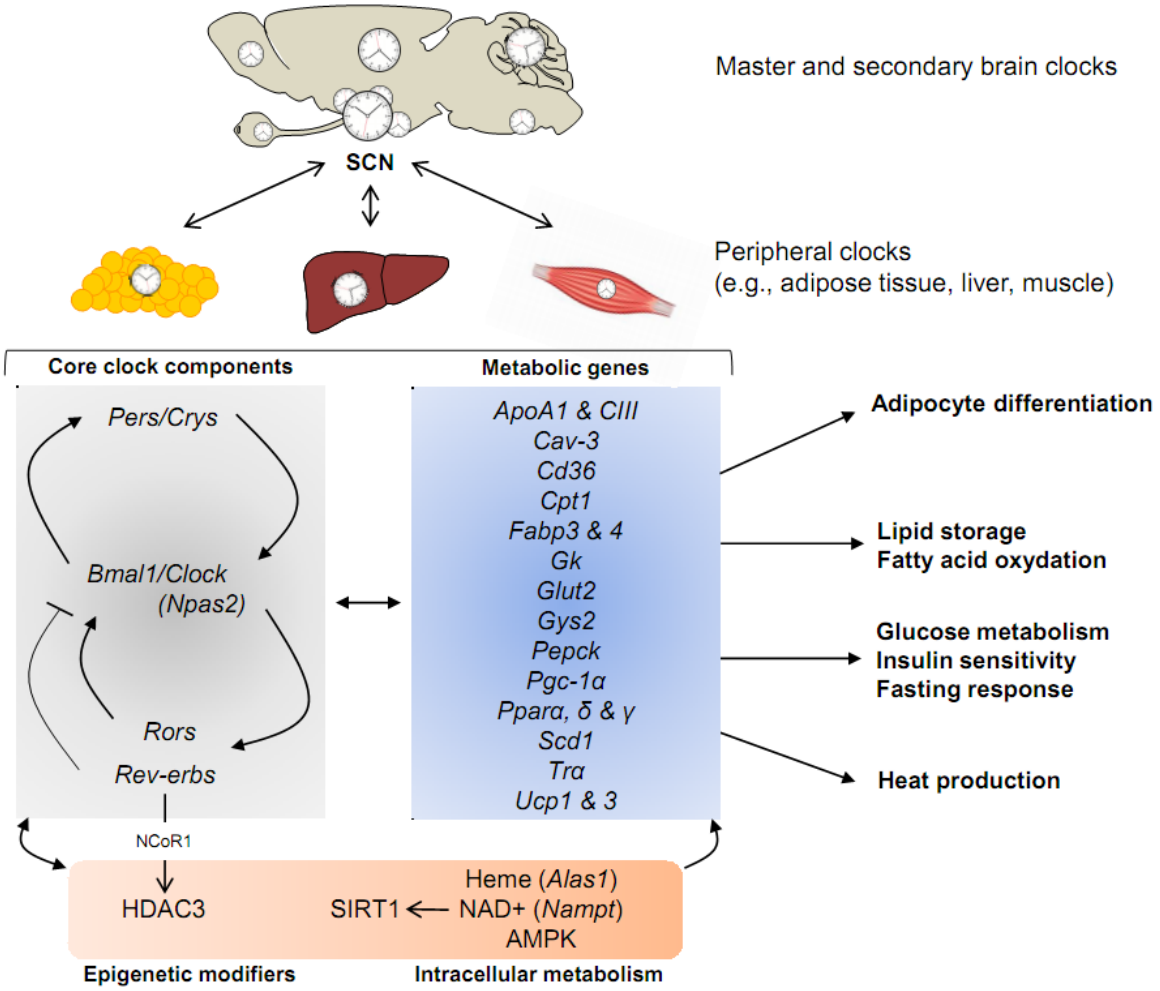


Figure 49. A schematic illustration of the cross-talk between circadian components and metabolic regulators

The master clock housed in the SCN is connected to several brain and peripheral clocks to orchestrate rhythmic activities. All clocks share a common molecular mechanism in which the clock genes are coexpressed, and the generation of circadian oscillations is made from transcriptional/translational interactions (represented in the gray box; for more details, see Fig. 17). Clock genes can influence, directly or indirectly, the rhythmic expression of many metabolic genes (listed in the blue box). Clock components can also control rate-limiting enzymes in the NAD^+ salvage pathway (i.e., *Nampt*) and heme biosynthesis (i.e., *Alas1*). Intracellular metabolism through the action of SIRT1, AMPK, and heme can impinge both metabolic genes and core clock machinery. In addition, epigenetic modifiers, such as HDAC3 recruited by REV-ERBα via NCoR1, modulate clock and metabolic gene transcription (orange box).

4.4.1 The nuclear receptors' dynamic network

Transcriptomal analyses have demonstrated that a large number of nuclear receptors are expressed in a circadian manner (Yang et al. 2006). Among them, *Rors, Rev-erbs* and peroxisome proliferator-activated receptor (*Ppars*) genes appear to be pivotal players at the interface between the circadian system and metabolism (Teboul et al. 2008).

As introduced earlier, RORs and REV-ERBs compete for binding RORE in clock gene promoters (Preitner et al. 2002; Guillaumond et al. 2005; Crumbley et al. 2010; Crumbley et al. 2011). *Rev-erbα* has also been shown to repress its own transcription through a Rev-erbα responsive element (RevRE) located in its promoter (Adelmant et al. 1996). Interestingly, RORα binds to this site, thus controlling the transcription of *Rev-erbα* (Raspe et al. 2002). Hence, genes containing RORE/RevRE in their promoter are activated by RORα and repressed by REV-ERBα. This highlights the occurrence of dynamic interactions within the core clock machinery.

In skeletal muscle, *Rorα* directly regulates the mouse *caveolin-3* (*Cav*-3) and *carnitine palmitoyltransferase-1* (*Cpt1*) genes, involved in fatty acid metabolism (Lau et al. 2004). *Rorα* also enhances activity of the human Apo C-III gene promoter (Raspe et al. 2001) and binds to the rodent *Apolipoprotein A-I* (*ApoA1*) RORE (Vu-Dac et al. 1997). Additional evidence for the role of *Rorα* in metabolism is provided by the fact that cholesterol is a putative ligand of RORα (Kallen et al. 2004). Numerous studies have shown that REV-ERBs also influence lipid and energy homeostasis. The expression of a dominant negative version of *Rev-erbβ* in transfected cell lines attenuates expression of genes involved in lipid metabolism, such as *fatty acid binding protein 3-4* (*Fabp3-4*)*, cluster of differentiation 36* (*Cd36*)*,*

stearoyl-Coenzyme A desaturase 1, and cellular energy balance (e.g., *uncoupling protein 3, Ucp3*). Similarly to *Rorα*, *Rev-erbα* can bind directly to the human Apo C-III promoter (Raspe et al. 2002) and the rat Apoa-I promoter RevRE (Vu-Dac et al. 1998). For the repressive action on *Bmal1* transcription, *Rev-erbα* has been shown to recruit the nuclear receptor corepressor (NCoR)/histone deacetylase 3 (HDAC3) complex (Yin and Lazar 2005). Of interest, genetic disruption of NCoR1-HDAC3 interaction in mice induces changes in *Bmal1* expression and circadian behavior with concomitant effects on metabolism (e.g., increased insulin sensitivity, altered expression of metabolic genes). Surprisingly, loss of a functional NCoR1-HDAC3 complex also protects mice from diet-induced obesity (Alenghat et al. 2008). On the other hand, a recent study has explored the role of the circadian genomic recruitment of HDAC3 in the mouse liver. They showed that REV-ERBα controls the circadian expression of lipid metabolism genes by recruiting the repressive chromatin modifier HDAC3 (and NCoR) to the genome during the light period, therefore preventing lipogenesis at a time when animals are resting. In addition, deletion of either Hdac3 or *Rev-erbα* results in hepatic steatosis indicating steady lipogenesis (Fig. 50) (Feng et al. 2011).

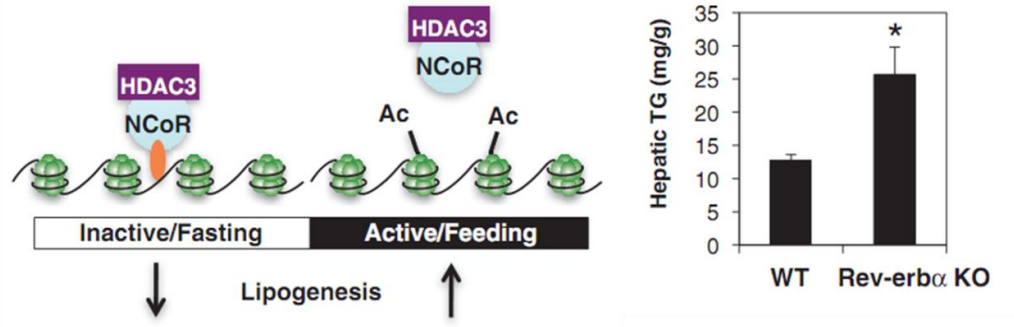


Figure 50. Regulation of hepatic lipid homeostasis by HDAC3

(*Left*) Hepatic TG levels in livers from 9-week-old WT and Rev-erbα KO. Values are mean ± SEM (*n* = 4). **P* < 0.05 by Student's *t* test. (*Right*) Model depicting the mechanistic links between the daily cycles of *Rev-erbα* expression (orange oval), HDAC3 genomic recruitment, epigenomic status, and hepatic lipogenesis. Adapted from (Feng et al. 2011)

These findings demonstrate that the circadian control of epigenetic modifiers by clock components is critical for normal metabolic processing. Additional evidence of the involvement of *Rev-erbs* in metabolism is provided by studies on heme which has diverse biological functions, including oxygen sensing, cell respiration and metabolism (Tsiftsoglou et al. 2006). Heme, whose expression occurs in a circadian manner (Kaasik and Lee 2004) can bind to both REV-ERB (Raghuram et al. 2007).

Heme binding to REV-ERBs facilitates recruitment of the nuclear corepressor NCoR (Yin et al. 2005; Raghuram et al. 2007). The rate-limiting enzyme in heme biosynthesis *Aminolevulinate synthase 1* (*Alas1*), is directly controlled by the clock (Zheng et al. 2001). Heme biosynthesis is also influenced by the nutritional status through the regulation of *Alas1* by the PPARγ coactivator 1α (PGC-1α) (Handschin et al. 2005). This nuclear receptor coactivator is induced by fasting (Handschin et al. 2005) and participates in a variety of metabolic pathways such as glucose and lipid regulation (Lin et al. 2005). Moreover, both PGC-1α and heme are able to modulate the expression of circadian genes (Dioum et al. 2002; Kaasik et al. 2004; Liu et al. 2007). These results illustrate how REV-ERBs can sense dynamic metabolic changes and transmit them to the core clock machinery.

PPARs are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors. The three PPARs (*α, δ, γ*) have been shown to regulate carbohydrate, lipid, lipoprotein and energy metabolism (Gervois et al. 2000; Yoon 2009). *PPARα* is rhythmically expressed in tissues with high fatty acid catabolism rates such as the liver and adipose tissue. *PPARγ* is rhythmically expressed in liver and WAT, while *PPARδ* expression oscillates in liver and brown adipose tissue (BAT) (Yang et al. 2006). PPARα is likely to play a role in connecting circadian physiology to metabolism. *Ppar*α-deficient mice have normal rhythmic behavior and clock gene expression in the SCN, but PPARα deficiency alters *Bmal1* and *Per3* expression in the liver. PPARα directly regulates hepatic *Bmal1* expression through a response element located in its promoter (Canaple et al. 2006). Besides, it has been shown that *Pparα* is a clock-controlled gene (Oishi et al. 2005) and daytime feeding inverts its circadian expression pattern in the liver (Canaple et al. 2006). Interestingly, PPARα is also connected to the nuclear receptor *Rev-erbα* because the PPARα agonists (fibrates) induce liver expression of *Rev-erbα*. In addition, PPARα (as well as PPARγ) can directly transactivate *Rev-erbα* via RevRE located in its promoter (Vu-Dac et al. 1998; Gervois et al. 1999; Fontaine et al. 2003). Clearly, all these data demonstrate that the metabolic sensor PPARα may play a prominent role in circadian functioning. Other results indicate that PER2 has the capacity to recruit PPARα or REV-ERBα for the modulation of *Bmal1* expression (Fig. 51) (Schmutz et al. 2010), again showing intimate interactions between clock and metabolic components in the clock circuitry.

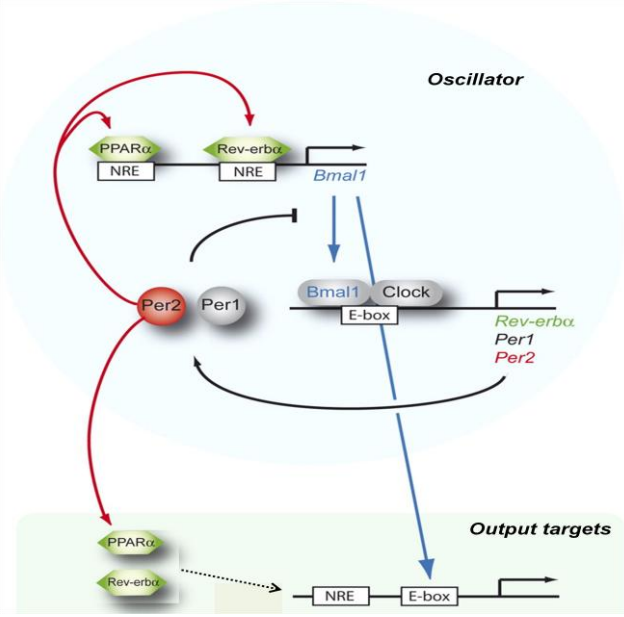


Figure 51. Model of how PER2 may couple E-box-driven and nuclear receptor-regulated gene expression.

In the oscillator, both PER proteins act as repressors of E-box-mediated circadian transcription via interaction with BMAL1 and CLOCK. In addition, PER2 can modulate NRE-mediated transcription via interaction with nuclear receptors. This affects expression of *Bmal1* in the oscillator and target genes. These output targets can be modulated via E-boxes, NREs, or both. PER2–nuclear receptor interactions may be involved in this regulatory process (hatched arrows) to coordinate clock output processes. Modified from (Schmutz et al. 2010)

PPARγ and *PPARδ* are both involved in energy homeostasis. A recent study reveals that PER2 can interact directly with *PPARγ* to repress its transcriptional activity. Ablation of *Per2* in cell culture increased induction of adipogenic genes, while deletion of *Per2* in mice results in slight changes in circadian expression of *PPARγ* target genes in WAT (Grimaldi et al. 2010). These results demonstrate that the control of *PPARγ* by a circadian actor is essential for normal lipid metabolism. Interestingly, PGC-1 (PPARγ coactivator 1, as introduced above), which is involved in thermogenesis and associated metabolic responses (Puigserver et al. 1998; Liu et al. 2007), is rhythmically expressed in phase with *PPARγ*, *PPARδ* and thermogenic genes such as *Ucp1* and *Thyroid receptor α* (*Trα*), in BAT (Yang et al. 2006). Furthermore, PGC-1 can specifically bind *PPARγ* (Puigserver et al. 1998), whereas the latter can also interact with *Ucp-1* promoter (Sears et al. 1996). All these findings suggest that PPARs, could transmit (diurnal) changes in heat dissipation to the circadian system.

Based on these data, nuclear receptors are controlling an amazing array of metabolic functions and are capable of communicating the body metabolic state to the core clock machinery. Incidentally, other nuclear receptor subgroups also participate in the interplay between metabolic and circadian physiology (e.g., *Retinoid X receptor, Liver X receptor* and *Estrogen-related receptor*) (for reviews, see Yang et al. 2006; Teboul et al. 2008; Asher and Schibler 2011).

4.4.2 Nutrient sensors interact with clock components

The tight connection between the circadian timing system and metabolism is also underscored by the role of nutrient sensors. Clock adaptation to metabolic reactions is likely to be achieved also by direct input of nutrient sensing regulators to the intrinsic clock machinery.

Recent evidence has demonstrated that local changes in cellular energy, such as redox reactions (portmanteau for reduction-oxidation), can influence the circadian expression of core clock genes and clock-related nuclear receptors. Nicotinamide adenine dinucleotide (NAD+) and its reduced form NADH, are coenzymes found in all living cells. NAD+ also exists in a phosphorylated form NADP+ and can be reduced to NADPH. NAD(P)+ and NAD(P)H are involved in cellular redox reactions. Interestingly, CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers can sense intracellular redox status. The DNA-binding activities of these dimers are influenced by both reduced and oxidized forms of NAD(H) and NADP(H) in an opposing manner. The reduced forms (NADH and NADPH) activate DNA binding of CLOCK:BMAL1 and NPAS2:BMAL1, whereas the oxidized forms (NAD+ and NADP+) inhibit it, consistent with a role as redox sensors (Fig. 52) (Rutter et al. 2001).

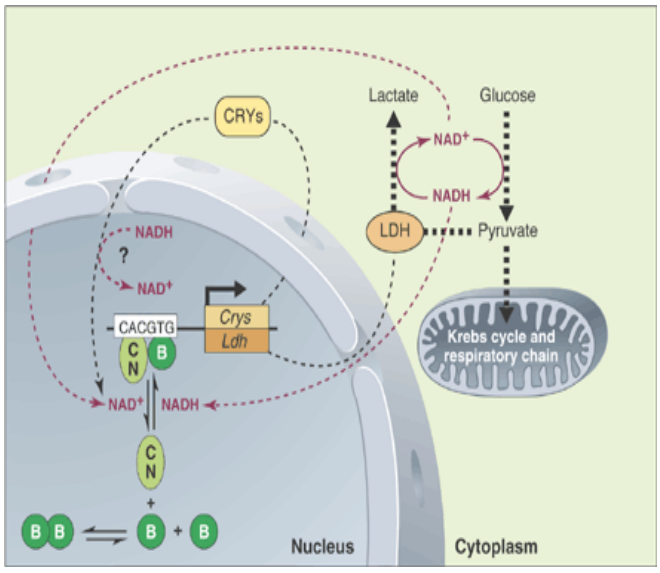


Figure 52. Metabolic states and the mammalian circadian feedback loop

BMAL1 can bind to Clock or NPAS2, and these heterodimers activate transcription. Alternatively, BMAL1 can form homodimers with itself that do not activate transcription. The formation of the Clock: BMAL1 and NPAS2:BMAL1 heterodimers and their binding to DNA is stimulated by reduced NADH and inhibited by oxidized NAD. These heterodimers enhance the expression of the clock genes *Cry* and *Per* (not shown) and the clock output gene *Ldh*. CRY proteins repress Clock: NPAS2-mediated gene activation, possibly by oxidizing the NAD^+ cofactors associated with these proteins. Conceivably, the negative action of CRY proteins on Clock-NPAS2 could be reinforced by lactate dehydrogenase (LDH), which may increase the cellular concentration of NAD^+. (Only unphosphorylated NAD electron carriers are shown.). From (Schibler et al. 2001)

These results raise the possibility that cell energy metabolism can influence circadian rhythmicity. For example, these clock protein sensors might participate in the rhythm imposed by the alternation of fasting-feeding. Indeed, NAD:NADH ratio is modified under fasting and feeding conditions since starvation lowers NADP:NADPH (i.e., shift toward a reduced state) in both rat liver mitochondria and cytoplasm (Williamson et al. 1967). Thus, for feeding synchronization, it is plausible that the cellular redox status could transmit changes in energy metabolism directly to the clock architecture. In this context, it is tempting to think that irregular mealtimes could also contribute to circadian misalignment by subtle perturbations of local circadian oscillator functioning.

Sirtuin 1 (SIRT1), another energy sensor, has recently been found to link circadian to metabolic physiology. SIRT1 is a NAD-dependent histone deacetylase that contributes to epigenetic gene silencing and a plethora of biological processes ranging from gluconeogenesis, insulin secretion and sensitivity, lipid regulation, mitochondrial activity and thermogenesis, adipogenesis and adipocyte differentiation, apoptosis, to caloric restriction-dependent life span extension (Blander and Guarente 2004; Dali-Youcef et al. 2007; Yu and Auwerx 2009). SIRT1, whose expression occurs in a circadian manner, has been shown to influence circadian transcription of several clock genes (e.g., *Per2, Cry1, Bmal1* and *Rorα*) (Asher et al. 2008). Moreover, SIRT1 can directly bind CLOCK:BMAL1 heterodimers and promote deacetylation of PER2 (Asher et al. 2008), BMAL1 and histone H3 (Nakahata et al. 2008). Interestingly, SIRT1 can interact with and deacetylate PGC-1α to control the expression of gluconeogenic and glycolytic genes (Rodgers et al. 2005). In addition, hepatic SIRT1 is able to modulate the expression of PPARα (Purushotham et al. 2009). Importantly, SIRT1 can also repress the fat regulator PPARγ and it is activated during fasting to promote fat mobilization in WAT, again demonstrating its clear implication in lipid homeostasis (Picard et al. 2004). Furthermore, SIRT1 has been shown to be decreased in adipose tissue endothelial cells from obese human subjects (Villaret et al. 2010). Of interest, since fat accumulation is associated with several adverse complications, such as diabetes and hypertension, caloric restriction (and consequent fat depletion) which has multiple biological and life-extending benefits (Barzilai and Gabriely 2001), may be used to prevent or treat metabolic disruptions.

In addition to NAD(P)+ and SIRT1, AMP-activated protein kinase (AMPK) is a further important nutrient sensor. AMPK is sensitive to fluctuations in the cellular AMP:ATP ratio and can be activated by various factors such as exercise, glucose deprivation or leptin treatment. The functions of AMPK cover the whole-body energy balance (e.g., food intake, body mass, lipid and glucose homeostasis, cholesterol and triglyceride synthesis, energy expenditure) (Kahn et al. 2005). In mouse skeletal muscle and cultured myotubes, AMPK has been demonstrated to regulate genes involved in energy metabolism, by acting in coordination with SIRT1. Interestingly, AMPK activation increases NAD+ levels, which in turn enhance SIRT1 activity, resulting in the deacetylation and activation of the downstream SIRT1 target PGC-1α (Canto et al. 2009). Deletion of the AMPKγ3 subunit in mice leads to impaired expression profiles of clock genes in skeletal muscle in response to the AMPK activator AICAR (5-amino-4-imidazole-carboxamide riboside), and attenuated daily variations of the respiratory exchange ratio (Vieira et al. 2008). AMPK also has direct actions on the clock machinery. AMPK phosphorylates and destabilizes CRY1 (Lamia et al. 2009). Casein kinase Iε, an important regulator of PER proteins stability, is also phosphorylated by AMPK which induces subsequent degradation of PER2 and phase-shifts of peripheral oscillators (Um et al. 2007). Recently, it has been demonstrated that mice deficient for either AMPKα1 or AMPKα2 have altered circadian feeding behavior and free-running period (Um et al. 2011). Surprisingly, the rhythmic gene expression of leptin, PGC-1α and nicotinamide phosphoryl-transferase (NAMPT), a rate-limiting enzyme in the NAD+ salvage pathway regulated by CLOCK:BMAL1 (Nakahata et al. 2009; Ramsey et al. 2009), was abolished in AMPK-deficient mice (Um et al. 2011). This study reveals that AMPK is to some extent involved in the cycling of NAMPT-(NAD+)-SIRT1-PGC-1α pathway. In addition, prolonged activation of SIRT1 by its agonist SRT1720 causes an indirect activation of AMPK, and both SIRT1 and AMPK respond to low-energy levels (Bordone and Guarente 2005; Kahn et al. 2005). Therefore, SIRT1 and AMPK may have overlapping functions to ensure the fine-tuning of metabolic and clock regulations.

Finally, it is important to mention that the mammalian molecular circadian clockwork (based on transcriptional–translational events) overviewed in this section, is not a unique cellular clock model. Indeed, circadian redox rhythms of peroxiredoxins, which are antioxidant enzymes, can occur independently of

transcription, thus defining a metabolic non-transcriptional oscillator (O'Neill and Reddy 2011).

4.5 General conclusion

At the molecular/cellular levels, the recent findings cited above highlight multiple transcriptional crossroads between circadian and metabolic pathways in organs involved in metabolism, namely liver, adipose tissue, pancreas and muscle. Nuclear receptors such as *Rors* and *Rev-erbs* are well-situated to receive metabolic signals and integrate them into the core clock architecture. In addition, fluctuations in cellular metabolism can directly influence the transcriptional activity of core clock components such as *Cry1*, *Per2*, *Clock* and *Bmal1*. This can be of importance to adapt behavior and physiology to situation where food availability is limited in time.

At the level of the organism, the circadian timing system provides internal temporal organization controlled locally by brain and peripheral oscillators—including the FEO network—that can be reset by feeding time, and supervised by the master SCN pacemaker mainly reset by ambient light. Impairment of this internal timing due to altered endogenous (local) clockwork or misalignment with external cues, light and/or mealtime, has deleterious impact on health.

> *[…} the hierarchically dominant position of the SCN is impressive, but limited. We like to think of it as an orchestra's conductor, using a large repertoire of gestures to set the timing of individual musicians in playing their instruments. However, the success of the symphony not only depends on the signs given by the director, but also on the capacity of the individual musicians to play their parts.* (Schibler and Sassone-Corsi 2002)

5. Aim of the thesis

5.1 Role of *Rev-erbα* in the clockwork of food anticipation

During my PhD thesis, which started in October 2008, I was involved in the study of the food-entrainable oscillator (FEO). In terms of molecular machinery, one of the current predictions is that food-entrainable oscillations are generated by transcriptional/translational feedback loops similar to those already described in the SCN. In this context, several mutant and KO mice models were used to test this hypothesis and Feillet and colleagues in 2006 gave strong evidence that *Per2* is a circadian gene critical for behavioral food anticipation. This finding was a first step to better understand the functioning of the FEO. However, the other key components of the clockwork involved in food anticipation remained to be identified.

Thus, we assessed the involvement of the nuclear receptor *Rev-erbα* as a putative circadian regulator of food-entrainable oscillations. As outlined in the introduction, *Rev-erbα* is a transcriptional repressor that is expressed in a circadian manner (mRNA acrophase ZT6-10, Fig. 53) in all tissues studied up to now. Furthermore, *Rev-erbα* has been demonstrated to regulate several clock components including, *Clock, Npas2* and *Bmal1*. In addition, the depletion of *Rev-erbα in vivo* was shown to shorten the SCN period length and disrupt the shifting response to light. All these findings thus support a role for *Rev-erbα* as a modulator of circadian-dependent functions and make it an ideal candidate for the integration of environmental changes into the core clock machinery during food synchronization.

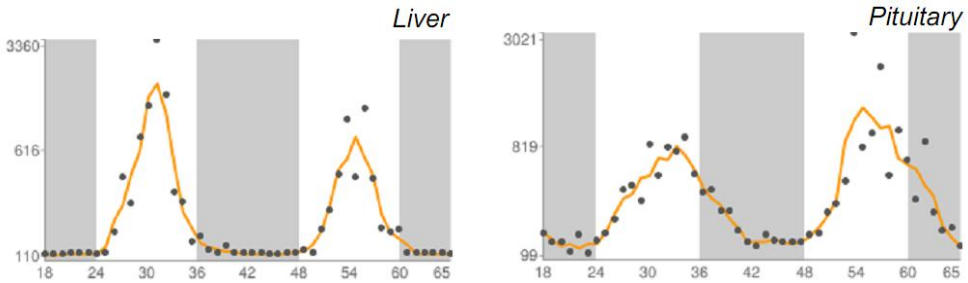


Figure 53. Daily expression of *Rev-erbα* mRNA

Note that the acrophase of *Rev-erbα* expression occurs in the middle of the light period. From http://bioinf.itmat.upenn.edu/circa

As a model to investigate further the FEO, we used mice KO for *Rev-erbα* (kindly provided by Pr. Ueli Schibler and raised in our facility). WT and KO mice, with or without a functional SCN, were exposed to schedules of restricted feeding in different lighting conditions. We analyzed multiple food-entrained rhythms such as general locomotor and wheel-running activities, body temperature and corticosterone. We also studied the expression of PER2, considered as a component of the FEO clockwork, and p-ERK proteins in different brain areas, both in *ad libitum* (AL) and restricted feeding (RF) conditions. Since the liver oscillator plays a role in food synchronization, we evaluated the hepatic expression of clock and clock-controlled genes in response to RF challenge (Fig. 54).

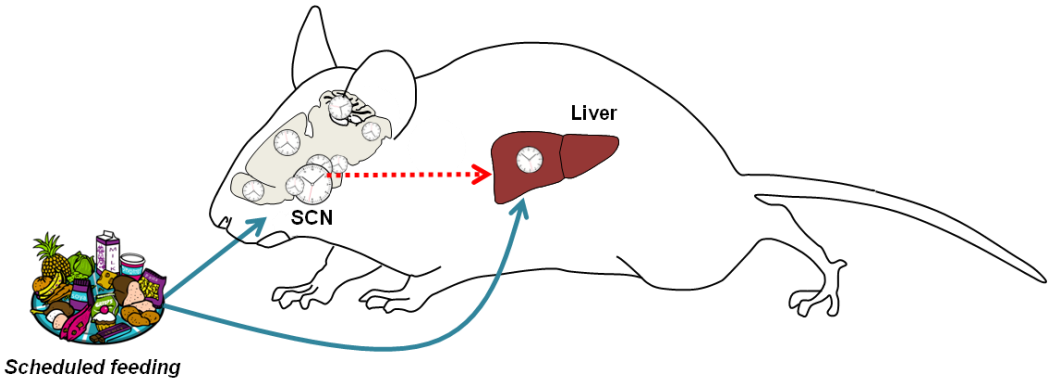


Figure 54. Study of the food-entrainable oscillator network in *Rev-erbα* KO mice

We assessed the effect of scheduled feeding on central oscillators as well as the liver oscillator. Several behavioral and physiological food-entrained rhythms were evaluated.

5.2 Role of *Rev-erbα* in energy homeostasis

As highlighted in the introduction, the nuclear receptor *Rev-erbα* participates in several molecular pathways, especially in the cross-talk between the circadian system and metabolism. Indeed, *Rev-erbα* is highly expressed in peripheral tissues essential for energy homeostasis such as the liver, adipose tissue, skeletal muscle. In addition, this gene was shown to regulate the transcription of metabolic actors involved in lipid metabolism.

Besides, REV-ERBα expression has been demonstrated to be stabilized by the *glycogen synthase kinase 3 beta* (GSK3β). Heme binding to REV-ERBs can facilitate the recruitment of the nuclear corepressor NCoR, which in turn increase the capacity of REV-ERBα to recruit the repressive chromatin modifier HDAC3 to the genome (Fig. 55). Of importance, GSK3β, heme and HDAC3 are all involved in particular in energy homeostasis.

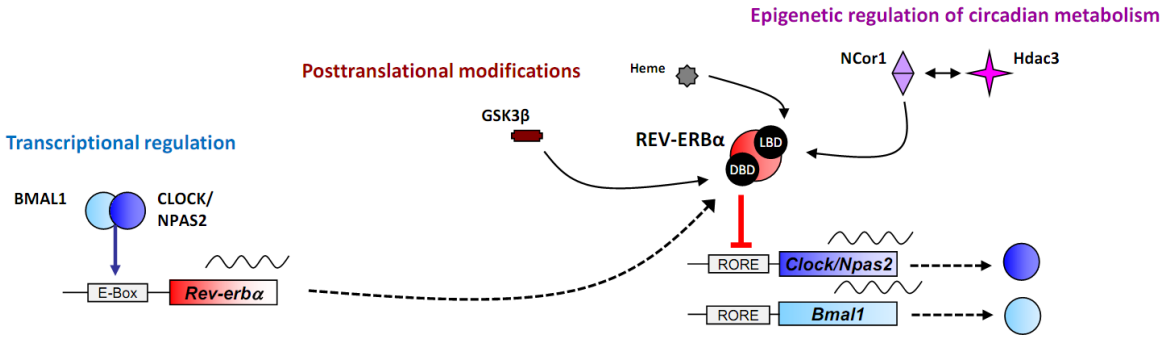


Figure 55. The nuclear receptor *Rev-erbα* and its partners

Rev-erbα transcription is controlled in particular by the CLOCK-BMAL1 heterodimer. REV-ERBα is regulated at the post-translational level by glycogen synthase kinase (GSK 3β), thereby regulating its stability. REV-ERBα protein can mediate repression by recruiting the nuclear corepressor NCoR1, which then activates the histone deacetylase (HDAC) 3. Heme binding to REV-ERBα facilitates the recruitment of NCor1. As a result, genes that contain a RORE sequence in their promoter are repressed.

Hence, we started in the early 2009 to gain insight into the role of *Rev-erbα* in energy homeostasis *in vivo*, since there was no report at this time on the metabolic adaptation of the *Rev-erbα*-deficient mice to various feeding challenges. Our investigation on energy balance was also justified by the fact that the use of clock mutants to study food entrainment implies a necessary exploration of their metabolic phenotype before drawing robust conclusions.

In this context, we explored daily metabolism of carbohydrates and lipids in chow-fed, fasted or high-fat-fed *Rev-erbα* KO mice and their WT littermates. In particular, we assessed the adaptation of both genotypes to different nutritional challenges by evaluating behavioral, physiological and metabolic parameters. We have also evaluated the functioning of metabolically active tissues that are themselves peripheral oscillators, including the liver, adipose tissue and muscle, at the molecular levels (Fig. 56).

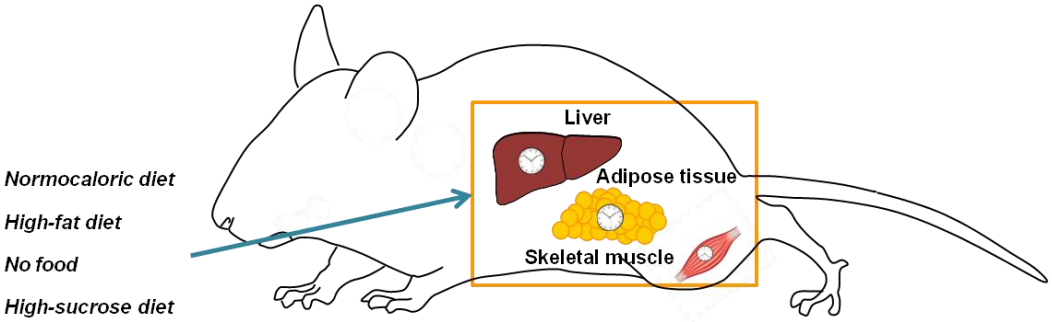


Figure 56. Study of energy homeostasis in *Rev-erbα* KO mice

We analyzed the physiological and molecular responses of multiple metabolic tissues in response to different feeding challenges (i.e., normocaloric versus high-fat feeding, fasting versus high-carbohydrate refeeding challenge).

Chapter 2 RESULTS

1. The *Rev-erbα* clock gene participates in the circadian clockwork of food anticipation

Title: The nuclear receptor *Rev-erbα* participates in the circadian clockwork of food anticipation

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Keywords: food-entrainable oscillator, restricted feeding, clock gene, hypothalamus

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In preparation…

SUMMARY

Limiting food access to a time window during the day (termed restricted feeding, RF) has profound synchronizing effects on circadian rhythms. A few hours before mealtime, rodents display food-anticipatory activity (FAA) as well as changes in physiological parameters (e.g. rise in body temperature). Food anticipation is thought to be controlled by a circadian food-entrainable oscillator (FEO), independent of the central clock in the suprachiasmatic nucleus (SCN). Recent studies showed that disruption of some of the well-known circadian clock genes fails to affect the FEO, therefore questioning the hypothesized SCN-like molecular mechanisms responsible for food anticipation. In this context, we focused on the *Rev-erbα* clock gene. We showed that *Rev-erbα*$^{-/-}$ mice subjected to RF exhibited reduced FAA compared to wild-type littermates as demonstrated by decreased locomotor activities, regardless of the lighting conditions. In accordance with behavioral data, the anticipatory thermogenesis was nearly absent and the food-anticipatory peak of corticosterone was also diminished in *Rev-erbα*$^{-/-}$ mice. Conversely, hepatic clock gene expression was shifted in response to daytime feeding in both genotypes, indicating that disrupted FAA is not the consequence of impaired liver synchronization in *Rev-erbα*$^{-/-}$ mice. On the other hand, PER2 expression was not phase-adjusted to mealtime in the cerebellum while p-ERK expression was not clearly responsive to scheduled RF in several hypothalamic regions. The present results indicate that mice lacking *Rev-erbα* exhibit altered food anticipation, suggesting that this gene may be a component of the FEO.

INTRODUCTION

In mammals, the circadian timing system is composed of several endogenous oscillators. The suprachiasmatic nucleus (SCN), the central clock located in the anterior hypothalamus, is principally synchronized to the environmental light-dark cycle. The SCN in turn controls the daily rhythmicity of many aspects of physiology and behavior. The molecular machinery of the SCN clock is based on transcription/translation feedback loops of clock gene expression. Among them, *Bmal1, Clock, Period 1-3* (*Per1-3*), *Cryptochrome 1-2* (*Cry1-2*), *Ror*α,β and *Rev-erb*α,β are essential (Crumbley and Burris, 2011; Guillaumond et al., 2005; Preitner et al., 2002). Secondary oscillators, whose molecular mechanisms are close to the SCN, are also present in multiple brain regions and peripheral tissues (Dibner et al., 2010).

Besides the light-dark cycle, feeding is a potent synchronizer for the circadian system as well (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001). When food availability is limited to a regular time window each day, rodents can readily adapt those schedules and predict the time of food access. They develop food-seeking behaviors a few hours in anticipation of meal access, concomitant with physiological and hormonal activations (Mistlberger, 1994). In addition, RF-induced adaptation is highlighted by the phase-adjustment of clock-related gene expression in several peripheral tissues (Damiola et al., 2000).

Food-anticipatory rhythms have the properties of a clock-controlled process: it occurs gradually (1 to 3 h before mealtime); it persists over days in the absence of food; it is expressed only if food access is given within the circadian range (22-31 h); and it shows transient cycles when food is presented at a new phase (Mistlberger, 1994). Besides, food anticipation survives the ablation of the SCN clock (Marchant and Mistlberger, 1997; Stephan et al., 1979). Altogether, these data indicate that food anticipation is an output of a circadian food-entrainable oscillator (FEO), likely located in the brain (Davidson et al., 2003).

The identification of the neural substrate and the molecular basis of the so-called FEO proved to be a real challenge these last 30 years (for reviews, see Challet et al., 2009; Mistlberger, 2011). In particular, the SCN-like molecular mechanism hypothesis at the basis

of food entrainment is currently not fully supported. Few studies gave evidence that clock components (i.e., *Per2* and *Bmal1*) may be essential for the FEO molecular clockwork (Feillet et al., 2006; Mieda and Sakurai, 2011). However, the implication of other canonical clock genes (e.g., *Per1, Clock, Npas2, Cry1-2*) has been revealed mild (Dudley et al., 2003; Horikawa et al., 2005; Iijima et al., 2005; Mendoza et al., 2010a) or even non significant (Feillet et al., 2006; Pitts et al., 2003; Storch and Weitz, 2009)

Therefore, in the current study we sought to evaluate the role of the nuclear receptor *Rev-erbα* in the molecular clockwork of the FEO. For that purpose, we used mice lacking the *Rev-erbα* clock gene (knockout, -/-) and their control littermates (wild-type, +/+). Both genotypes were maintained in a regular 12 h:12 h light-dark cycle (LD) or constant darkness (DD) with food available *ad libitum* (AL) and then challenged with scheduled RF. Wheel-running behavior, general activity and body temperature were recorded. We also investigated the effect of the daily feeding schedules on energy balance and hepatic clock genes oscillations. The expression of PER2 and p-ERK proteins was also determined in brain regions such as the dorsomedial hypothalamus and cerebellum, assumed to play a role in food entrainment (Acosta-Galvan et al., 2011; Gooley et al., 2006; Mendoza et al., 2010b).

METHODS

Animals and housing conditions

The founder *Rev-erbα*$^{+/-}$ mice kindly provided by Prof. Ueli Schibler (University of Geneva, Switzerland) were rederived on a C57BL6/J background (Charles River Laboratories, France) and backcrossed until N5 in our local animal care facilities (Chronobiotron UMS 3415, Strasbourg). The *Rev-erbα* deletion strategy is described in (Preitner et al., 2002). Mice were maintained under a 12 h light and 12 h dark (LD 12:12) conditions or in constant darkness (DD), in a temperature controlled room (22 ± 1 °C). Regular chow (SAFE 105, Augy, France) and water were provided *ad libitum* (AL) except during the period of RF (see experimental procedures). Mice were housed individually in transparent plastic cages equipped with a running wheel (12.5 cm in diameter). All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996), the French National Law (implementing the European Communities Council Directive 86/609/EEC) and approved by the Regional Ethical Committee of Strasbourg for Animal Experimentation (CREMEAS).

Genotype determination

Genotyping was performed by PCR using tail biopsy. Briefly, tissue was digested with NaOH (25 mM) in the presence of EDTA (0.2 mM) at 95 °C for 25 min. 2µL of digested tissue was added to a DNA amplification reaction containing (in final concentration): 1X Taq Reaction buffer (Fermentas), MgCl2 2.8 mM (Fermentas), DMSO (Hybri-Max, Sigma D2650), dNTP 0.39 mM and Taq DNA polymerase 1.25 U (Fermentas). Three primers were added to the mixture: a forward primer located in exon 2 (5'-CCAGGAAGTCTACAAGTGGCCATGGAAGA-3', 0.79 µM) and a reverse primer located in exon 3 (5'- CACCTTACACAGTAGCACCATGCCATTC-3', 0.55 µM) amplified a 340 bp band corresponding to the wild-type allele; and a forward primer located in the LacZ cassette (5' AAACCAGGCAAAGCGCCATTCGCCATTCA-3', > 0.55 µM) amplified a band about 200 bp from the targeted allele. The PCR protocol consisted of 5 min at 95°, 35 cycles of amplification (each cycle consisting of 1 min 15 s at 95°C, 1 min at 62°C and 1 min 30 s at 72°C) and 10 min at 72°C. Products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide and visualized under UV light.

Locomotor activity and body temperature recordings

Mice were implanted intraperitoneally (i.p.) under constant gaseous anesthesia (oxygen-nitrogen protoxide with a constant rate at 0.2 L/min and isoflurane at 2%) with a small transponder (G2 E-Mitter, Mini Mitter, Bend, Oregon, USA) allowing the continuous recordings of gross locomotor activity (general activity hereafter) and core body temperature. A PC-based acquisition system (VitalView, MiniMitter) recorded the aforementioned parameters plus the wheel-running activity 24 hours a day.

Experimental procedures

Independent experiments were conducted with distinct cohorts of mice (sex ratio about 1:1). Mice were 2- to 5-month old at the beginning of each experiment and body mass and food intake were determined weekly.

LD conditions: *Rev-erbα*$^{+/+}$ and $^{-/-}$ mice (4-month old, n = 8-12 / genotype) fed on an AL basis for several weeks post-weaning were exposed to RF schedules in which food availability was reduced to 6 h per day, from ZT6 to ZT12 (ZT refers to Zeitgeber Time and ZT0 and ZT12 are defined as time of lights-on and lights-off, respectively) for up to 3 weeks. Note that this experiment was reproduced with another set of mice (n = 9 / genotype) and that additional age matched AL-fed animals (n = 20 / genotype) were kept in LD for further study of the rhythmic expression of metabolic parameters, clock gene expression in the liver and PER2 and p-ERK expression in the brain (see results).

DD conditions: *Rev-erbα*$^{+/+}$ and $^{-/-}$ mice (5-months old, n = 6 / genotype) were placed in DD conditions and fed AL and then challenged with a RF paradigm in which food was given for 6 h from 02:00 p.m. to 08:00 p.m for 4 weeks.

DD conditions and SCN lesions: *Rev-erbα*$^{+/+}$ and $^{-/-}$ mice (2 to 4-months old, n = 9-12 / genotype) maintained in LD conditions, were SCN-lesioned and immediately placed several weeks in DD for recovery. Mice were then exposed to temporal RF for which the food access was limited to 6 h per day for up to 3 weeks (from 02:00 p.m. to 08:00 p.m.).

SCN lesions

2 to 4-month old mice (n = 9-12 / genotype) were anesthetized with ketamine-xylazine (100 mg/kg-10 mg/kg, respectively) and placed in a stereotaxic apparatus. A lesioning

electrode (0.25 mm shaft diameter, 0.1 mm lead/contact diameter; SNEX-300, Rhodes Medical Instruments) was inserted into the SCN (0.5 mm posterior to bregma, 0.2 mm lateral to midline, 5.7 mm ventral to dura, incisor bar 0 mm below ears bars). Bilateral electrolytic lesions were generated with constant current (1.5 mA, 20 s; Lesion making device 53500, Ugo Basile, Italy). During the surgery procedure, animals were placed on heating pad (Harvard Apparatus, France) and treated with antiseptics and lidocaine 5%. After recovery from anesthesia, mice were weighted and injected i.p. with Metacam (1 mg/kg) and transferred back into their cages. Arrhytmicity was later assessed by evaluating locomotor activity with X^2 periodogram. Animals were killed at the end of the RF paradigm, and brains were removed and immediately frozen on dry ice. Lesions were confirmed by cresyl violet staining and only mice with complete SCN lesions were included in the analysis.

Procedure for tissue and blood collections

Mice (6-month old) from LD experiments, either on AL or RF conditions, were sacrificed at ZT0, ZT6, ZT12 and ZT18. Briefly, mice were injected with a lethal dose of pentobarbital, a blood sample was taken intracardially and two pieces of liver were cut and immediately flash-frozen in liquid nitrogen. Then animals were perfused transcardially with 50 mL PBS 1X (Phosphate-buffered saline, pH 7.2) and 50 mL PFA buffer (4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4). Brains were removed and post-fixed in 4% PFA for 24 h at 4°C and cryoprotected successively for 24 h at 4°C into 10, 20 and 30% sucrose solution in 0.1M PB. Brains were then frozen in isopentane at −30 to -40°C and then stored at −80°C.

Metabolic measurements

The blood glucose rhythm was determined in mice from repeated tail blood microsamples (<1 µL) using a Glucotrend®Premium (Roche Diagnostics, Germany). Plasma glucose was evaluated with GOD-PAP Kit (BIOLABO, Maizy, FRANCE). Plasma corticosterone was measured using an EIA kit (AC-14F1, IDS, Paris, FRANCE). Plasma β-Hydroxybutyrate concentrations were determined with a Cyclic Enzymatic Method (Autokit 3-HB, Wako, Japan). Hepatic glycogen was determined following the methods of Murat and Serfaty (Murat and Serfaty, 1974).

Immunohistochemistry

Coronal frozen brain sections (30 µm thick) of the hypothalamus and the cerebellum were made using a cryostat. Free-floating sections were rinsed in PBS 1X and incubated in a solution of 3% H_2O_2 (Sigma-Aldrich) in PBS for 30 min at room temperature. Sections were then rinsed in PBS, and incubated for 2 h in a blocking solution containing 10% goat serum in PBS with 0.3% Tween 20 in PBS. Then sections were incubated in the primary antibody solution (in PBS + 0.3% Tween 20 + 10% goat serum) for 24 h with gentle agitation at 4°C. We used a rabbit polyclonal anti-PER2 (1:2000; Alpha Diagnostic International, Cat. PER21-A; #869900A1) and a rabbit anti-p44/42 MAPK (1:20000; Cell Signaling #4370). Sections were then rinsed in PBS with 0.05% Tween 20 and incubated for 2 h at 4°C with a biotinylated anti-rabbit IgG made in goat (Vectastain ABC peroxydase kit PK6101), diluted 1:500 with 0.3% Tween 20 in PBS on plate agitation at 4°C. Thereafter, sections were rinsed in PBS + 0.05% Tween 20 and incubated for 1 h at room temperature with an avidin-biotin-peroxidase complex (1:250; Vectastain Kit, PK6101; Vector Laboratories) in PBS + 0.05% Tween 20. Next, sections were rinsed in PBS, and incubated with 3,3'-diaminobenzidine (0.5 mg/mL; Sigma) with 0.015% H_2O_2 in H_2O. Thereafter, sections were rinsed with PBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohol, soaked in toluene, and coverslipped with EUKITT. Photomicrographs were taken on Leica DMRB microscope (Leica Microsystems) with an Olympus DP50 digital camera (Olympus France). The intensity and number of immunoreactive cells in hypothalamic nuclei and the Purkinje layer of the cerebellum, respectively, were determined using NIH ImageJ software (Rasband, W.S., U. S. National Institutes of Health, Bethesda MD, USA). The average intensity or cell numbers was determined, as far as possible, from three brain sections per animal. The number of animals / genotype / ZT was comprised between 2 to 6.

mRNA extraction and quantitative Real-time PCR

Pieces of frozen livers were homogenized in lysis buffer supplemented with *β*-mercaptoethanol and total RNA was extracted according to the manufacturer's protocol (Absolutely RNA Miniprep Kit, Stratagene, Agilent technologies). The RNA samples were further purified by precipitation with sodium acetate and isopropyl alcohol. RNA quality was evaluated with the Bioanalyseur 2100 (Agilent Technolgies; RNA integrity number > 6 for all samples). RNA quantity was measured using NanoDrop ND-1000 Spectrophotometer

(NanoDrop Technologies; A_{260}/A_{280} and A_{260}/A_{230} values were >1.7). cDNAs were synthesized from 1 µg of total RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative Real-time PCR was performed and analyzed using an Applied Biosystems 7300 Real-time PCR System with 1X TaqMan Gene Expression Master Mix (Applied Biosystems), 1X TaqMan Gene Expression Assay (Applied Biosystems, see references below) and 1 µL of cDNA in a total volume of 20 µL. PCR conditions were 10 min at 95°C followed by 40 cycles of 15 s at 95°C, 1 min at 60°C. PCR reactions were done in duplicate and negative controls (i.e., no-reverse transcription and no-template controls) were added to the reactions. Relative expression levels were determined using the comparative ΔC_T method to normalize target gene mRNA to *β-actin* (reference below). A dilution curve of the pool of all cDNA samples was used to calculate the amplification efficiency for each assay (values were between 1.9 and 2). The following TaqMan Gene Expression Assays were used: *β-actin* (Mm01205647_g1), *Bmal1* (Arnt1, Mm00500226_m1), *Clock* (Mm00455950_m1), *Dbp* (Mm01194021_m1), *Per1* (Mm00501813_m1) and *Per2* (Mm00478113_m1).

Analysis of locomotor activity and temperature data

Locomotor activity data were double-plotted in actograms using a 5 min block size. Daily rhythms of general activity, wheel-running activity and body temperature were analyzed using a Clocklab software (Actimetrics, Evanston, IL, USA) associated to MatLab (MathWorks France). Mean activity profiles were quantified every 1 h during the last 10 days of the AL and RF conditions in the LD experiments, whereas for the DD experiment only a period of 5 days was taken into account to avoid overlap between the nocturnal activity controlled by the SCN and the food-anticipatory activity. To determine the free-running period in animals in DD conditions, with or without SCN, X^2 periodogram was used.

Statistical analysis

All values are expressed as mean ± SEM. Normality and homogeneity of variance were assessed with Kolmogorov–Smirnov & Lilliefors test and Levene's test, respectively. Data non-normal and/or heteroscedastic were subjected to logarithmic transformation before analysis. Alpha was set at 0.05. Data collected successively at different time points within each group were compared with ANOVA for repeated measures. Simple main effects approach was conducted if the interaction term was significant. Statistical analyses were performed with Statistica version 10 (StatSoft, Maisons-Alfort, France).

RESULTS

Food anticipation is reduced in LD conditions in *Rev-erbα*$^{-/-}$ mice

We first determined if *Rev-erbα* deletion led to an impaired ability of mice to anticipate mealtime in LD conditions. No significant differences were found between genotypes fed AL regarding body mass and food intake (Figure S1A), 24-h body temperature rhythm (Figure 1B) and daily general locomotor activity (data not shown). The amount of daily wheel-running revolutions was not significantly different in *Rev-erbα*$^{+/+}$ vs. *Rev-erbα*$^{-/-}$ mice exposed to LD (11665 ± 2382 vs. 7536 ± 2784, respectively; $P = 0.27$). For comparison purpose, data points were expressed as a percentage of total daily activity. The normalized circadian pattern of activity was similar in both genotypes placed in LD and fed AL (Figure 1A,B). During the scheduled RF, both genotypes showed a similar body mass (Figure S1A). Moreover, body mass loss after one-week of RF was not different in both genotypes (+/+ = -8.09 ± 2.12% vs. -/- = -9.52 ± 3.07%; *n.s.*). As expected, *Rev-erbα*$^{+/+}$ mice expressed a strong FAA 1 h (from ZT5 to ZT6) before meal access, illustrated by significant increases of wheel-running and general activities. In contrast, *Rev-erbα*$^{-/-}$ mice showed a reduced, albeit non significant ($P = 0.09$), percent elevation of wheel-activity 1 h prior to mealtime (Figure 1C,D). This result was further confirmed by the amount of general activity in *Rev-erbα*$^{-/-}$ mice, that did not vary significantly with time and was significantly decreased in anticipation, in comparison to *Rev-erbα*$^{+/+}$ controls (Figure 1C,D). Moreover, a clear rise in body temperature a few hours before food arrival was observed in control animals, whereas in *Rev-erbα*$^{-/-}$ mice, thermogenesis in anticipation was nearly absent (Figure 1C,D). Of note, LD experiment was reproduced with an additional series of mice for the metabolic and mRNA/protein expression analyses presented thereafter. Their results as well as those of *Rev-erbα*$^{+/-}$ mice, which demonstrated similar increases of food-anticipatory components than *Rev-erbα*$^{+/+}$ mice, are shown in Figure S2.

FAA is nearly absent in *Rev-erbα*$^{-/-}$ mice exposed to constant darkness

Under LD conditions, it was difficult to exclude a potential masking effect of light that could contribute to the reduced food anticipation in *Rev-erbα*$^{-/-}$ mice. We therefore evaluated food-anticipatory parameters in DD conditions. During AL access to food, body mass and food intake (Figure S1B), and total daily amount of general activity were similar between both genotypes (data not shown), while total wheel-running activity was

significantly decreased in *Rev-erbα*$^{-/-}$ mice compared to *Rev-erbα*$^{+/+}$ animals (5912 ± 2440 vs. 15886 ± 2753, respectively; *P* = 0.018). As in LD conditions, *Rev-erbα*$^{+/+}$ mice displayed a significant elevation of both wheel-running and general activities 1 h prior to food access, whereas *Rev-erbα*$^{-/-}$ mice demonstrated a significant reduction of FAA (Figure 2A,B). Moreover, the sharp preprandial rise in body temperature seen in control animals was not observed in *Rev-erbα*$^{-/-}$ mice, although the postprandial peak of body temperature occurred in both genotypes, thus indicating active food processing (Figure 2B). To further confirm the failure of *Rev-erbα*$^{-/-}$ mice to express a strong FAA, we performed SCN lesions (SCNx) in order to abolish the nocturnal activity component controlled by the SCN that could obscure food anticipation in DD. In control animals with successful SCN lesion, FAA was recognizable in some *Rev-erbα*$^{+/+}$ animals (n = 4, Figure 2C), but not all (n = 4). However, none of SCNx *Rev-erbα*$^{-/-}$ mice displayed significant FAA in response to food restriction (n = 5, Figure 2C). Of note, food-anticipatory wheel-running activity was also diminished in *Rev-erbα*$^{-/-}$ mice exposed to a skeleton photoperiod (data not shown). Thus, it was clear that, regardless of the lighting conditions, the time of the day when food was provided and the presence of a functional SCN pacemaker, the expression of FAA in *Rev-erbα*$^{-/-}$ mice was markedly reduced.

Corticosterone and ketone body levels are decreased in anticipation in *Rev-erbα*$^{-/-}$ mice

We then decided to evaluate the energy balance in both genotypes fed AL or exposed to RF in LD conditions. 24-h plasma glucose levels were significantly higher in *Rev-erbα*$^{-/-}$ mice compared to their control littermates fed AL. This significant hyperglycemia was not detected when food access was restricted to 6 h during daytime, despite elevated plasma glucose levels at ZT6 and ZT18 in *Rev-erbα*$^{-/-}$ mice (Figure 3A). In addition, we observed that hepatic glycogen levels were also significantly higher in *Rev-erbα*$^{-/-}$ mice at ZT12, which correspond to the end of the resting period (Figure 3B). While RF induced a change in the daily rhythm of glycogen levels in both genotypes, significant higher values were also detected at ZT6 (i.e., prior to mealtime) in *Rev-erbα*$^{-/-}$ mice compared to *Rev-erbα*$^{+/+}$ mice (Figure 3B). We then measured plasma ketone bodies, an important source of energy during starvation, which have been demonstrated to increase prior to food access in food-restricted rats (Escobar et al., 1998). In contrast to the absence of significant difference between both populations fed AL, however, plasma ketone bodies concentrations were significantly reduced in *Rev-erbα*$^{-/-}$ mice in RF conditions, in particular, at ZT6 (i.e., before food arrival)

and at ZT18 (i.e., after robust nocturnal activity) (Figure 3C). Taken together, these results could suggest that *Rev-erbα*$^{-/-}$ mice do not mobilize energy to the same extent as their control littermates. Previous studies have shown that a second peak of corticosterone appears in anticipation of food access in wild-type animals (Nelson et al., 1975). There was no significant difference in the 24-h corticosterone profiles between both genotypes, in spite of lower rhythm amplitude in *Rev-erbα*$^{-/-}$ mice (Figure 3D). In RF conditions, a strong rise of plasma corticosterone concentration emerged prior to food access in *Rev-erbα*$^{+/+}$ mice. On the opposite, there was no significant change in corticosterone values of *Rev-erbα*$^{-/-}$ mice during scheduled RF at ZT6 when both AL and RF conditions are compared. As a result, ZT6 corticosterone levels were significantly lower in *Rev-erbα*$^{-/-}$ mice when compared to control animals (Figure 3D). Since the anticipatory peak of corticosterone is considered as an output of the FEO, the absence of a rise in corticosterone in *Rev-erbα*$^{-/-}$ mice further substantiates our behavioral observations.

Hepatic clock gene expression is shifted by RF in both genotypes in LD conditions

Since daytime feeding changes the phase of circadian gene expression in peripheral tissues (Damiola et al., 2000), we also evaluated whether the synchronizing effects of RF on the liver oscillator involves *Rev-erbα.* For that purpose, we analyzed the mRNA expression of key actors of circadian molecular oscillations in both genotypes. In line with a previous report (Preitner et al., 2002), we found that *Bmal1* and *Clock* mRNA levels were up-regulated in *Rev-erbα*$^{-/-}$ mice (Figure 4A). As a consequence, *Clock* mRNA 24-h rhythmicity was even abolished in the absence of *Rev-erbα*. However, the circadian temporal pattern of *Per1, Per2* and *Dbp* mRNA were not different between genotypes in AL conditions (Preitner et al., 2002; Figure 4C-E), in accordance with previous results. As expected, feeding time induced a 6-h phase shift of clock-related gene expression in control animals (Damiola et al., 2000; Stokkan et al., 2001) (Figure 4A-E). Food synchronization of hepatic oscillations also occurred in *Rev-erbα*$^{-/-}$ mice, however, no phase-shift of mRNA expression could be detected for *Bmal1* (Figure 4A-E). This can be explained by the fact that the mRNA level of this clock component is severely up-regulated in the absence of *Rev-erbα,* and could therefore mask a change in their temporal expression. On the other side, *Clock* and *Bmal1* genes may not be essential for hepatic food entrainment. In that respect, *Rev-erbα* does not appear either essential for phase-adjustment of peripheral oscillations, since 6-h phase-shifts or the core clock

components *Per1, Per2,* as well as the crucial clock output *Dbp* were observed when it is lacking (Figure 4A-E).

Feeding time differently affected brain PER2 and p-ERK expression in *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice

A number of extra-SCN oscillators that could be part of the FEO network are sensitive to changes in the temporal organization of feeding behavior as well as those in energy balance (Dibner et al., 2010; Feillet et al., 2008; Mendoza et al., 2010b). We therefore investigated PER2 expression in key hypothalamic areas in mice fed AL and under RF paradigm. We also used as a marker of neuronal activation, the phosphorylated form of the Extracellular signal Regulated Kinases I/II (p-ERK), to assess its expression in both genotypes in response to a scheduled feeding. We found that all hypothalamic structures showed daily rhythmic expression of PER2 in both genotypes (Figure 5A). Of note, the amplitude rhythm of PER2 expression in the PVN and VMH was fairly diminished in *Rev-erbα*$^{-/-}$ mice, while PER2 expression was 6-h phase-advanced in the DMH and ARC of *Rev-erbα*$^{-/-}$ mice (Figure 5A). No change was detected in the SCN which serve as an internal control to evaluate the reliability of our immunohistochemistry experiments (Figure 5A). Unexpectedly, adaptation of hypothalamic PER2 oscillations to food restriction, in particular in the DMH, was observed in both genotypes, as evidenced by an elevation of PER2 expression at time of food access (Figure 5A). Hence, as for the liver oscillator, feeding synchronization of hypothalamic oscillators, involved in particular in feeding regulation, does not seem to require the clock gene *Rev-erbα*. On the other hand, we analyzed p-ERK expression to identify relevant structures that may directly respond to food restriction. In AL conditions, we could not detect significant rhythmic pattern of p-ERK expression in hypothalamic regions when both genotypes were considered in the analysis (Figure 5B). However, our results indicate that the acrophase of p-ERK expression occurred around ZT12 in all hypothalamic nuclei in control animals, while p-ERK peak expression was less evident in the hypothalamus of *Rev-erbα*$^{-/-}$ mice. Interestingly, in response to RF, p-ERK expression peaked at mealtime in *Rev-erbα*$^{+/+}$ mice. On the opposite, temporal changes in the expression of p-ERK in the hypothalamus of *Rev-erbα*$^{-/-}$ mice were hardly detected. Indeed, daily expression patterns were quite similar to those of AL conditions, in spite of a noticeable acrophase of p-ERK at the night-day transition in the SCN, PVN, VMH and DMH of *Rev-erbα*$^{-/-}$ mice. Besides that, only the ARC

nucleus seemed to adjust to food access in *Rev-erbα*$^{-/-}$ mice (Figure 5B). In the cerebellum, implicated in food anticipation (Mendoza et al., 2010b), the temporal pattern of PER2 and p-ERK expression was undistinguishable in the two genotypes fed AL (Figure 5A,B). In response to daytime RF, PER2 expression was severely phase-advanced in control mice. Conversely, the shift in PER2 expression was not observed in *Rev-erbα*$^{-/-}$ mice (Figure 5A). In addition, p-ERK expression was clearly up-regulated during food access in control animals, while the trough of p-ERK expression occurred prior to mealtime in *Rev-erbα*$^{-/-}$ mice (Figure 5B).

DISCUSSION

In the present study, we evaluated the role of an essential actor of the circadian timing system, the nuclear receptor *Rev-erbα*, which has not been considered in the context of food anticipation. We show that *Rev-erbα*-deficient mice have disrupted food-entrainable circadian rhythms, in particular those of locomotor activity, body temperature and plasma corticosterone.

Our results indicate that *Rev-erbα*$^{-/-}$ mice display a significant reduction of FAA in LD conditions compared to control animals. This is further confirmed by our investigations in DD conditions, in animals bearing or not SCN lesions. The lack of food-seeking behaviors in *Rev-erbα*$^{-/-}$ mice was not linked to larger body mass loss, reduced food intake or delayed food intake during the 6-h daily access (data not shown). In accordance with behavioral results, a sharp rise in body temperature before food access occurred in *Rev-erbα*$^{+/+}$ mice, whereas this anticipatory thermogenesis was nearly absent in the *Rev-erbα*$^{-/-}$ mice. Indeed, while control mice decreased their body temperature during food withdrawal, *Rev-erbα*$^{-/-}$ maintained body temperature values close to their respective values in AL feeding (Figure 1B,C). The absence of phase- and amplitude-adjustment of the body temperature rhythm in *Rev-erbα*$^{-/-}$ mice could reflect altered FEO influence on thermoregulation. However, relative normothermia was not observed in *Per2* mutant mice that showed a drastic reduction of FAA and preprandial thermogenesis. On the contrary, *Per2* mutant mice showed similar RF-induced drop of body temperature in late night hours than that in control littermates (Feillet et al., 2006). Hence, relative normothermia in *Rev-erbα*$^{-/-}$ mice could, instead, indicate that RF does not trigger to the same degree adaptive mechanisms to reduce energy expenditure as in wild-type littermates.

To further substantiate altered food entrainment in the absence of *Rev-erbα*, we measured hormonal and metabolic parameters, important for energy balance and that have been shown to increase prior to mealtime. In AL conditions, the 24-h plasma corticosterone profiles were not different between genotypes, suggesting that the adrenal peripheral oscillator (Oster et al., 2006) is likely unaltered in the absence of *Rev-erbα*. Interestingly, scheduled feeding affects the 24-h plasma corticosterone rhythm, with a shift of the acrophase of corticosterone at the night-day transition in *Rev-erbα*$^{-/-}$ mice, without changing

the peak at the day-night transition in control animals. However, the food-anticipatory peak of corticosterone, considered as a strong output of the FEO (Nelson et al., 1975) was absent in *Rev-erbα*$^{-/-}$ mice. Plasma glucose and hepatic glycogen levels were higher in *Rev-erbα*$^{-/-}$ mice in LD conditions. Of note, REV-ERBα has been suggested to play a role in hepatic gluconeogenesis *in vitro* (Yin et al., 2007). Hence, altered expression of gluconeogenesic actors could contribute to the elevated plasma glucose levels in *Rev-erbα*$^{-/-}$ mice. Mild hyperglycemia and increased hepatic glycogen levels in *Rev-erbα*$^{-/-}$ mice were also observed in RF conditions prior to food access. Of interest, dietary glucose can affect the circadian properties of the FEO (Stephan and Davidson, 1998). Thus, abnormal circulating blood glucose levels could alter the FEO functioning. However, rats rendered diabetic by streptozotocine injection exhibit normal FAA (Davidson et al., 2002). Therefore, the higher blood glucose levels and hepatic glycogen stores in *Rev-erbα*$^{-/-}$ mice could rather reflect decreased utilization of energy substrates to feed FAA—since the latter is clearly reduced in these mice. Nonetheless, we could not detect an elevation of ketone bodies levels in *Rev-erbα*$^{-/-}$ mice prior to mealtime in RF conditions. Since *Rev-erbα*$^{-/-}$ mice are not hypoactive indicated by measurements of general activity and maintain relative normothermia in RF conditions, whether the absence of a rise in ketone bodies results from an increased utilization or a decreased mobilization of this energy source has to be further investigated.

Our findings also confirm that the molecular functioning of the liver oscillator is not impaired in LD conditions with food provided on an AL basis in *Rev-erbα*$^{-/-}$ mice, as demonstrated by undistinguishable *Per1-2, Cry1-2* and *Dbp* circadian pattern (present results; Preitner et al., 2002). In addition, we demonstrate that central clock components can be phase-shifted in response to limited food access in the absence of *Rev-erbα*, despite its suggested role in the acute response of the liver clockwork to feeding (Tahara et al., 2011). Indeed, synchronization of the liver oscillator to feeding time was preserved in *Rev-erbα*$^{-/-}$ mice as demonstrated by a shift in *Per1-2* and *Dbp* expression, suggesting that the disrupted FAA is not the consequence of impaired entrainment of the liver to scheduled feeding. Of note, the expression of clock-related genes in the liver of *Per2* mutant mice was also shifted in response to daytime feeding (Feillet et al., 2006). Furthermore, mice with streptozotocin-induced diabetes which demonstrate drastic elevation and reduction of serum glucose and insulin levels, respectively, displayed a similar phase shift of clock gene expression in

peripheral tissues such as liver in response to RF (Oishi et al., 2004). Thus, if liver dysfunctions contribute to altered energy status (i.e., high plasma glucose and low ketone bodies levels) in RF conditions, it is unlikely due to altered circadian oscillations.

We then investigated the expression of the core clock protein PER2 in key hypothalamic regions, involved in particular in feeding behavior and that exhibit circadian oscillations (Dibner et al., 2010; Horvath and Diano, 2004). Indeed, on one hand, PER2 expression has been previously demonstrated to strongly respond to feeding time (Feillet et al., 2008). On the other hand, the DMH oscillator has been proposed as important for the expression of food-entrainable oscillations (Acosta-Galvan et al., 2011; Gooley et al., 2006). Our results show that PER2 expression in all hypothalamic nuclei studied (i.e., SCN, PVN, VMH, DMH, ARC) was not significantly different between both genotypes either fed AL or challenged with RF. Therefore, the diminished capability of food entrainment in *Rev-erbα*$^{-/-}$ mice does not appear to be correlated with altered molecular functioning of central hypothalamic areas. Conversely, increased expression of a marker of neuronal activation such as c-FOS, before and after mealtime, has been observed in several hypothalamic nuclei, including the DMH and the VMH (Angeles-Castellanos et al., 2004; Gooley et al., 2006; Ribeiro et al., 2007). Of interest, our data on p-ERK expression, which is a marker of neuronal activation, indicate that all hypothalamic nuclei, including the SCN, demonstrated a phase-adjustment of p-ERK oscillations in control animals. On the contrary, the circadian pattern of p-ERK was only in phase with mealtime in the ARC of *Rev-erbα*$^{-/-}$ mice. Taken together, these results on p-ERK expression can suggest that hypothalamic regions are not activated to the same extent in *Rev-erbα*$^{-/-}$ mice compared to control animals during temporal food restriction.

It was reported recently that the cerebellum harbors a circadian oscillator sensitive to feeding schedules and that genetic and pharmacological impairment of the cerebellar function led to reduced or lacking FAA (Mendoza et al., 2010b). In this context, we also determined the expression pattern of PER2 and p-ERK proteins in the cerebellum of *Rev-erbα*$^{-/-}$ mice, since the latter exhibited reduced food-anticipatory components. Interestingly, we found no phase-adjustment of PER2 oscillations as well as a slight diminution of p-ERK expression in anticipation of eating in *Rev-erbα*$^{-/-}$ mice. Of interest, *Per2* mRNA was not increased in anticipation to mealtime in *Grid2*$^{ho/ho}$ mice that have genetic cerebellar deficits,

and which demonstrated a lack of FAA (Mendoza et al., 2010b). Therefore, these results on PER2 and p-ERK expression in the cerebellum of *Rev-erbα*$^{-/-}$ mice could be a direct correlate to their defect to display strong food-anticipatory components.

The present findings indicate that mice lacking the *Rev-erbα* clock gene exhibit altered behavioral and physiological food anticipation, in addition to impaired p-ERK response to RF, implying that *Rev-erbα* may be a key component of the FEO. Our results could thus support the hypothesis that the basis of the FEO molecular mechanism is likely similar to those of well-known circadian oscillators, in line with previous studies (Dudley et al., 2003; Feillet et al., 2006; Iijima et al., 2005; Mieda and Sakurai, 2011). However, considering the strong involvement of *Rev-erbα* in metabolic regulations (Duez et al., 2008; Raspe et al., 2002; Yin et al., 2007), our results do not fully exclude that the decrease of FAA in *Rev-erbα*$^{-/-}$ mice could be linked to a metabolic defect at central or peripheral levels beyond preserved core clock gene oscillations. Therefore, the implication of *Rev-erbα* in metabolic processes *in vivo* has to be further explored.

REFERENCES

Acosta-Galvan, G., Yi, C.X., van der Vliet, J., Jhamandas, J.H., Panula, P., Angeles-Castellanos, M., Del Carmen Basualdo, M., Escobar, C., and Buijs, R.M. (2011). Interaction between hypothalamic dorsomedial nucleus and the suprachiasmatic nucleus determines intensity of food anticipatory behavior. Proc Natl Acad Sci U S A 108, 5813-5818.

Angeles-Castellanos, M., Aguilar-Roblero, R., and Escobar, C. (2004). c-Fos expression in hypothalamic nuclei of food-entrained rats. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 286, R158-R165.

Challet, E., Mendoza, J., Dardente, H., and Pevet, P. (2009). Neurogenetics of food anticipation. European Journal of Neuroscience 30, 1676-1687.

Crumbley, C., and Burris, T.P. (2011). Direct regulation of CLOCK expression by REV-ERB. PLoS One 6, e17290.

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., and Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev 14, 2950-2961.

Davidson, A.J., Poole, A.S., Yamazaki, S., and Menaker, M. (2003). Is the food-entrainable circadian oscillator in the digestive system? Genes Brain Behav 2, 32-39.

Davidson, A.J., Stokkan, K.A., Yamazaki, S., and Menaker, M. (2002). Food-anticipatory activity and liver per1-luc activity in diabetic transgenic rats. Physiol Behav 76, 21-26.

Dibner, C., Schibler, U., and Albrecht, U. (2010). The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annu Rev Physiol 72, 517-549.

Dudley, C.A., Erbel-Sieler, C., Estill, S.J., Reick, M., Franken, P., Pitts, S., and McKnight, S.L. (2003). Altered patterns of sleep and behavioral adaptability in NPAS2-deficient mice. Science 301, 379-383.

Duez, H., van der Veen, J.N., Duhem, C., Pourcet, B., Touvier, T., Fontaine, C., Derudas, B., Bauge, E., Havinga, R., Bloks, V.W., Wolters, H., van der Sluijs, F.H., Vennstrom, B., Kuipers, F., and Staels, B. (2008). Regulation of bile acid synthesis by the nuclear receptor Rev-erbalpha. Gastroenterology 135, 689-698.

Escobar, C., Diaz-Munoz, M., Encinas, F., and Aguilar-Roblero, R. (1998). Persistence of metabolic rhythmicity during fasting and its entrainment by restricted feeding schedules in rats. Am J Physiol 274, R1309-1316.

Feillet, C.A., Mendoza, J., Albrecht, U., Pevet, P., and Challet, E. (2008). Forebrain oscillators ticking with different clock hands. Mol Cell Neurosci 37, 209-221.

Feillet, C.A., Ripperger, J.A., Magnone, M.C., Dulloo, A., Albrecht, U., and Challet, E. (2006). Lack of food anticipation in Per2 mutant mice. Curr Biol 16, 2016-2022.

Gooley, J.J., Schomer, A., and Saper, C.B. (2006). The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms. Nat Neurosci 9, 398-407.

Guillaumond, F., Dardente, H., Giguere, V., and Cermakian, N. (2005). Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. J Biol Rhythms 20, 391-403.

Hara, R., Wan, K., Wakamatsu, H., Aida, R., Moriya, T., Akiyama, M., and Shibata, S. (2001). Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. Genes Cells 6, 269-278.

Horikawa, K., Minami, Y., Iijima, M., Akiyama, M., and Shibata, S. (2005). Rapid damping of food-entrained circadian rhythm of clock gene expression in clock-defective peripheral tissues under fasting conditions. Neuroscience 134, 335-343.

Horvath, T.L., and Diano, S. (2004). The floating blueprint of hypothalamic feeding circuits. Nature Reviews Neuroscience 5, 662-667.

Iijima, M., Yamaguchi, S., van der Horst, G.T., Bonnefont, X., Okamura, H., and Shibata, S. (2005). Altered food-anticipatory activity rhythm in Cryptochrome-deficient mice. Neurosci Res 52, 166-173.

Marchant, E.G., and Mistlberger, R.E. (1997). Anticipation and entrainment to feeding time in intact and SCN-ablated C57BL/6j mice. Brain Res 765, 273-282.

Mendoza, J., Albrecht, U., and Challet, E. (2010a). Behavioural food anticipation in clock genes deficient mice: confirming old phenotypes, describing new phenotypes. Genes Brain Behav 9, 467-477.
Mendoza, J., Pevet, P., Felder-Schmittbuhl, M.P., Bailly, Y., and Challet, E. (2010b). The cerebellum harbors a circadian oscillator involved in food anticipation. J Neurosci 30, 1894-1904.
Mieda, M., and Sakurai, T. (2011). Bmal1 in the nervous system is essential for normal adaptation of circadian locomotor activity and food intake to periodic feeding. J Neurosci 31, 15391-15396.
Mistlberger, R.E. (1994). Circadian food-anticipatory activity: formal models and physiological mechanisms. Neurosci Biobehav Rev 18, 171-195.
Mistlberger, R.E. (2011). Neurobiology of food anticipatory circadian rhythms. Physiol Behav 104, 535-545.
Murat, J.C., and Serfaty, A. (1974). Simple enzymatic determination of polysaccharide (glycogen) content of animal tissues. Clin Chem 20, 1576-1577.
Nelson, W., Scheving, L., and Halberg, F. (1975). Circadian rhythms in mice fed a single daily meal at different stages of lighting regimen. J Nutr 105, 171-184.
Oishi, K., Kasamatsu, M., and Ishida, N. (2004). Gene- and tissue-specific alterations of circadian clock gene expression in streptozotocin-induced diabetic mice under restricted feeding. Biochem Biophys Res Commun 317, 330-334.
Oster, H., Damerow, S., Kiessling, S., Jakubcakova, V., Abraham, D., Tian, J., Hoffmann, M.W., and Eichele, G. (2006). The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock. Cell Metab 4, 163-173.
Pitts, S., Perone, E., and Silver, R. (2003). Food-entrained circadian rhythms are sustained in arrhythmic Clk/Clk mutant mice. Am J Physiol Regul Integr Comp Physiol 285, R57-67.
Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110, 251-260.
Raspe, E., Duez, H., Mansen, A., Fontaine, C., Fievet, C., Fruchart, J.C., Vennstrom, B., and Staels, B. (2002). Identification of Rev-erbalpha as a physiological repressor of apoC-III gene transcription. J Lipid Res 43, 2172-2179.
Ribeiro, A.C., Sawa, E., Carren-LeSauter, I., LeSauter, J., Silver, R., and Pfaff, D.W. (2007). Two forces for arousal: Pitting hunger versus circadian influences and identifying neurons responsible for changes in behavioral arousal. Proc Natl Acad Sci U S A 104, 20078-20083.
Stephan, F.K., and Davidson, A.J. (1998). Glucose, but not fat, phase shifts the feeding-entrained circadian clock. Physiol Behav 65, 277-288.
Stephan, F.K., Swann, J.M., and Sisk, C.L. (1979). Entrainment of circadian rhythms by feeding schedules in rats with suprachiasmatic lesions. Behav Neural Biol 25, 545-554.
Stokkan, K.A., Yamazaki, S., Tei, H., Sakaki, Y., and Menaker, M. (2001). Entrainment of the circadian clock in the liver by feeding. Science 291, 490-493.
Storch, K.F., and Weitz, C.J. (2009). Daily rhythms of food-anticipatory behavioral activity do not require the known circadian clock. Proceedings of the National Academy of Sciences of the United States of America 106, 6808-6813.
Tahara, Y., Otsuka, M., Fuse, Y., Hirao, A., and Shibata, S. (2011). Refeeding after fasting elicits insulin-dependent regulation of Per2 and Rev-erbalpha with shifts in the liver clock. J Biol Rhythms 26, 230-240.
Yin, L., Wu, N., Curtin, J.C., Qatanani, M., Szwergold, N.R., Reid, R.A., Waitt, G.M., Parks, D.J., Pearce, K.H., Wisely, G.B., and Lazar, M.A. (2007). Rev-erbalpha, a heme sensor that coordinates metabolic and circadian pathways. Science 318, 1786-1789.

FIGURE LEGENDS

Figure 1. Food-anticipatory components are reduced in *Rev-erbα*$^{-/-}$ mice exposed to a 12:12 light-dark cycle.

(A) Representative double-plotted actograms (left, middle) and thermograms (right) of *Rev-erbα*$^{+/+}$ (upper panel) and $^{-/-}$ mice (lower panel) under LD conditions with food provided AL and then restricted to 6 h per day (grey box, food was provided from ZT6 to ZT12). (B-C) Normalized activity profiles and body temperature raw data representing the average of 10 days during AL conditions (B) and RF conditions (C). Daytime and nighttime are indicated by white and black bars, respectively. The period of food access during RF is represented by the grey rectangle. (D) Percent of FAA over total daily activity from 4 h to 1 h before mealtime (left, middle) and rise in body temperature in anticipation (right). * significantly different from control animals ($P < 0.05$). Note that the main effect of time was not represented (see text).

Figure 2. Food-anticipatory components are reduced in *Rev-erbα*$^{-/-}$ mice exposed to constant darkness.

(A) Representative double-plotted actograms (left, middle) and thermograms (right) of *Rev-erbα*$^{+/+}$ (upper panel) and $^{-/-}$ mice (lower panel) under DD conditions with food provided AL and then restricted to 6 h per day (grey box, food was provided from 02:00 p.m. to 08:00 p.m.). Constant darkness is indicated by a black bar. (B) Percent of FAA over total daily activity (average of 5 days) from 4 h before mealtime and 2 h after mealtime (left, middle) and rise in body temperature in anticipation (right). (C) Representative double-plotted actograms (left, middle) and corresponding χ^2 periodograms (right) from SCN-X *Rev-erbα*$^{+/+}$ (upper panel) and $^{-/-}$ mice (lower panel) under LD conditions and DD conditions (DD onset indicated by the black arrow) with food provided AL and then restricted to 6 h per day (grey box, food was provided from 02:00 p.m. to 08:00 p.m.). * significantly different from control animals ($P < 0.05$). Note that the main effect of time was not represented (see text).

Figure 3. Plasma ketone bodies and corticosterone peaks in *Rev-erbα*$^{-/-}$ mice are decreased in anticipation of feeding time.

24-h (A) plasma glucose, (B) hepatic glycogen, (C) plasma ketone bodies and (D) plasma corticosterone levels in both AL (left) and RF conditions (right). Daytime and nighttime are indicated by white and black bars, respectively, on the X axis. Food access during RF schedules is depicted by a grey rectangle. Data for ZT0 are double-plotted. * significantly different from control animals ($P < 0.05$). ~ for main effect of time (top left corner) or *vs.* control animals (above data point) ($P < 0.05$).

Figure 4. mRNA expression of clock-related genes in the liver oscillator are synchronized by feeding time in both genotypes.

Expression of *Bmal1* (A), *Clock* (B), *Per1* (C), *Per2* (D), *Dbp* (E) under AL conditions (left panel) and RF conditions (right panel). Daytime and nighttime are indicated by white and black rectangles, respectively, on the X axis. Food access during RF schedules is depicted by a grey rectangle. Data for ZT0 are double-plotted. * significantly different from control animals ($P <$

0.05). ~ for main effect of time (top left corner) or *vs.* control animals (above data point) ($P < 0.05$).

Figure 5. PER2 and p-ERK expression in the hypothalamus and cerebellum of *Rev-erbα*$^{-/-}$ mice entrained by food.

(A) PER2 and (B) p-ERK immunoreactive (ir) cells in hypothalamic nuclei and cerebellum of mice under AL conditions or exposed to RF schedules. Note that staining intensity was taken into consideration for all hypothalamic nuclei; while for the cerebellum, the number of labeled purkinje cells was counted. Data for ZT0 are double-plotted. ~ for main effect of time (top left corner) ($P < 0.05$).

Supplemental Figure 1. Food intake and body mass of mice maintained in LD or DD conditions before and during scheduled feeding.

Food intake (left) and body mass (right) determined weekly in (A) LD conditions and (B) DD conditions. The period of restricted feeding is indicated by the hatched bars.

Supplemental Figure 2. Food-anticipatory parameters in mice maintained in LD or DD conditions under restricted feeding.

(A) Percent of wheel-running activity and (B) general activity over total daily activity 1 h prior to meal access in three independent series of animals (i.e., LD1, DD and LD2). (C) Note that heterozygous mice (+/-) for *Rev-erbα* were added to the results. Results of *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice depicted on figure A, B and C for the LD1 experiment are reproduced from Figure 1C-D and Figure 2B. An average of 10 days was also taken into account during the second LD experiment (LD2) to determine the amount of FAA at the end of the RF period.

Fig.1

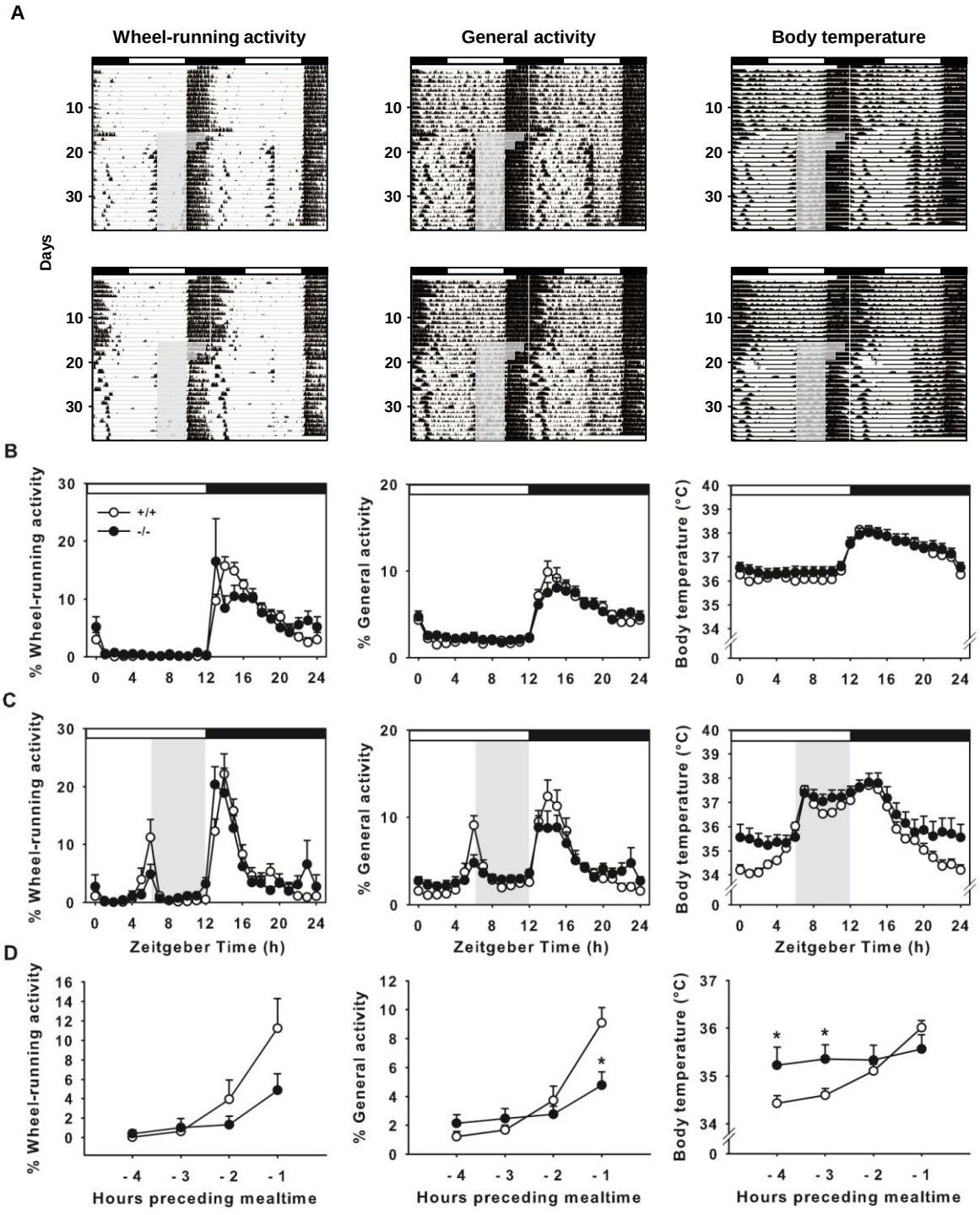

A
Wheel-running activity
General activity
Body temperature
Days
10
20
30
B
% Wheel-running activity
% General activity
Body temperature (°C)
+/+
-/-
0 4 8 12 16 20 24
C
Zeitgeber Time (h)
D
Hours preceding mealtime
- 4 - 3 - 2 - 1
*

Fig.2

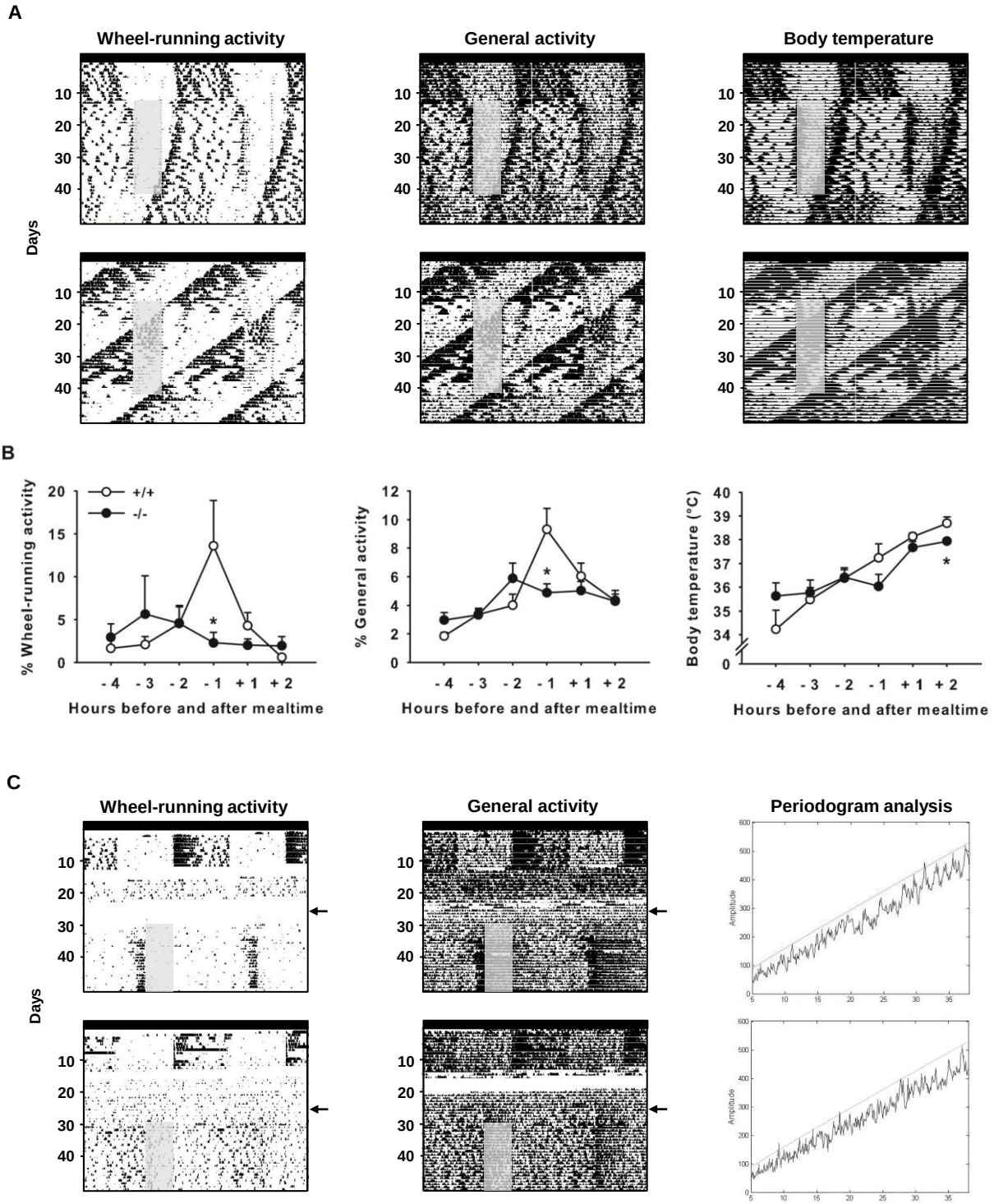

A
Wheel-running activity
General activity
Body temperature
Days
10
20
30
40
B
% Wheel-running activity
+/+
-/-
% General activity
Body temperature (°C)
- 4
- 3
- 2
- 1
+ 1
+ 2
Hours before and after mealtime
C
Wheel-running activity
General activity
Periodogram analysis
Days
Amplitude

Fig.3

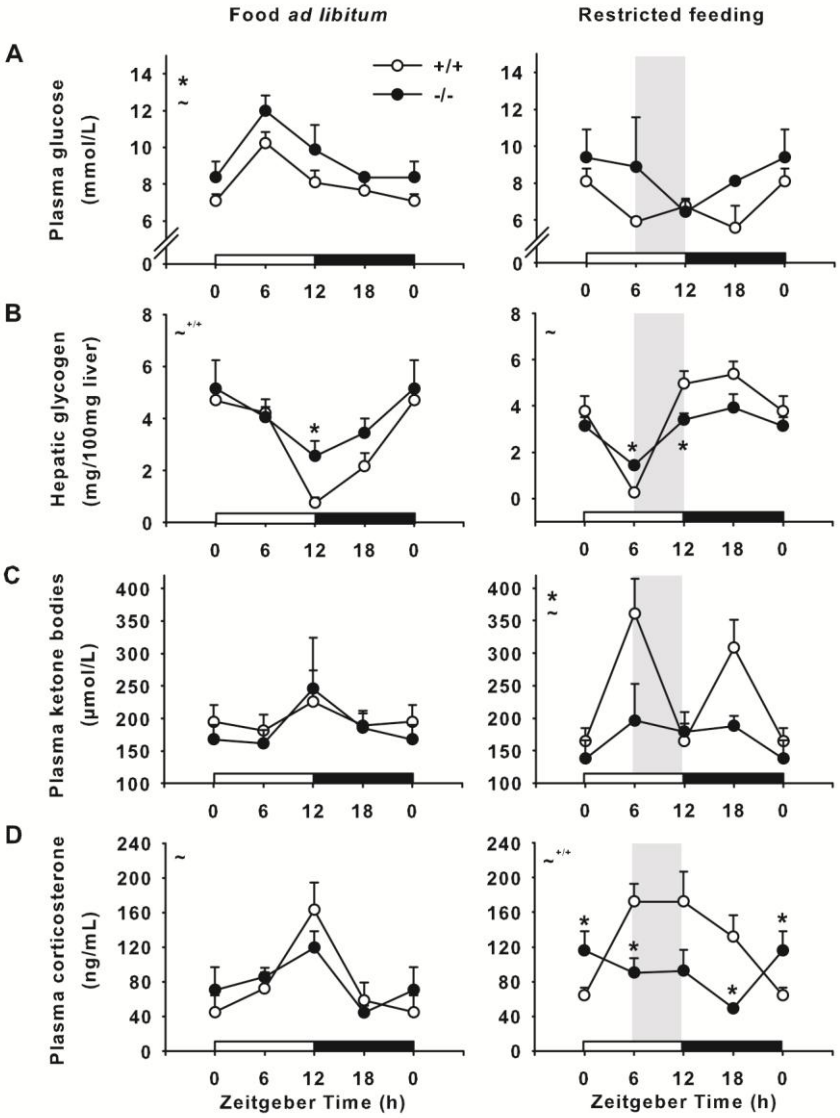

Food ad libitum
Restricted feeding
A
B
C
D
+/+
-/-
Plasma glucose (mmol/L)
Hepatic glycogen (mg/100mg liver)
Plasma ketone bodies (µmol/L)
Plasma corticosterone (ng/mL)
Zeitgeber Time (h)
Zeitgeber Time (h)

Fig.4

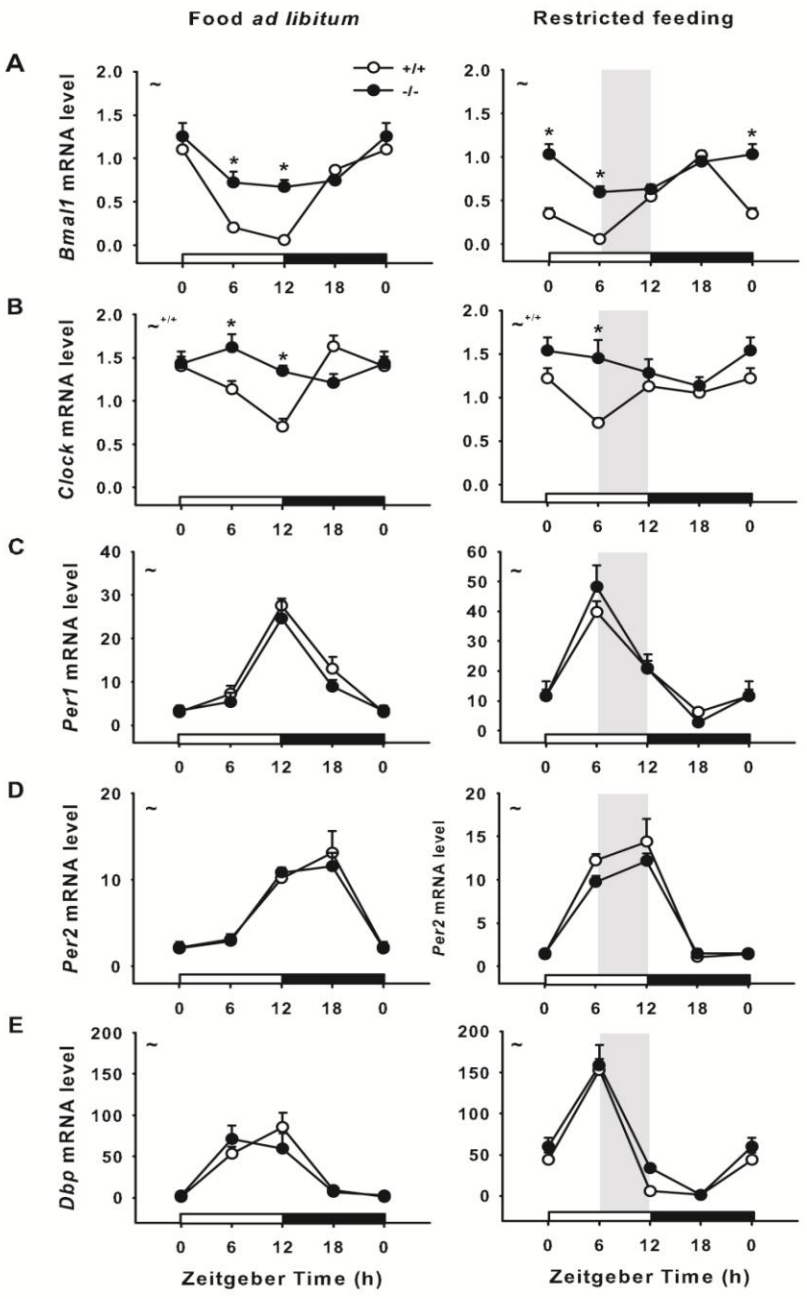
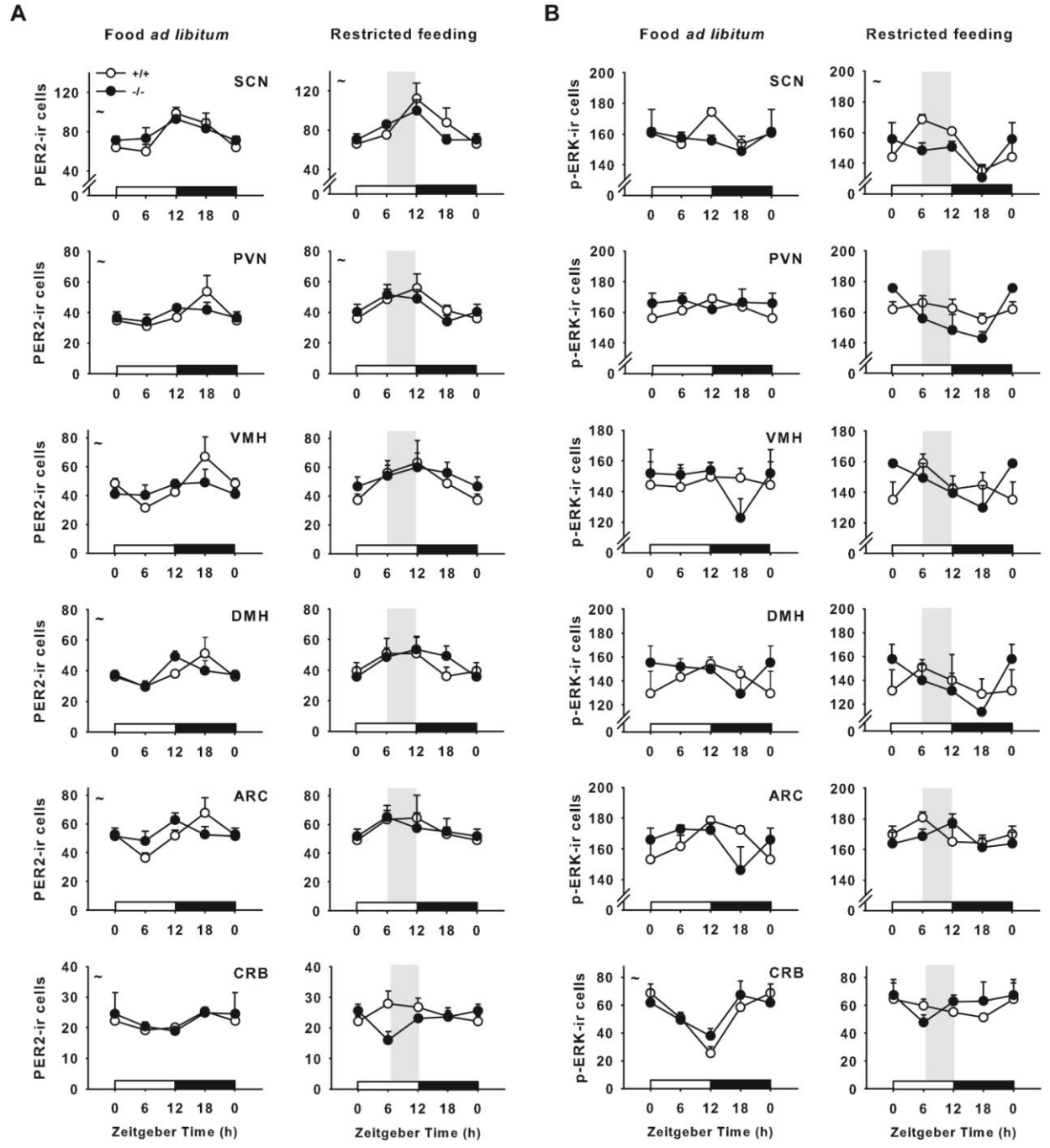
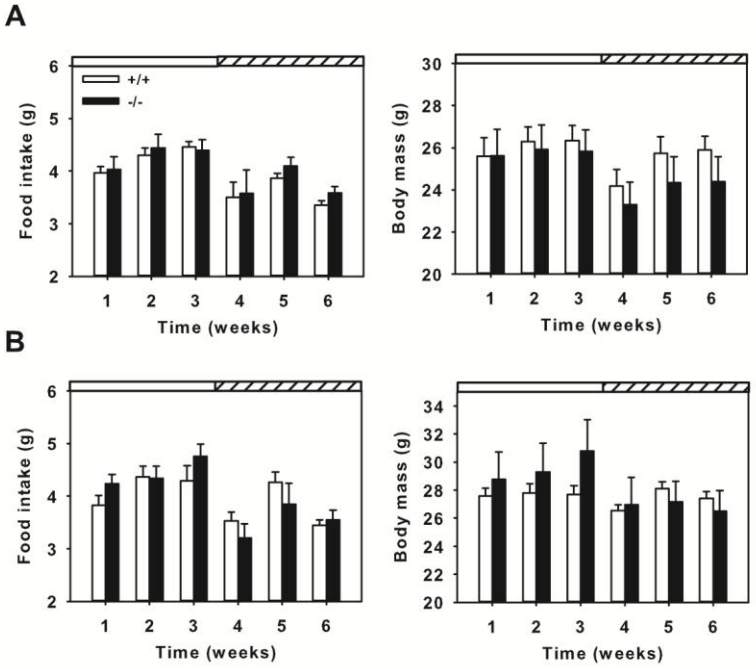

Food ad libitum
Restricted feeding
A
B
C
D
E
+/+
-/-
Bmal1 mRNA level
Clock mRNA level
Per1 mRNA level
Per2 mRNA level
Dbp mRNA level
Zeitgeber Time (h)
Zeitgeber Time (h)

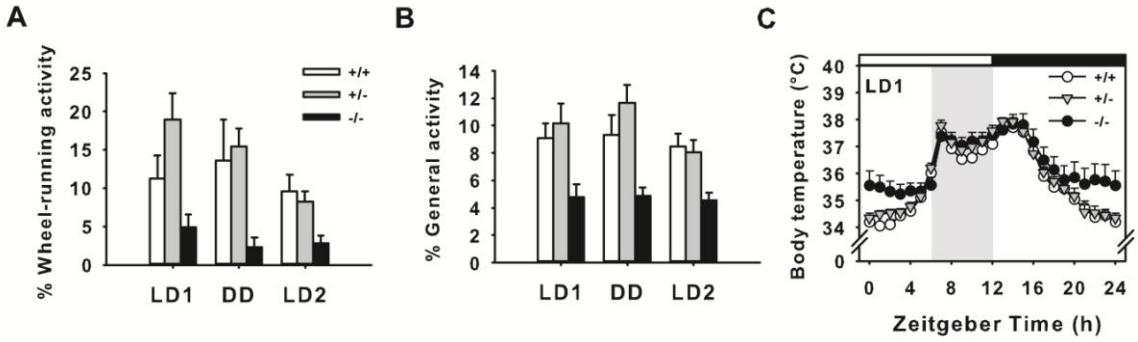
Fig.5


A
Food ad libitum
Restricted feeding
+/+
-/-
SCN
PVN
VMH
DMH
ARC
CRB
PER2-ir cells
Zeitgeber Time (h)
B
Food ad libitum
Restricted feeding
p-ERK-ir cells
Zeitgeber Time (h)

Supp. Fig.1



Supp. Fig.2



2. The nuclear receptor REV-ERBα is required for the daily balance of carbohydrate and lipid metabolism

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The nuclear receptor REV-ERBα is required for the daily balance of carbohydrate and lipid metabolism

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ABSTRACT **Mutations of clock genes can lead to diabetes and obesity. REV-ERBα, a nuclear receptor involved in the circadian clockwork, has been shown to control lipid metabolism. To gain insight into the role of REV-ERBα in energy homeostasis *in vivo*, we explored daily metabolism of carbohydrates and lipids in chow-fed, unfed, or high-fat-fed *Rev-erbα*$^{-/-}$ mice and their wild-type littermates. Chow-fed *Rev-erbα*$^{-/-}$ mice displayed increased adiposity (2.5-fold) and mild hyperglycemia (~10%) without insulin resistance. Indirect calorimetry indicates that chow-fed *Rev-erbα*$^{-/-}$ mice utilize more fatty acids during daytime. A 24-h nonfeeding period in *Rev-erbα*$^{-/-}$ animals favors further fatty acid mobilization at the expense of glycogen utilization and gluconeogenesis, without triggering hypoglycemia and hypothermia. High-fat feeding in *Rev-erbα*$^{-/-}$ mice amplified metabolic disturbances, including expression of lipogenic factors. *Lipoprotein lipase* (*Lpl*) gene, critical in lipid utilization/storage, is triggered in liver at night and constitutively up-regulated (~2-fold) in muscle and adipose tissue of *Rev-erbα*$^{-/-}$ mice. We show that CLOCK, up-regulated (2-fold) at night in *Rev-erbα*$^{-/-}$ mice, can transactivate *Lpl*. Thus, overexpression of *Lpl* facilitates muscle fatty acid utilization and contributes to fat overload. This study demonstrates the importance of clock-driven *Lpl* expression in energy balance and highlights circadian disruption as a potential cause for the metabolic syndrome.—Delezie, J., Dumont, S., Dardente, H., Oudart, H., Gréchez-Cassiau, A., Klosen, P., Teboul, M., Delaunay, F., Pévet, P., Challet, E. The nuclear receptor REV-ERBα is required for the daily balance of carbohydrate and lipid metabolism. *FASEB J.* 26, 000–000 (2012). www.fasebj.org**

Key Words: circadian · obesity · hyperglycemia · respiratory quotient · lipoprotein lipase

Altered circadian rhythmicity is a newly identified determinant of metabolic disorders in humans (1). Most aspects of behavior and metabolism display daily rhythms, including sleep-wake and feeding-nonfeeding cycles (2). These daily variations are controlled by a circadian timing system made of interconnected clocks and oscillators. In mammals, the master circadian clock, located in the suprachiasmatic nuclei of the hypothalamus, is mainly reset by ambient light and synchronizes peripheral oscillations to 24 h. Secondary oscillators are present in many brain regions and peripheral organs (*e.g.*, liver), and can be shifted by feeding-related cues (3–5).

The molecular clockwork is based on autoregulatory transcriptional and translational feedback loops involving clock genes and proteins that generate a rhythmic transcriptional activity with a ~24 h period. In this clock network, two transcriptional activators, CLOCK and BMAL1, stimulate the expression of *Period* (*Per1–3*) and *Cryptochrome* (*Cry1, 2*) genes, whose proteins in turn repress the CLOCK-BMAL1 transactivation (6). In addition to these main components, the nuclear receptors ROR(α,β,γ) and REV-ERB(α,β) compete to activate and repress, respectively, the transcription of the *Bmal1* and *Clock* genes, thereby reinforcing the robustness of circadian oscillations (7–9).

Approximately 10% of the mammalian transcriptome is under circadian regulation (10). In particular,

Abbreviations: AUC, area under the curve; DMEM, Dulbecco's modified Eagle's medium; GST, glucagon stimulation test; HDL, high-density lipoprotein; HFD, high-fat diet; IPIST, intraperitoneal insulin sensitivity test; LDL, low-density lipoprotein; MyHC, myosin heavy chain; NEFA, nonesterified fatty acid; OGTT, oral glucose tolerance test; PTT, pyruvate tolerance test; RLU, relative luminescence unit; RQ, respiratory quotient; VLDL, very low density lipoprotein; WAT, white adipose tissue; ZT, zeitgeber time

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metabolic processes are closely linked to circadian oscillations, as evidenced by the transcriptional control of genes involved in lipid and glucose metabolism by clock-related factors (3, 11). Consequently, disruption of the circadian clockwork leads to profound changes in energy balance. Mutation of *Clock* leads to obesity (12), while pancreas-specific *Bmal1*-mutant mice develop diabetes (13). On the other hand, diet-induced obesity affects the master clock as well as peripheral oscillations in wild-type mice (14, 15) and exacerbates metabolic phenotypes in mice with deficient clocks (12, 16).

As outlined above, REV-ERBα is a component of the circadian clockwork that is expressed in the brain and peripheral tissues, such as liver, pancreas, adipose tissue, and muscle (8, 17, 18). Furthermore, REV-ERBα participates in the regulation of diverse metabolic pathways, including adipocyte differentiation, gluconeogenesis, bile acid synthesis, and cholesterol homeostasis (19–22). Besides, REV-ERBα can modulate the expression of its own ligand, heme (23, 24), implicated in cellular metabolism. Daily expression of REV-ERBα has also been shown to control the circadian transcription of various lipid metabolism genes by recruiting the repressive chromatin modifier histone deacetylase 3 in the liver (25). Taken together, these data suggest that REV-ERBα may tightly connect the circadian system to energy metabolism on a daily basis.

Here we show that mice lacking *Rev-erb*α display changes in daily energy homeostasis, leading to enhanced lipid fuel utilization and production during daytime and nighttime, respectively, and predisposing to diet-induced obesity. This occurs with constitutive elevation of the *Lpl* gene in peripheral tissues, likely due to the defective clock regulation of this gene.

MATERIALS AND METHODS

Animals and housing conditions

Rev-erb$\alpha^{+/-}$ mice, kindly provided by Prof. Ueli Schibler (University of Geneva, Geneva, Switzerland) were rederived on a C57BL6/J background (Charles River Laboratories, L'Arbresle, France) and backcrossed until N5 in our local animal care facilities (Chronobiotron, Strasbourg, France). Characterization of the genetic background of the strain by Charles River Laboratories indicated that mice were >95% C57BL6/J. The *Rev-erb*α-deletion strategy is described in ref. 8. All mice were maintained under a 12-h light-dark cycle in a temperature-controlled room (22 ± 1°C). Normocaloric chow diet (2.89 kcal/g, 14% kcal from fat, 27% kcal from protein and 59% from carbohydrates; SAFE 105; SAFE, Augy, France) and water were provided *ad libitum* unless specified otherwise. All experiments were performed in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996), the French National Law (implementing the European Communities Council Directive 86/609/EEC) and approved by the Regional Ethical Committee of Strasbourg for Animal Experimentation (CREMEAS).

Animal experiments

Behavior, physiology, and glucose homeostasis

Mice (4 mo old, n=10/genotype, sex ratio 1:1) were used for measuring general locomotor activity and body temperature after intraperitoneal implantation with a small transponder (G2 E-Mitter; MiniMitter, Sunriver, OR, USA) under gaseous anesthesia (2% isoflurane in 50:50 O_2/N_2O). Locomotor activity and temperature data were recorded every 5 min, 24 h/d, with a PC-based acquisition system (VitalView; MiniMitter) and analyzed with Clocklab (Actimetrics, Evanston, IL, USA). Indirect calorimetry was performed on other mice (n=10/genotype, sex ratio 1:1) as described previously (26) as well as the hepatocyte study (see procedure below). For glucose, insulin measurements and pancreas studies, mice (n=12/genotype, sex ratio 1:1) were sampled at zeitgeber time 0 (ZT0; lights on, onset of the resting/nonfeeding period) and ZT12 (lights off, onset of the active/feeding period).

Food withdrawal and refeeding experiments

Mice (4 mo old) were divided into 2 groups (4 males and 3 females/group/genotype); one group was unfed for 24 h starting at ZT0, and the other was unfed for 24 h and then refed for the next 24 h with a high-carbohydrate diet (3.63 kcal/g, 7.4% kcal from fat, 14.1% kcal from protein, and 78.5% kcal from carbohydrate; SAFE U8960v2). Both groups were killed by lethal injection of pentobarbital at ZT0. Blood samples were collected with 4% EDTA and centrifuged for 10 min at 5000 rpm at 4°C. Liver samples were flash-frozen in liquid nitrogen. Locomotor activity and body temperature were recorded as above.

High-fat-diet (HFD) challenge

Mice (4 wk old, sex ratio 1:1) were fed either with a chow diet (as described above; n=12/genotype) or an HFD (4.65 kcal/g, 53.2% kcal from fat, 14.6% kcal from protein and 32.2% kcal from carbohydrate, SAFE U8955v3; n = 12/genotype) for up to 12 wk. Then, animals were sacrificed at ZT0 and ZT12. Mice were unfed for 1 h before injection of a lethal dose of pentobarbital. Blood samples were collected as above. Liver, white adipose tissue (WAT; retroperitoneal and perigonadal), and rectus femoris muscle were taken and immediately flash-frozen in liquid nitrogen.

In vivo glucose homeostasis

The blood glucose rhythm was determined in mice fed *ad libitum* from repeated tail-blood microsamples (<0.5 μl) using an Accu-Check glucometer (Roche Diagnostics, Meylan, France). For all metabolic tests, mice were unfed for 14 h (from ZT12 to ZT2) and injected at ZT2. For the oral glucose tolerance test (OGTT), mice received a glucose load *via* gavage (2 g/kg; D-glucose; Sigma, Saint Quentin Fallavier, France). For the glucagon stimulation test (GST) and pyruvate tolerance test (PTT), glucagon (1 mg/kg; GlucaGen; Novo Nordisk, Bagsvaerd, Denmark) and pyruvate (1.5 g/kg; P5280; Sigma) were administered intraperitoneally. Note that metabolic tests were separated by several days. The euglycemic clamp study was done by the Mouse Clinical Institute (MCI; Strasbourg, France) from ZT6 in animals previously unfed from ZT0 to ZT6 (see method in ref. 27).

Pancreatic histology and immunohistochemistry

Pancreases were fixed in Bouin's solution for 24 h and dehydrated successively in 70% ethanol, 2-ethoxyethanol, and

butanol. The tissue was embedded in paraffin, and 8-μm-thick serial sections were cut on a microtome and collected on gelatin-coated slides. For determination of islet area and circularity, sections were stained with Carrazzi's hematoxylin. To assess insulin and glucagon content, sections were washed with Tris buffered saline and incubated overnight with either mouse anti-insulin (1:50,000; clone HUI018; Novo Nordisk) or mouse anti-glucagon (1:60,000; clone GlU001; Novo Nordisk) antibodies. The sections were then rinsed and incubated for 1 h with a biotinylated secondary antibody (1:2000; Jackson ImmunoResearch, West Grove, PA, USA). Finally, streptavidin-peroxidase conjugate (1:2000; Roche) was added for 1 h after washing. Peroxidase activity was visualized with 0.5 mg/ml 3,3′-diaminobenzidine (Sigma) in the presence of 0.003% H_2O_2 in 50 mM Tris buffer containing 10 mM imidazole (pH 7.6). Micrographs were taken on a Leica DMRB microscope (Leica Microsystems, Nanterre, France) with an Olympus DP50 digital camera (Olympus, Rungis, France) and analyzed with ImageJ software (W. S. Rasband, U. S. National Institutes of Health, Bethesda MD, USA).

Plasma metabolic parameters

Total, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterol levels were determined by a direct colorimetric method (Biolabo, Maizy, France). Plasma leptin was assayed with an ELISA kit (Leptin EZML-82K; Millipore, Molsheim, France). Plasma glucose was evaluated with a GOD-PAP kit (Biolabo). Plasma insulin was determined with an ultrasensitive mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA). Plasma glucagon was assayed with a glucagon RIA (GL-32K, Millipore) after adding aprotinin (250 kIU/ml) when blood samples were taken. Triglyceride concentration was determined by a triglyceride determination kit (TR-0100; Sigma). The ACS-ACOD method (NEFA-HR2; Wako, Osaka, Japan) was used for assaying plasma nonesterified fatty acids (NEFAs). Plasma β-hydroxybutyrate concentrations were determined with a cyclic enzymatic method (Autokit 3-HB; Wako).

Hepatic glycogen and triglycerides

Hepatic glycogen and triglycerides were assayed following the methods of Murat and Serfaty (28) and Miao *et al.* (29), respectively.

Primary hepatocyte culture

Livers sampled from adult mice at ZT2 to ZT4 were immediately perfused at the flow rate of 3 ml/min *via* the portal vein for 5 min with a calcium-free perfusion buffer (10 mM HEPES, 140 mM NaCl, and 6.7 mM KCl, pH 7.65), supplemented by 0.6 mM EGTA, followed by an additional 2 min with the Ca^{2+}-free buffer, then 5 min with the same buffer containing 5 mM $CaCl_2$ and 15 mg/ml thermolysin (Liberase, medium research grade; Roche). All solutions were kept at 37°C. The livers were then excised, and hepatocytes were released by mechanical disruption of the liver capsule into 20 ml of Leibovitz L-15 medium (Invitrogen, Cergy-Pontoise, France) supplemented with 100 IU penicillin and 100 mg/ml streptomycin. The cells were filtered through a 70-μm nylon mesh and centrifuged at 800 rpm for 2 min. The supernatant and cell debris were aspirated, and the cell pellet was washed 2 times into 10 ml of the same medium. The resulting cell pellet was finally resuspended in William's medium E with Glutamax (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 100 IU penicillin, 100 mg/ml streptomycin, and 0.2 μM insulin. Hepatocyte viability was assessed by trypan blue exclusion. Hepatocytes were placed at 2.5×10^4 cells/cm^2 in collagen-coated 35-mm plates and cultured for up to 6 h in the resuspended medium at 37°C in 5% CO_2 chamber. After cell attachment, the medium was renewed without fetal calf serum and supplemented with 10 nM dexamethasone and 20 nM insulin for 12–16 h. Glucose production was measured 2 and 24 h after incubation of hepatocytes in glucose-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 16 mM lactate, 4 mM pyruvate, 10 nM dexamethasone, and 20 nM insulin. Glucose release in the medium was determined by the GOD-POD colorimetric method (Sclavo Diagnostics International, Sovicille, Italy) and normalized to the protein concentration determined by the Bradford method.

mRNA extraction and quantitative real-time PCR

Samples of frozen livers were homogenized in lysis buffer supplemented with β-mercaptoethanol, and total RNA was extracted (Absolutely RNA Miniprep Kit; Stratagene; Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. The RNA samples were further purified by precipitation with sodium acetate and isopropyl alcohol. For retroperitoneal WAT and rectus femoris muscle samples, homogenization was done with QIAzol Lysis Reagent (Qiagen, Courtaboeuf, France), and total RNA was extracted using an RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's protocol. RNA quality was evaluated with the Bioanalyzer 2100 (Agilent Technologies; RNA integrity number for all samples was >7). RNA quantity was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA; A_{260}/A_{280} and A_{260}/A_{230} values were >1.8). cDNAs were synthesized from 1 μg of total RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Courtaboeuf, France). Quantitative Real-time PCR was performed and analyzed using a 7300 Real-Time PCR System with 1× SYBR Green PCR Master Mix (Applied Biosystems), 0.9 μM primers (Invitrogen), and 1 μl of cDNA in a total volume of 20 μl. PCR conditions were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. PCR reactions were done in duplicate and negative controls (*i.e.*, no reverse transcription and no template controls) were added to the reactions. Relative expression levels were determined using the comparative ΔC_T method to normalize target gene mRNA to *36b4*. Primers were designed and optimized for an annealing temperature of 60°C. To assess primer specificity and product uniformity, sequences were tested with the Basic Local Alignment Search Tool (BLAST; U.S. National Center for Biotechnology Information; http://blast.ncbi.nlm.nih.gov/), and postamplification dissociation curves were determined. A dilution curve of the pool of all cDNA samples was used to calculate the amplification efficiency for each assay (values were between 1.85 and 2). Primer sequences are summarized in Supplemental Table S1.

Cell culture, transfection, and luciferase assay

COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc.; Invitrogen), 1% penicillin/streptomycin (Life Technologies) mix and sodium pyruvate in a humidified atmosphere with 5% CO_2 at 37°C. Cells were plated in 24-well plates and transfected with GeneJuice (Novagen; Merck, Darmstadt, Germany). Depending on the experiment, hRORα and hREV-ERBα expression vectors (7) were used at either 100 or 200 and 200 ng/well, respectively. The o*Bmal1-luc* and *mPer1-luc* reporter construct and m*Clock*/m*Bmal1* expression vectors have been used pre-

viously (30,31). The proximal promoter region of m*Lpl* (−143 to +186) was cloned in pGL3 basic (see Supplemental Data for further information). All reporter constructs and β-galactosidase reporter construct were used at 50 and 100 ng/well, respectively. Total transfected DNA amount was set to an equal amount between all conditions by addition of the corresponding empty vector. Luciferase assay was performed 48 h after transfection. Briefly, cells were rinsed twice in cold PBS and lysed for 15 min in lysis buffer (25 mM Tris, 2 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 1% TritonX-100). The luciferase assay was performed using a luciferase assay system kit (Promega, Charbonnieres, France) and a PolarStar Optima luminometer (BMG Labtech, Ortenberg, Germany). Results were normalized to β-galactosidase activity. Data [in relative luminescence units (RLU)] represent fold induction once normalized to β-galactosidase. Experiments were repeated 3 times, each condition in triplicate.

Statistical analysis

All values are presented as means ± SE. Area under the curve (AUC) was determined as incremental area (*i.e.*, above baseline; see ref. 27). The 48-h respiratory quotient (RQ) data were fitted to a cosinor function [$f = a + (b * \cos(2 * \pi * (x - c)/24))$], where x indicates the time, a the mean value, b the amplitude, and c the acrophase. Statistical difference between genotypes for a given parameter (*e.g.*, amplitude) was tested in an analysis of covariance. Normality and homogeneity of variance were assessed with Kolmogorov-Smirnov and Lilliefors test and Levene's test, respectively. Data that were non-normal and/or heteroscedastic were subjected to logarithmic transformation before analysis. Value of α was set at 0.05. Unpaired Student's *t* test was used to compare 2 groups. Luciferase assay data were analyzed using 1-way analyses of variance (ANOVA) followed by Tukey HSD *post hoc* analysis. Data collected successively at different time points (*e.g.*, locomotor activity) within each group were compared with ANOVA for repeated measures. Two-way ANOVA was performed to assess the effects of genotype and feeding condition and the interaction between these factors. Three-way ANOVA design was employed to test the aforementioned factors plus the effect of time. Simple main effects approach was conducted to examine each factor separately if the interaction term was significant. Statistical analyses were performed with Statistica 10 (StatSoft, Maisons-Alfort, France).

RESULTS

Rev-erbα$^{-/-}$ mice are slightly hyperglycemic without insulin resistance

We first evaluated whether *Rev-erbα* deficiency affects general physiology and behavior. No significant difference was found between *Rev-erbα*$^{-/-}$ mice and their wild-type (+/+) littermates regarding the amount and timing of chow intake (**Fig. 1*A***). Body mass was not significantly different between 4-mo-old *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice (24.02 ± 1.11 *vs.* 25.58 ± 1.20 g, respectively). The daily patterns of general locomotor activity and body temperature were very close in the two genotypes (Fig. 1*B*, *C*). In sharp contrast, 24-h blood glucose rhythm showed higher values in *Rev-erbα*$^{-/-}$ mice compared to *Rev-erbα*$^{+/+}$ mice (Fig. 1*D*), suggesting altered glucose homeostasis. Of note, mild hyperglycemia at the day-night transition has been previously observed in *Rev-erbα*$^{-/-}$ mice (20). To understand the origin of this hyperglycemia in *Rev-erbα*$^{-/-}$ mice, we determined the day-night levels of insulin, glucagon, and hepatic glycogen in both genotypes. Plasma insulin was increased at ZT0 (lights on, onset of the resting period; **Fig. 2*A***), while hepatic glycogen content was higher at ZT12 (lights off, onset of the active period; Fig. 2*B*). These higher insulin and glycogen levels in *Rev-erbα*$^{-/-}$ mice are consistent with previous results (20, 32). Plasma glucagon levels showed no difference between genotypes (Fig. 2*C*). According to standardized guidelines (27), we then compared the responses

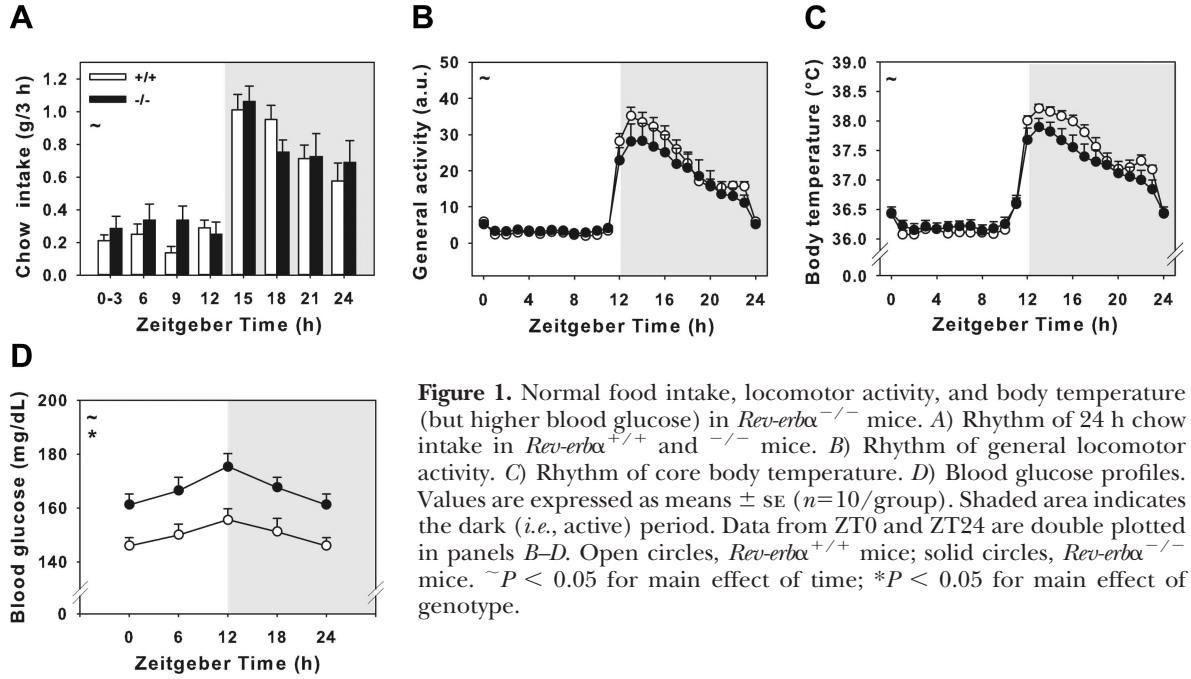

Figure 1. Normal food intake, locomotor activity, and body temperature (but higher blood glucose) in *Rev-erbα*$^{-/-}$ mice. *A*) Rhythm of 24 h chow intake in *Rev-erbα*$^{+/+}$ and $^{-/-}$ mice. *B*) Rhythm of general locomotor activity. *C*) Rhythm of core body temperature. *D*) Blood glucose profiles. Values are expressed as means ± SE (n=10/group). Shaded area indicates the dark (*i.e.*, active) period. Data from ZT0 and ZT24 are double plotted in panels *B–D*. Open circles, *Rev-erbα*$^{+/+}$ mice; solid circles, *Rev-erbα*$^{-/-}$ mice. $^{\sim}P < 0.05$ for main effect of time; $^{*}P < 0.05$ for main effect of genotype.

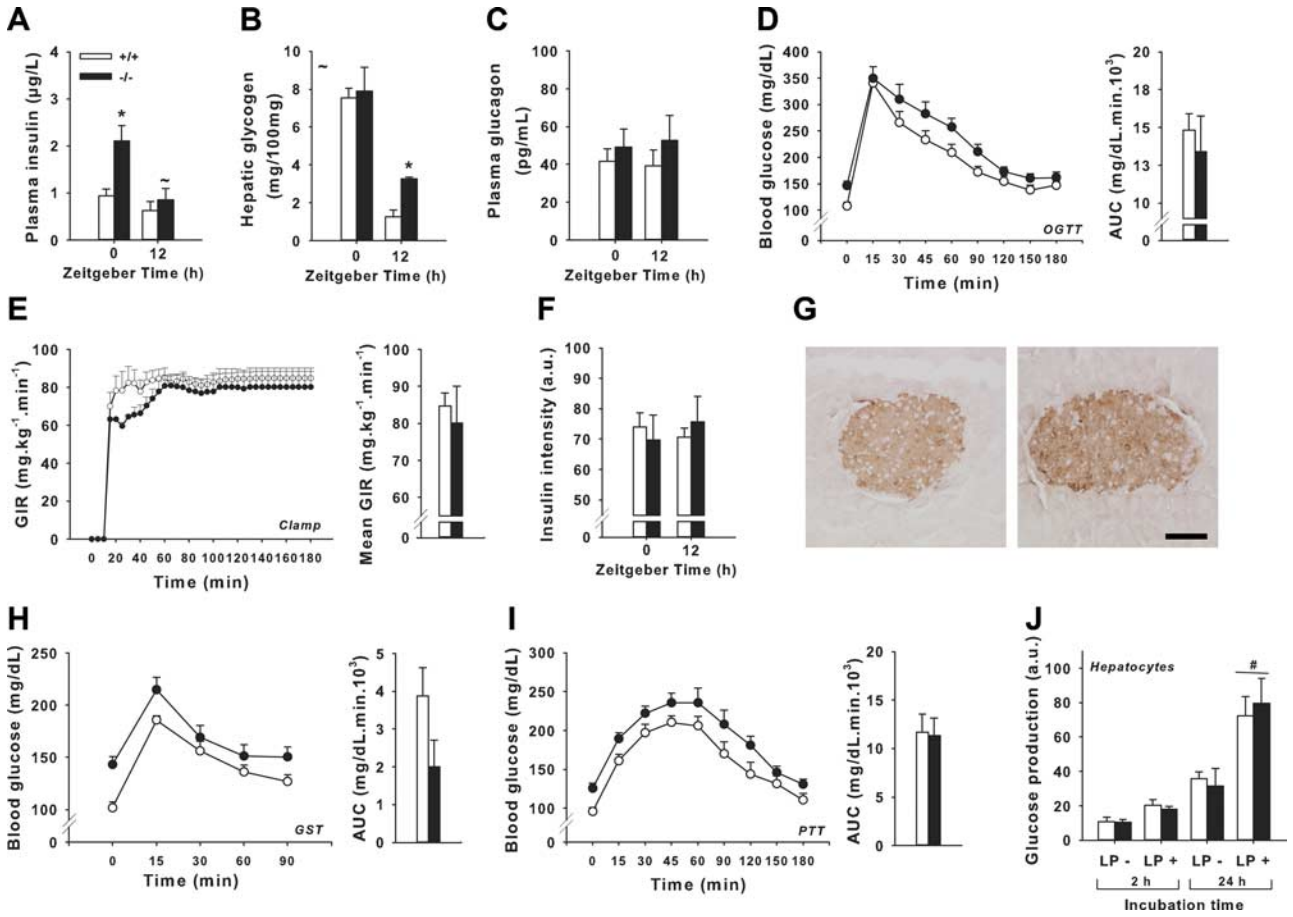

Figure 2. Lack of whole insulin resistance or increased gluconeogenesis in *Rev-erbα*$^{-/-}$ mice. *A–C*) Plasma insulin levels (*A*), hepatic glycogen concentration (*B*), and plasma glucagon levels (*C*); $n = 6$/group. *D*) OGTT in mice unfed overnight (*i.e.*, from ZT12 to ZT2) and the resulting incremental AUC (n=12/group). *E*) Hyperinsulinemic-euglycemic clamp and the resulting glucose infusion rates averaged over the last 60 min of the clamp (n=6/group). *F*) Insulin immunostaining from pancreas sections of +/+ and −/− animals and their respective quantification (n=6/group). *G*) Pancreas sections of a *Rev-erbα*$^{+/+}$ (left panel) and a *Rev-erbα*$^{-/-}$ mouse (right panel) stained with insulin antibody. Scale bar = 50 µm. *H, I*) GST (*H*) and PTT (*I*) in mice unfed overnight and the resulting AUCs (n=12/group). *J*) Glucose production in *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ primary hepatocytes incubated with (+) or without (−) lactate-pyruvate (LP) for 2 or 24 h (n=7/group). Data are means ± SE. Open circles, *Rev-erbα*$^{+/+}$ mice; solid circles, *Rev-erbα*$^{-/-}$ mice. $^{\sim}P < 0.05$ for main effect of time (top left corner) or *vs.* corresponding ZT0 group of same genotype (above bar); $^{*}P < 0.05$ *vs.* +/+ genotype at same ZT; $^{\#}P < 0.05$ *vs.* 24 h LP− treatment.

of both genotypes to metabolic challenges, by comparing their respective AUC values, calculated as incremental area (above baseline, to exclude the effect of differences in fasting glucose levels between animals). The OGTT (performed at the same ZTs as in ref. 13) showed no significant difference between genotypes either at ZT2 (Fig. 2*D*) or at ZT14 (Supplemental Fig. S1*C*). Insulin sensitivity, assessed by the intraperitoneal insulin sensitivity test (IPIST), was not significantly modified in *Rev-erbα*$^{-/-}$ mice compared to wild-type mice, either at ZT2 (despite a trend toward increased sensitivity; P=0.07 for AUC; Supplemental Fig. S1*B*), or at ZT14 (Supplemental Fig. S1*D*). When *Rev-erbα*$^{-/-}$ mice were challenged with an OGTT after overnight food withdrawal, their glucose-stimulated insulin secretion was also similar to that of control mice (Supplemental Fig. S1*A*). Similarly, a hyperinsulinemic-euglycemic clamp study performed from ZT6 in animals previously unfed for 6 h indicated that the glucose infusion rate, averaged over the last 60 min of the clamp, was similar between genotypes (Fig. 2*E*), ruling out any major alteration in whole-body insulin sensitivity. In addition, pancreatic islets from both genotypes were identical in area, circularity, and insulin content (Fig. 2*F, G* and Supplemental Fig. S2*A*). Altogether, these observations suggest that the high blood glucose seen in chow-fed *Rev-erbα*$^{-/-}$ mice is not the result of either insulin resistance or a defect in insulin secretion.

Gluconeogenesis is not enhanced in *Rev-erbα*$^{-/-}$ mice

The following experiments were conducted to determine whether the hyperglycemia in *Rev-erbα*$^{-/-}$ mice was of hepatic origin. For that purpose, we investigated glycogenolysis by challenging mice with a GST. *Rev-erbα*$^{-/-}$ mice did not show a larger hyperglycemic response compared to that in controls (Fig. 2*H*), excluding hypersensitivity to glucagon as a potential cause of hyperglycemia. We then challenged mice with a PTT at ZT2 to investigate a potentially greater contribution

of gluconeogenesis in *Rev-erbα*$^{-/-}$ mice. No significant difference was detected between the genotypes (Fig. 2*I*). Metformin, known to decrease hepatic glucose production by inhibiting gluconeogenesis and increasing insulin sensitivity, improved glucose tolerance to the same degree in both genotypes (data not shown). To confirm these *in vivo* results, we compared glucose production *in vitro* in primary hepatocytes from *Rev-erbα*$^{-/-}$ and control animals. Following 24 h incubation in the presence of lactate-pyruvate, *Rev-erbα*$^{-/-}$ hepatocytes did not produce more glucose than wild-type cells (Fig. 2*J*). We conclude that the chronic hyperglycemic phenotype of *Rev-erbα*$^{-/-}$ mice cannot be explained by increased gluconeogenesis. As REV-ERBα is a potential link between the circadian timing system and metabolism, we hypothesized that these mice may exhibit a circadian misalignment of energy utilization.

Daily balance in energy stores utilization is exacerbated in *Rev-erbα*$^{-/-}$ mice

To test whether the daily cycle of lipid and glucose utilization is modified in *Rev-erbα*$^{-/-}$ mice, we assessed the 24-h variations of energy metabolism *in vivo* by using indirect calorimetry. Basal metabolism, determined from the 5 lowest O_2 consumption values during the second 24-h cycle, was not significantly different between genotypes (Supplemental Fig. S3). The RQ (Vco_2/Vo_2) tracks the type of fuel oxidized. High (*i.e.*, close to 1) and low (*i.e.*, close to 0.7) RQ values indicate preferential utilization of carbohydrates and lipids, respectively. Of interest, *Rev-erbα*$^{-/-}$ mice showed altered daily variations in RQ values compared to control mice, with lower and higher values during the day and the night, respectively (**Fig. 3*A*, *B***). Cosinor fitting followed by analysis of covariance of 48 h RQ data revealed that the acrophase of RQ occurred 1.4 h later (*i.e.*, was phase delayed) in *Rev-erbα*$^{-/-}$ compared to control mice (ZT 17.4 ± 0.1 *vs.* 16.0 ± 0.2, respectively; $P < 0.001$). Furthermore, daily changes of RQ in *Rev-erbα*$^{-/-}$ mice displayed larger amplitude than in control littermates (0.11 ± 0.003 *vs.* 0.06 ± 0.003, respectively; $P < 0.001$), whereas the daily mean RQ, which reflects energy homeostasis, did not differ between genotypes (+/+ *vs.* −/−: 0.85 ± 0.002 *vs.* 0.85 ± 0.002, respectively).

These data demonstrate that *Rev-erbα*$^{-/-}$ mice have increased fatty acid utilization during the resting period compared to littermate controls, while their glucose utilization (*i.e.*, glucose supply to active organs and/or conversion of dietary carbohydrates to fat) is delayed and increased during the active period.

Increased utilization of lipid fuels in unfed *Rev-erbα*$^{-/-}$ mice

To test a preferential use of fatty acids over glucose in *Rev-erbα*$^{-/-}$ mice, we analyzed the physiological and hepatic molecular responses of both genotypes after 24 h of food withdrawal (*i.e.*, to trigger mobilization of energy stores) and 24 h of food withdrawal followed by 24 h refeeding with high-carbohydrate diet (*i.e.*, to trigger lipogenesis). Changes in body mass associated with food withdrawal and refeeding did not differ significantly between the genotypes (**Fig. 4*A***). Compared to controls, *Rev-erbα*$^{-/-}$ mice exhibited a significantly lower hypothermia response to 24 h of food deprivation, resulting in higher body temperature at the end of the nonfeeding period (Fig. 4*B*). This lack of hypothermia was not due to changes in the level of locomotor activity (Supplemental Fig. S4*A*). In addition, fasting blood glucose levels were still significantly higher in *Rev-erbα*$^{-/-}$ mice compared to *Rev-erbα*$^{+/+}$ animals (Fig. 4*C*). This confirms the mild hyperglycemia after overnight food withdrawal before metabolic tests. However, there was no difference between the genotypes in the refeeding condition (Fig. 4*C*). The absence of hyperglycemia in refed *Rev-erbα*$^{-/-}$ mice was not linked to differential changes in insulin levels (Fig. 4*D*) or food intake (+/+ *vs.* −/−: 6.09 ± 0.16 *vs.* 6.33 ± 0.17 g, respectively). One possible explanation is that previously unfed *Rev-erbα*$^{+/+}$ mice are more sensitive than *Rev-erbα*$^{-/-}$ animals to the hyperglycemic effect of high-carbohydrate diet. In other words, *Rev-erbα*$^{-/-}$ mice may be more efficient to process high-carbohydrate intake for fatty acid synthesis.

While most metabolic parameters studied did not differ between the two genotypes in the refed condition (Fig. 4*D–I*), many of them were differentially affected by 24 h of food withdrawal in *Rev-erbα*$^{+/+}$ *vs.* $^{-/-}$ animals. In particular, hepatic glycogen was significantly more elevated in *Rev-erbα*$^{-/-}$ mice (Fig. 4*E*), indicating that blood glucose is not used as a primary source of energy in these mice. Interestingly, plasma

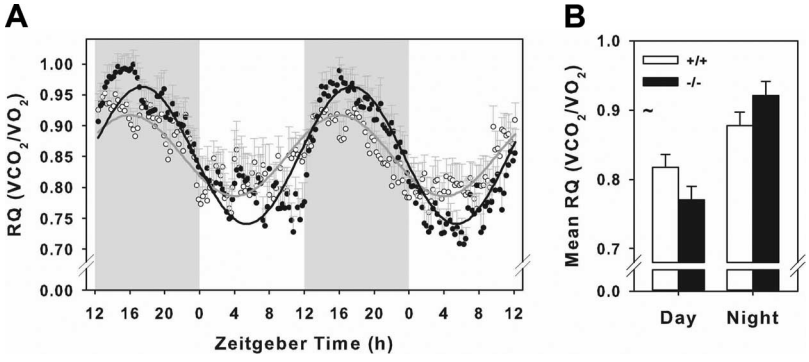

Figure 3. Changes in daily fuel utilization in *Rev-erbα*$^{-/-}$ mice. *A*) RQ (48 h; *i.e.*, Vco_2/Vo_2 values) of *Rev-erbα*$^{+/+}$ and $^{-/-}$ mice, determined by indirect calorimetry. Shaded and solid traces show fitting of the 48-h RQ data of *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice, respectively, to the cosine function. Note that data were collected every 15 min. Shaded area indicates the dark (*i.e.*, active/feeding) period. Open circles, *Rev-erbα*$^{+/+}$ mice; solid circles, *Rev-erbα*$^{-/-}$ mice. *B*) Day and night averages of RQ. Values are expressed as means ± SE (n=10/group). $^{\sim}P < 0.05$ for main effect of time.

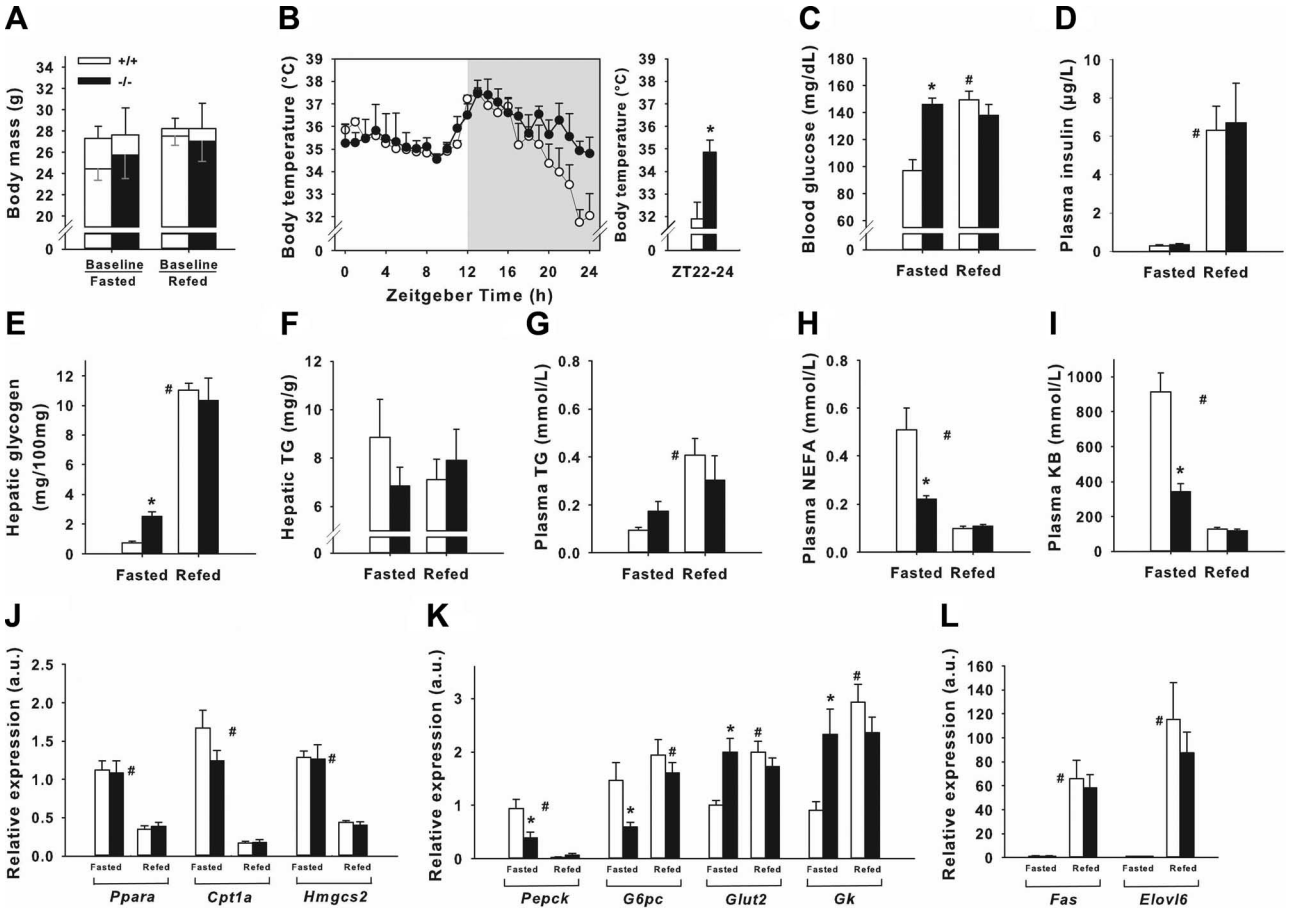

Figure 4. Shift from glucose to fatty acid utilization in unfed *Rev-erbα*$^{-/-}$ mice. *A*) Body mass changes determined before (left, upper values) and after a 24 h fast (left, lower values), and before (right, upper values) and after 24 h of food withdrawal + 24 h refeeding (right, lower values) in *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice. *B*) Body temperature during 24 h of food withdrawal and the adjacent ZT22-24 average. Open circles, *Rev-erbα*$^{+/+}$ mice; solid circles, *Rev-erbα*$^{-/-}$ mice. *C–I*) Blood glucose (*C*), plasma insulin (*D*), hepatic glycogen (*E*), hepatic triglyceride (TG; *F*), plasma TG (*G*), plasma NEFA (*H*), and plasma ketone body (KB; *I*) levels in unfed (fasted) and refed animals. *J–L*) mRNA expression levels of ketogenic genes (*J*), glucose metabolism genes (*K*), and lipogenic genes (*L*). Animals were sampled at ZT0. Values are expressed as means ± SE (n=7/group). See Supplemental Table S1 for gene names. $^{\#}P < 0.05$ for main effect of feeding condition (midpanel) or *vs.* corresponding unfed (fasted) group of same genotype (above bar); $^{*}P < 0.05$ *vs.* +/+ genotype of same feeding condition.

NEFA and ketone body levels were significantly decreased in *Rev-erbα*$^{-/-}$ mice compared to control mice (Fig. 4*H, I*). This suggests that fatty acids are used as the main source of energy during 24 h of food withdrawal in *Rev-erbα*-deficient animals.

To substantiate this possibility, we analyzed the mRNA expression of *peroxisome proliferator-activated receptor* α (*Pparα*) and *Pparα*-target genes known to be involved in ketogenesis: *carnitine palmitoyltransferase 1a* (*Cpt1a*) and *3-hydroxy-3-methylglutaryl-CoA synthase 2* (*Hmgcs2*). Food withdrawal similarly enhanced the mRNA levels of *Pparα*, *Cpt1a*, and *Hmgcs2* in both genotypes (Fig. 4*J*), ruling out an abnormal ketogenesis in *Rev-erbα*$^{-/-}$ mice, which could have explained the reduction of their ketone body levels. In addition, the expression of *phosphoenolpyruvate carboxykinase 2* (*Pepck*) and *glucose-6-phosphatase* (*G6pc*), two key actors of gluconeogenesis, was reduced in unfed *Rev-erbα*$^{-/-}$ mice (Fig. 4*K*). Furthermore, the mRNA levels of *glucose transporter 2* (*Glut2*) and *glucokinase* (*Gk*), involved in glucose transport and both glycogen synthesis and glycolysis, respectively, were higher after food withdrawal in *Rev-erbα*$^{-/-}$ mice than in wild-type mice (Fig. 4*K*). In contrast, loss of *Rev-erbα* did not block the refeeding-induced increase of lipogenic genes (Fig. 4*L* and Supplemental Fig. S4*C*). The mRNA levels of other regulators of glucose and lipid metabolism were similar between the genotypes (Supplemental Fig. S4*B, C*). To summarize, unfed *Rev-erbα*$^{-/-}$ mice maintain relative hyperglycemia, concomitant with available glycogen stores and reduced gluconeogenesis. In agreement with these findings, the lower RQ values in chow-fed *Rev-erbα*$^{-/-}$ mice during daytime demonstrate that *Rev-erbα* deletion *in vivo* leads to a greater mobilization and oxidation of fatty acids as well as a greater ketolysis during the resting period.

Rev-erbα$^{-/-}$ mice are more prone to HFD-induced obesity

The experiments reported above indicate that the absence of *Rev-erbα* leads to a shift from glucose to fatty

acid utilization for energy supply, at least during the resting period and 24 h of food withdrawal. During nighttime (*i.e.*, period of dietary carbohydrate intake), however, mild hyperglycemia, normal insulin sensitivity, and high RQ values in chow-fed *Rev-erbα*$^{-/-}$ mice indicate an active absorption of carbohydrates. We wondered whether *de novo* lipogenesis from dietary carbohydrates could be increased at night in *Rev-erbα*$^{-/-}$ mice. We first assessed day-night parameters related to lipid metabolism in 4-wk-old *Rev-erbα*$^{+/+}$ and $^{-/-}$ mice fed chow or HFD for up to 12 wk. *Rev-erbα*$^{-/-}$ mice fed HFD gained much more body mass than HFD-fed control animals (**Fig. 5*A***). This cannot be attributed to biased (*i.e.*, different) values of baseline body mass, because they were similar between the genotypes at 4 wk of age. Moreover, all groups had a similar body size at the end of the experiments (data not shown). Mean calorie intake did not significantly differ between the genotypes, despite a trend for increased calorie consumption in the *Rev-erbα*$^{-/-}$ group (P=0.08; Supplemental Fig. S5*A*). *Rev-erbα*$^{-/-}$ mice had a significantly higher body mass index, especially in the HFD group (Supplemental Fig. S5*B*). In accordance with this observation, the amount of WAT (*i.e.*, perigonadal and retroperitoneal) was also significantly greater in *Rev-erbα*$^{-/-}$ mice compared to controls, regardless of the feeding conditions (Fig. 5*B*, *C*). As expected (33), HFD-fed control animals had significantly higher body mass and adiposity than chow-fed control animals, and in addition, they reached a similar adiposity as in chow-fed *Rev-erbα*$^{-/-}$ mice (Fig. 5*B*, *C*).

When fed HFD, both genotypes expressed an attenuated diurnal feeding rhythm (data not shown) and showed significant hyperleptinemia (Fig. 5*D*), increased hepatic triglycerides (Fig. 5*F*), decreased plasma triglycerides (Fig. 5*G*), increased plasma LDL and plasma NEFAs (Fig. 5*H*, *I*), hypercholesterolemia (Supplemental Fig. S5*D*) and hyperinsulinemia (Supplemental Fig. S5*G*), compared to their chow-fed controls. Interestingly, irrespective of the feeding conditions, *Rev-erbα*$^{-/-}$ mice had significantly increased plasma leptin, glucose, NEFA, LDL, ketone body, cholesterol, insulin, and adiponectin levels compared to *Rev-erbα*$^{+/+}$ mice (Fig. 5*D*, *E*, *H–J* and Supplemental Fig. S5*D*, *G*, *H*). Incidentally, HFD-induced hyperglycemia was only detected with blood sampling by tail incision (Supplemental Fig. S5*K*, *L*). The high levels of adiponectin and leptin in chow- and HFD-fed *Rev-erbα*$^{-/-}$ mice could have prevented the development of insulin resistance (34). Corticosterone levels, important for carbohydrate metabolism, were not significantly enhanced in chow-fed *Rev-erb*$^{-/-}$ mice (Supplemental Fig. S5*I*). Hepatic triglycerides in chow-fed *Rev-erbα*$^{-/-}$ mice were found to be increased at ZT10 (20) and reduced at ZT12 (25). However, we noticed a trend toward elevated hepatic triglycerides at ZT12 in *Rev-erbα*$^{-/-}$ mice, regardless of the feeding conditions (P=0.06; Fig. 5*F*). Of interest, plasma glycerol in *Rev-erbα*$^{-/-}$ mice was enhanced at ZT12 in chow-fed conditions and at both time points in HFD-fed conditions (Supplemental Fig. S5*F*), which may indicate enhanced hydrolysis of triglycerides. Taken together, our data indicate that *Rev-erbα* deletion leads to a fat phenotype in chow-fed conditions, which is severely amplified in HFD-fed conditions.

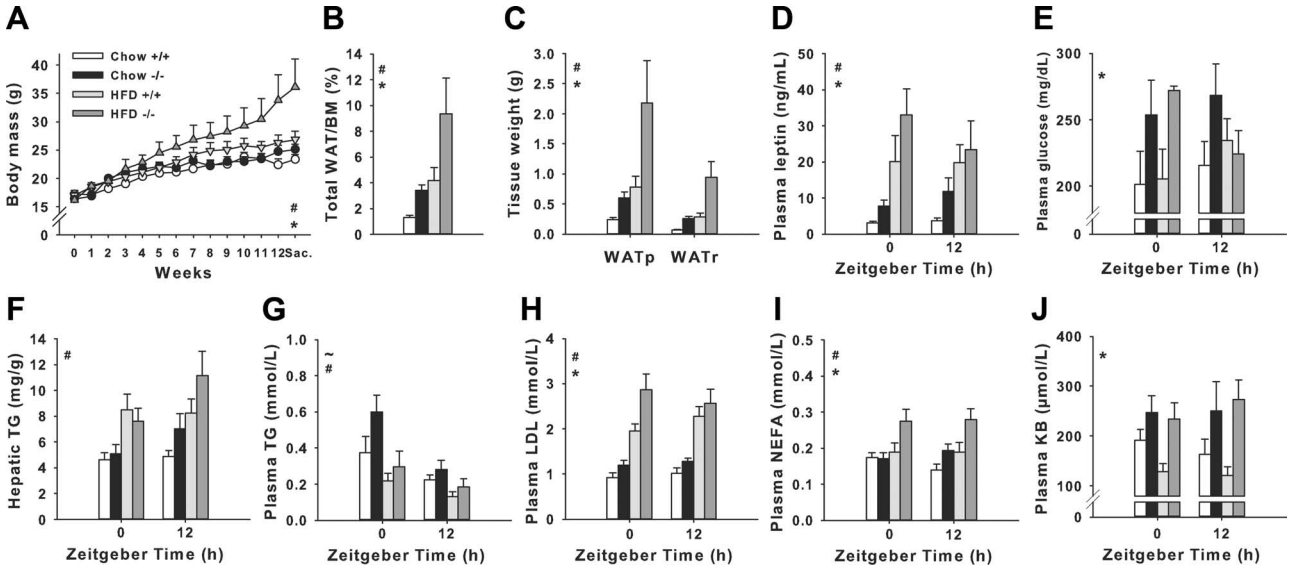

Figure 5. *Rev-erbα*$^{-/-}$ mice exhibit a fat phenotype amplified on prolonged high-fat feeding. *A*) Body mass change over time in chow-fed and HFD-fed +/+ and −/− animals (n=12/group). Sac., sacrifice. Note that statistical analysis was performed on the last week of feeding. Open circles, chow-fed *Rev-erbα*$^{+/+}$ mice; solid circles, chow-fed *Rev-erbα*$^{-/-}$ mice; light shaded triangles, HFD-fed *Rev-erbα*$^{+/+}$ mice; dark shaded triangles, HFD-fed *Rev-erbα*$^{-/-}$ mice. *B*) Total adiposity, expressed as a percentage of body mass (n=12/group). *C*) Amount of perigonadal and retroperitoneal WAT (WATp and WATr, respectively; n=12/group). *D*, *E*) Plasma leptin (*D*) and glucose (*E*) levels (n=6/group). *F*, *G*) Hepatic triglyceride (TG; *F*) and plasma TG (*G*) levels (n=6/group). *H–J*) Plasma LDL (*H*), NEFA (*I*), and ketone body (KB; *J*) levels (n=6/group). Values are expressed as means ± SE. $^{\sim}P < 0.05$ for main effect of time; $^{*}P < 0.05$ for main effect of genotype; $^{\#}P < 0.05$ for main effect of feeding condition.

Expression of metabolic genes is disrupted in chow- and HFD-fed *Rev-erbα*$^{-/-}$ mice

To gain further insight into the role of REV-ERBα as transcriptional repressor for the daily control of energy balance, we examined the day-night levels of glucose and lipid-related genes in the liver, WAT, and skeletal muscle of chow- and HFD-fed *Rev-erbα*$^{+/+}$ and $^{-/-}$ mice. Of note, day-night expression patterns of most metabolic genes studied in *Rev-erbα*$^{+/+}$ mice were in accordance with expression profiling from transcriptome data (10,35). We first determined whether the defective energy homeostasis in *Rev-erbα*$^{-/-}$ mice was the consequence of altered hepatic gene expression. Notably, hepatic *glycogen synthase 2* (*Gys2*) and *Glut2* expression was significantly diminished at ZT12 in *Rev-erbα*$^{-/-}$ mice in both feeding conditions (**Fig. 6*A***), while no significant differences were found for *Gk*, *Pepck*, and *G6pc* (Supplemental Fig. S6*A*), in keeping with a previous report (20). Hepatic expression of lipogenic genes [*e.g.*, *sterol regulatory element binding transcription factor 1* (*Srebp1c*) and *fatty acid synthase* (*Fas*)] showed decreased levels at ZT12 in chow-fed *Rev-erbα*$^{-/-}$ mice (Fig. 6*A*). However, while HFD did not markedly change the expression of *Srebp1c*, the hepatic mRNA levels of *Fas, acetyl-coenzyme A carboxylase* (*Acc*), and *ELOVL fatty acid elongase 6* (*Elovl6*) in the *Rev-erbα*$^{-/-}$ group were ~2-fold higher than in control animals at both time points (Fig. 6*A*). Similarly, we found that the fatty acid transporter *cluster of differentiation 36* (*Cd36*) was significantly induced by HFD exclusively in *Rev-erbα*$^{-/-}$ animals (Fig. 6*A*). Of special interest, *Lpl* mRNA, which encodes the rate-limiting enzyme in triglyceride hydrolysis (36), was significantly increased at ZT12 in the liver of *Rev-erbα*$^{-/-}$ mice

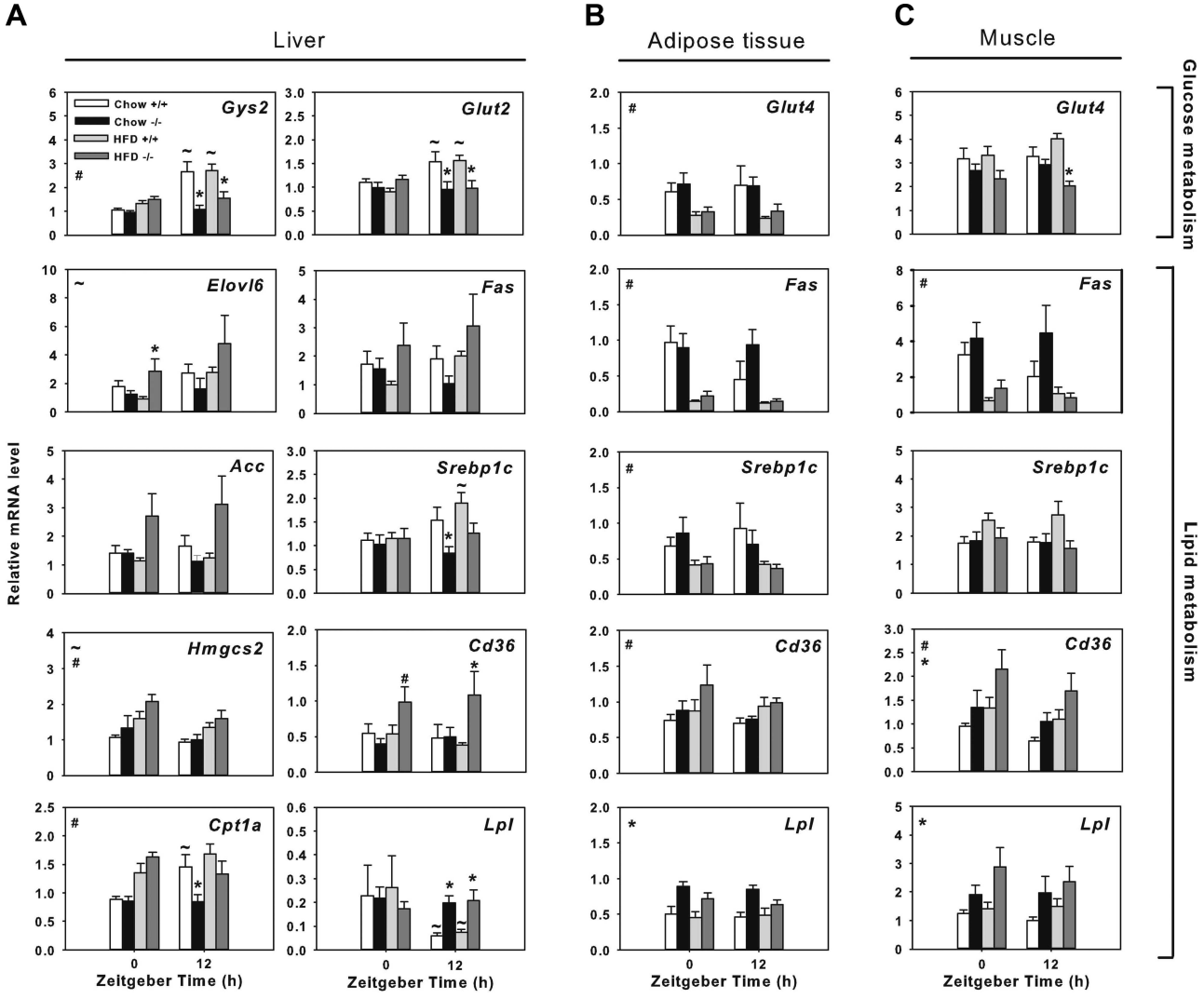

Figure 6. Lipid metabolism gene expression is modified in the absence of *Rev-erbα*. mRNA expression of glucose and lipid metabolism genes in liver (*A*), retroperitoneal WAT (*B*), and rectus femoris skeletal muscle (*C*). Data are means ± SE (n=6/group). See Supplemental Table S1 for gene names. $^{\sim}P < 0.05$ for main effect of time (top left corner) or *vs.* corresponding ZT0 group of same genotype (above bar); $^{*}P < 0.05$) for main effect of genotype (top left corner) or *vs.* corresponding +/+ genotype group at same ZT (above bar); $^{\#}P < 0.05$ for main effect of feeding condition (top left corner) or *vs.* corresponding chow-fed group at same ZT (above bar).

compared to controls (Fig. 6*A*). Taken together, the elevated expression of these metabolic actors may contribute to obesity.

To further specify the obesity phenotype of *Rev-erbα*$^{-/-}$ mice, we analyzed the mRNA levels of metabolic actors in WAT and skeletal muscle. *Glut4*, *Fas*, *Acc*, and *Srebp1c* were significantly down-regulated by HFD in both genotypes (Fig. 6*B* and Supplemental Fig. S6*B*). Incidentally, this HFD-dependent regulation of the lipogenic genes *Acc* and *Fas* has also been observed in obese humans and rodents (37). While there was no difference in *Cd36* expression between the two genotypes, the deletion of *Rev-erbα* also increased *Lpl* mRNA expression in WAT (Fig. 6*B*). This suggests that in *Rev-erbα*$^{-/-}$ mice, WAT *Lpl* enhanced uptake of NEFA for fat storage, thus contributing to fat overload. In skeletal muscle, an overall tendency to down-regulate *Glut4* was seen in *Rev-erbα*$^{-/-}$ mice, which only reached statistical significance in HFD-fed *Rev-erbα*$^{-/-}$ animals at ZT12 (Fig. 6*C*), when massive body fat overload started to impede physical activity (data not shown). Expression of *Fas* and *Acc* in the muscle was also down-regulated in response to HFD in both genotypes, whereas the expression of *Cd36* was increased by HFD in both genotypes (Fig. 6*C*, Supplemental S6*C*). Of note, *Cd36* and *Lpl* expression was significantly increased in *Rev-erbα*$^{-/-}$ mice, irrespective of the feeding conditions (Fig. 6*C*). This further supports our hypothesis of an enhanced utilization of fatty acids as energy substrate for the muscle.

Altogether, these results clearly indicate that *Rev-erbα*$^{-/-}$ mice display a defective molecular control of energy homeostasis which contributes to HFD-induced obesity. Both liver and skeletal muscle appear to play a role in the production and preferential use of fatty acids at the expense of peripheral utilization of glucose, thus leading to a chronic mild hyperglycemia.

CLOCK drives transcriptional activation of *Lpl*

The overexpression of *Lpl* mRNA that we found in 3 peripheral tissues of *Rev-erbα*$^{-/-}$ mice led us to test whether this gene could be clock controlled. Since several putative RORE motifs were found in the proximal promoter region of *Lpl* (see Supplemental Data), we first decided to check whether the latter would be responsive to REV-ERBα and RORα. Neither RORα nor REV-ERBα had any effect toward this reporter construct (**Fig. 7*A***). In contrast, RORα consistently activated *Bmal1-luc*, an effect that could be partially overcome by the addition of REV-ERBα (Fig. 7*B*).

However, the transcriptions of *Rev-erbα* and *Clock/Bmal1* are interlocked: The heterodimer CLOCK/BMAL1 drives *Rev-erbα* transcription (38), and REV-ERBα inhibits both *Bmal1* and *Clock* transcriptions, as previously mentioned. This explains why the mRNA levels of both *Bmal1* and *Clock* are higher in the *Rev-erbα*$^{-/-}$ mice than in their wild-type littermates (Supplemental Fig. S7). Therefore, it seems plausible that at least a subset of CLOCK/BMAL1 targets will

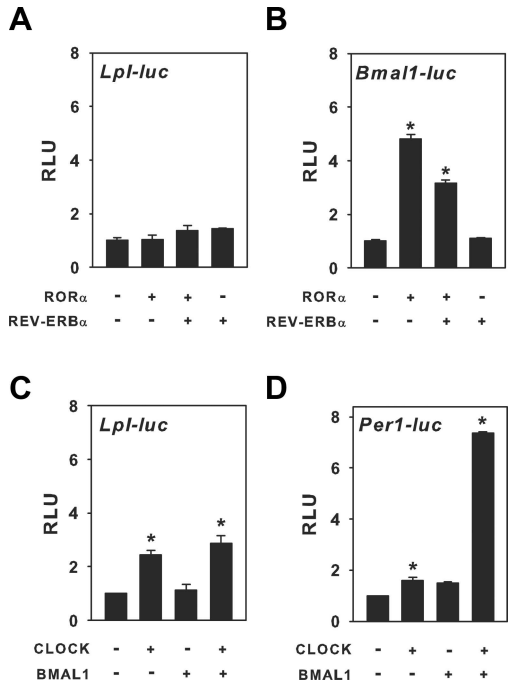

Figure 7. Transactivation by CLOCK of the *Lpl* promoter. *A*, *B*) Effects of RORα and REV-ERBα on the transcription of *Lpl-luc* (*A*) and *Bmal1-luc* (*B*) promoter reporter constructs. *C*, *D*) Effects of CLOCK and BMAL1 on the transcription of *Lpl-luc* (*C*) and *Per1-luc* (*D*) promoter reporter constructs. Luciferase assay was performed on COS-7 cells as indicated in Materials and Methods. Data are means ± SE of a representative experiment performed in triplicate wells. RLU, relative luminescence units. $*P < 0.05$ *vs.* control (empty vector).

display enhanced expression levels in these *Rev-erbα*$^{-/-}$ mice. We thus tested the potential implication of a putative E-box, which is located in the most proximal promoter region of m*Lpl*. This reporter construct significantly responded to CLOCK/BMAL1, although the amplitude of the response was not different from that elicited by CLOCK alone (Fig. 7*C*). These results demonstrate that the circadian clockwork is somehow involved in the transcriptional activation of the *Lpl* gene.

DISCUSSION

Energy metabolism can be affected by circadian disturbance, as evidenced by epidemiologic studies in shift workers and genetic disruption of clock components in mice (1, 11). REV-ERBα is a transcription factor involved both in the molecular clockwork and in several metabolic pathways. Therefore, dissecting REV-ERBα contribution to the daily balance between glucose and lipid metabolism is an important step toward understanding the functional crosstalk between the circadian and metabolic systems. Here we show that the absence of REV-ERBα *in vivo* leads to a preferential use of fatty acids as energy substrate during daytime, in association with non-insulin-dependent hyperglycemia.

During the daily resting period, liver and muscle derive most of their energy from fatty acids released

from WAT. Chow-fed *Rev-erbα*$^{-/-}$ mice rely more on lipid fuels during the inactive period than do control mice, as evidenced by low (*i.e.*, close to 0.7) RQ values. Accordingly, hepatic glycogen is less mobilized at the end of the resting period, while the hyperglycemia persists. A defect of glucose entry due to tissue-specific impaired insulin signaling would have explained the increased fatty acid utilization in *Rev-erbα*$^{-/-}$ mice. Deficiency in *Glut4* expression has been associated with insulin resistance (39). However, chow-fed *Rev-erbα*$^{-/-}$ mice have normal whole-body insulin sensitivity without obvious changes in *Glut4* mRNA levels in peripheral tissues.

Another explanation for the increased fatty acid utilization stems from the implication of REV-ERBα in the regulation of myosin heavy chain (MyHc) isoform expression in the skeletal muscle, based in particular on a significant fast-to-slow MyHC isoform transformation in skeletal muscle of *Rev-erbα*$^{-/-}$ mice (40). Thus, the preponderance of this slow myosin may have increased aerobic lipid metabolism, sparing glucose use in the muscles of *Rev-erbα*-deficient mice. In agreement with this hypothesis, the membrane protein *Cd36* involved in the binding and transport of fatty acids (36) is up-regulated in *Rev-erbα*$^{-/-}$ mice in the rectus femoris muscle, which has both oxidative and glycolytic capabilities (41). Muscle-specific overexpression of *Cd36* in mice promotes clearance of circulating fatty acids and increases plasma glucose and insulin (42). Furthermore, *Lpl* mRNA in the muscle of *Rev-erbα*-deficient animals is up-regulated both in the morning and the evening. Of note, LPL activity is higher in muscles composed predominantly of high-oxidative slow-twitch fibers (43) and is inversely phased to daily variation of RQ (44). Moreover, *Rev-erbα*$^{-/-}$ mice are resistant to cold-induced hypothermia (Supplemental Fig. S8), as are transgenic mice that overexpress *Lpl* in skeletal muscles (45). This improved cold tolerance could be linked to changes in muscle physiology (*i.e.*, enhanced oxidative capacity) due to LPL overexpression (45). Taken together, these findings show that increased *Cd36* and *Lpl* expression reflects increased muscular uptake of fatty acids in *Rev-erbα*$^{-/-}$ mice and can contribute not only to hyperglycemia but also to the absence of food withdrawal-induced drops in blood glucose and body temperature.

The physiological responses to acute food withdrawal include depletion of glycogen stores, hepatic gluconeogenesis, and mobilization of triglyceride in adipose tissues, which together provide energy supply. After 24 h of food withdrawal, glycogen depletion in *Rev-erbα*$^{-/-}$ mice remains incomplete and the hepatic expression of gluconeogenic genes much less increased in their liver. Strikingly, blood glucose levels in unfed *Rev-erbα*$^{-/-}$ mice are not decreased as in control animals, but instead show similar values to those of fed *Rev-erbα*$^{-/-}$ mice. In sharp contrast, plasma NEFA and ketone bodies levels in unfed *Rev-erbα*$^{-/-}$ mice are lower than in unfed control mice, reflecting acute utilization of fatty acids. This conclusion is substantiated by the facts that plasma NEFA and ketone body levels are either similar or greater in chow-fed *Rev-erbα*$^{-/-}$ mice compared to their controls, and that ketogenesis at the molecular level is induced by food withdrawal. Thus, it is unlikely that reduced NEFA and ketone bodies levels are due to decreased lipolysis and ketogenesis. Hence, the relative hyperglycemia and normothermia in starved *Rev-erbα*$^{-/-}$ mice result from a combination of lower activation of gluconeogenesis, diminished glucose utilization, and increased production and utilization of lipid fuels.

During the daily activity period, feeding allows the restoration of energy stores depleted over the previous daytime food withdrawal. Nocturnal RQ values in *Rev-erbα*$^{-/-}$ mice are higher than in control mice, suggesting a greater utilization of glucose. This feature, however, cannot be explained by increased *Glut4* mRNA levels, fast glycolytic fibers (40), or physical activity. Alternatively, higher nocturnal RQ values in *Rev-erbα*$^{-/-}$ mice can result from increased *de novo* lipogenesis, leading to enhanced production of lipids. Indeed, when fatty acids are synthesized from glucose, for instance during the absorption of dietary carbohydrates, the average RQ remains close to or below 1 (46). *Rev-erbα*$^{-/-}$ mice exhibit increased adiposity even when fed chow. Of note, body fat can derive from dietary fat and *de novo* lipogenesis, mainly from carbohydrates (46). *Fas* mRNA expression shows a 2-fold increase at ZT12 in the muscle and WAT of *Rev-erbα*$^{-/-}$ mice, indicating elevated fat synthesis in these tissues. However, no increased expression of lipogenic genes has been detected in the liver of chow-fed *Rev-erbα*$^{-/-}$ mice. This is explained by our sampling times, because expression of *Srebp1c*, *Fas*, and *Elovl6* is phase shifted in the liver of *Rev-erbα*-deficient mice (20), suggesting a delay in the occurrence of fat synthesis. This was confirmed by Feng *et al.* (25), who demonstrated that REV-ERBα controls the expression of lipid metabolism genes by recruiting the repressive chromatin modifier histone deacetylase 3, ensuring a temporal control of lipogenesis. In line with this study, we also show a trend toward elevated hepatic triglycerides, an important source of lipids for very low density lipoprotein (VLDL) assembly at the end of the day. Hepatic *de novo* lipogenesis contributes to VLDL production, the latter being increased in *Rev-erbα*-deficient mice (47). In addition, our calorimetry data indicating a possible nocturnal elevation of fat synthesis from dietary glucose in chow-fed *Rev-erbα*$^{-/-}$ mice are in accordance with an increased *de novo* lipogenesis found in these mice following the administration of deuterated water (25). The improved ability to convert dietary glucose to fat in the absence of *Rev-erbα* may explained the diminished differences in blood glucose levels between both genotypes fed high-carbohydrate diet or HFD.

In addition, our study demonstrates that the altered circadian control of lipid homeostasis due to the absence of REV-ERBα *in vivo* facilitates HFD-induced obesity. The metabolic phenotype in *Rev-erbα*$^{-/-}$ mice is robust, as increased adiposity occurs even on chow

diet without significant hyperphagia. Interestingly, an opposite phenotype (*i.e.*, resistance to HFD) has been described in *Rora*-deficient mice (48). While *Rev-erbα*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice challenged with HFD become overweight and develop hyperleptinemia, hyperlipidemia, hypercholesterolemia, hyperinsulinemia, and fatty liver, these metabolic responses are all significantly greater in *Rev-erbα*$^{-/-}$ mice. Besides, high-fat-fed *Rev-erbα*$^{-/-}$ mice display increased ketonemia, which is likely a response to an overload of the citric acid cycle, blood glucose being converted to acetyl-CoA in parallel to the production of acetyl-CoA from fat breakdown. This indicates that the enhancement of the fat oxidation capacity of the skeletal muscle of *Rev-erbα*$^{-/-}$ mice is not sufficient to cope with the adverse effects of prolonged HFD feeding. In addition, hepatic expression of *Acc*, *Fas*, and *Elovl6* increases only in *Rev-erbα*$^{-/-}$ mice at both time points on HFD, in keeping with increased *de novo* lipogenesis.

The overexpression of *Lpl* observed in *Rev-erbα*$^{-/-}$ mice is not restricted to the skeletal muscle. Indeed, expression of *Lpl*, which is barely detectable in the adult liver (49), is significantly up-regulated at ZT12 in the liver of *Rev-erbα*$^{-/-}$ mice. This is in line with the 24-h up-regulation of the *Lpl* transcript found in these mice by Le Martelot *et al.* (20). Of note, hepatic *endothelial lipase* mRNA levels are decreased in the liver (20) while *hepatic lipase* mRNA expression is similar in *Rev-erbα*$^{-/-}$ mice (Supplemental Fig. S6). Interestingly, the metabolic phenotype of chow-fed *Rev-erbα*$^{-/-}$ animals is quite similar to that of liver-only *Lpl*-expressing mice, which show increased liver triglyceride content and ketone body levels (50, 51). However, *Rev-erbα*$^{-/-}$ mice do not exhibit insulin resistance like mice overexpressing *Lpl* in liver or skeletal muscle (51). Of importance, conflicting results have been reported regarding insulin resistance induced by *Lpl* overexpression (52). In addition, our results show that the overexpression of

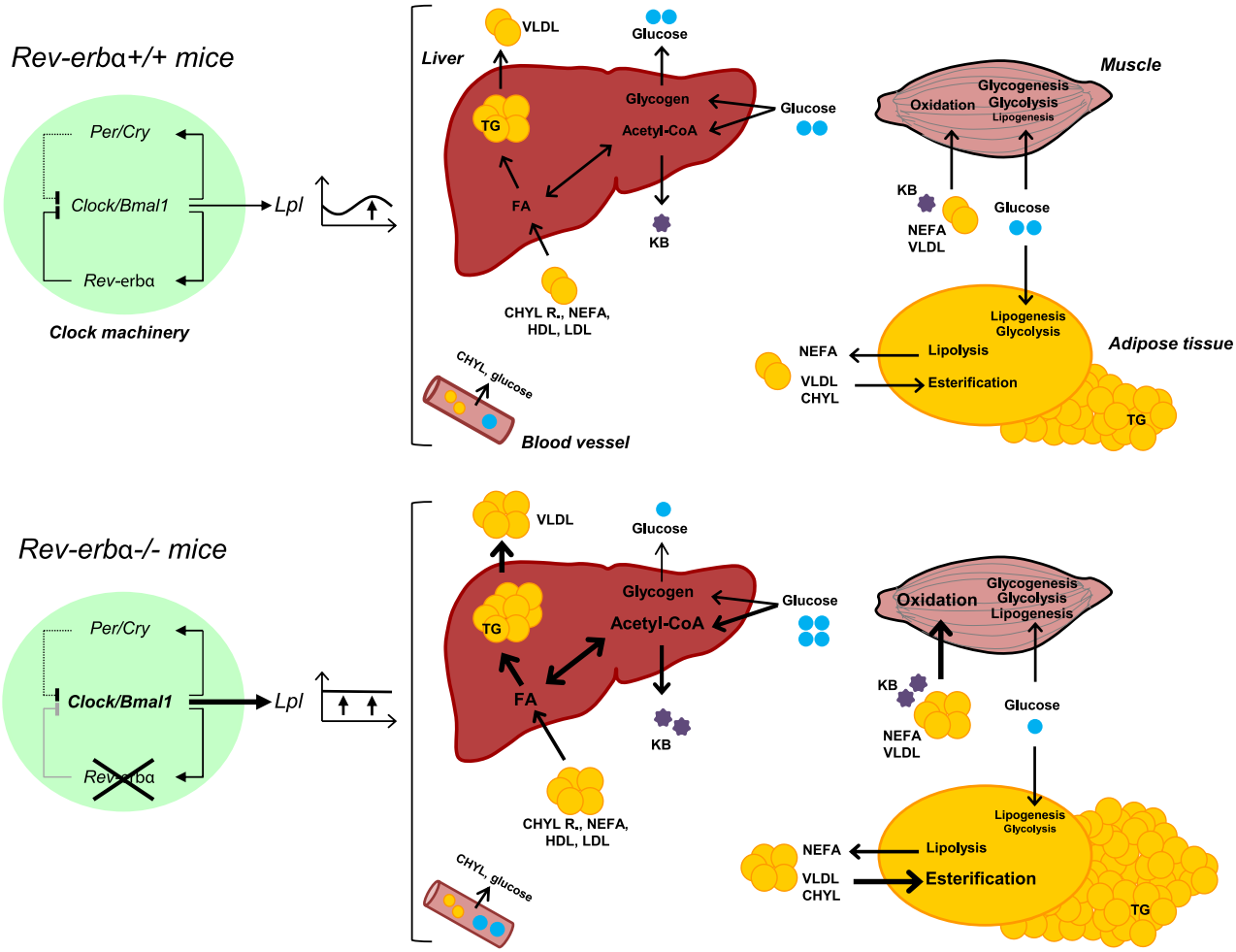

Figure 8. Hypothetical model illustrating the consequence of altered *Lpl* expression in *Rev-erbα*$^{-/-}$ mice. In addition to altered daily hepatic expression of lipogenic genes (20, 25), *Rev-erbα* deletion impaired circadian control of *Lpl*, leading to its overexpression in peripheral tissues. As a result, skeletal muscle may adapt by preferentially utilizing fatty acids (FA), thus sparing glucose. The surplus of glucose is therefore converted to acetyl-CoA through *de novo* lipogenesis in parallel to the generation of acetyl-CoA from fat breakdown. Acetyl-CoA production exceeding the cellular energy needs, ketone bodies (KB) are synthesized to make use of the energy available (*e.g.*, by muscle). At the same time, fat storage is increased as triglycerides (TG) in both liver and adipose tissue. This leads to a frail energy balance in which greater uptake of fatty acids by the muscle may relatively protect from severe hepatic steatosis and body fat overload. CHYL, chylomicrons; CHYL R, chylomicron remnants.

Lpl also occurs in WAT of *Rev-erbα*$^{-/-}$ mice. Specific overexpression of *Lpl* in adipose tissue does not lead to increased adiposity, an effect that could be due to down-regulation of LPL in other tissues or increase in energy expenditure (53). Interestingly, *Rev-erbα*$^{-/-}$ mice do not show increased body temperature, basal metabolism, or thermogenic response to noradrenaline (data not shown). Moreover, a direct correlation between adipocyte-derived LPL expression and lipid storage has been proposed (54). Therefore, it is likely that increased *Lpl* transcription in WAT of *Rev-erbα*$^{-/-}$ mice reflects increased activity of LPL, leading to enhanced hydrolysis of circulating triglycerides and facilitated NEFA uptake for storage.

The potential implication of REV-ERBα in the transcriptional control of *Lpl* was assessed by luciferase assay using the proximal promoter region of this gene. In contrast to a *Bmal1-luc* reporter, *mLpl-luc* was unresponsive to either REV-ERBα or RORα. Of interest, an E-box has been found in the mouse *Lpl* promoter (55). Instead of a direct repression by REV-ERBα, activation by CLOCK/BMAL1 could drive the temporal expression of the *Lpl* gene. Indeed, REV-ERBα expression is crucial on a daily basis for the transcriptional control of *Clock* and *Bmal1*. In the absence of *Rev-erbα*, *Clock* and *Bmal1* mRNA are overexpressed in peripheral tissues, which can affect the expression of CLOCK/BMAL1 target genes. Our results show that *Lpl* expression can be elicited by CLOCK alone. Whether this effect depends on the acetyltransferase activity of CLOCK (56,57) remains to be established. Such a positive regulation of *Lpl* transcription by CLOCK/BMAL1 has been previously mentioned (J. M. Gimble and Z. E. Floyd, Pennington Biomedical Research Center, Baton Rouge, LA, USA; unpublished results mentioned in ref. 58). Thus, the overexpression of *Lpl* appears to be the result of a defective clockwork, keeping in mind that the regulation of *Lpl* transcription can also be achieved by other pathways (59, 60).

This study provides strong insight into the role of REV-ERBα in the regulation of *in vivo* energy balance. Our findings do not fully exclude the possibility that impaired development of skeletal muscles (40) and adipocytes (19) in *Rev-erbα*-deficient mice may participate in the observed metabolic phenotype. Alternatively, altered clock functioning in the absence of REV-ERBα could account for impaired temporal coordination of developmental processes related to muscle and adipose physiology (58, 61). We propose that altered circadian control of metabolic pathways across peripheral tissues is likely the main cause of the metabolic phenotype of the *Rev-erbα*$^{-/-}$ mice. Our data are in agreement with the involvement of REV-ERBα in the timing of hepatic lipid metabolism genes (20, 25). Furthermore, we show that altered clock machinery leads to up-regulated transcription of *Lpl*. In line with a clock-regulated timing of *Lpl* mRNA expression, we could not detect day-night variation of *Lpl* in the liver of *Rev-erbα*$^{-/-}$ mice. Circadian variations of *Lpl* mRNA and LPL activity have been observed in various tissues including the liver, with hepatic mRNA acrophase in the early morning (4, 10, 20, 44, 59). Therefore, in the context of glucose and fat regulation, circadian control of *Lpl* appears to be essential for a balanced energy metabolism, as suggested by Gimble and Floyd (58). If *Lpl* is expressed continuously around the clock, fat overload can ensue, while at the same time muscles adapt toward a more oxidative metabolism (**Fig. 8**).

In summary, the present study uncovers a molecular pathway that ties clock-driven *Lpl* expression to energy homeostasis and highlights circadian disruption as a potential cause for the etiology of the metabolic syndrome. FJ

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REFERENCES

1. Froy, O. (2010) Metabolism and circadian rhythms–implications for obesity. *Endocr. Rev.* **31,** 1–24
2. Kalsbeek, A., Yi, C. X., La Fleur, S. E., and Fliers, E. (2010) The hypothalamic clock and its control of glucose homeostasis. *Trends Endocrinol. Metab.* **21,** 402–410
3. Asher, G., and Schibler, U. (2011) Crosstalk between components of circadian and metabolic cycles in mammals. *Cell Metab.* **13,** 125–137
4. Zvonic, S., Ptitsyn, A. A., Conrad, S. A., Scott, L. K., Floyd, Z. E., Kilroy, G., Wu, X., Goh, B. C., Mynatt, R. L., and Gimble, J. M. (2006) Characterization of peripheral circadian clocks in adipose tissues. *Diabetes* **55,** 962–970
5. Shibata, S., Tahara, Y., and Hirao, A. (2010) The adjustment and manipulation of biological rhythms by light, nutrition, and abused drugs. *Adv. Drug Deliv. Rev.* **62,** 918–927
6. Ko, C. H., and Takahashi, J. S. (2006) Molecular components of the mammalian circadian clock. *Hum. Mol. Genet.* **15**(Spec. 2), R271–R277
7. Guillaumond, F., Dardente, H., Giguere, V., and Cermakian, N. (2005) Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *J. Biol. Rhythms.* **20,** 391–403
8. Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110,** 251–260
9. Crumbley, C., and Burris, T. P. (2011) Direct regulation of CLOCK expression by REV-ERB. *PLoS One* **6,** e17290
10. Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., Schultz, P. G., Kay, S. A., Takahashi, J. S., and Hogenesch, J. B. (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* **109,** 307–320

11. Delezie, J., and Challet, E. (2011) Interactions between metabolism and circadian clocks: reciprocal disturbances. *Ann. N. Y. Acad. Sci.* **1243,** 30–46
12. Turek, F. W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olson, S., Easton, A., Jensen, D. R., Eckel, R. H., Takahashi, J. S., and Bass, J. (2005) Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* **308,** 1043–1045
13. Marcheva, B., Ramsey, K. M., Buhr, E. D., Kobayashi, Y., Su, H., Ko, C. H., Ivanova, G., Omura, C., Mo, S., Vitaterna, M. H., Lopez, J. P., Philipson, L. H., Bradfield, C. A., Crosby, S. D., JeBailey, L., Wang, X., Takahashi, J. S., and Bass, J. (2010) Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature* **466,** 627–631
14. Mendoza, J., Pevet, P., and Challet, E. (2008) High-fat feeding alters the clock synchronization to light. *J. Physiol.* **586,** 5901–5910
15. Kohsaka, A., Laposky, A. D., Ramsey, K. M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F. W., and Bass, J. (2007) High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab.* **6,** 414–421
16. Dallmann, R., and Weaver, D. R. (2010) Altered body mass regulation in male mPeriod mutant mice on high-fat diet. *Chronobiol. Int.* **27,** 1317–1328
17. Lazar, M. A., Hodin, R. A., Darling, D. S., and Chin, W. W. (1989) A novel member of the thyroid/steroid hormone receptor family is encoded by the opposite strand of the rat c-erbA alpha transcriptional unit. *Mol. Cell. Biol.* **9,** 1128–1136
18. Vieira, E., Marroqui, L., Batista, T. M., Caballero-Garrido, E., Carneiro, E. M., Boschero, A. C., Nadal, A., and Quesada, I. (2012) The clock gene Rev-erbalpha regulates pancreatic beta-cell function: modulation by leptin and high-fat diet. *Endocrinology* **153,** 592–601
19. Wang, J., and Lazar, M. A. (2008) Bifunctional role of Rev-erbalpha in adipocyte differentiation. *Mol. Cell. Biol.* **28,** 2213–2220
20. Le Martelot, G., Claudel, T., Gatfield, D., Schaad, O., Kornmann, B., Sasso, G. L., Moschetta, A., and Schibler, U. (2009) REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. *PLoS Biol* **7,** e1000181
21. Duez, H., van der Veen, J. N., Duhem, C., Pourcet, B., Touvier, T., Fontaine, C., Derudas, B., Bauge, E., Havinga, R., Bloks, V. W., Wolters, H., van der Sluijs, F. H., Vennstrom, B., Kuipers, F., and Staels, B. (2008) Regulation of bile acid synthesis by the nuclear receptor Rev-erbalpha. *Gastroenterology* **135,** 689–698
22. Yin, L., Wu, N., Curtin, J. C., Qatanani, M., Szwergold, N. R., Reid, R. A., Waitt, G. M., Parks, D. J., Pearce, K. H., Wisely, G. B., and Lazar, M. A. (2007) Rev-erbalpha, a heme sensor that coordinates metabolic and circadian pathways. *Science* **318,** 1786–1789
23. Wu, N., Yin, L., Hanniman, E. A., Joshi, S., and Lazar, M. A. (2009) Negative feedback maintenance of heme homeostasis by its receptor, Rev-erbalpha. *Genes Dev.* **23,** 2201–2209
24. Raghuram, S., Stayrook, K. R., Huang, P., Rogers, P. M., Nosie, A. K., McClure, D. B., Burris, L. L., Khorasanizadeh, S., Burris, T. P., and Rastinejad, F. (2007) Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-erbbeta. *Nat. Struct. Mol. Biol.* **14,** 1207–1213
25. Feng, D., Liu, T., Sun, Z., Bugge, A., Mullican, S. E., Alenghat, T., Liu, X. S., and Lazar, M. A. (2011) A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism. *Science* **331,** 1315–1319
26. Fergani, A., Eschbach, J., Oudart, H., Larmet, Y., Schwalenstocker, B., Ludolph, A. C., Loeffler, J. P., and Dupuis, L. (2011) A mutation in the dynein heavy chain gene compensates for energy deficit of mutant SOD1 mice and increases potentially neuroprotective IGF-1. *Mol. Neurodegener.* **6,** 26
27. Heikkinen, S., Argmann, C. A., Champy, M. F., and Auwerx, J. (2007) Evaluation of glucose homeostasis. *Curr. Protoc. Mol. Biol.* Chap. 29, Unit 29B 23
28. Murat, J. C., and Serfaty, A. (1974) Simple enzymatic determination of polysaccharide (glycogen) content of animal tissues. *Clin. Chem.* **20,** 1576–1577
29. Miao, B., Zondlo, S., Gibbs, S., Cromley, D., Hosagrahara, V. P., Kirchgessner, T. G., Billheimer, J., and Mukherjee, R. (2004) Raising HDL cholesterol without inducing hepatic steatosis and hypertriglyceridemia by a selective LXR modulator. *J. Lipid Res.* **45,** 1410–1417
30. Dardente, H., Fustin, J. M., and Hazlerigg, D. G. (2009) Transcriptional feedback loops in the ovine circadian clock. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **153,** 391–398
31. Travnickova-Bendova, Z., Cermakian, N., Reppert, S. M., and Sassone-Corsi, P. (2002) Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 7728–7733
32. Schmutz, I., Ripperger, J. A., Baeriswyl-Aebischer, S., and Albrecht, U. (2010) The mammalian clock component PERIOD2 coordinates circadian output by interaction with nuclear receptors. *Genes Dev.* **24,** 345–357
33. Gallou-Kabani, C., Vige, A., Gross, M. S., Rabes, J. P., Boileau, C., Larue-Achagiotis, C., Tome, D., Jais, J. P., and Junien, C. (2007) C57BL/6J and A/J mice fed a high-fat diet delineate components of metabolic syndrome. *Obesity (Silver Spring)* **15,** 1996–2005
34. Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., Ezaki, O., Akanuma, Y., Gavrilova, O., Vinson, C., Reitman, M. L., Kagechika, H., Shudo, K., Yoda, M., Nakano, Y., Tobe, K., Nagai, R., Kimura, S., Tomita, M., Froguel, P., and Kadowaki, T. (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat. Med.* **7,** 941–946
35. Hughes, M. E., DiTacchio, L., Hayes, K. R., Vollmers, C., Pulivarthy, S., Baggs, J. E., Panda, S., and Hogenesch, J. B. (2009) Harmonics of circadian gene transcription in mammals. *PLoS Genet.* **5,** e1000442
36. Goldberg, I. J., Eckel, R. H., and Abumrad, N. A. (2009) Regulation of fatty acid uptake into tissues: lipoprotein lipase- and CD36-mediated pathways. *J. Lipid Res.* **50**(Suppl), S86–S90
37. Ortega, F. J., Mayas, D., Moreno-Navarrete, J. M., Catalan, V., Gomez-Ambrosi, J., Esteve, E., Rodriguez-Hermosa, J. I., Ruiz, B., Ricart, W., Peral, B., Fruhbeck, G., Tinahones, F. J., and Fernandez-Real, J. M. (2010) The gene expression of the main lipogenic enzymes is downregulated in visceral adipose tissue of obese subjects. *Obesity (Silver Spring)* **18,** 13–20
38. Triqueneaux, G., Thenot, S., Kakizawa, T., Antoch, M. P., Safi, R., Takahashi, J. S., Delaunay, F., and Laudet, V. (2004) The orphan receptor Rev-erbalpha gene is a target of the circadian clock pacemaker. *J. Mol. Endocrinol.* **33,** 585–608
39. Stenbit, A. E., Tsao, T. S., Li, J., Burcelin, R., Geenen, D. L., Factor, S. M., Houseknecht, K., Katz, E. B., and Charron, M. J. (1997) GLUT4 heterozygous knockout mice develop muscle insulin resistance and diabetes. *Nat. Med.* **3,** 1096–1101
40. Pircher, P., Chomez, P., Yu, F., Vennstrom, B., and Larsson, L. (2005) Aberrant expression of myosin isoforms in skeletal muscles from mice lacking the Rev-erbAalpha orphan receptor gene. *Am. J. Physiol.* **288,** R482–490
41. Manttari, S., and Jarvilehto, M. (2005) Comparative analysis of mouse skeletal muscle fibre type composition and contractile responses to calcium channel blocker. *BMC Physiol.* **5,** 4
42. Ibrahimi, A., Bonen, A., Blinn, W. D., Hajri, T., Li, X., Zhong, K., Cameron, R., and Abumrad, N. A. (1999) Muscle-specific overexpression of FAT/CD36 enhances fatty acid oxidation by contracting muscle, reduces plasma triglycerides and fatty acids, and increases plasma glucose and insulin. *J. Biol. Chem.* **274,** 26761–26766
43. Ferraro, R. T., Eckel, R. H., Larson, D. E., Fontvieille, A. M., Rising, R., Jensen, D. R., and Ravussin, E. (1993) Relationship between skeletal muscle lipoprotein lipase activity and 24-hour macronutrient oxidation. *J. Clin. Invest.* **92,** 441–445
44. Tsutsumi, K., Inoue, Y., and Kondo, Y. (2002) The relationship between lipoprotein lipase activity and respiratory quotient of rats in circadian rhythms. *Biol. Pharm. Bull.* **25,** 1360–1363
45. Jensen, D. R., Knaub, L. A., Konhilas, J. P., Leinwand, L. A., MacLean, P. S., and Eckel, R. H. (2008) Increased thermoregulation in cold-exposed transgenic mice overexpressing lipoprotein lipase in skeletal muscle: an avian phenotype? *J. Lipid Res.* **49,** 870–879
46. Schutz, Y. (2004) Concept of fat balance in human obesity revisited with particular reference to de novo lipogenesis. *Int. J. Obes. Relat. Metab. Disord.* **28**(Suppl. 4), S3–S11
47. Raspe, E., Duez, H., Mansen, A., Fontaine, C., Fievet, C., Fruchart, J. C., Vennstrom, B., and Staels, B. (2002) Identifica-

tion of Rev-erbalpha as a physiological repressor of apoC-III gene transcription. *J. Lipid Res.* **43,** 2172–2179
48. Lau, P., Fitzsimmons, R. L., Raichur, S., Wang, S. C., Lechtken, A., and Muscat, G. E. (2008) The orphan nuclear receptor, RORalpha, regulates gene expression that controls lipid metabolism: staggerer (SG/SG) mice are resistant to diet-induced obesity. *J. Biol. Chem.* **283,** 18411–18421
49. Staels, B., and Auwerx, J. (1992) Perturbation of developmental gene expression in rat liver by fibric acid derivatives: lipoprotein lipase and alpha-fetoprotein as models. *Development* **115,** 1035–1043
50. Merkel, M., Weinstock, P. H., Chajek-Shaul, T., Radner, H., Yin, B., Breslow, J. L., and Goldberg, I. J. (1998) Lipoprotein lipase expression exclusively in liver. A mouse model for metabolism in the neonatal period and during cachexia. *J. Clin. Invest.* **102,** 893–901
51. Kim, J. K., Fillmore, J. J., Chen, Y., Yu, C., Moore, I. K., Pypaert, M., Lutz, E. P., Kako, Y., Velez-Carrasco, W., Goldberg, I. J., Breslow, J. L., and Shulman, G. I. (2001) Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 7522–7527
52. Voshol, P. J., Jong, M. C., Dahlmans, V. E., Kratky, D., Levak-Frank, S., Zechner, R., Romijn, J. A., and Havekes, L. M. (2001) In muscle-specific lipoprotein lipase-overexpressing mice, muscle triglyceride content is increased without inhibition of insulin-stimulated whole-body and muscle-specific glucose uptake. *Diabetes* **50,** 2585–2590
53. Hensley, L. L., Ranganathan, G., Wagner, E. M., Wells, B. D., Daniel, J. C., Vu, D., Semenkovich, C. F., Zechner, R., and Kern, P. A. (2003) Transgenic mice expressing lipoprotein lipase in adipose tissue. Absence of the proximal 3′-untranslated region causes translational upregulation. *J. Biol. Chem.* **278,** 32702–32709
54. Gonzales, A. M., and Orlando, R. A. (2007) Role of adipocyte-derived lipoprotein lipase in adipocyte hypertrophy. *Nutr. Metab. (Lond.)* **4,** 22
55. Bey, L., Etienne, J., Tse, C., Brault, D., Noe, L., Raisonnier, A., Arnault, F., Hamilton, M. T., and Galibert, F. (1998) Cloning, sequencing and structural analysis of 976 base pairs of the promoter sequence for the rat lipoprotein lipase gene. Comparison with the mouse and human sequences. *Gene* **209,** 31–38
56. Doi, M., Hirayama, J., and Sassone-Corsi, P. (2006) Circadian regulator CLOCK is a histone acetyltransferase. *Cell* **125,** 497–508
57. Nader, N., Chrousos, G. P., and Kino, T. (2009) Circadian rhythm transcription factor CLOCK regulates the transcriptional activity of the glucocorticoid receptor by acetylating its hinge region lysine cluster: potential physiological implications. *FASEB J.* **23,** 1572–1583
58. Gimble, J. M., and Floyd, Z. E. (2009) Fat circadian biology. *J. Appl. Physiol.* **107,** 1629–1637
59. Gachon, F., Leuenberger, N., Claudel, T., Gos, P., Jouffe, C., Fleury Olela, F., de Mollerat du Jeu, X., Wahli, W., and Schibler, U. (2011) Proline- and acidic amino acid-rich basic leucine zipper proteins modulate peroxisome proliferator-activated receptor alpha (PPARalpha) activity. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 4794–4799
60. Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A. M., Heyman, R. A., Briggs, M., Deeb, S., Staels, B., and Auwerx, J. (1996) PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J.* **15,** 5336–5348
61. Andrews, J. L., Zhang, X., McCarthy, J. J., McDearmon, E. L., Hornberger, T. A., Russell, B., Campbell, K. S., Arbogast, S., Reid, M. B., Walker, J. R., Hogenesch, J. B., Takahashi, J. S., and Esser, K. A. (2010) CLOCK and BMAL1 regulate MyoD and are necessary for maintenance of skeletal muscle phenotype and function. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 19090–19095

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SUPPLEMENTAL DATA

Animal experiments: For the intra-peritoneal insulin sensitivity test (IPIST), mice (n = 10 / genotype, sex ratio 1:1) were fasted for 14 h (either from ZT12 to ZT2 or from ZT22 to ZT14) and injected i.p. with 0.5 IU/kg insulin (Umuline®NPH, Lilly, France). For the oral glucose tolerance test (OGTT), mice were fasted for 14 h (from ZT22 to ZT14) and received a glucose load via gavage (2 g/kg; D-Glucose Sigma). Adaptive thermogenesis was assessed in 4-month-old mice (n = 6 / genotype) exposed to 4°C for 6 h from ZT2 (see Argmann et al. (2006) *Curr Protoc Mol Biol*) and recorded with rectal thermometer (Harvard apparatus, MA, USA). Note that the animals were transferred from their housing room to a 4°C room.

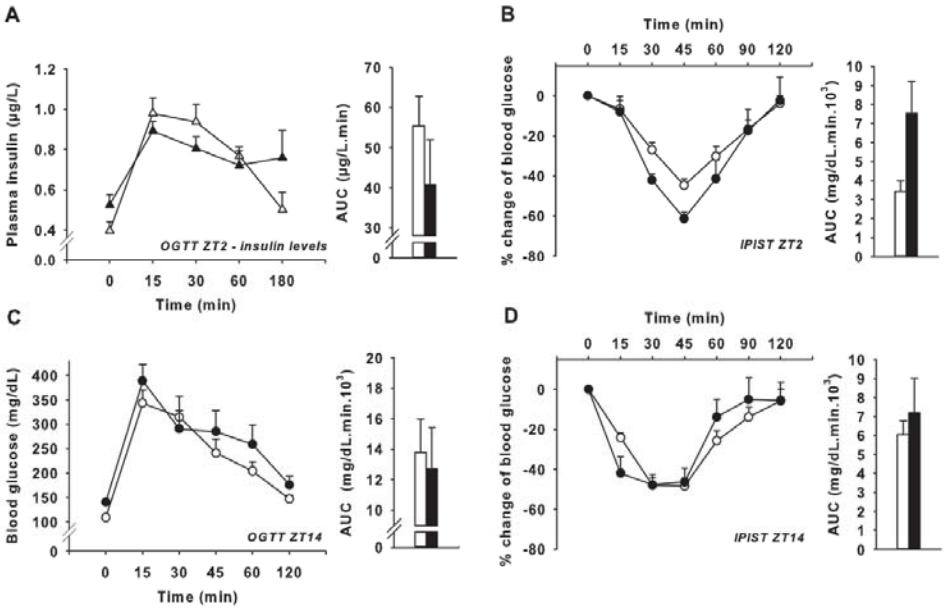
Plasma metabolic parameters: Plasma adiponectin was assayed with ELISA Kits (Adiponectin EZMADP-60K). Plasma corticosterone was measured using an EIA kit (AC-14F1, IDS EURL).

Cell Culture, Transfection, and Luciferase Assay: The entire locus of the *Lpl* gene (including 10kb upstream of the transcription start site and 10kb downstream of the last exon) was searched for the presence of consensus ROREs (WAWNTRGGTCA, where W is A/T, R is A/G and N corresponds to any nucleotide). A single consensus RORE was found in the *Lpl* gene within the 46.4kb region examined (NC_000074, nt 71394454..71440831); this corresponds to an intergenic region, located 3.4kb downstream of the last *Lpl* exon (minus strand, position 39788-39778 of the above-mentioned genomic fragment). Due to its location, the functional relevance of this element is difficult to evaluate and was consequently not investigated. Proximal promoter regions often bear functionally important response elements and most transcription factors can bind sequences slightly divergent from their consensus DNA binding motif. Indeed, several potential non-consensus ROREs were found in the proximal promoter regions of *Lpl*. We therefore decided to check whether this proximal promoter region was responsive to REV-ERBα/RORα (see results).

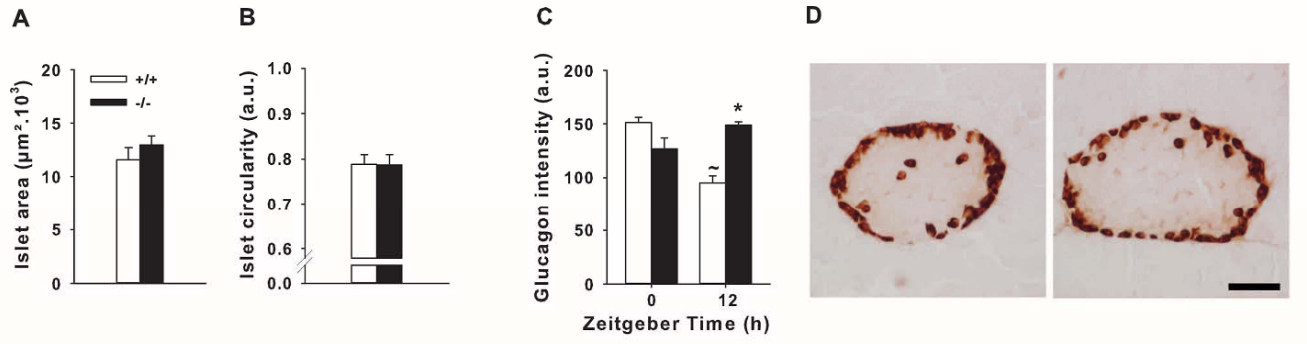
Supplemental Table S1. Sequences of primers used for Quantitative Real-Time PCR

Genes	Forward	Reverse
36b4	GCTGATGGGCAAGAACACCA	CCCAAAGCCTGGAAGAAGGA
Acc (Acetyl-Coenzyme A carboxylase alpha)	GCCTCTTCCTGACAAACGAG	TGACTGCCGAAACATCTCTG
Acly (ATP citrate lyase)	ATCAACCCCCTTGTGGTGA	GCTTCAAGCTTGCTCCACTT
ApocIII (Apolipoprotein CIII)	ACATGGAACAAGCCTCCAAG	TGGTTGGTCCTCAGGGTTAG
Bmal1 (Brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like)	CTCATTGATGCCAAGACTGG	GGTGGCCAGCTTTTCAAATA
Cd36 (Cluster of Differentiation 36)	AAGAGGTCCTTACACATACAGAGTTC	AGCTGCTACAGCCAGATTCA
Clock (Circadian Locomotor Output Cycles Kaput)*	ACCACAGCAACAGCAACAAC	GGCTGCTGAACTGAAGGAAG
Cpt1a (Carnitine palmitoyltransferase 1A (liver))	TACTACGCCATGGAGATGCT	TGACGTGTTGGATGGTGTCT
Cpt1b (Carnitine palmitoyltransferase 1B (muscle))	GTCGCTTCTTCAAGGTCTGG	AAGAAAGCAGCACGTTCGAT
Elovl6 (ELOVL fatty acid elongase 6)	GAGCAGAGGCGCAGAGAAC	ATGCCGACCACCAAAGATAA
Fas (Fatty acid synthase)	TCGACTTCAAAGGACCAAGC	TTCATGAACTGCACAGAGGTG
Fgf21 (Fibroblast growth factor 21)	AGATCAGGGAGGATGGAACA	TCAAAGTGAGGCGATCCATA
G6pc (Glucose-6-phosphatase, catalytic subunit)	CTCGTCTTCAAGTGGATTCTGT	TGGCTTTTTCTTTCCTCGAA
Gk (Glucokinase)	CCAGAAGGCTCAGAAGTTGG	TGCTTGTCCAGGAAGTCAGA
Glut2 (Glucose transporter 2)	GGACAAACTTGGAAGGATCA	CAGTCCTGAAATTAGCCCACA
Glut4 (Glucose transporter 4)	GATTCTGCTGCCCTTCTGTC	ATTGGACGCTCTCTCTCCAA
Gys2 (Glycogen synthase 2)	CCAGCTTGACAAGTTCGACA	CCTCTTCAGCATGTGCTCTG
Hmgcs2 (3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial))	AAGACCAAGGCCTCCCTTTA	TAAGCCTGAGCCGTAGGAGA
Insig2a (Insulin induced gene 2a)	CACGCCAGTGCTAAAGTAGAC	GGGTGACAACGGTTGCTAAG
L-Pk (Liver-pyruvate kinase)	CGGAAAATTGGCCCAGA	CACCACATCACTGGCTTTTC
Lipc (Hepatic lipase)	GCTGCTGGGAACAAAAGAAG	AATGAGGCCAGAGTGGTGAG
Lpl (Lipoprotein lipase)	AGGGCTCTGCCTGAGTTGTA	CCATCCTCAGTCCCAGAAAA
Pepck (Phosphoenolpyruvate carboxykinase 2 (mitochondrial))	TTTGATGCCCAAGGCAACTT	ATCGATGCCTTCCCAGTAAA
Ppara (Peroxisome proliferator-activated receptor alpha)	GGATGTCACACAATGCAATTC	GGCCTTGACCTTGTTCATGT
Pparbp (Ppar binding protein)	GAGAATCCTGTGAGCTGTCC	CGTTGGTTGCCTTCCAGTA
Pparg (Peroxisome proliferator-activated receptor gamma)	AGACCACTCGCATTCCTTTGACAT	TCCCCACAGACTCGGCACTCAATG
Srebp1c (Sterol regulatory element binding transcription factor 1)	GGCACTGAAGCAAAGCTGAA	TCATGCCCTCCATAGACACA

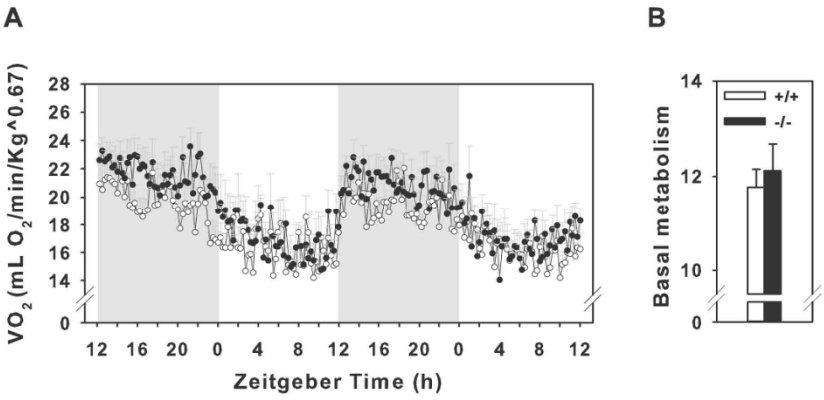
* Primers from Kohsaka et al. (2007) *Cell Metab*



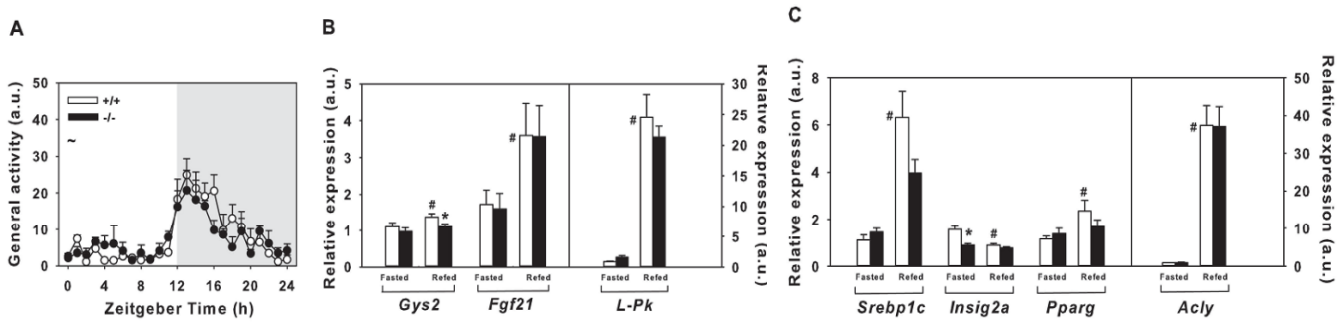
Supplemental Figure 1. *Rev-erbα-/-* mice are hyperglycemic without whole insulin resistance.
(A) Determination of plasma insulin levels during an oral glucose tolerance test (OGTT) in mice fasted overnight (i.e., from ZT12 to ZT2) and the resulting incremental area under the curve (AUC) based on insulin data (*n* = 8 / group). (B) Intraperitoneal insulin sensitivity test (IPIST) in mice fasted overnight (i.e., from ZT12 to ZT2) and the resulting AUC (*n* = 12 / group). (C) OGTT and (D) IPIST in daytime fasted mice (i.e., from ZT22 to ZT14) and the resulting AUC (*n* = 12 / group). Values are expressed as mean ± SEM. Note for the IPIST that percents change from the baseline are depicted. * significant difference between genotypes for a given time point ($P < 0.05$). *Rev-erbα*+/+ (open symbols) and -/- mice (closed symbols).



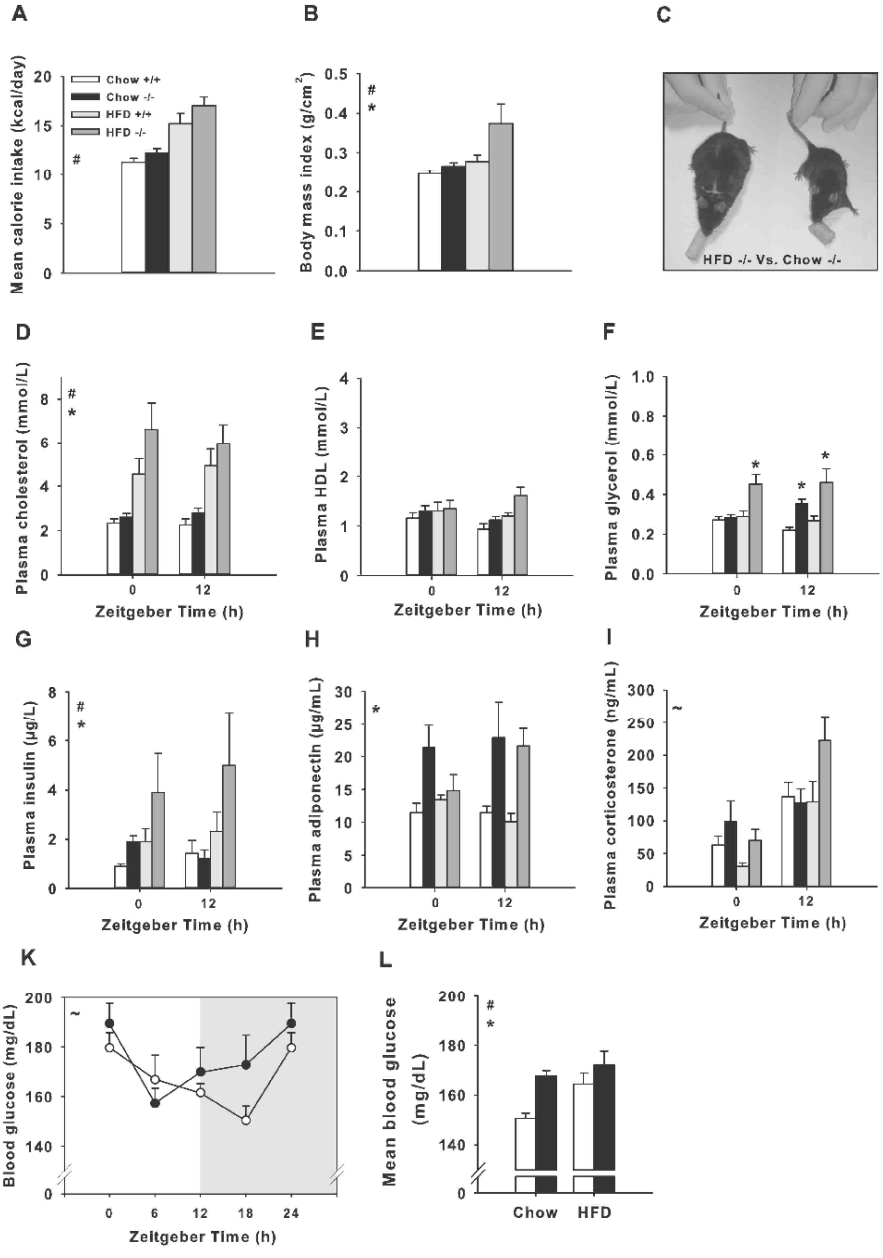
Supplemental Figure 2. Pancreatic islets are normal in *Rev-erbα-/-* mice.
(A) Islet area and (B) circularity (*n* = 12 / group). (C) Glucagon immunostaining of pancreatic sections in *Rev-erbα*+/+ and -/- mice (*n* = 6 / group). (D) Pancreas sections of a *Rev-erbα*+/+ (left) and a -/- mouse (right) stained with glucagon antibody; Scale bar: 50 µm.



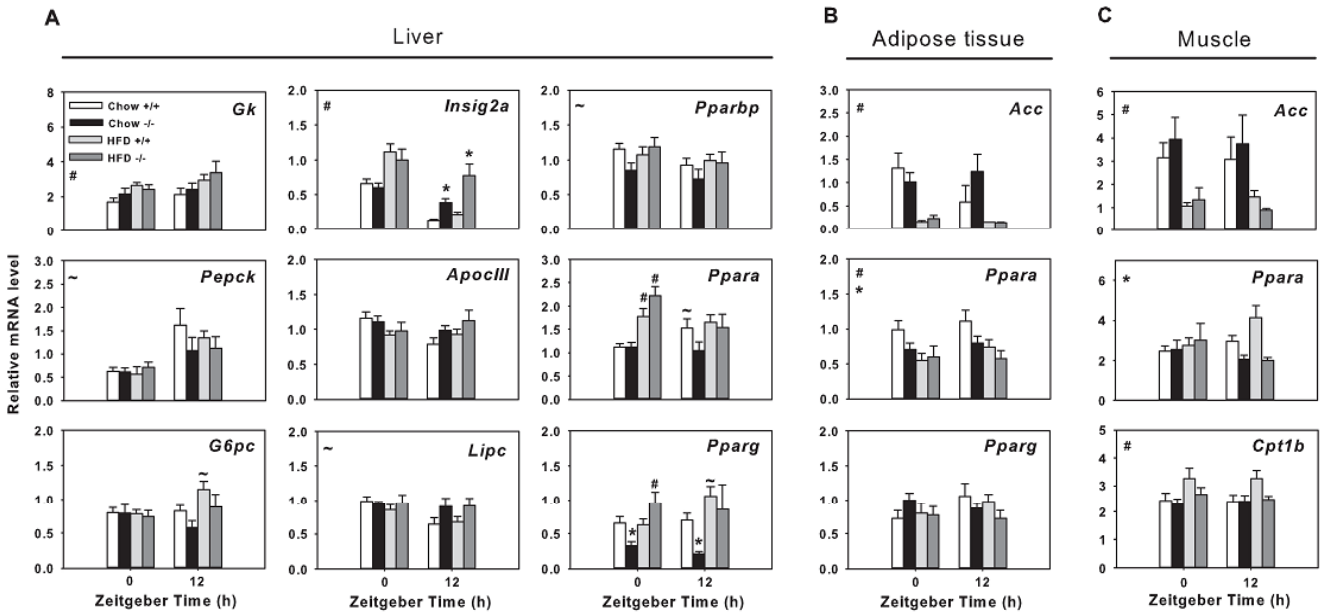
Supplemental Figure 3. Changes in daily fuel utilization in *Rev-erbα-/-* mice.
(A) 48-h O_2 consumption of *Rev-erbα+/+* and *-/-* mice determined by indirect calorimetry.
(B) Basal metabolism calculated from the five lowest O_2 values. The grey rectangle indicates the dark (i.e., active/feeding) period. *Rev-erbα*+/+ (open circles) and -/- mice (closed circles).



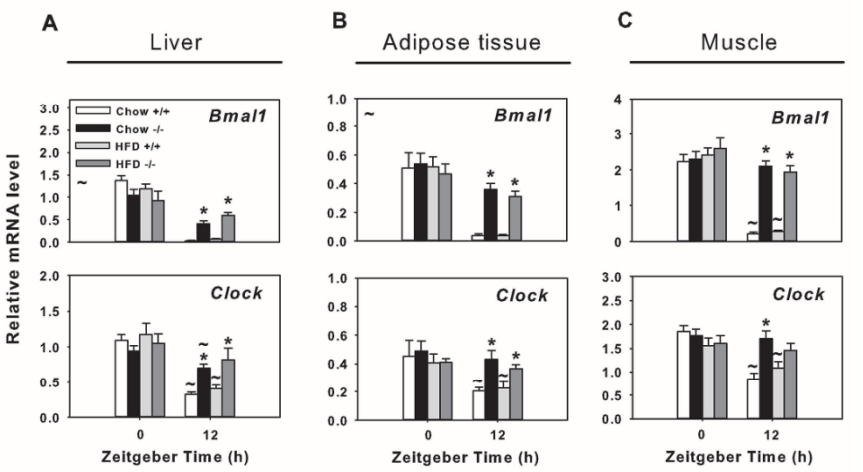
Supplemental Figure 4. General activity and hepatic gene expression in fasted or refed *Rev-erbα*+/+ and -/- mice.
(A) General locomotor activity during a 24-h fast. (B) mRNA expression levels of glucose metabolism genes and (C) lipid metabolism genes. Animals were sampled at ZT0. Values are expressed as mean ± SEM (n = 7 / group). ˜ main effect of time; # main effect of feeding condition (middle panel) or significant difference between feeding conditions for a given genotype (above bar graph) (P < 0.05); * significant difference between genotypes for a given feeding condition (P < 0.05). For abbreviations of the different genes, see table S1. *Rev-erbα*+/+ (open circles) and -/- mice (closed circles).



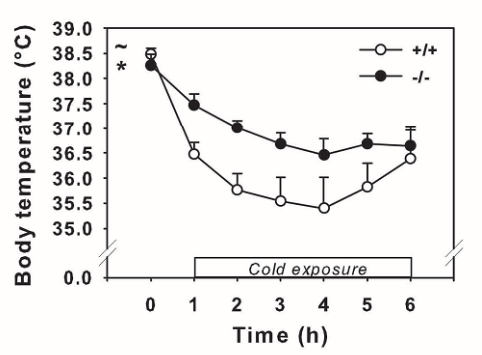
Supplemental Figure 5. *Rev-erbα*-/- mice exhibit a fat phenotype amplified upon prolonged High-fat feeding.
(A) Daily calorie intake in chow-fed and high-fat fed (HFD) +/+ and -/- animals (n = 12 / group). (B) Body mass index (n = 12 / group). (C) Picture representing two 3-month old *Rev-erbα-/-* mice under HFD and chow conditions.
(D) Plasma cholesterol, (E) high-density lipoprotein (HDL), (F) glycerol, (G) insulin, (H) adiponectin and (I) corticosterone levels (n = 12 / group).
(K) 24-h blood glucose profiles from tail bleed in HFD conditions. (L) Mean blood glucose levels over 24 h from chow (data from Figure 1D) and HFD conditions (data from Figure S5K). *Rev-erbα*+/+ (open circles) and -/- mice (closed circles).
Note that mice from each feeding conditions were age-matched at the time of sampling. Values are represented as mean ± SEM. # main effect of feeding condition (P < 0.05); ˜ main effect of time (P < 0.05); * main effect of genotype (upper left corner) or significant difference between genotypes for a given time point and feeding condition (above bar graph) (P < 0.05).



Supplemental Figure 6. Gene expression in chow- and high-fat fed *Rev-erbα+/+* and -/- animals.
mRNA expression of glucose and lipid metabolism genes in (A) liver, (B) retroperitoneal white adipose tissue, (C) *rectus femoris* skeletal muscle. Data are means ± SEM (*n* = 6 / group). # main effect of feeding condition (upper left corner) or significant difference between feeding conditions for a given genotype and time point (above bar graph) ($P < 0.05$); ˜ main effect of time (upper left corner) or significant difference between time points for a given genotype and feeding condition (above bar graph) ($P < 0.05$); * main effect of genotype (upper left corner) or significant difference between genotypes for a given time point and feeding condition (above bar graph) ($P < 0.05$). For abbreviations of the different genes, see table S1.



Supplemental Figure 7. Clock gene expression in chow- and high-fat fed *Rev-erbα+/+* and -/- animals.
mRNA expression of clock genes in (A) liver, (B) retroperitoneal white adipose tissue, (C) *rectus femoris* skeletal muscle. Data are means ± SEM (*n* = 6 / group). ˜ main effect of time (upper left corner) or significant difference between time points for a given genotype and feeding condition (above bar graph) ($P < 0.05$); * significant difference between genotypes for a given time point and feeding condition (above bar graph) ($P < 0.05$).



Supplemental Figure 8. Enhanced adaptive thermoregulation in *Rev-erbα-/-* mice.
Body temperature before (Time 0, ZT2) and during exposure to 4°C (*n* = 6 / group). Values are expressed as mean ± SEM. ˜ main effect of time (upper left corner); * main effect of genotype (upper left corner) ($P < 0.05$).

Chapter 3 DISCUSSION

1. The FEO in *Rev-erbα* knockout mice

Numerous studies have explored the role of canonical clock genes in the functioning of the FEO. Some have suggested that the latter is a true circadian oscillator and that clock genes such as *Per2* or *Bmal1* are essential for its clockwork. Others have indicated that the molecular mechanism of the FEO could either involve non-classical clockwork yet to be discovered or that clock gene deletion fails to deeply affect food entrainment due to the involvement of metabolic and cognitive cues.

1.1 Key findings discussed

In 12:12 light-dark conditions, *Rev-erbα*$^{-/-}$ mice exhibit reduced FAA prior to food access. This result is further confirmed in DD conditions in animals with or without SCN. However, contrary to *Per2* mutant mice (Feillet et al. 2006; Mendoza et al. 2010), *Rev-erbα* deletion does not lead to a total disappearance of FAA[31].

In contrast to slight reductions of behavioral components, thermogenesis in anticipation is almost abolished in *Rev-erbα*$^{-/-}$ mice. The anticipatory corticosterone peak and the rise in plasma ketone bodies are not observed either. However, 24-h hepatic clock mRNA oscillations and 24-h hypothalamic PER2 oscillations are RF-entrained in *Rev-erbα*$^{-/-}$ mice as in their control littermates. Nevertheless, we found that the daily PER2 oscillations in the cerebellum are not similarly changed between *Rev-erb*$^{+/+}$ and *Rev-erbα*$^{-/-}$ mice in response to RF.

At first glance, our results could support the circadian nature of the FEO functioning. Some matters, however, remain unsettled.

1.1.1 Rev-erbα deletion and locomotor behaviors

By evaluating *Rev-erbα* contribution to food entrainment, we give a new evidence that clock genes are important for the ability of mice to properly anticipate

[31] On the other hand, not all control mice show strong FAA in our studies, in line with previous results (Pendergast et al. 2009).

mealtime, in line with previous reports (Dudley et al. 2003; Horikawa et al. 2005; Iijima et al. 2005; Feillet et al. 2006; Mendoza et al. 2010; Mieda et al. 2011). Indeed, the expression of general and wheel-running locomotor activities is significantly impacted prior to food access in *Rev-erbα*$^{-/-}$ mice. Nevertheless, in our study, the absence of *Rev-erbα* leads to an overall reduction of wheel-running activity. Interestingly, this has been observed in some (LopezMolina et al. 1997; Bunger et al. 2000), but not all circadian mutants (Vitaterna et al. 1994; Zheng et al. 1999). Thus, it is not excluded that mice that do not behave similarly in the presence of a running wheel can eventually influence the accurate evaluation of food entrainment.

As previously mentioned, not all studies agree on the involvement of clock genes for the expression of FAA or for a proper MASCO functioning (Mohawk et al. 2009; Storch et al. 2009), disputing the reliance of the putative FEO and MASCO oscillators on a SCN-like molecular mechanism. Most often, however, these studies did not investigate other parameters than wheel-running behaviors. In addition, FAA is rarely evaluated: in different lighting conditions[32]; following mealtime jet-lag test; or in T-cycle experiments (period of feeding different from 24 h, but in the circadian range). We have evaluated FAA in constant dark conditions, in a skeleton photoperiod (i.e., 1 h light-pulse at the beginning of the resting period and 1 h light-pulse at the end of the resting period) as well as following mealtime jet-lag test, without revealing more FAA than in LD conditions (unpublished data; see appendix 1). Incidentally, considering the fact that the FEO and MASCO may be a unique entity, the use of MAP injections could be informative to further substantiate the involvement of *Rev-erbα* in circadian pathways.

1.1.2 Rev-erbα deletion and clock gene oscillations

As outlined above, the SCN functioning is altered in the absence of *Rev-erbα* (Preitner et al. 2002). Regarding the FEO, since its location/network has not been yet clearly revealed, we have to search rather blindly for altered *molecular* oscillations. At least, we found neither major changes of core clock mRNA expression (i.e., *Per1-2*) in liver nor of PER2 expression in key hypothalamic nuclei known to be circadian oscillators, in both AL and RF conditions. It is thus plausible that the circadian

[32] It has been recently shown that FAA in *Bmal1*$^{-/-}$ mice could be observed in 18L:6D lighting cycles or in constant darkness, while it was nearly absent in 12L:12D conditions (Pendergast et al. 2009)

network (probably including the FEO) could still work in spite of genetic perturbations induced by the depletion of *Rev-erbα*—thus contributing to the expression of some FAA. For instance, active compensatory mechanisms in the clock network have been recently demonstrated *in vitro* (Baggs et al. 2009). In that study, knockdown of *Rev-erbβ* was shown to increase expression of its paralog *Rev-erbα,* ensuring a "genetic backup". In this regard, more recently, *Rev-erbβ* has been proposed to compensate for the absence of *Rev-erbα* in mouse embryonic fibroblasts and to protect circadian clock function (Bugge et al. 2012)[33]. At least in the liver (an organ which demonstrates significant metabolic changes in the absence of *Rev-erbα*, see below), 24-h *Rev-erbβ* levels are unchanged in adult *Rev-erbα*$^{-/-}$ mice (Schmutz et al. 2010).

On the other hand, without strong modifications in the core clock functioning—apart from altered *Bmal1* and *Clock* levels—of hypothalamic and peripheral oscillators, it is possible that downstream targets of the FEO and of the *Rev-erbα* gene are differentially impacted. This could therefore explained why some physiological and metabolic parameters, considered as FEO outputs, such as thermogenesis (mainly controlled by the preoptic area), corticosterone release (controlled by the adrenal), ketone bodies production (by the liver), are severely impaired in *Rev-erbα*$^{-/-}$ mice under scheduled food restriction, although we did not investigate clock and metabolic gene expression in the adrenal gland and the preoptic area (for investigations on the liver, see part 2.1).

As for the CRB oscillator, our data on PER2 expression in *Rev-erbα*$^{-/-}$ mice under RF are intriguing in the light of a recent study demonstrating that a functional cerebellar oscillator is essential to ensure proper FAA—but not for the development of the food-anticipatory peak of corticosterone and the phase-adjustment of hypothalamic *Per1-2* expression to mealtime (Mendoza et al. 2010). However, it seems premature to correlate the altered PER2 oscillations in the cerebellum of *Rev-erbα*$^{-/-}$ mice to their defect to show robust food anticipation. Our view of the oscillator properties of the CRB of *Rev-erbα*$^{-/-}$ mice is limited since we did not evaluate the daily pattern of expression of additional clock proteins. Indeed, mutation of clock-related factors such as *Overtime* or *Cry1* can significantly affect the circadian expression of some, but not all, clock transcripts/proteins in the CRB (Miyamoto and

[33] This study reveals a close identity of the *Rev-erbα* and *Rev-erbβ* cistromes.

Sancar 1999; Siepka et al. 2007). Moreover, the role of the CRB in circadian timing processes such as food-entrainable oscillations is not yet defined. There is evidence of clock mRNA/protein oscillations in this brain region (Siepka et al. 2007; Farnell et al. 2008) that can be entrained by RF (present results and Mendoza et al. 2010). However, the CRB fine-tunes motor activity, but does not initiate it; thus, decreased food-anticipatory activities cannot be solely imputed to impaired clockwork in this structure. Besides that, the CRB represents rather a weak oscillator compared to the SCN, since the slight PER1 oscillations in tissue explants damped out relatively quickly (Mendoza et al. 2010). In this regard, it is not known if the coordinated daily clock gene expression in the CRB requires extrinsic factors from the brain (e.g., indirect coupling with the SCN pacemaker) and/or the periphery (e.g., feedback from feeding-related signals). Furthermore, since daytime RF challenge leads to the appearance of locomotor activity prior to mealtime and can shorten the main bout of nocturnal activity (Challet et al. 1998; Holmes and Mistlberger 2000), to what level these changes are interpreted by the CRB oscillator? This is especially important considering that locomotor activity, particularly the access to a running wheel, can feedback to the circadian system (Turek 1989; Lax et al. 1998; Campuzano et al. 1999; Cambras et al. 2000; van der Veen et al. 2011). Since *Rev-erbα*$^{-/-}$ mice show decreased wheel-running activity, this could render difficult the establishment and expression of food-entrained circadian rhythms[34]. To summarize, beyond the need to evaluate the property of CRB cells (i.e., the Purkinje cells) to exhibit for instance rhythmic neuronal activity as a putative circadian electrical output signal to modulate food anticipation, it would be informative to study the degree of sensitivity of clock gene oscillations to diverse manipulations, such as following SCN removal or in the absence of a feedback from the periphery[35]. Likewise, instead of pharmacological/genetic impairments of the cerebellar architecture, the use of inducible circadian mutant to only affect the oscillator properties of the CRB could be enlightening.

[34] Of note, the absence of *Rev-erbα* leads to several defects during the postnatal development of the cerebellar cortex (e.g., delayed differentiation of Purkinje cells), yet with no reported deficits in motor coordination in adult mice (Chomez et al. 2000)—a phenotype which contrasts to *Rorα*$^{-/-}$ mice that suffer from cerebellar ataxia (Hamilton et al. 1996).

[35] Interestingly, PER1 expression in the DMH has been shown to increase in response to food that was presented at variable and unpredictable times each day, in the absence of entrained FAA. In addition, peak of PER2 expression is observed in the DMH after mealtime. These data suggest that oscillations in the DMH are sensitive to feeding-related signals rather than synchronized by mealtime (Verwey and Amir 2009).

1.1.3 Rev-erbα deletion and noncircadian effects

It is also important not to exclude impaired additional biological functions in the observed phenotype. Circadian oscillators are considered ubiquitous and the nuclear receptor *Rev-erbα* is no exception to the rule.

In this context, oligophrenin-1, important for dendritic spine morphogenesis and hippocampus-dependent spatial memory (Khelfaoui et al. 2007), has been recently shown to interact with *Rev-erbα* (Valnegri et al. 2011). Lack of FAA in *Rev-erbα*$^{-/-}$ mice could hence result from disrupted higher order cognitive processes and associative learning—and their interactions with the circadian system[36]. This view is supported by studies on *Cry1*$^{-/-}$*;Cry2*$^{-/-}$ mice, which show altered onset and robustness of FAA (Iijima et al. 2005) concomitant to a lack of their ability to achieve time-place learning (Van der Zee et al. 2008). This is also supported by reports on *Npas2*$^{-/-}$ mice that demonstrate deficits in contextual and cues memory (Garcia et al. 2000) as well as delayed development of FAA in response to RF (unpublished data and Dudley et al. 2003). We have not investigated yet, however, cognitive defects (such as the ability to discriminate and remember a circadian phase) as a potential confounding effect of the disrupted FAA in *Rev-erbα*$^{-/-}$ mice[37].

Another element that could account for the reduced FAA in *Rev-erbα*$^{-/-}$ mice concerns mood and anxiety disorders. Mice harboring mutations in clock-related genes have interesting phenotypes (Easton et al. 2003; Roybal et al. 2007; Li et al. 2009). In particular, mice overexpressing GSK3β are considered as a good model of hyperactivity and mania (Prickaerts et al. 2006). At least, what we have noticed is that *Rev-erbα* deficient mice do not suffer from a loss of motivation to eat after a period of food withdrawal or an aversion to consume a palatable snack (personal observations). However, we still have to assess more specifically motivation-oriented behaviors. On the other hand, we have evaluated anxiety and depressive-like behaviors in *Rev-erbα*$^{-/-}$ mice and found no noteworthy difference in comparison to wild-type littermates (unpublished data, see appendix 2).

[36] And vice-versa; indeed, the recourse to higher order cognitive processes and associative learning could contribute to the apparition of residual FAA in the presence of a dysfunctional FEO (network).

[37] As an aside, *Per2*^{Brdm1} mutant mice that exhibit abolished FAA (Feillet et al. 2006), show normal spatial and contextual learning (Zueger et al. 2006). However, the time-of-day regulation of memory has not yet been studied in *Per2* mutant mice.

1.1.4 Rev-erbα deletion and energy balance

As aforementioned in the introduction, studies on the circadian nature of the FEO can also be inconclusive due to the strong interactions between the circadian network and metabolism.

In our study *Rev-erbα*-deficient mice were healthy as evidenced by similar body mass, water/food intake, and general locomotor activity. Nevertheless, we revealed significant changes in bodily functions such as glucose metabolism, ketone bodies utilization and heat production, either on AL or on RF conditions. Moreover, the responses of several hypothalamic nuclei to food access were not clearly observed at the level of p-ERK expression.

These elements could contribute to altered food entrainment as well, and are discussed in the following part.

2. The energy homeostasis of *Rev-erbα* knockout mice

In the last decades, tight connections between clock and metabolic cycles have been revealed. Body's functions and body's energy levels cycle along the day. The SCN pacemaker and slave oscillators timely coordinate metabolic processes to ensure their occurrence at the right time of the day. *Rev-erbα,* a gear of the circadian clockwork, holds a prominent role in this regard.

2.1 Key findings discussed

Rev-erbα$^{-/-}$ mice show higher blood glucose values than those of control mice across the whole 24-h cycle and after a fasting period as well. However, this is not due to decreased sensitivity to exogenous insulin, decreased production of endogenous insulin or increased hepatic glucose production in the absence of *Rev-erbα*[38]. Instead, indirect calorimetric measurements of resting energy expenditure in AL conditions and a 24-h fast challenge indicate that *Rev-erbα*$^{-/-}$ mice rely heavily on fatty acids as a source of energy.

In addition, *Rev-erbα*$^{-/-}$ mice fed with a standard normocaloric diet exhibit increased adiposity that can be severely enhanced under high-fat diet regimen. They also develop hyperlipidemia and hyperleptinemia, regardless of the feeding conditions. Their defective energy homeostasis was a consequence of abnormal timing in the liver clockwork, as evidenced by a slight delayed in lipogenesis during the night period and altered expression of lipogenic genes. Moreover, *lipoprotein lipase* (*Lpl*) gene expression is significantly enhanced in peripheral oscillators following *Rev-erbα* depletion, thus contributing to altered lipid homeostasis (i.e., lipid utilization and storage).

These results highlight the significance of daily variations in clock gene expression for the control of metabolic gene expression, essential for fuel balance.

[38] Of note, no sex differences were observed regarding our *in vivo* glucose homeostasis studies, apart from higher blood glucose values in *Rev-erbα*$^{-/-}$ males than in *Rev-erbα*$^{-/-}$ females, which was also observed between wild-type males and females. Moreover, physiological and molecular data following the 24-h fasting and refeeding experiments were analyzed with no sex differences, apart from *Pepck* mRNA expression that was stronger in *Rev-erbα*$^{-/-}$ females than *Rev-erbα*$^{-/-}$ males (unpublished data).

2.1.1 Rev-erbα and glucose metabolism

Depletion of *Rev-erbα* by using small interfering RNA (siRNA) molecules has been shown to increase the expression of *G6pc* and *Pepck* mRNA in a human hepatoma cell line (Yin et al. 2007). Of note, *Rev-erbα* overexpression has the opposite effect. The human *G6pc* promoter contains a RORE site, important for the repressive action of *Rev-erbα.* Moreover, hemin treatment also diminishes gluconeogenic gene expression and glucose production *in vitro*, demonstrating the metabolic relevance of heme binding to *Rev-erbα*. These results suggest that *Rev-erbα* may play a role in the daily variation of gluconeogenesis (Yin et al. 2007). In that regard, we observed that *Rev-erbα* deletion affects glucose levels *in vivo*, in animals fed AL[39], fasted overnight, fasted for 4 h (from ZT16 to ZT0; unpublished data) or for 24 h. However, this is not due to increased gluconeogenesis as first hypothesized. Indeed, neither *Pepck* nor *G6pc* mRNA are significantly elevated in the liver of *Rev-erbα*$^{-/-}$ mice (our results and Le Martelot et al. 2009) or decreased in the liver of *Rev-erbα* overexpressing mice (Le Martelot et al. 2009). Likewise, *in vitro* hepatic glucose production from *Rev-erbα*$^{-/-}$ hepatocytes is not enhanced in the presence of lactate and pyruvate.

Recently, the pancreatic islets have been shown to exhibit robust oscillations of clock gene, and deletion of *Clock* or *Bmal1* has deleterious consequence on *β*-cells function (Marcheva et al. 2010). Interestingly, *Rev-erbα* depletion by siRNA treatment in islets cells impairs glucose-induced insulin secretion and *β*-cell proliferation (Vieira et al. 2011). However, we did not detect alterations in pancreas physiology that could explain the mild hyperglycemia in *germline* KO mice. It is thus not improbable that metabolic regulations (in relation with the circadian system) are reorganized to some extent during development (i.e., compensatory mechanisms as discussed above) in the absence of *Rev-erbα.*

Since we have done metabolic tests around the clock to evaluate glucose homeostasis and have explored the hormonal system (e.g., insulin, glucagon and glucocorticoïds) that regulate glucose balance, without being able to find strong

[39] Review of the literature and our data indicate strong inter-study variations of the blood glucose acrophase in AL-fed C57BL6/J wild-type mice under 12:12 LD cycle, making difficult to assess alterations in the *daily pattern* of blood glucose. We did not investigate the daily/circadian blood glucose rhythm in fasted animals.

defects that could account for the recurring mild hyperglycemia seen in *Rev-erbα*$^{-/-}$ mice, we favor the presence of altered timing in energy substrate utilization.

2.1.2 Rev-erbα and lipid metabolism

As mentioned in the introduction, the nuclear receptor *Rev-erbα* has been shown to play a role in bile acid, cholesterol and lipoprotein metabolism, particularly by controlling the expression of key components of metabolic pathways.

Regarding lipoprotein metabolism, liver *ApoA1* expression[40] can be regulated by *Rev-erbα in vitro* (Vu-Dac et al. 1998) and *in vivo* (Le Martelot et al. 2009). *Apoc3* transcript[41] has also been demonstrated to be controlled by *Rev-erbα in vitro* and to be up-regulated in the liver of *Rev-erbα*$^{-/-}$ mice (Raspe et al. 2002)[42], an observation, however, not confirmed by others (our results and Le Martelot et al. 2009). Further in the lipoprotein metabolism, we found that the *Lpl* transcript is consistently elevated in three peripheral tissues of *Rev-erbα*$^{-/-}$ mice. The up-regulation of *Lpl* is not directly a consequence of abolished repressive action of *Rev-erbα* on *Lpl* transcription, but involves enhanced CLOCK-BMAL1 transactivation on the *Lpl* promoter. Intriguingly, the expression of *Lpl-luc* reporter could be elicited by CLOCK alone. Whether CLOCK acts directly (as an E-Box binding partner for a protein endogenously present in COS7 cells) or indirectly (e.g. through its acetyltransferase activity; see Doi et al. 2006; Nader et al. 2009) remains to be established. At least, co-transfection with CLOCK-BMAL2 does not induce more luciferase activity than transfection with CLOCK alone (unpublished data, Dardente Hugues). Conversely, the role of CLOCK alone in the modulation of clock and metabolic gene transcription has been previously observed (Oishi et al. 2005; Cai et al. 2008; Nader et al. 2009; Doi et al. 2010; Shi et al. 2010). It is also important to mention that a recent study demonstrate that small hairpin RNA-mediated knockdown of *Rev-erbβ* potentiates the up-regulation of *Lpl* in the liver of *Rev-erbα*$^{-/-}$ mice (Bugge et al. 2012)—although *Clock* mRNA level has not been evaluated in this study.

[40] APOA1 is a major protein component of high density lipoprotein in plasma.

[41] APOC3 is a protein component of very low density lipoprotein. It is has been shown to inhibit lipoprotein lipase and hepatic lipase *in vitro*, however, APOC3 could have rather little effect *in vivo* (Mendivil et al. 2010).

[42] It is important to mention that genetic variations between mouse stains can contribute to different metabolic phenotype in mutant mice.

Worth to mention, as well, is the induction of *Lpl* in other circadian mutants (Shimba et al. 2011). Indeed, *Bmal1*$^{-/-}$ and liver-specific *Bmal1*$^{-/-}$ mice display a startling up-regulation of hepatic *Lpl* mRNA expression[43], while muscle *Lpl* expression remains unchanged in both mutant mice—however, again, without data on *Clock* mRNA expression. In addition, in primary hepatocytes from control animals, a two-fold reduction of *Bmal1* expression via siRNA knockdown, which is associated with a two-fold decrease of *Rev-erbα* mRNA levels without affecting *Clock* expression, has a slight effect on *Lpl* transcript level. None mechanistic explanation has been proposed in that study (Shimba et al. 2011). Additional studies show: *1)* that BMAL1 overexpression in 3T3-L1 adipocytes has no consequence on the expression of the *Lpl* gene (Shimba et al. 2005); *2)* that *Rev-erbα* and *Rev-erbβ* are nearly undetectable at all time points in *Bmal1*$^{-/-}$ animals (Kondratov et al. 2006; Grechez-Cassiau et al. 2008) and *3)* that the circadian oscillations of the nuclear accumulation and degradation of CLOCK have been suggested to depend on BMAL1 (Kondratov et al. 2003; Kwon et al. 2006). Thus, further studies are needed to shed more light on the importance of the molecular clockwork for the daily control of *Lpl* expression with regard to the possible contribution of other pathways (Schoonjans et al. 1996; Schoonjans et al. 2000; Gachon et al. 2011).

> "Once we succeed in finding the clock gene … boy, we'd lick the problem of mechanisms …" C. Pittendrigh to M.K. Chandrashekaran in Berkeley, 1968 (mentioned in Chandrashekaran 1998).

[43] In the study of Shimba et al. 2011 - Figure 7, the normalized *Lpl* mRNA levels to the 36b4 levels are quite similar among peripheral tissues. In addition, the acrophase of *Lpl* mRNA (see the daily and circadian patterns of *Lpl* expression) are 6-h phase-delayed compared to our results, those of Le Martelot et al. 2009 and Gachon et al. 2011. Besides, *Lpl* levels are similar to those of *Bmal1* in control animals, while when *L-Bmal*$^{-/-}$ and control mice are compared, *Lpl* levels are just above 0 in control animals. All these elements are hard to understand and raise concerns about the normalization method and the *Lpl* primer sequences. Furthermore, regardless of *Lpl* overexpression, the body mass of *Bmal1*$^{-/-}$ mice was much lower than that of control littermates, both in chow-fed and high-fat-fed conditions, which deeply contrasts with independent studies (Lamia et al. 2008; Guo et al. 2012). Discrepancies between studies that specifically evaluate adjustment to environmental changes and energy metabolism can result from the development of diseases due to pathogen exposition. All our experiments were done with mice raised in a pathogen-free facility.

As evidenced by the evaluation of energy expenditure in chow-fed mice and their circulating energy substrates following a 24-h challenge, *Lpl* overexpression leads to increased utilization of fatty acids as energy source in *Rev-erbα*$^{-/-}$ mice, thus sparing glucose use as shown by higher blood glucose levels and glycogen stores in chow-fed, RF and fasting conditions[44]. Since the resting muscle is a major contributor of fatty acid oxidation, we analyzed metabolic gene expression in this tissue. We showed no significant decrease in the capacity of the muscle of *Rev-erbα*$^{-/-}$ mice to oxidize fatty acids, regarding *Cpt1b* mRNA expression. Along with overexpressed *Lpl* mRNA, we found that *Cd36* transcript is also up-regulated in the muscle of *Rev-erbα*$^{-/-}$ mice. These findings thus support enhanced fatty acids entry into muscular cells, although we did not evaluate LPL activity in muscle tissues of *Rev-erbα*$^{-/-}$ mice or *in vivo* metabolic flux. Our results are consistent with the previously described shift towards an oxidative muscle phenotype in *Rev-erbα*$^{-/-}$ mice. Indeed, the type I MyHc isoform in the slow-twitch soleus muscle (mainly oxidative) and the type I fiber area in the fast-twitch extensor digitorum longus muscle (oxidative and highly glycolytic) were found to be increased in *Rev-erbα*$^{-/-}$ mice (Pircher et al. 2005). Incidentally, the importance of the circadian nuclear receptors in skeletal muscle cells is also highlighted by the role of *Rorα* in lipid homeostasis[45] (Lau et al. 2004) and the control of genes important for lipid absorption (e.g., *Cd36*) by *Rev-erbβ* (Ramakrishnan et al. 2005).

The overexpression of *Lpl* also favors fat accumulation. We found that chow-fed *Rev-erbα*$^{-/-}$ mice suffer from dyslipidemia, in line with previous reports (Raspe et al. 2002; Le Martelot et al. 2009). In addition, *Rev-erbα*$^{-/-}$ mice have severe adipose accumulation in chow-fed conditions and are more prone to HFD-induced obesity compared to control mice[46], likely due to enhanced LPL-mediated fatty acids uptake. The level of hepatic triglycerides at the day-night transition is also elevated in these mice (our results and Feng et al. 2011; Bugge et al. 2012). Whether increased hepatic TG is due to increased *Lpl* mRNA expression remains unclear, considering that the capacity of the normal adult liver to synthesize LPL and its role are still a

[44] We did not, however, evaluate energy expenditure in fasted *Rev-erbα*$^{-/-}$ animals.
[45] Of note, *Rorα*$^{-/-}$ mice expressed similar levels of *Lpl* mRNA in skeletal muscle in comparison to wild-type animals.
[46] Interestingly, 3 mo. old *Rev-erbα*$^{-/-}$ mice high-fat-fed for 12 w also developed an obesity phenotype and severe dyslipedemia in contrast to their controls that did not (i.e., high-fed *Rev-erbα*$^{+/+}$ mice and chow-fed *Rev-erbα*$^{-/-}$ mice; unpublished data).

subject of debate (Vilaro et al. 1986; Vilaro et al. 1988; Benavides et al. 1998; Merkel et al. 1998; Merkel et al. 2002). However, several works have shown that hepatic *Lpl* up-regulation can contribute to hepatic steatosis in humans and rodents (Kim et al. 2001; Westerbacka et al. 2007; Pardina et al. 2009; Barclay et al. 2011). Of note, the fatty acid transporter *Fabp5* is up-regulated in the liver of *Rev-erbα*$^{-/-}$ mice (Le Martelot et al. 2009). Moreover, we showed that HFD induces an up-regulation of *Cd36* mRNA only in *Rev-erbα*$^{-/-}$ mice. Hence, it is clear that hepatic fatty acid transport is altered in the absence of *Rev-erbα*.

Emblematic of significant changes in lipid homeostasis in the liver of *Rev-erbα-/-* mice, its capacity of lipogenesis is also modified. We demonstrate *in vivo* that *Rev-erbα* depletion results in a change in RQ values during the active/feeding period—in line with the described phase-shifted expression of lipogenic genes in the late night (Le Martelot et al. 2009) and the importance of genomic recruitment of HDAC3 by *Rev-erbα* on a daily basis to ensure fatty acid synthesis at the most appropriate time of the day (Feng et al. 2011). Indeed, the acrophase of RQ values is slightly delayed in *Rev-erbα*$^{-/-}$ mice and RQ values are higher compared to control littermates during nighttime, indicative of enhanced hepatic *de novo* lipogenesis[47]. Interestingly, the importance of *Rev-erbα* in the control of lipogenesis is highlighted by several studies. The circadian transcription activity of *Elovl3,* which plays a role in fatty acid chain elongation, has been suggested to be mediated by *Rev-erbα* (Anzulovich et al. 2006; Le Martelot et al. 2009). Additionally, *Elovl6* mRNA expression is phase-shifted in the liver of *Rev-erbα*$^{-/-}$ mice (Le Martelot et al. 2009). These results support a modulating role for *Rev-erbα* in the synthesis of very long chain fatty acids. In this context, we showed that *Elovl6* mRNA is severely increased in the liver of high-fat fed *Rev-erbα*$^{-/-}$ mice. Additional lipogenic genes (i.e., *Fas, Acc*) are up-regulated as well. Incidentally, this elevated expression of lipogenic genes in *Rev-erbα*$^{-/-}$ mice could also be an adaptive response to energy surplus (Tepperman and Tepperman 1965) and reflect a remodeling of hepatic fatty acids (Oosterveer et al. 2009).

[47] The liver is the main organ that removes the glucose from the blood. It is the principle site of *de novo* lipogenesis.

2.1.3 Rev-erbα and developmental defects

We have demonstrated that the consistent overexpression of *Lpl* in liver, adipose tissue and skeletal muscle, results from a defective clock control of this gene. However, beyond clock impairment, we do not entirely exclude growth and developmental defects that could contribute to the metabolic phenotype of *Rev-erbα*$^{-/-}$ mice.

As previously introduced, both REV-ERBs are crucial during developmental stages. In particular, REV-ERBα is essential for the appropriate balance of transcriptional activators and repressors during postnatal cerebellar development (Chomez et al. 2000). REV-ERBβ has been suggested to be important for latter aspects of neuronal development, such as axon guidance, and at later stages of muscle differentiation (Bonnelye et al. 1994). REV-ERBα expression was linked to muscle fiber composition (Pircher et al. 2005) and both *Rev-erbα* and *Rev-erbβ* have been shown to play a role in skeletal muscle growth and differentiation, the former notably by regulating the *MyoD* and *Myogenin* genes (Downes et al. 1995; Burke et al. 1996). Besides, *Rev-erbα* mRNA has been shown to increase during differentiation of 3T3-L1 cells into adipocytes (Chawla and Lazar 1993; Fontaine et al. 2003), although this is more likely the proteasomal degradation of the REV-ERBα protein that is critical for late stages of adipocyte differentiation (Wang et al. 2008).

Regarding muscle development, additional studies give evidence that interactions between clocks component and actors of muscle physiology are crucial for proper muscle function. Indeed, *MyoD*, a regulator of myogenesis, is regulated by CLOCK and BMAL1 (Andrews et al. 2010). Moreover, *Bmal1*-deficient mice develop sarcopenia (Kondratov et al. 2006). *Clock*$^{\Delta 19}$ and *Bmal1*$^{-/-}$ mice exhibit reduced skeletal muscle specific tension concomitant to altered myofilament architecture (Andrews et al. 2010). Muscle performance and metabolism are also affected in m*Per2*$^{-/-}$ mice (Bae et al. 2006). On the other hand, the importance of circadian genes and adipose functions are also highlighted by several studies. High-fat-fed *Clock*$^{\Delta 19}$ mice develop adipocyte hypertrophy relative to wild-type controls (Turek et al. 2005). Lack of PER2 results in enhanced adipocyte differentiation of cultured fibroblasts and increased TG contents in WAT in mice (Grimaldi et al. 2010). Disruption of the *Bmal1* gene also leads to increased total body fat content (Lamia et al. 2008; Shi et al.

2010) that is amplified under HFD due to up-regulation of adipogenic factors and down-regulation of genes of the canonical Wnt signaling pathway, important to suppress adipogenesis (Ross et al. 2000; Guo et al. 2012).

The implication of *Rev-erbα* in specific developmental pathways and its capacity to modulate adipogenesis and muscle development were first considered with no overt effects on WAT expansion and the organization of aerobic, intermediate and anaerobic muscle fibers in adult *Rev-erbα*$^{-/-}$ mice (Chomez et al. 2000). However, these mice do display increased adiposity, and altered MyHC isoform expression in skeletal muscle[48] (Pircher et al. 2005), that contribute to enhanced oxidative metabolism. To what extent the involvement of *Rev-erbα* in clock-independent functions has contributed to the phenotype that we and other groups have observed remains to be further investigated. Indeed, while we privilege altered circadian molecular oscillations *per se*, we could not fully exclude that aberration in the early stages of adipose tissue development, such as hyperplastic growth, could have favored lipid storage, beyond *Lpl* overexpression. Besides, it is plausible that the fast-to-slow transition during muscle development in the absence of *Rev-erbα* could underlie *Lpl* up-regulation. In that perspective, *Lpl* overexpression could rather appear as a consequence of developmental defects and the role of *Clock* transactivation on the *Lpl* promoter that we demonstrate *in vitro* could rather be negligible *in vivo*. However, why does *Lpl* overexpression also occur in the liver oscillator? Indeed, in the presence of an enhanced muscle oxidative metabolism, the liver would have just likely compensated for the unused glucose by eliciting the expression of lipogenic genes, but not *Lpl.* In this context, the evaluation of *Lpl* expression in other tissues could be informative to confirm defective clock control of *Lpl*.

[48] It is not excluded that the skeletal muscle phenotype or the delayed maturation of Purkinje cells in *Rev-erbα-/-* mice may have, to some extent, contributed to their reduced wheel-running activity levels (e.g., altered energy-mobilization or altered fine motor coordination).

2.1.4 Lpl in central and peripheral tissues

"[…] *the whole is something besides the parts*" Aristotle, Metaphysics

The etiology for the mild hyperglycemia seen in chow fed, high-fat fed and fasted *Rev-erbα*$^{-/-}$ mice is believed to be caused by a greater reliance on fatty acids as energy source, particularly by the muscle. But it remains uncertain why liver or muscle *Lpl* overexpression has not induced insulin resistance (IR) in chow-fed *Rev-erbα*$^{-/-}$ mice, as in previous reports (Ferreira et al. 2001; Kim et al. 2001)[49]. This is all the more surprising because insulin sensitivity (assessed by the intraperitoneal insulin sensitivity test) was either similar or greater in high-fat-fed *Rev-erbα*$^{-/-}$ mice compared to high-fat-fed control littermates or chow-fed *Rev-erbα*$^{-/-}$ mice (unpublished data), thus excluding an evolution toward diabetes. However, not all studies show that *Lpl* overexpression induces a state of IR in mice. For instance, muscle TG content is increased without inhibition of insulin-stimulated whole-body and muscle-specific glucose uptake in muscle-specific *Lpl*-overexpressing mice. In addition, these mice exhibited a strong decrease in whole-body glucose oxidation, whereas nonoxidative glucose disposal (i.e., for glycogen and lipid synthesis) was increased during hyperinsulinemic-euglycemic clamp conditions (Voshol et al. 2001). In this study, the liver was suggested to have compensated by increasing insulin sensitivity. This is intriguing since we show in *Rev-erbα*$^{-/-}$ mice that whole-glucose insulin sensitivity is normal and that the liver may have enhanced its capacity to process glucose for fat synthesis, which is supported by a recent study (Feng et al. 2011).

Pancreatic islet cells can express *Lpl* transcript and show enzyme LPL activity (i.e., delivery of fatty acids to *β*-cells), which are regulated by glucose and insulin (Cruz et al. 2001). However, higher LPL activity in *β*-cell has been predicted to impair cell function such as insulin release and to promote lipotoxicity and apoptosis (Cruz et al. 2001). More recently, the effect of *Lpl* depletion and overexpression in pancreatic islets has been investigated. *β*-cell-specific *Lpl*-overexpressing mice had normal glucose tolerance until 5 months of age, while *β*LPL-KO mice display

[49] Of note, in Kim et al. 2001, no effect of liver-*Lpl* overexpression was observed on total body insulin-stimulated glucose uptake. However, the steady-state glucose infusion rate (average rate from 90 to 120 min) required to maintain euglycemia was significantly decreased in the liver-LPL mice during hyperinsulinemic–euglycemic clamp.

defective glucose tolerance from 1 month of age. Additionally, the increased LPL activity has been shown to reduce glucose oxidation in islets (Pappan et al. 2005). At least, our observations of unaltered glucose-induced insulin secretion, plasma insulin levels, as well as number, morphology and insulin content of pancreatic islets in *Rev-erbα*$^{-/-}$ mice, do not support a strong effect of a putative *Lpl* overexpression in pancreas. Incidentally, in rabbits, systemic overexpression of human LPL transgene (under the control of the actin promoter) has been shown to increase whole body insulin sensitivity (Liu et al. 2005) and protect from HFD-induced IR (Kitajima et al. 2004). It has been also suggested that LPL could modulate insulin signal pathway (e.g., in muscle), via a direct action on the insulin receptor substrate 1 (Liu et al. 2005). However, we did not evaluate total plasma LPL or the intracellular handling of glucose in *Rev-erbα*$^{-/-}$ mice (e.g., in the absence of altered insulin-mediated glucose transport, a disrupted glycolytic pathway could have spared glucose in favor of lipid utilization[50]). Besides, we have to consider that the adverse effect of *Lpl*/LPL up-regulation may be species-dependent—especially considering that systemic *Lpl* overexpression can have beneficial effects by correcting hyperlipidemia and reducing body fat accumulation in high-fat-fed rabbits (Koike et al. 2004).

The role of *Lpl* in the nervous system has been particularly highlighted recently. Mice with neuron-specific deletion of *Lpl* have reduced *Lpl* mRNA and LPL enzyme activity in both the hypothalamus and hippocampus and display altered energy balance, as reflected by increased fat mass and lower daily average metabolic rate—correlated with a substantial reduction in physical activity. Interestingly, LPL deficiency promotes the expression of the AgRP orexigenic peptide in the hypothalamus. From their findings, the authors proposed that TG-rich lipoproteins are sensed in the brain by a LPL-dependent mechanism (Wang et al. 2011). What would happen in the hypothalamus of *Rev-erbα*$^{-/-}$ mice if *Lpl* was also up-regulated? Does hypothalamic sensing of fatty acids would have been affected? This is interesting since central administration of oleic acid has been shown to inhibit food intake (Obici et al. 2002). Regarding the hypothalamus, we observed that food restriction does not elicit the same metabolic responses in *Rev-erbα*$^{-/-}$ mice as in control littermates. Indeed, daily p-ERK expression was shown to be slightly altered

[50] In addition, disrupted brain control of daily glucose homeostasis may also have contributed to steady hyperglycemia in *Rev-erbα*$^{-/-}$ mice.

in several hypothalamic areas in both AL and RF conditions, which can suggest that circulating signals are not integrated similarly or are different in *Rev-erbα*$^{-/-}$ mice. In this context, we observed that plasma leptin levels are not severely decreased following 24-h fast in *Rev-erbα*$^{-/-}$ mice (unpublished data). Of note, this absence of fasting-induced reduction of leptin levels has been suggested to be associated with low corticosterone levels (Jeong et al. 2004)—which are observed in *Rev-erbα*$^{-/-}$ mice under RF schedules. Considering that low leptin levels signal low energy stores and facilitate entry into torpor (Ahima et al. 1996; Gavrilova et al. 1999), the relative normothermia in food-deprived *Rev-erbα*$^{-/-}$ mice finds a first explanation[51]. Besides, the loss of hunger due to an altered starvation-sensing system may explain their defect to develop strong food-seeking behaviors—without altered food consumption after food deprivation. It would have been interesting to assess the time course of energy substrate utilization during starvation. Additionally, the characterization of hypothalamic neurons implicated in feeding and energy balance (e.g., in the ARC neurons, which are particularly sensitive to feeding-related signals such as leptin and ghrelin) could be informative, more especially as clock mutation can modify the levels and temporal expression of neuropeptides (see part 4.3.1). In that regard, AgRP is a good candidate considering that it is activated by ghrelin (Nogueiras et al. 2008) and that its expression is increased prior to mealtime (Yoshihara et al. 1996; Crowley et al. 2005). Moreover, AgRP neurons are sufficient to orchestrate feeding behavior (Aponte et al. 2011; Krashes et al. 2011). Besides AgRP neuronal activity has been shown to adapt in response to signals from hormones and metabolites such as ghrelin, leptin, glucose and fatty acids (mentioned in Sternson 2011).

[51] Of note, we cannot exclude either an up-regulation of *Lpl* transcript in the BAT of *Rev-erbα*$^{-/-}$ mice to explain their greater capacity to sustain normal body temperature levels during a period of food deprivation or during cold exposition. Indeed, LPL supplies BAT with fatty acids to sustain nonshivering thermogenesis (Klinegenspor et al. 1996).

3. Conclusion and perspectives

The involvement of the nuclear receptor *Rev-erbα* in circadian functions is undeniable. Indeed, its absence *in vivo* leads to a reduction of the SCN period length (Preitner et al. 2002; Cho et al. 2012) and to an enhanced shifting response to light exposure, along with disrupted SCN molecular clockwork due to blunted *Bmal1* expression (Preitner et al. 2002). *Rev-erbα* expression has been shown to be differentially sensitive, in regard to the peripheral tissue considered, to jet-lag (Kiessling et al. 2010). Moreover, food schedules differentially affect *Rev-erbα* expression in normal mice (Damiola et al. 2000; Filipski et al. 2005; Feillet et al. 2006).

In this *circadian* context, we further evaluate the involvement of well-known canonical clock genes in the molecular mechanisms of the FEO. We showed that *Rev-erbα* deletion *in vivo* leads to altered food-entrainable physiology, as evidenced by decreased locomotor activities as well as an absence of a rise in body temperature, corticosterone and ketone bodies prior to mealtime. These findings, however, were not correlated with disrupted molecular clockwork in the liver or DMH, the latter being likely a candidate for the development and expression of food anticipation (Gooley et al. 2006; Acosta-Galvan et al. 2011; Landry et al. 2011; Verhagen et al. 2011). Incidentally, the actual view privileges rather a spatially distributed FEO particularly at the central level. In that respect, our evaluation of PER2 oscillations in other hypothalamic nuclei essential to regulate energy balance and feeding behavior, did not allow us to detect altered phase-adjustment of PER2 expression in response to RF. Activation of hypothalamic neurons as measured through c-FOS expression in phase with feeding time has been described in several studies (Angeles-Castellanos et al. 2004; Gooley et al. 2006; Ribeiro et al. 2007). Our data on p-ERK activation in control mice, for the first time used to assess the effect of RF, further suggest that the FEO is integrated into a network of oscillators. Of interest, in *Rev-erbα*$^{-/-}$ mice the peak of p-ERK in phase with mealtime is not clearly observed as in their control littermates. In addition, we uncover a potential role for *Rev-erbα* in the food synchronization of the cerebellar clockwork. Yet, the relative importance of clock genes as well as the CRB structure for food entrainment remains to be further explored. Hence, an approach could be to selectively disrupt the *Rev-*

erbα clock gene in the Purkinje cells of the CRB, to evaluate the exact participation of the CRB oscillator to food entrainment and its capacity to generate an output signal to synchronize the activity of other components of the FEO network. In addition, since *Rev-erbα* depletion leads only to a mild reduction of FAA, and considering the recently revealed cooperative role of both *Rev-erbs* isoforms in circadian clock function (Cho et al. 2012), it would be enlightening to evaluate the phenotype of double KO mice (i.e., for both *Rev-erbα* and *Rev-erbβ*) regarding food entrainment.

The involvement of the nuclear receptor *Rev-erbα* in *metabolism* is also patent. Indeed, *Rev-erbα* acts to fine-tune metabolic pathways, by modulating SREBP target genes and by recruiting HDAC3 to the genome on a daily basis, both being important for the control of lipid metabolism (Le Martelot et al. 2009; Feng et al. 2011). Our results demonstrate that *in vivo* deletion of the circadian component *Rev-erbα* results in profound metabolic changes. Chow-fed *Rev-erbα*$^{-/-}$ mice display increased adiposity, mild hyperglycemia concomitant to a greater reliance on fatty acids as energy source with non-significant daily changes in energy intake or energy expenditure. We uncover a molecular pathway that ties together CLOCK expression to the 24-h rhythmic accumulation of the *Lpl* transcript. Disruption of this CLOCK-*Lpl* interaction due to elevated CLOCK expression in the absence of *Rev-erbα* is likely to have caused higher oxidative muscle metabolism and enhanced fat storage in the WAT and the liver of *Rev-erbα*$^{-/-}$ mice. Furthermore, this imbalance in fuel utilization predisposes *Rev-erbα*$^{-/-}$ mice to HFD-induced obesity, as supported by enhanced hepatic *de novo* lipogenesis and adipose fat storage in these mice. These results should be considered in the light of the recent study demonstrating that *in vivo* administration of synthetic REV-ERB agonists have positive effects on lipid and glucose oxidation. Moreover, these drugs are effective to reduce lipogenesis and to protect mice from the detrimental effects of HFD (Solt et al. 2012). Collectively, these findings and ours demonstrate that *Rev-erbα* is a key gene for the regulation of energy balance. In the future, it would be attractive to target *Rev-erbα* to limit circadian disorders and metabolic defects. In the same context, the relevance of clock-dependent and -independent pathways for the modulation of (daily) *Lpl* expression—and fuel balance—have to be strengthened by further investigations.

To summarize, our two studies give novel evidence that *Rev-erbα* is fundamental for an accurate crosstalk between the circadian system and metabolism. The use of germline KO for our investigations of food entrainment cannot rule out that the significant changes in the internal energy status of our mice may be a confounding effect for the observed reduction of food-anticipatory components. Indeed, we cannot fully argue that *Rev-erbα* is essential to shape the mice's 24-hour pattern of activity in limited food access conditions, since these mice do not display decreased hepatic glycogen reserves, plasma glucose and leptin levels, and body temperature values during periods of food deprivation. These body signals are likely essential to convey the energy status of an organism for instance to the hypothalamus, where nutrient-dependent signals are integrated and transduced into efferent signals—including food-seeking behaviors. Thus, it seems necessary to make use of brain-specific and inducible knockout, to solve the long-lasting and unresolved enigma regarding the (circadian) nature of the FEO (Fig. 57)—a strategy that we have already adopted.

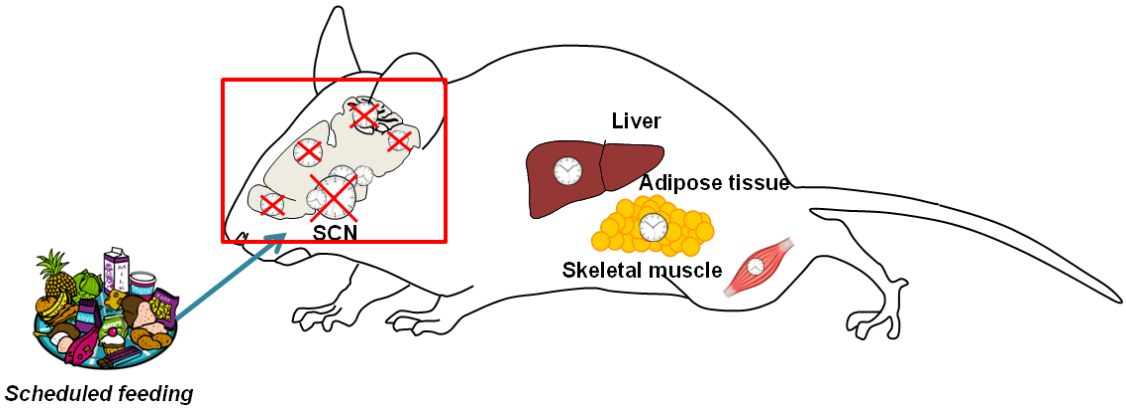


Figure 57. Study of the food-entrainable oscillator network in brain-specific *Rev-erbα* KO mice

We will evaluate several behavioral and physiological food-entrained rhythms in mice lacking *Rev-erbα* in neurons. The effect of scheduled feeding on central oscillators will be evaluated as well.

Abe, H. and B. Rusak (1992). "Anticipatory activity and entrainment of circadian rhythms in Syrian hamsters exposed to restricted palatable diets." Am J Physiol **263**(1 Pt 2): R116-24.

Abe, M., E. D. Herzog, S. Yamazaki, M. Straume, H. Tei, Y. Sakaki, M. Menaker and G. D. Block (2002). "Circadian rhythms in isolated brain regions." J Neurosci **22**(1): 350-6.

Acosta-Galvan, G., C. X. Yi, J. van der Vliet, J. H. Jhamandas, P. Panula, M. Angeles-Castellanos, M. Del Carmen Basualdo, C. Escobar and R. M. Buijs (2011). "Interaction between hypothalamic dorsomedial nucleus and the suprachiasmatic nucleus determines intensity of food anticipatory behavior." Proc Natl Acad Sci U S A **108**(14): 5813-8.

Adelmant, G., A. Begue, D. Stehelin and V. Laudet (1996). "A functional Rev-erb alpha responsive element located in the human Rev-erb alpha promoter mediates a repressing activity." Proc Natl Acad Sci U S A **93**(8): 3553-8.

Ahima, R. S. and M. A. Lazar (2008). "Adipokines and the peripheral and neural control of energy balance." Mol Endocrinol **22**(5): 1023-31.

Ahima, R. S., D. Prabakaran, C. Mantzoros, D. Qu, B. Lowell, E. Maratos-Flier and J. S. Flier (1996). "Role of leptin in the neuroendocrine response to fasting." Nature **382**(6588): 250-2.

Akhtar, R. A., A. B. Reddy, E. S. Maywood, J. D. Clayton, V. M. King, A. G. Smith, T. W. Gant, M. H. Hastings and C. P. Kyriacou (2002). "Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus." Curr Biol **12**(7): 540-50.

Akiyama, M., T. Yuasa, N. Hayasaka, K. Horikawa, T. Sakurai and S. Shibata (2004). "Reduced food anticipatory activity in genetically orexin (hypocretin) neuron-ablated mice." Eur J Neurosci **20**(11): 3054-62.

Albrecht, U., Z. S. Sun, G. Eichele and C. C. Lee (1997). "A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light." Cell **91**(7): 1055-64.

Albrecht, U., B. Zheng, D. Larkin, Z. S. Sun and C. C. Lee (2001). "MPer1 and mper2 are essential for normal resetting of the circadian clock." J Biol Rhythms **16**(2): 100-4.

Alenghat, T., K. Meyers, S. E. Mullican, K. Leitner, A. Adeniji-Adele, J. Avila, M. Bucan, R. S. Ahima, K. H. Kaestner and M. A. Lazar (2008). "Nuclear receptor corepressor and histone deacetylase 3 govern circadian metabolic physiology." Nature **456**(7224): 997-1000.

Altimus, C. M., A. D. Guler, N. M. Alam, A. C. Arman, G. T. Prusky, A. P. Sampath and S. Hattar (2010). "Rod photoreceptors drive circadian photoentrainment across a wide range of light intensities." Nat Neurosci **13**(9): 1107-12.

Ando, H., M. Kumazaki, Y. Motosugi, K. Ushijima, T. Maekawa, E. Ishikawa and A. Fujimura (2011). "Impairment of peripheral circadian clocks precedes metabolic abnormalities in ob/ob mice." Endocrinology **152**(4): 1347-54.

Ando, H., H. Yanagihara, Y. Hayashi, Y. Obi, S. Tsuruoka, T. Takamura, S. Kaneko and A. Fujimura (2005). "Rhythmic messenger ribonucleic acid expression of clock genes and adipocytokines in mouse visceral adipose tissue." Endocrinology **146**(12): 5631-6.

Andrews, J. L., X. Zhang, J. J. McCarthy, E. L. McDearmon, T. A. Hornberger, B. Russell, K. S. Campbell, S. Arbogast, M. B. Reid, J. R. Walker, J. B. Hogenesch, J. S. Takahashi and K. A. Esser (2010). "CLOCK and BMAL1 regulate MyoD and are necessary for maintenance of skeletal muscle phenotype and function." Proc Natl Acad Sci U S A **107**(44): 19090-5.

Angeles-Castellanos, M., R. Aguilar-Roblero and C. Escobar (2004). "c-Fos expression in hypothalamic nuclei of food-entrained rats." American Journal of Physiology-Regulatory Integrative and Comparative Physiology **286**(1): R158-R165.

Angeles-Castellanos, M., J. Mendoza and C. Escobar (2007). "Restricted feeding schedules phase shift daily rhythms of c-Fos and protein Per1 immunoreactivity in corticolimbic regions in rats." Neuroscience **144**(1): 344-55.

Angeles-Castellanos, M., R. Salgado-Delgado, K. Rodriguez, R. M. Buijs and C. Escobar (2008). "Expectancy for food or expectancy for chocolate reveals timing systems for metabolism and reward." Neuroscience **155**(1): 297-307.

Anzulovich, A., A. Mir, M. Brewer, G. Ferreyra, C. Vinson and R. Baler (2006). "Elovl3: a model gene to dissect homeostatic links between the circadian clock and nutritional status." J Lipid Res **47**(12): 2690-700.

Aponte, Y., D. Atasoy and S. M. Sternson (2011). "AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training." Nature Neuroscience **14**(3): 351-355.

Arble, D. M., J. Bass, A. D. Laposky, M. H. Vitaterna and F. W. Turek (2009). "Circadian timing of food intake contributes to weight gain." Obesity (Silver Spring) **17**(11): 2100-2.

Arendt, J. (2010). "Shift work: coping with the biological clock." Occup Med (Lond) **60**(1): 10-20.

Aschoff, J. (1960). "Exogenous and endogenous components in circadian rhythms." Cold Spring Harb Symp Quant Biol **25**: 11-28.

Aschoff, J. (1965). "Circadian Rhythms in Man." Science **148**(3676): 1427-32.

Asher, G., D. Gatfield, M. Stratmann, H. Reinke, C. Dibner, F. Kreppel, R. Mostoslavsky, F. W. Alt and U. Schibler (2008). "SIRT1 regulates circadian clock gene expression through PER2 deacetylation." Cell **134**(2): 317-28.

Asher, G. and U. Schibler (2011). "Crosstalk between components of circadian and metabolic cycles in mammals." Cell Metab **13**(2): 125-37.

Aton, S. J., C. S. Colwell, A. J. Harmar, J. Waschek and E. D. Herzog (2005). "Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons." Nat Neurosci **8**(4): 476-83.

Bae, K., K. Lee, Y. Seo, H. Lee, D. Kim and I. Choi (2006). "Differential effects of two period genes on the physiology and proteomic profiles of mouse anterior tibialis muscles." Mol Cells **22**(3): 275-84.

Baez-Ruiz, A., D. Luna-Moreno, O. Vazquez-Martinez, J. Ramirez-Salcedo and M. D. Munoz (2005). "The food entrainable oscillator studied by DNA microarrays: What is the liver doing during food anticipatory activity?" Biological Rhythm Research **36**(1-2): 83-97.

Baggs, J. E., T. S. Price, L. DiTacchio, S. Panda, G. A. FitzGerald and J. B. Hogenesch (2009). "Network Features of the Mammalian Circadian Clock." Plos Biology **7**(3): 563-575.

Balsalobre, A., S. A. Brown, L. Marcacci, F. Tronche, C. Kellendonk, H. M. Reichardt, G. Schutz and U. Schibler (2000). "Resetting of circadian time in peripheral tissues by glucocorticoid signaling." Science **289**(5488): 2344-7.

Barclay, J. L., C. N. Nelson, M. Ishikawa, L. A. Murray, L. M. Kerr, T. R. McPhee, E. E. Powell and M. J. Waters (2011). "GH-Dependent STAT5 Signaling Plays an Important Role in Hepatic Lipid Metabolism." Endocrinology **152**(1): 181-192.

Bargiello, T. A. and M. W. Young (1984). "Molecular genetics of a biological clock in Drosophila." Proc Natl Acad Sci U S A **81**(7): 2142-6.

Barnea, M., Z. Madar and O. Froy (2009). "High-fat diet delays and fasting advances the circadian expression of adiponectin signaling components in mouse liver." Endocrinology **150**(1): 161-8.

Barzilai, N. and I. Gabriely (2001). "The role of fat depletion in the biological benefits of caloric restriction." J Nutr **131**(3): 903S-906S.

Bassareo, V. and G. Di Chiara (1999). "Differential responsiveness of dopamine transmission to food-stimuli in nucleus accumbens shell/core compartments." Neuroscience **89**(3): 637-641.

Benavides, A., M. Siches and M. Llobera (1998). "Circadian rhythms of lipoprotein lipase and hepatic lipase activities in intermediate metabolism of adult rat." Am J Physiol **275**(3 Pt 2): R811-7.

Berson, D. M., F. A. Dunn and M. Takao (2002). "Phototransduction by retinal ganglion cells that set the circadian clock." Science **295**(5557): 1070-3.

Blander, G. and L. Guarente (2004). "The Sir2 family of protein deacetylases." Annu Rev Biochem **73**: 417-35.

Blum, I. D., Z. Patterson, R. Khazall, E. W. Lamont, M. W. Sleeman, T. L. Horvath and A. Abizaid (2009). "Reduced anticipatory locomotor responses to scheduled meals in ghrelin receptor deficient mice." Neuroscience **164**(2): 351-9.

Bodosi, B., J. Gardi, I. Hajdu, E. Szentirmai, F. Obal and J. M. Krueger (2004). "Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation." American Journal of Physiology-Regulatory Integrative and Comparative Physiology **287**(5): R1071-R1079.

Bolles, R. C. and S. A. Moot (1973). "The rat's anticipation of two meals a day." J Comp Physiol Psychol **83**(3): 510-4.

Bolles, R. C. and L. W. Stokes (1965). "Rat's anticipation of diurnal and a-diurnal feeding." J Comp Physiol Psychol **60**(2): 290-4.

Bonnelye, E., J. M. Vanacker, X. Desbiens, A. Begue, D. Stehelin and V. Laudet (1994). "Rev-erb beta, a new member of the nuclear receptor superfamily, is expressed in the nervous system during chicken development." Cell Growth Differ **5**(12): 1357-65.

Bordone, L. and L. Guarente (2005). "Calorie restriction, SIRT1 and metabolism: understanding longevity." Nat Rev Mol Cell Biol **6**(4): 298-305.

Boulos, Z., D. M. Frim, L. K. Dewey and M. C. Moore-Ede (1989). "Effects of restricted feeding schedules on circadian organization in squirrel monkeys." Physiol Behav **45**(3): 507-15.

Boulos, Z., A. M. Rosenwasser and M. Terman (1980). "Feeding schedules and the circadian organization of behavior in the rat." Behav Brain Res **1**(1): 39-65.

Bretzl, H. (1903). Botanische Forschungen des Alexanderzuges. (Leipzig, Germany: B.G. Teubner).

Bugge, A., D. Feng, L. J. Everett, E. R. Briggs, S. E. Mullican, F. Wang, J. Jager and M. A. Lazar (2012). "Rev-erbalpha and Rev-erbbeta coordinately protect the circadian clock and normal metabolic function." Genes Dev **26**(7): 657-67.

Buijs, R. M., S. J. Chun, A. Niijima, H. J. Romijn and K. Nagai (2001). "Parasympathetic and sympathetic control of the pancreas: a role for the suprachiasmatic nucleus and other hypothalamic centers that are involved in the regulation of food intake." J Comp Neurol **431**(4): 405-23.

Buijs, R. M., J. Wortel, J. J. Van Heerikhuize, M. G. Feenstra, G. J. Ter Horst, H. J. Romijn and A. Kalsbeek (1999). "Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway." Eur J Neurosci **11**(5): 1535-44.

Bunger, M. K., L. D. Wilsbacher, S. M. Moran, C. Clendenin, L. A. Radcliffe, J. B. Hogenesch, M. C. Simon, J. S. Takahashi and C. A. Bradfield (2000). "Mop3 is an essential component of the master circadian pacemaker in mammals." Cell **103**(7): 1009-1017.

Bunning, E. and I. Moser (1969). "Interference of moonlight with the photoperiodic measurement of time by plants, and their adaptive reaction." Proc Natl Acad Sci U S A **62**(4): 1018-22.

Burke, L., M. Downes, A. Carozzi, V. Giguere and G. E. Muscat (1996). "Transcriptional repression by the orphan steroid receptor RVR/Rev-erb beta is dependent on the signature motif and helix 5 in the E region: functional evidence for a biological role of RVR in myogenesis." Nucleic Acids Res **24**(18): 3481-9.

Caba, M. and G. Gonzalez-Mariscal (2009). "The rabbit pup, a natural model of nursing-anticipatory activity." Eur J Neurosci **30**(9): 1697-706.

Cai, W., J. Rambaud, M. Teboul, I. Masse, G. Benoit, J. A. Gustafsson, F. Delaunay, V. Laudet and I. Pongratz (2008). "Expression levels of estrogen receptor beta are modulated by components of the molecular clock." Molecular and Cellular Biology **28**(2): 784-793.

Caldelas, I., V. J. Poirel, B. Sicard, P. Pevet and E. Challet (2003). "Circadian profile and photic regulation of clock genes in the suprachiasmatic nucleus of a diurnal mammal Arvicanthis ansorgei." Neuroscience **116**(2): 583-91.

Cambras, T., J. Vilaplana, A. Campuzano, M. M. Canal-Corretger, M. Carulla and A. Diez-Noguera (2000). "Entrainment of the rat motor activity rhythm: effects of the light-dark cycle and physical exercise." Physiol Behav **70**(3-4): 227-32.

Campuzano, A., J. Vilaplana, T. Cambras and A. Diez-Noguera (1999). "The role of wheel running in the coupling of two simultaneous circadian rhythms of motor activity in the rat." Biological Rhythm Research **30**(5): 497-507.

Canaple, L., J. Rambaud, O. Dkhissi-Benyahya, B. Rayet, N. S. Tan, L. Michalik, F. Delaunay, W. Wahli and V. Laudet (2006). "Reciprocal regulation of brain and muscle Arnt-like protein 1 and peroxisome proliferator-activated receptor alpha defines a novel positive feedback loop in the rodent liver circadian clock." Mol Endocrinol **20**(8): 1715-27.

Canto, C., Z. Gerhart-Hines, J. N. Feige, M. Lagouge, L. Noriega, J. C. Milne, P. J. Elliott, P. Puigserver and J. Auwerx (2009). "AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity." Nature **458**(7241): 1056-60.

Castillo, M. R., K. J. Hochstetler, R. J. Tavernier, Jr., D. M. Greene and A. Bult-Ito (2004). "Entrainment of the master circadian clock by scheduled feeding." Am J Physiol Regul Integr Comp Physiol **287**(3): R551-5.

Cavallari, N., E. Frigato, D. Vallone, N. Frohlich, J. F. Lopez-Olmeda, A. Foa, R. Berti, F. J. Sanchez-Vazquez, C. Bertolucci and N. S. Foulkes (2011). "A Blind Circadian Clock in Cavefish Reveals that Opsins Mediate Peripheral Clock Photoreception." Plos Biology **9**(9).

Cha, M. C., C. J. Chou and C. N. Boozer (2000). "High-fat diet feeding reduces the diurnal variation of plasma leptin concentration in rats." Metabolism **49**(4): 503-7.

Challet, E. (2010). "Interactions between light, mealtime and calorie restriction to control daily timing in mammals." J Comp Physiol B **180**(5): 631-44.

Challet, E., J. Mendoza, H. Dardente and P. Pevet (2009). "Neurogenetics of food anticipation." European Journal of Neuroscience **30**(9): 1676-1687.

Challet, E., P. Pevet, B. Vivien-Roels and A. Malan (1997). "Phase-advanced daily rhythms of melatonin, body temperature, and locomotor activity in food-restricted rats fed during daytime." J Biol Rhythms **12**(1): 65-79.

Challet, E., L. C. Solberg and F. W. Turek (1998). "Entrainment in calorie-restricted mice: conflicting zeitgebers and free-running conditions." Am J Physiol **274**(6 Pt 2): R1751-61.

Chandrashekaran, M. K. (1998). "Biological rhythms research: A personal account." Journal of Biosciences **23**(5): 545-555.

Chawla, A. and M. A. Lazar (1993). "Induction of Rev-ErbA alpha, an orphan receptor encoded on the opposite strand of the alpha-thyroid hormone receptor gene, during adipocyte differentiation." J Biol Chem **268**(22): 16265-9.

Cheng, M. Y., E. L. Bittman, S. Hattar and Q. Y. Zhou (2005). "Regulation of prokineticin 2 expression by light and the circadian clock." BMC Neurosci **6**: 17.

Cheng, M. Y., C. M. Bullock, C. Li, A. G. Lee, J. C. Bermak, J. Belluzzi, D. R. Weaver, F. M. Leslie and Q. Y. Zhou (2002). "Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus." Nature **417**(6887): 405-10.

Cho, H., X. Zhao, M. Hatori, R. T. Yu, G. D. Barish, M. T. Lam, L. W. Chong, L. DiTacchio, A. R. Atkins, C. K. Glass, C. Liddle, J. Auwerx, M. Downes, S. Panda and R. M. Evans (2012). "Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta." Nature **485**(7396): 123-127.

Chomez, P., I. Neveu, A. Mansen, E. Kiesler, L. Larsson, B. Vennstrom and E. Arenas (2000). "Increased cell death and delayed development in the cerebellum of mice lacking the rev-erbA(alpha) orphan receptor." Development **127**(7): 1489-98.

Chou, T. C., T. E. Scammell, J. J. Gooley, S. E. Gaus, C. B. Saper and J. Lu (2003). "Critical role of dorsomedial hypothalamic nucleus in a wide range of behavioral circadian rhythms." J Neurosci **23**(33): 10691-702.

Clarke, J. D. and G. J. Coleman (1986). "Persistent meal-associated rhythms in SCN-lesioned rats." Physiol Behav **36**(1): 105-13.

Clement, K., C. Vaisse, N. Lahlou, S. Cabrol, V. Pelloux, D. Cassuto, M. Gourmelen, C. Dina, J. Chambaz, J. M. Lacorte, A. Basdevant, P. Bougneres, Y. Lebouc, P. Froguel and B. Guy-Grand

(1998). "A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction." Nature **392**(6674): 398-401.

Coleman, G. J., S. Harper, J. D. Clarke and S. Armstrong (1982). "Evidence for a separate meal-associated oscillator in the rat." Physiol Behav **29**(1): 107-15.

Coleman, G. J. and M. Hay (1990). "Anticipatory Wheel-Running in Behaviorally Anosmic Rats." Physiology & Behavior **47**(6): 1145-1151.

Colwell, C. S. (2011). "Linking neural activity and molecular oscillations in the SCN." Nat Rev Neurosci **12**(10): 553-69.

Comperatore, C. A. and F. K. Stephan (1990). "Effects of vagotomy on entrainment of activity rhythms to food access." Physiol Behav **47**(4): 671-8.

Crowley, W. R., G. Ramoz, K. A. Keefe, R. Torto, S. P. Kalra and G. R. Hanson (2005). "Differential effects of methamphetamine on expression of neuropeptide Y mRNA in hypothalamus and on serum leptin and ghrelin concentrations in ad libitum-fed and schedule-fed rats." Neuroscience **132**(1): 167-73.

Crumbley, C. and T. P. Burris (2011). "Direct regulation of CLOCK expression by REV-ERB." PLoS One **6**(3): e17290.

Crumbley, C., Y. Wang, D. J. Kojetin and T. P. Burris (2010). "Characterization of the core mammalian clock component, NPAS2, as a REV-ERBalpha/RORalpha target gene." J Biol Chem **285**(46): 35386-92.

Cruz, W. S., G. Kwon, C. A. Marshall, M. L. McDaniel and C. F. Semenkovich (2001). "Glucose and insulin stimulate heparin-releasable lipoprotein lipase activity in mouse islets and INS-1 cells. A potential link between insulin resistance and beta-cell dysfunction." J Biol Chem **276**(15): 12162-8.

Dali-Youcef, N., M. Lagouge, S. Froelich, C. Koehl, K. Schoonjans and J. Auwerx (2007). "Sirtuins: the 'magnificent seven', function, metabolism and longevity." Ann Med **39**(5): 335-45.

Dallmann, R., C. Touma, R. Palme, U. Albrecht and S. Steinlechner (2006). "Impaired daily glucocorticoid rhythm in Per1 (Brd) mice." J Comp Physiol A Neuroethol Sens Neural Behav Physiol **192**(7): 769-75.

Dallmann, R. and D. R. Weaver (2010). "Altered body mass regulation in male mPeriod mutant mice on high-fat diet." Chronobiol Int **27**(6): 1317-28.

Damiola, F., N. Le Minh, N. Preitner, B. Kornmann, F. Fleury-Olela and U. Schibler (2000). "Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus." Genes Dev **14**(23): 2950-61.

Davidson, A. J. (2006). "Search for the feeding-entrainable circadian oscillator: a complex proposition." Am J Physiol Regul Integr Comp Physiol **290**(6): R1524-6.

Davidson, A. J., B. J. Aragona, T. A. Houpt and F. K. Stephan (2001). "Persistence of meal-entrained circadian rhythms following area postrema lesions in the rat." Physiology & Behavior **74**(3): 349-354.

Davidson, A. J., S. L. Cappendijk and F. K. Stephan (2000). "Feeding-entrained circadian rhythms are attenuated by lesions of the parabrachial region in rats." Am J Physiol Regul Integr Comp Physiol **278**(5): R1296-304.

Davidson, A. J., A. S. Poole, S. Yamazaki and M. Menaker (2003). "Is the food-entrainable circadian oscillator in the digestive system?" Genes Brain Behav **2**(1): 32-9.

Davidson, A. J. and F. K. Stephan (1998). "Circadian food anticipation persists in capsaicin deafferented rats." Journal of Biological Rhythms **13**(5): 422-429.

De Coursey, P. J. (1960). "Daily light sensitivity rhythm in a rodent." Science **131**(3392): 33-5.

De Coursey, P. J., J. R. Krulas, G. Mele and D. C. Holley (1997). "Circadian performance of suprachiasmatic nuclei (SCN)-lesioned antelope ground squirrels in a desert enclosure." Physiol Behav **62**(5): 1099-108.

de Mairan, J. (1729). Observation botanique. Hist. Acad. Roy. Sci. 35–36.

Diaz-Munoz, M., O. Vazquez-Martinez, R. Aguilar-Roblero and C. Escobar (2000). "Anticipatory changes in liver metabolism and entrainment of insulin, glucagon, and corticosterone in food-restricted rats." American Journal of Physiology-Regulatory Integrative and Comparative Physiology **279**(6): R2048-R2056.

Dibner, C., U. Schibler and U. Albrecht (2010). "The mammalian circadian timing system: organization and coordination of central and peripheral clocks." Annu Rev Physiol **72**: 517-49.

Dioum, E. M., J. Rutter, J. R. Tuckerman, G. Gonzalez, M. A. Gilles-Gonzalez and S. L. McKnight (2002). "NPAS2: a gas-responsive transcription factor." Science **298**(5602): 2385-7.

Doi, M., J. Hirayama and P. Sassone-Corsi (2006). "Circadian regulator CLOCK is a histone acetyltransferase." Cell **125**(3): 497-508.

Doi, R., K. Oishi and N. Ishida (2010). "CLOCK regulates circadian rhythms of hepatic glycogen synthesis through transcriptional activation of Gys2." J Biol Chem **285**(29): 22114-21.

Downes, M., A. J. Carozzi and G. E. Muscat (1995). "Constitutive expression of the orphan receptor, Rev-erbA alpha, inhibits muscle differentiation and abrogates the expression of the myoD gene family." Mol Endocrinol **9**(12): 1666-78.

Dragunow, M. and R. Faull (1989). "The Use of C-Fos as a Metabolic Marker in Neuronal Pathway Tracing." Journal of Neuroscience Methods **29**(3): 261-265.

Dudley, C. A., C. Erbel-Sieler, S. J. Estill, M. Reick, P. Franken, S. Pitts and S. L. McKnight (2003). "Altered patterns of sleep and behavioral adaptability in NPAS2-deficient mice." Science **301**(5631): 379-83.

Dunlap, J. C. (1999). "Molecular bases for circadian clocks." Cell **96**(2): 271-90.

Earnest, D. J. and C. D. Sladek (1986). "Circadian rhythms of vasopressin release from individual rat suprachiasmatic explants in vitro." Brain Res **382**(1): 129-33.

Easton, A., J. Arbuzova and F. W. Turek (2003). "The circadian Clock mutation increases exploratory activity and escape-seeking behavior." Genes Brain and Behavior **2**(1): 11-19.

Edgar, D. M., W. C. Dement and C. A. Fuller (1993). "Effect of SCN lesions on sleep in squirrel monkeys: evidence for opponent processes in sleep-wake regulation." J Neurosci **13**(3): 1065-79.

Edmonds, S. C. (1977). "Food and light as entrainers of circadian running activity in the rat." Physiol Behav **18**(5): 915-9.

Engelmann, W. (2002). Rhythms of Life, An introduction using selected topics and examples. http://www.uni-tuebingen.de/plantphys/bioclox/

Escobar, C., M. Angeles-Castellanos, T. Solis-Salazar and J. Mendoza (2005). "Metabolic parameters are food-entrained in rats made cirrhotic by chronic CCl4 treatment." Biological Rhythm Research **36**(1-2): 39-45.

Escobar, C., J. Y. Mendoza, A. Salazar-Juarez, J. Avila, R. Hernandez-Munoz, M. Diaz-Munoz and R. Aguilar-Roblero (2002). "Rats made cirrhotic by chronic CCl(4) treatment still exhibit anticipatory activity to a restricted feeding schedule." Chronobiology International **19**(6): 1073-1086.

Farnell, Y. Z., G. C. Allen, S. S. Nahm, N. Neuendorff, J. R. West, W. J. A. Chen and D. J. Earnest (2008). "Neonatal alcohol exposure differentially alters clock gene oscillations within the suprachiasmatic nucleus, cerebellum, and liver of adult rats." Alcoholism-Clinical and Experimental Research **32**(3): 544-552.

Feillet, C. A., J. Mendoza, U. Albrecht, P. Pevet and E. Challet (2008). "Forebrain oscillators ticking with different clock hands." Mol Cell Neurosci **37**(2): 209-21.

Feillet, C. A., J. A. Ripperger, M. C. Magnone, A. Dulloo, U. Albrecht and E. Challet (2006). "Lack of food anticipation in Per2 mutant mice." Curr Biol **16**(20): 2016-22.

Feng, D., T. Liu, Z. Sun, A. Bugge, S. E. Mullican, T. Alenghat, X. S. Liu and M. A. Lazar (2011). "A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism." Science **331**(6022): 1315-9.

Ferreira, L. D. M. C. B., L. K. Pulawa, D. R. Jensen and R. H. Eckel (2001). "Overexpressing human lipoprotein lipase in mouse skeletal muscle is associated with insulin resistance." Diabetes **50**(5): 1064-1068.

Filipski, E., P. F. Innominato, M. W. Wu, X. M. Li, S. Iacobelli, L. J. Xian and F. Levi (2005). "Effects of light and food schedules on liver and tumor molecular clocks in mice." Journal of the National Cancer Institute **97**(7): 507-517.

Fonken, L. K., J. L. Workman, J. C. Walton, Z. M. Weil, J. S. Morris, A. Haim and R. J. Nelson (2010). "Light at night increases body mass by shifting the time of food intake." Proc Natl Acad Sci U S A **107**(43): 18664-9.

Fontaine, C., G. Dubois, Y. Duguay, T. Helledie, N. Vu-Dac, P. Gervois, F. Soncin, S. Mandrup, J. C. Fruchart, J. Fruchart-Najib and B. Staels (2003). "The orphan nuclear receptor Rev-Erbalpha is a peroxisome proliferator-activated receptor (PPAR) gamma target gene and promotes PPARgamma-induced adipocyte differentiation." J Biol Chem **278**(39): 37672-80.

Freedman, M. S., R. J. Lucas, B. Soni, M. von Schantz, M. Munoz, Z. David-Gray and R. Foster (1999). "Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors." Science **284**(5413): 502-4.

Fukagawa, K., T. Sakata, H. Yoshimatsu, K. Fujimoto, K. Uchimura and C. Asano (1992). "Advance shift of feeding circadian rhythm induced by obesity progression in Zucker rats." Am J Physiol **263**(6 Pt 2): R1169-75.

Fuller, P. M., J. Lu and C. B. Saper (2008). "Differential rescue of light- and food-entrainable circadian rhythms." Science **320**(5879): 1074-7.

Fuller, P. M., J. Lu and C. B. Saper (2009). "Standards of evidence in chronobiology: A response." J Circadian Rhythms **7**: 9.

Gachon, F., N. Leuenberger, T. Claudel, P. Gos, C. Jouffe, F. Fleury Olela, X. de Mollerat du Jeu, W. Wahli and U. Schibler (2011). "Proline- and acidic amino acid-rich basic leucine zipper proteins modulate peroxisome proliferator-activated receptor alpha (PPARalpha) activity." Proc Natl Acad Sci U S A **108**(12): 4794-9.

Garaulet, M., J. M. Ordovas, P. Gomez-Abellan, J. A. Martinez and J. A. Madrid (2011). "An approximation to the temporal order in endogenous circadian rhythms of genes implicated in human adipose tissue metabolism." J Cell Physiol **226**(8): 2075-80.

Garcia, J. A., D. Zhang, S. J. Estill, C. Michnoff, J. Rutter, M. Reick, K. Scott, R. Diaz-Arrastia and S. L. McKnight (2000). "Impaired cued and contextual memory in NPAS2-deficient mice." Science **288**(5474): 2226-30.

Gavrilova, O., L. R. Leon, B. Marcus-Samuels, M. M. Mason, A. L. Castle, S. Refetoff, C. Vinson and M. L. Reitman (1999). "Torpor in mice is induced by both leptin-dependent and -independent mechanisms." Proc Natl Acad Sci U S A **96**(25): 14623-8.

Gerozissis, K. (2008). "Brain insulin, energy and glucose homeostasis; genes, environment and metabolic pathologies." Eur J Pharmacol **585**(1): 38-49.

Gervois, P., S. Chopin-Delannoy, A. Fadel, G. Dubois, V. Kosykh, J. C. Fruchart, J. Najib, V. Laudet and B. Staels (1999). "Fibrates increase human REV-ERBalpha expression in liver via a novel peroxisome proliferator-activated receptor response element." Mol Endocrinol **13**(3): 400-9.

Gervois, P., I. P. Torra, J. C. Fruchart and B. Staels (2000). "Regulation of lipid and lipoprotein metabolism by PPAR activators." Clin Chem Lab Med **38**(1): 3-11.

Ginty, D. D., J. M. Kornhauser, M. A. Thompson, H. Bading, K. E. Mayo, J. S. Takahashi and M. E. Greenberg (1993). "Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock." Science **260**(5105): 238-41.

Gooley, J. J., A. Schomer and C. B. Saper (2006). "The dorsomedial hypothalamic nucleus is critical for the expression of food- entrainable circadian rhythms." Nature Neuroscience **9**(3): 398-407.

Gooley, J. J., A. Schomer and C. B. Saper (2006). "The dorsomedial hypothalamic nucleus is critical for the expression of food-entrainable circadian rhythms." Nat Neurosci **9**(3): 398-407.

Granados-Fuentes, D., A. Tseng and E. D. Herzog (2006). "A circadian clock in the olfactory bulb controls olfactory responsivity." J Neurosci **26**(47): 12219-25.

Grechez-Cassiau, A., B. Rayet, F. Guillaumond, M. Teboul and F. Delaunay (2008). "The circadian clock component BMAL1 is a critical regulator of p21WAF1/CIP1 expression and hepatocyte proliferation." J Biol Chem **283**(8): 4535-42.

Green, D. J. and R. Gillette (1982). "Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic brain slice." Brain Res **245**(1): 198-200.

Grimaldi, B., M. M. Bellet, S. Katada, G. Astarita, J. Hirayama, R. H. Amin, J. G. Granneman, D. Piomelli, T. Leff and P. Sassone-Corsi (2010). "PER2 controls lipid metabolism by direct regulation of PPARgamma." Cell Metab **12**(5): 509-20.

Guilding, C., A. T. Hughes and H. D. Piggins (2010). "Circadian oscillators in the epithalamus." Neuroscience **169**(4): 1630-9.

Guilding, C. and H. D. Piggins (2007). "Challenging the omnipotence of the suprachiasmatic timekeeper: are circadian oscillators present throughout the mammalian brain?" Eur J Neurosci **25**(11): 3195-216.

Guillaumond, F., H. Dardente, V. Giguere and N. Cermakian (2005). "Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors." J Biol Rhythms **20**(5): 391-403.

Guler, A. D., J. L. Ecker, G. S. Lall, S. Haq, C. M. Altimus, H. W. Liao, A. R. Barnard, H. Cahill, T. C. Badea, H. Zhao, M. W. Hankins, D. M. Berson, R. J. Lucas, K. W. Yau and S. Hattar (2008). "Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision." Nature **453**(7191): 102-5.

Guo, B., S. Chatterjee, L. Li, J. M. Kim, J. Lee, V. K. Yechoor, L. J. Minze, W. Hsueh and K. Ma (2012). "The clock gene, brain and muscle Arnt-like 1, regulates adipogenesis via Wnt signaling pathway." FASEB J.

Guo, H., J. M. Brewer, M. N. Lehman and E. L. Bittman (2006). "Suprachiasmatic regulation of circadian rhythms of gene expression in hamster peripheral organs: effects of transplanting the pacemaker." J Neurosci **26**(24): 6406-12.

Hakansson, M. L., H. Brown, N. Ghilardi, R. C. Skoda and B. Meister (1998). "Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus." J Neurosci **18**(1): 559-72.

Halberg, F., F. Carandente, G. Cornelissen and G. S. Katinas (1977). "[Glossary of chronobiology (author's transl)]." Chronobiologia **4 Suppl 1**: 1-189.

Halberg, F., G. Cornelissen, G. Katinas, E. V. Syutkina, R. B. Sothern, R. Zaslavskaya, Y. Watanabe, O. Schwartzkopff, K. Otsuka, R. Tarquini, P. Frederico and J. Siggelova (2003). "Transdisciplinary unifying implications of circadian findings in the 1950s." J Circadian Rhythms **1**(1): 2.

Hall, F. S., M. Goeb, X. F. Li, L. Sora and G. R. Uhl (2004). "mu-opioid receptor knockout mice display reduced cocaine conditioned place preference but enhanced sensitization of cocaine-induced locomotion." Molecular Brain Research **121**(1-2): 123-130.

Handschin, C., J. Lin, J. Rhee, A. K. Peyer, S. Chin, P. H. Wu, U. A. Meyer and B. M. Spiegelman (2005). "Nutritional regulation of hepatic heme biosynthesis and porphyria through PGC-1alpha." Cell **122**(4): 505-15.

Hannibal, J. (2006). "Roles of PACAP-containing retinal ganglion cells in circadian timing." Int Rev Cytol **251**: 1-39.

Hattar, S., H. W. Liao, M. Takao, D. M. Berson and K. W. Yau (2002). "Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity." Science **295**(5557): 1065-70.

Havel, P. J., R. Townsend, L. Chaump and K. Teff (1999). "High-fat meals reduce 24-h circulating leptin concentrations in women." Diabetes **48**(2): 334-41.

Hems, D. A., E. A. Rath and T. R. Verrinder (1975). "Fatty acid synthesis in liver and adipose tissue of normal and genetically obese (ob/ob) mice during the 24-hour cycle." Biochem J **150**(2): 167-73.

Herzog, E. D., M. E. Geusz, S. B. Khalsa, M. Straume and G. D. Block (1997). "Circadian rhythms in mouse suprachiasmatic nucleus explants on multimicroelectrode plates." Brain Res **757**(2): 285-90.

Holmes, M. M. and R. E. Mistlberger (2000). "Food anticipatory activity and photic entrainment in food-restricted BALB/c mice." Physiol Behav **68**(5): 655-66.

Honma, K., S. Honma and T. Hiroshige (1987). "Activity rhythms in the circadian domain appear in suprachiasmatic nuclei lesioned rats given methamphetamine." Physiol Behav **40**(6): 767-74.

Honma, K., Y. Noe, S. Honma, Y. Katsuno and T. Hiroshige (1992). "Roles of Paraventricular Catecholamines in Feeding-Associated Corticosterone Rhythm in Rats." American Journal of Physiology **262**(6): E948-E955.

Honma, S., K. I. Honma and T. Hiroshige (1989). "Methamphetamine Induced Locomotor Rhythm Entrains to Restricted Daily Feeding in Scn Lesioned Rats." Physiology & Behavior **45**(5): 1057-1065.

Honma, S., W. Nakamura, T. Shirakawa and K. Honma (2004). "Diversity in the circadian periods of single neurons of the rat suprachiasmatic nucleus depends on nuclear structure and intrinsic period." Neurosci Lett **358**(3): 173-6.

Horikawa, K., Y. Minami, M. Iijima, M. Akiyama and S. Shibata (2005). "Rapid damping of food-entrained circadian rhythm of clock gene expression in clock-defective peripheral tissues under fasting conditions." Neuroscience **134**(1): 335-43.

Hsu, C. T., D. F. Patton, R. E. Mistlberger and A. D. Steele (2010). "Palatable meal anticipation in mice." PLoS One **5**(9).

Huang, T. C., J. Tu, T. J. Chow and T. H. Chen (1990). "Circadian Rhythm of the Prokaryote Synechococcus sp. RF-1." Plant Physiol **92**(2): 531-3.

Hughes, M. E., L. DiTacchio, K. R. Hayes, C. Vollmers, S. Pulivarthy, J. E. Baggs, S. Panda and J. B. Hogenesch (2009). "Harmonics of circadian gene transcription in mammals." PLoS Genet **5**(4): e1000442.

Iijima, M., T. Nikaido, M. Akiyama, T. Moriya and S. Shibata (2002). "Methamphetamine-induced, suprachiasmatic nucleus-independent circadian rhythms of activity and mPer gene expression in the striatum of the mouse." Eur J Neurosci **16**(5): 921-9.

Iijima, M., S. Yamaguchi, G. T. van der Horst, X. Bonnefont, H. Okamura and S. Shibata (2005). "Altered food-anticipatory activity rhythm in Cryptochrome-deficient mice." Neurosci Res **52**(2): 166-73.

Inouye, S. I. T. (1982). "Ventromedial Hypothalamic-Lesions Eliminate Anticipatory Activities of Restricted Daily Feeding Schedules in the Rat." Brain Research **250**(1): 183-187.

Inouye, S. T. and H. Kawamura (1979). "Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus." Proc Natl Acad Sci U S A **76**(11): 5962-6.

Ishida, A., T. Mutoh, T. Ueyama, H. Bando, S. Masubuchi, D. Nakahara, G. Tsujimoto and H. Okamura (2005). "Light activates the adrenal gland: timing of gene expression and glucocorticoid release." Cell Metab **2**(5): 297-307.

Jenkins, J. B., T. Omori, Z. Guan, A. N. Vgontzas, E. O. Bixler and J. Fang (2006). "Sleep is increased in mice with obesity induced by high-fat food." Physiol Behav **87**(2): 255-62.

Jeong, K. H., S. Sakihara, E. P. Widmaier and J. A. Majzoub (2004). "Impaired leptin expression and abnormal response to fasting in corticotropin-releasing hormone-deficient mice." Endocrinology **145**(7): 3174-81.

Jiang, Z. G., Y. Q. Yang and C. N. Allen (1997). "Tracer and electrical coupling of rat suprachiasmatic nucleus neurons." Neuroscience **77**(4): 1059-66.

Johnson, C. H., S. S. Golden, M. Ishiura and T. Kondo (1996). "Circadian clocks in prokaryotes." Mol Microbiol **21**(1): 5-11.

Kaasik, K. and C. C. Lee (2004). "Reciprocal regulation of haem biosynthesis and the circadian clock in mammals." Nature **430**(6998): 467-71.

Kahn, B. B., T. Alquier, D. Carling and D. G. Hardie (2005). "AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism." Cell Metab **1**(1): 15-25.

Kallen, J., J. M. Schlaeppi, F. Bitsch, I. Delhon and B. Fournier (2004). "Crystal structure of the human RORalpha Ligand binding domain in complex with cholesterol sulfate at 2.2 A." J Biol Chem **279**(14): 14033-8.

Kalsbeek, A. and R. M. Buijs (2002). "Output pathways of the mammalian suprachiasmatic nucleus: coding circadian time by transmitter selection and specific targeting." Cell Tissue Res **309**(1): 109-18.

Kalsbeek, A., R. M. Buijs, J. J. van Heerikhuize, M. Arts and T. P. van der Woude (1992). "Vasopressin-containing neurons of the suprachiasmatic nuclei inhibit corticosterone release." Brain Res **580**(1-2): 62-7.

Kalsbeek, A., E. Fliers, J. A. Romijn, S. E. La Fleur, J. Wortel, O. Bakker, E. Endert and R. M. Buijs (2001). "The suprachiasmatic nucleus generates the diurnal changes in plasma leptin levels." Endocrinology **142**(6): 2677-85.

Kalsbeek, A., I. F. Palm, S. E. La Fleur, F. A. Scheer, S. Perreau-Lenz, M. Ruiter, F. Kreier, C. Cailotto and R. M. Buijs (2006). "SCN outputs and the hypothalamic balance of life." J Biol Rhythms **21**(6): 458-69.

Kalsbeek, A., R. Teclemariam-Mesbah and P. Pevet (1993). "Efferent projections of the suprachiasmatic nucleus in the golden hamster (Mesocricetus auratus)." J Comp Neurol **332**(3): 293-314.

Kalsbeek, A., J. J. van Heerikhuize, J. Wortel and R. M. Buijs (1996). "A diurnal rhythm of stimulatory input to the hypothalamo-pituitary-adrenal system as revealed by timed intrahypothalamic administration of the vasopressin V1 antagonist." J Neurosci **16**(17): 5555-65.

Kalsbeek, A., C. X. Yi, S. E. La Fleur and E. Fliers (2010). "The hypothalamic clock and its control of glucose homeostasis." Trends Endocrinol Metab **21**(7): 402-10.

Kaneko, K., T. Yamada, S. Tsukita, K. Takahashi, Y. Ishigaki, Y. Oka and H. Katagiri (2009). "Obesity alters circadian expressions of molecular clock genes in the brainstem." Brain Res **1263**: 58-68.

Karatsoreos, I. N., S. Bhagat, E. B. Bloss, J. H. Morrison and B. S. McEwen (2011). "Disruption of circadian clocks has ramifications for metabolism, brain, and behavior." Proc Natl Acad Sci U S A **108**(4): 1657-62.

Kas, M. J. H., R. van den Bos, A. M. Baars, M. Lubbers, H. M. B. Lesscher, J. J. G. Hillebrand, A. G. Schuller, J. E. Pintar and B. M. Spruijt (2004). "Mu-opioid receptor knockout mice show diminished food-anticipatory activity." European Journal of Neuroscience **20**(6): 1624-1632.

Kaur, S., S. Thankachan, S. Begum, C. Blanco-Centurion, T. Sakurai, M. Yanagisawa and P. J. Shiromani (2008). "Entrainment of temperature and activity rhythms to restricted feeding in orexin knock out mice." Brain Res **1205**: 47-54.

Kennaway, D. J., J. A. Owens, A. Voultsios, M. J. Boden and T. J. Varcoe (2007). "Metabolic homeostasis in mice with disrupted Clock gene expression in peripheral tissues." Am J Physiol Regul Integr Comp Physiol **293**(4): R1528-37.

Khelfaoui, M., C. Denis, E. van Galen, F. de Bock, A. Schmitt, C. Houbron, E. Morice, B. Giros, G. Ramakers, L. Fagni, J. Chelly, M. Nosten-Bertrand and P. Billuart (2007). "Loss of X-linked mental retardation gene oligophrenin1 in mice impairs spatial memory and leads to ventricular enlargement and dendritic spine immaturity." J Neurosci **27**(35): 9439-50.

Kiessling, S., G. Eichele and H. Oster (2010). "Adrenal glucocorticoids have a key role in circadian resynchronization in a mouse model of jet lag." Journal of Clinical Investigation **120**(7): 2600-2609.

Kim, J. K., J. J. Fillmore, Y. Chen, C. Yu, I. K. Moore, M. Pypaert, E. P. Lutz, Y. Kako, W. Velez-Carrasco, I. J. Goldberg, J. L. Breslow and G. I. Shulman (2001). "Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance." Proc Natl Acad Sci U S A **98**(13): 7522-7.

Kim, S. M., A. Power, T. M. Brown, C. M. Constance, S. L. Coon, T. Nishimura, H. Hirai, T. Cai, C. Eisner, D. R. Weaver, H. D. Piggins, D. C. Klein, J. Schnermann and A. L. Notkins (2009). "Deletion of the secretory vesicle proteins IA-2 and IA-2beta disrupts circadian rhythms of cardiovascular and physical activity." FASEB J **23**(9): 3226-32.

King, D. P., Y. Zhao, A. M. Sangoram, L. D. Wilsbacher, M. Tanaka, M. P. Antoch, T. D. Steeves, M. H. Vitaterna, J. M. Kornhauser, P. L. Lowrey, F. W. Turek and J. S. Takahashi (1997). "Positional cloning of the mouse circadian clock gene." Cell **89**(4): 641-53.

Kitajima, S., M. Morimoto, E. Liu, T. Koike, Y. Higaki, Y. Taura, K. Mamba, K. Itamoto, T. Watanabe, K. Tsutsumi, N. Yamada and J. Fan (2004). "Overexpression of lipoprotein lipase improves insulin resistance induced by a high-fat diet in transgenic rabbits." Diabetologia **47**(7): 1202-9.

Ko, C. H. and J. S. Takahashi (2006). "Molecular components of the mammalian circadian clock." Hum Mol Genet **15 Spec No 2**: R271-7.

Kohsaka, A., A. D. Laposky, K. M. Ramsey, C. Estrada, C. Joshu, Y. Kobayashi, F. W. Turek and J. Bass (2007). "High-fat diet disrupts behavioral and molecular circadian rhythms in mice." Cell Metab **6**(5): 414-21.

Koike, T., J. Liang, X. Wang, T. Ichikawa, M. Shiomi, G. Liu, H. Sun, S. Kitajima, M. Morimoto, T. Watanabe, N. Yamada and J. Fan (2004). "Overexpression of lipoprotein lipase in transgenic Watanabe heritable hyperlipidemic rabbits improves hyperlipidemia and obesity." J Biol Chem **279**(9): 7521-9.

Kondratov, R. V., M. V. Chernov, A. A. Kondratova, V. Y. Gorbacheva, A. V. Gudkov and M. P. Antoch (2003). "BMAL1-dependent circadian oscillation of nuclear CLOCK: posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system." Genes Dev **17**(15): 1921-32.

Kondratov, R. V., A. A. Kondratova, V. Y. Gorbacheva, O. V. Vykhovanets and M. P. Antoch (2006). "Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock." Genes Dev **20**(14): 1868-73.

Kondratov, R. V., R. K. Shamanna, A. A. Kondratova, V. Y. Gorbacheva and M. P. Antoch (2006). "Dual role of the CLOCK/BMAL1 circadian complex in transcriptional regulation." Faseb Journal **20**(1): 530-+.

Konopka, R. J. and S. Benzer (1971). "Clock mutants of Drosophila melanogaster." Proc Natl Acad Sci U S A **68**(9): 2112-6.

Kornhauser, J. M., D. E. Nelson, K. E. Mayo and J. S. Takahashi (1990). "Photic and circadian regulation of c-fos gene expression in the hamster suprachiasmatic nucleus." Neuron **5**(2): 127-34.

Krashes, M. J., S. Koda, C. Ye, S. C. Rogan, A. C. Adams, D. S. Cusher, E. Maratos-Flier, B. L. Roth and B. B. Lowell (2011). "Rapid, reversible activation of AgRP neurons drives feeding behavior in mice." J Clin Invest **121**(4): 1424-8.

Kreier, F., E. Fliers, P. J. Voshol, C. G. Van Eden, L. M. Havekes, A. Kalsbeek, C. L. Van Heijningen, A. A. Sluiter, T. C. Mettenleiter, J. A. Romijn, H. P. Sauerwein and R. M. Buijs (2002). "Selective parasympathetic innervation of subcutaneous and intra-abdominal fat--functional implications." J Clin Invest **110**(9): 1243-50.

Krieger, D. T. (1974). "Food and water restriction shifts corticosterone, temperature, activity and brain amine periodicity." Endocrinology **95**(5): 1195-201.

Kudo, T., M. Akiyama, K. Kuriyama, M. Sudo, T. Moriya and S. Shibata (2004). "Night-time restricted feeding normalises clock genes and Pai-1 gene expression in the db/db mouse liver." Diabetologia **47**(8): 1425-36.

Kudo, T., M. Kawashima, T. Tamagawa and S. Shibata (2008). "Clock mutation facilitates accumulation of cholesterol in the liver of mice fed a cholesterol and/or cholic acid diet." Am J Physiol Endocrinol Metab **294**(1): E120-30.

Kudo, T., T. Tamagawa, M. Kawashima, N. Mito and S. Shibata (2007). "Attenuating effect of clock mutation on triglyceride contents in the ICR mouse liver under a high-fat diet." J Biol Rhythms **22**(4): 312-23.

Kwon, I., J. Lee, S. H. Chang, N. C. Jung, B. J. Lee, G. H. Son, K. Kim and K. H. Lee (2006). "BMAL1 shuttling controls transactivation and degradation of the CLOCK/BMAL1 heterodimer." Mol Cell Biol **26**(19): 7318-30.

La Fleur, S. E., A. Kalsbeek, J. Wortel and R. M. Buijs (1999). "A suprachiasmatic nucleus generated rhythm in basal glucose concentrations." J Neuroendocrinol **11**(8): 643-52.

la Fleur, S. E., A. Kalsbeek, J. Wortel and R. M. Buijs (2000). "Polysynaptic neural pathways between the hypothalamus, including the suprachiasmatic nucleus, and the liver." Brain Res **871**(1): 50-6.

Lagud, M. and S. G. Reebs (2000). "Food-anticipatory activity of groups of golden shiners during both day and night." Canadian Journal of Zoology-Revue Canadienne De Zoologie **78**(5): 886-889.

Lall, G. S., V. L. Revell, H. Momiji, J. Al Enezi, C. M. Altimus, A. D. Guler, C. Aguilar, M. A. Cameron, S. Allender, M. W. Hankins and R. J. Lucas (2010). "Distinct contributions of rod, cone, and melanopsin photoreceptors to encoding irradiance." Neuron **66**(3): 417-28.

Lamia, K. A., U. M. Sachdeva, L. DiTacchio, E. C. Williams, J. G. Alvarez, D. F. Egan, D. S. Vasquez, H. Juguilon, S. Panda, R. J. Shaw, C. B. Thompson and R. M. Evans (2009). "AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation." Science **326**(5951): 437-40.

Lamia, K. A., K. F. Storch and C. J. Weitz (2008). "Physiological significance of a peripheral tissue circadian clock." Proc Natl Acad Sci U S A **105**(39): 15172-7.

Lamont, E. W., B. Robinson, J. Stewart and S. Amir (2005). "The central and basolateral nuclei of the amygdala exhibit opposite diurnal rhythms of expression of the clock protein Period2." Proceedings of the National Academy of Sciences of the United States of America **102**(11): 4180-4184.

Landry, G. J., B. A. Kent, D. F. Patton, M. Jaholkowski, E. G. Marchant and R. E. Mistlberger (2011). "Evidence for Time-of-Day Dependent Effect of Neurotoxic Dorsomedial Hypothalamic Lesions on Food Anticipatory Circadian Rhythms in Rats." PLoS One **6**(9).

Landry, G. J., M. M. Simon, I. C. Webb and R. E. Mistlberger (2006). "Persistence of a behavioral food-anticipatory circadian rhythm following dorsomedial hypothalamic ablation in rats." Am J Physiol Regul Integr Comp Physiol **290**(6): R1527-34.

Landry, G. J., G. R. Yamakawa, I. C. Webb, R. J. Mear and R. E. Mistlberger (2007). "The dorsomedial hypothalamic nucleus is not necessary for the expression of circadian food-anticipatory activity in rats." J Biol Rhythms **22**(6): 467-78.

Landry, G. J., G. R. S. Yamakawa and R. E. Mistlberger (2007). "Robust food anticipatory circadian rhythms in rats with complete ablation of the thalamic paraventricular nucleus." Brain Research **1141**: 108-118.

Lau, P., S. J. Nixon, R. G. Parton and G. E. Muscat (2004). "RORalpha regulates the expression of genes involved in lipid homeostasis in skeletal muscle cells: caveolin-3 and CPT-1 are direct targets of ROR." J Biol Chem **279**(35): 36828-40.

Lax, P., S. Zamora and J. A. Madrid (1998). "Coupling effect of locomotor activity on the rat's circadian system." American Journal of Physiology-Regulatory Integrative and Comparative Physiology **275**(2): R580-R587.

Le Martelot, G., T. Claudel, D. Gatfield, O. Schaad, B. Kornmann, G. L. Sasso, A. Moschetta and U. Schibler (2009). "REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis." PLoS Biol **7**(9): e1000181.

Lee, C., J. P. Etchegaray, F. R. Cagampang, A. S. Loudon and S. M. Reppert (2001). "Posttranslational mechanisms regulate the mammalian circadian clock." Cell **107**(7): 855-67.

LeSauter, J., N. Hoque, M. Weintraub, D. W. Pfaff and R. Silver (2009). "Stomach ghrelin-secreting cells as food-entrainable circadian clocks." Proc Natl Acad Sci U S A **106**(32): 13582-7.

Li, J. D., W. P. Hu, L. Boehmer, M. Y. Cheng, A. G. Lee, A. Jilek, J. M. Siegel and Q. Y. Zhou (2006). "Attenuated circadian rhythms in mice lacking the prokineticin 2 gene." J Neurosci **26**(45): 11615-23.

Li, J. D., W. P. Hu and Q. Y. Zhou (2009). "Disruption of the Circadian Output Molecule Prokineticin 2 Results in Anxiolytic and Antidepressant-like Effects in Mice." Neuropsychopharmacology **34**(2): 367-373.

Lima, F. B., N. S. Hell, C. Timoiaria, R. Scivoletto, M. S. Dolnikoff and A. A. Pupo (1981). "Metabolic Consequences of Food Restriction in Rats." Physiology & Behavior **27**(1): 115-123.

Lin, J., C. Handschin and B. M. Spiegelman (2005). "Metabolic control through the PGC-1 family of transcription coactivators." Cell Metab **1**(6): 361-70.

Liu, A. C., D. K. Welsh, C. H. Ko, H. G. Tran, E. E. Zhang, A. A. Priest, E. D. Buhr, O. Singer, K. Meeker, I. M. Verma, F. J. Doyle, 3rd, J. S. Takahashi and S. A. Kay (2007). "Intercellular coupling confers robustness against mutations in the SCN circadian clock network." Cell **129**(3): 605-16.

Liu, C., S. Li, T. Liu, J. Borjigin and J. D. Lin (2007). "Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism." Nature **447**(7143): 477-81.

Liu, E., S. Kitajima, Y. Higaki, M. Morimoto, H. Sun, T. Watanabe, N. Yamada and J. Fan (2005). "High lipoprotein lipase activity increases insulin sensitivity in transgenic rabbits." Metabolism **54**(1): 132-8.

Long, M. A., M. J. Jutras, B. W. Connors and R. D. Burwell (2005). "Electrical synapses coordinate activity in the suprachiasmatic nucleus." Nat Neurosci **8**(1): 61-6.

Lopez-Olmeda, J. F., E. V. Tartaglione, H. O. de la Iglesia and F. J. Sanchez-Vazquez (2010). "Feeding entrainment of food-anticipatory activity and per1 expression in the brain and liver of zebrafish under different lighting and feeding conditions." Chronobiol Int **27**(7): 1380-400.

LopezMolina, L., F. Conquet, M. DuboisDauphin and U. Schibler (1997). "The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior." Embo Journal **16**(22): 6762-6771.

Lowrey, P. L. and J. S. Takahashi (2004). "Mammalian circadian biology: elucidating genome-wide levels of temporal organization." Annu Rev Genomics Hum Genet **5**: 407-41.

Malek, Z. S., D. Sage, P. Pevet and S. Raison (2007). "Daily rhythm of tryptophan hydroxylase-2 messenger ribonucleic acid within raphe neurons is induced by corticoid daily surge and modulated by enhanced locomotor activity." Endocrinology **148**(11): 5165-72.

Marcheva, B., K. M. Ramsey, E. D. Buhr, Y. Kobayashi, H. Su, C. H. Ko, G. Ivanova, C. Omura, S. Mo, M. H. Vitaterna, J. P. Lopez, L. H. Philipson, C. A. Bradfield, S. D. Crosby, L. JeBailey, X. Wang, J. S. Takahashi and J. Bass (2010). "Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes." Nature **466**(7306): 627-31.

Martinez-Merlos, M. T., M. Angeles-Castellanos, M. Diaz-Munoz, R. Aguilar-Roblero, J. Mendoza and C. Escobar (2004). "Dissociation between adipose tissue signals, behavior and the food-entrained oscillator." J Endocrinol **181**(1): 53-63.

McClung, C. R. (2006). "Plant circadian rhythms." Plant Cell **18**(4): 792-803.

Meijer, J. H. and W. J. Schwartz (2003). "In search of the pathways for light-induced pacemaker resetting in the suprachiasmatic nucleus." J Biol Rhythms **18**(3): 235-49.

Menaker, M. (1996). "Colin S. Pittendrigh (1918-96)." Nature **381**(6577): 24.

Mendoza, J., U. Albrecht and E. Challet (2010). "Behavioural food anticipation in clock genes deficient mice: confirming old phenotypes, describing new phenotypes." Genes Brain Behav **9**(5): 467-77.

Mendoza, J., M. Angeles-Castellanos and C. Escobar (2005). "A daily palatable meal without food deprivation entrains the suprachiasmatic nucleus of rats." European Journal of Neuroscience **22**(11): 2855-2862.

Mendoza, J., M. Angeles-Castellanos and C. Escobar (2005). "Differential role of the accumbens Shell and Core subterritories in food-entrained rhythms of rats." Behav Brain Res **158**(1): 133-42.

Mendoza, J., M. Angeles-Castellanos and C. Escobar (2005). "Entrainment by a palatable meal induces food-anticipatory activity and c-Fos expression in reward-related areas of the brain." Neuroscience **133**(1): 293-303.

Mendoza, J., P. Pevet and E. Challet (2008). "High-fat feeding alters the clock synchronization to light." J Physiol **586**(Pt 24): 5901-10.

Mendoza, J., P. Pevet, M. P. Felder-Schmittbuhl, Y. Bailly and E. Challet (2010). "The cerebellum harbors a circadian oscillator involved in food anticipation." J Neurosci **30**(5): 1894-904.

Merkel, M., R. H. Eckel and I. J. Goldberg (2002). "Lipoprotein lipase: genetics, lipid uptake, and regulation." J Lipid Res **43**(12): 1997-2006.

Merkel, M., P. H. Weinstock, T. Chajek-Shaul, H. Radner, B. Yin, J. L. Breslow and I. J. Goldberg (1998). "Lipoprotein lipase expression exclusively in liver. A mouse model for metabolism in the neonatal period and during cachexia." J Clin Invest **102**(5): 893-901.

Merkestein, M., M. A. Brans, M. C. Luijendijk, J. W. de Jong, E. Egecioglu, S. L. Dickson and R. A. Adan (2012). "Ghrelin Mediates Anticipation to a Palatable Meal in Rats." Obesity (Silver Spring).

Meyer-Bernstein, E. L., A. E. Jetton, S. I. Matsumoto, J. F. Markuns, M. N. Lehman and E. L. Bittman (1999). "Effects of suprachiasmatic transplants on circadian rhythms of neuroendocrine function in golden hamsters." Endocrinology **140**(1): 207-18.

Michel, S., M. E. Geusz, J. J. Zaritsky and G. D. Block (1993). "Circadian rhythm in membrane conductance expressed in isolated neurons." Science **259**(5092): 239-41.

Mieda, M. and T. Sakurai (2011). "Bmal1 in the nervous system is essential for normal adaptation of circadian locomotor activity and food intake to periodic feeding." J Neurosci **31**(43): 15391-6.

Mieda, M., S. C. Williams, J. A. Richardson, K. Tanaka and M. Yanagisawa (2006). "The dorsomedial hypothalamic nucleus as a putative food-entrainable circadian pacemaker." Proceedings of the National Academy of Sciences of the United States of America **103**(32): 12150-12155.

Mieda, M., S. C. Williams, C. M. Sinton, J. A. Richardson, T. Sakurai and M. Yanagisawa (2004). "Orexin neurons function in an efferent pathway of a food-entrainable circadian oscillator in eliciting food-anticipatory activity and wakefulness." J Neurosci **24**(46): 10493-501.

Miller, B. H., E. L. McDearmon, S. Panda, K. R. Hayes, J. Zhang, J. L. Andrews, M. P. Antoch, J. R. Walker, K. A. Esser, J. B. Hogenesch and J. S. Takahashi (2007). "Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation." Proc Natl Acad Sci U S A **104**(9): 3342-7.

Mistlberger, R. and B. Rusak (1987). "Palatable daily meals entrain anticipatory activity rhythms in free-feeding rats: dependence on meal size and nutrient content." Physiol Behav **41**(3): 219-26.

Mistlberger, R. E. (1994). "Circadian food-anticipatory activity: formal models and physiological mechanisms." Neurosci Biobehav Rev **18**(2): 171-95.

Mistlberger, R. E. (2011). "Neurobiology of food anticipatory circadian rhythms." Physiol Behav **104**(4): 535-45.

Mistlberger, R. E. and M. C. Antle (1999). "Neonatal monosodium glutamate alters circadian organization of feeding, food anticipatory activity and photic masking in the rat." Brain Research **842**(1): 73-83.

Mistlberger, R. E., M. C. Antle, T. S. Kilduff and M. Jones (2003). "Food- and light-entrained circadian rhythms in rats with hypocretin-2-saporin ablations of the lateral hypothalamus." Brain Research **980**(2): 161-168.

Mistlberger, R. E., R. M. Buijs, E. Challet, C. Escobar, G. J. Landry, A. Kalsbeek, P. Pevet and S. Shibata (2009). "Food anticipation in Bmal1-/- and AAV-Bmal1 rescued mice: a reply to Fuller et al." J Circadian Rhythms **7**: 11.

Mistlberger, R. E., R. M. Buijs, E. Challet, C. Escobar, G. J. Landry, A. Kalsbeek, P. Pevet and S. Shibata (2009). "Standards of evidence in chronobiology: critical review of a report that restoration of Bmal1 expression in the dorsomedial hypothalamus is sufficient to restore circadian food anticipatory rhythms in Bmal1-/- mice." J Circadian Rhythms **7**: 3.

Mistlberger, R. E., T. A. Houpt and M. C. Moore-Ede (1990). "Food-anticipatory rhythms under 24-hour schedules of limited access to single macronutrients." J Biol Rhythms **5**(1): 35-46.
Mistlberger, R. E., B. A. Kent, S. Chan, D. F. Patton, A. Weinberg and M. Parfyonov (2012). "Circadian clocks for all meal-times: anticipation of 2 daily meals in rats." PLoS One **7**(2): e31772.
Mistlberger, R. E., H. Lukman and B. G. Nadeau (1998). "Circadian rhythms in the Zucker obese rat: assessment and intervention." Appetite **30**(3): 255-67.
Mistlberger, R. E. and E. G. Marchant (1999). "Enhanced food-anticipatory circadian rhythms in the genetically obese Zucker rat." Physiol Behav **66**(2): 329-35.
Mistlberger, R. E. and D. G. Mumby (1992). "The Limbic System and Food-Anticipatory Circadian-Rhythms in the Rat - Ablation and Dopamine Blocking Studies." Behavioural Brain Research **47**(2): 159-168.
Mistlberger, R. E. and A. Rechtschaffen (1984). "Recovery of Anticipatory Activity to Restricted Feeding in Rats with Ventromedial Hypothalamic-Lesions." Physiology & Behavior **33**(2): 227-235.
Mistlberger, R. E. and B. Rusak (1988). "Food-Anticipatory Circadian-Rhythms in Rats with Paraventricular and Lateral Hypothalamic Ablations." Journal of Biological Rhythms **3**(3): 277-291.
Mistlberger, R. E., S. Yamazaki, J. S. Pendergast, G. J. Landry, T. Takumi and W. Nakamura (2008). "Comment on "Differential Rescue of Light- and Food-Entrainable Circadian Rhythms"." Science **322**(5902).
Miyamoto, Y. and A. Sancar (1999). "Circadian regulation of cryptochrome genes in the mouse." Brain Res Mol Brain Res **71**(2): 238-43.
Mohawk, J. A., M. L. Baer and M. Menaker (2009). "The methamphetamine-sensitive circadian oscillator does not employ canonical clock genes." Proc Natl Acad Sci U S A **106**(9): 3519-24.
Moore, D. (2001). "Honey bee circadian clocks: behavioral control from individual workers to whole-colony rhythms." Journal of Insect Physiology **47**(8): 843-857.
Moore, D. and M. A. Rankin (1983). "Diurnal Changes in the Accuracy of the Honeybee Foraging Rhythm." Biological Bulletin **164**(3): 471-482.
Moore, R. Y. (1983). "Organization and function of a central nervous system circadian oscillator: the suprachiasmatic hypothalamic nucleus." Fed Proc **42**(11): 2783-9.
Moore, R. Y. and V. B. Eichler (1972). "Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat." Brain Res **42**(1): 201-6.
Moore, R. Y. and D. C. Klein (1974). "Visual pathways and the central neural control of a circadian rhythm in pineal serotonin N-acetyltransferase activity." Brain Res **71**(1): 17-33.
Moore, R. Y. and N. J. Lenn (1972). "A retinohypothalamic projection in the rat." J Comp Neurol **146**(1): 1-14.
Moore, R. Y., J. C. Speh and R. K. Leak (2002). "Suprachiasmatic nucleus organization." Cell Tissue Res **309**(1): 89-98.
Moriya, T., R. Aida, T. Kudo, M. Akiyama, M. Doi, N. Hayasaka, N. Nakahata, R. Mistlberger, H. Okamura and S. Shibata (2009). "The dorsomedial hypothalamic nucleus is not necessary for food-anticipatory circadian rhythms of behavior, temperature or clock gene expression in mice." Eur J Neurosci **29**(7): 1447-60.
Morse, D. and P. Sassone-Corsi (2002). "Time after time: inputs to and outputs from the mammalian circadian oscillators." Trends Neurosci **25**(12): 632-7.
Motzkus, D., E. Maronde, U. Grunenberg, C. C. Lee, W. Forssmann and U. Albrecht (2000). "The human PER1 gene is transcriptionally regulated by multiple signaling pathways." FEBS Lett **486**(3): 315-9.
Murakami, D. M., B. A. Horwitz and C. A. Fuller (1995). "Circadian rhythms of temperature and activity in obese and lean Zucker rats." Am J Physiol **269**(5 Pt 2): R1038-43.
Nader, N., G. P. Chrousos and T. Kino (2009). "Circadian rhythm transcription factor CLOCK regulates the transcriptional activity of the glucocorticoid receptor by acetylating its hinge region lysine cluster: potential physiological implications." FASEB J **23**(5): 1572-83.

Nagai, K., T. Nishio, H. Nakagawa, S. Nakamura and Y. Fukuda (1978). "Effect of bilateral lesions of the suprachiasmatic nuclei on the circadian rhythm of food-intake." Brain Res **142**(2): 384-9.

Nakahara, K., K. Fukui and N. Murakami (2004). "Involvement of thalamic paraventricular nucleus in the anticipatory reaction under food restriction in the rat." Journal of Veterinary Medical Science **66**(10): 1297-1300.

Nakahata, Y., M. Kaluzova, B. Grimaldi, S. Sahar, J. Hirayama, D. Chen, L. P. Guarente and P. Sassone-Corsi (2008). "The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control." Cell **134**(2): 329-40.

Nakahata, Y., S. Sahar, G. Astarita, M. Kaluzova and P. Sassone-Corsi (2009). "Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1." Science **324**(5927): 654-7.

Nelson, W., L. Scheving and F. Halberg (1975). "Circadian rhythms in mice fed a single daily meal at different stages of lighting regimen." J Nutr **105**(2): 171-84.

Nishida, T., M. Saito and M. Suda (1978). "Parallel between Circadian-Rhythms of Intestinal Disaccharidases and Food-Intake of Rats under Constant Lighting Conditions." Gastroenterology **74**(2): 224-227.

Nogueiras, R., M. H. Tschop and J. M. Zigman (2008). "Central nervous system regulation of energy metabolism: ghrelin versus leptin." Ann N Y Acad Sci **1126**: 14-9.

O'Neill, J. S. and A. B. Reddy (2011). "Circadian clocks in human red blood cells." Nature **469**(7331): 498-503.

Obici, S., Z. H. Feng, Y. Morgan, D. Stein, G. Karkanias and L. Rossetti (2002). "Central administration of oleic acid inhibits glucose production and food intake." Diabetes **51**(2): 271-275.

Oishi, K., N. Amagai, H. Shirai, K. Kadota, N. Ohkura and N. Ishida (2005). "Genome-wide expression analysis reveals 100 adrenal gland-dependent circadian genes in the mouse liver." DNA Res **12**(3): 191-202.

Oishi, K., G. Atsumi, S. Sugiyama, I. Kodomari, M. Kasamatsu, K. Machida and N. Ishida (2006). "Disrupted fat absorption attenuates obesity induced by a high-fat diet in Clock mutant mice." FEBS Lett **580**(1): 127-30.

Oishi, K., K. Miyazaki, K. Kadota, R. Kikuno, T. Nagase, G. Atsumi, N. Ohkura, T. Azama, M. Mesaki, S. Yukimasa, H. Kobayashi, C. Iitaka, T. Umehara, M. Horikoshi, T. Kudo, Y. Shimizu, M. Yano, M. Monden, K. Machida, J. Matsuda, S. Horie, T. Todo and N. Ishida (2003). "Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes." J Biol Chem **278**(42): 41519-27.

Oishi, K., N. Ohkura, M. Wakabayashi, H. Shirai, K. Sato, J. Matsuda, G. Atsumi and N. Ishida (2006). "CLOCK is involved in obesity-induced disordered fibrinolysis in ob/ob mice by regulating PAI-1 gene expression." J Thromb Haemost **4**(8): 1774-80.

Oishi, K., H. Shirai and N. Ishida (2005). "CLOCK is involved in the circadian transactivation of peroxisome-proliferator-activated receptor alpha (PPARalpha) in mice." Biochem J **386**(Pt 3): 575-81.

Oosterveer, M. H., T. H. van Dijk, U. J. Tietge, T. Boer, R. Havinga, F. Stellaard, A. K. Groen, F. Kuipers and D. J. Reijngoud (2009). "High fat feeding induces hepatic fatty acid elongation in mice." PLoS One **4**(6): e6066.

Oster, H., S. Damerow, S. Kiessling, V. Jakubcakova, D. Abraham, J. Tian, M. W. Hoffmann and G. Eichele (2006). "The circadian rhythm of glucocorticoids is regulated by a gating mechanism residing in the adrenal cortical clock." Cell Metab **4**(2): 163-73.

Ottenweller, J. E., D. L. Pitman and B. H. Natelson (1987). "Repeated stress at the same time of day does not mimic timed feeding in its effects on the plasma corticosterone rhythm in rats." Chronobiologia **14**(1): 1-6.

Otway, D. T., S. Mantele, S. Bretschneider, J. Wright, P. Trayhurn, D. J. Skene, M. D. Robertson and J. D. Johnston (2011). "Rhythmic diurnal gene expression in human adipose tissue from individuals who are lean, overweight, and type 2 diabetic." Diabetes **60**(5): 1577-81.

Panda, S., I. Provencio, D. C. Tu, S. S. Pires, M. D. Rollag, A. M. Castrucci, M. T. Pletcher, T. K. Sato, T. Wiltshire, M. Andahazy, S. A. Kay, R. N. Van Gelder and J. B. Hogenesch (2003). "Melanopsin

is required for non-image-forming photic responses in blind mice." Science **301**(5632): 525-527.

Panda, S., T. K. Sato, A. M. Castrucci, M. D. Rollag, W. J. DeGrip, J. B. Hogenesch, I. Provencio and S. A. Kay (2002). "Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting." Science **298**(5601): 2213-6.

Pappan, K. L., Z. Pan, G. Kwon, C. A. Marshall, T. Coleman, I. J. Goldberg, M. L. McDaniel and C. F. Semenkovich (2005). "Pancreatic beta-cell lipoprotein lipase independently regulates islet glucose metabolism and normal insulin secretion." J Biol Chem **280**(10): 9023-9.

Pardina, E., J. A. Baena-Fustegueras, R. Llamas, R. Catalan, R. Galard, A. Lecube, J. M. Fort, M. Llobera, H. Allende, V. Vargas and J. Peinado-Onsurbe (2009). "Lipoprotein Lipase Expression in Livers of Morbidly Obese Patients Could be Responsible for Liver Steatosis." Obesity Surgery **19**(5): 608-616.

Pate, J.R (1937). Transneural atrophy of the nucleus ovidens following eye remocal in cats. Anat. Rec. 67, 39

Paul, K. N., T. B. Saafir and G. Tosini (2009). "The role of retinal photoreceptors in the regulation of circadian rhythms." Rev Endocr Metab Disord **10**(4): 271-8.

Pecoraro, N., F. Gomez, K. Laugero and M. F. Dallman (2002). "Brief access to sucrose engages food-entrainable rhythms in food-deprived rats." Behav Neurosci **116**(5): 757-76.

Pendergast, J. S., W. Nakamura, R. C. Friday, F. Hatanaka, T. Takumi and S. Yamazaki (2009). "Robust Food Anticipatory Activity in BMAL1-Deficient Mice." PLoS One **4**(3).

Picard, F., M. Kurtev, N. Chung, A. Topark-Ngarm, T. Senawong, R. Machado De Oliveira, M. Leid, M. W. McBurney and L. Guarente (2004). "Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma." Nature **429**(6993): 771-6.

Pircher, P., P. Chomez, F. Yu, B. Vennstrom and L. Larsson (2005). "Aberrant expression of myosin isoforms in skeletal muscles from mice lacking the rev-erbAalpha orphan receptor gene." Am J Physiol Regul Integr Comp Physiol **288**(2): R482-90.

Pittendrigh, C. S. (1954). "On Temperature Independence in the Clock System Controlling Emergence Time in Drosophila." Proc Natl Acad Sci U S A **40**(10): 1018-29.

Pitts, S., E. Perone and R. Silver (2003). "Food-entrained circadian rhythms are sustained in arrhythmic Clk/Clk mutant mice." Am J Physiol Regul Integr Comp Physiol **285**(1): R57-67.

Preitner, N., F. Damiola, L. Lopez-Molina, J. Zakany, D. Duboule, U. Albrecht and U. Schibler (2002). "The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator." Cell **110**(2): 251-60.

Prickaerts, J., D. Moechars, K. Cryns, I. Lenaerts, H. van Craenendonck, I. Goris, G. Daneels, J. A. Bouwknecht and T. Steckler (2006). "Transgenic mice overexpressing glycogen synthase kinase 3 beta: A putative model of hyperactivity and mania." Journal of Neuroscience **26**(35): 9022-9029.

Provencio, I., I. R. Rodriguez, G. Jiang, W. P. Hayes, E. F. Moreira and M. D. Rollag (2000). "A novel human opsin in the inner retina." J Neurosci **20**(2): 600-5.

Puigserver, P., Z. Wu, C. W. Park, R. Graves, M. Wright and B. M. Spiegelman (1998). "A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis." Cell **92**(6): 829-39.

Purushotham, A., T. T. Schug, Q. Xu, S. Surapureddi, X. Guo and X. Li (2009). "Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation." Cell Metab **9**(4): 327-38.

Rada, P., N. M. Avena and B. G. Hoebel (2005). "Daily bingeing on sugar repeatedly releases dopamine in the accumbens shell." Neuroscience **134**(3): 737-744.

Raghuram, S., K. R. Stayrook, P. Huang, P. M. Rogers, A. K. Nosie, D. B. McClure, L. L. Burris, S. Khorasanizadeh, T. P. Burris and F. Rastinejad (2007). "Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-ERBbeta." Nat Struct Mol Biol **14**(12): 1207-13.

Ralph, M. R., R. G. Foster, F. C. Davis and M. Menaker (1990). "Transplanted suprachiasmatic nucleus determines circadian period." Science **247**(4945): 975-8.

Ralph, M. R. and M. Menaker (1988). "A mutation of the circadian system in golden hamsters." Science **241**(4870): 1225-7.

Ramakrishnan, S. N., P. Lau, L. J. Burke and G. E. Muscat (2005). "Rev-erbbeta regulates the expression of genes involved in lipid absorption in skeletal muscle cells: evidence for cross-talk between orphan nuclear receptors and myokines." J Biol Chem **280**(10): 8651-9.

Ramsey, K. M., J. Yoshino, C. S. Brace, D. Abrassart, Y. Kobayashi, B. Marcheva, H. K. Hong, J. L. Chong, E. D. Buhr, C. Lee, J. S. Takahashi, S. Imai and J. Bass (2009). "Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis." Science **324**(5927): 651-4.

Raspe, E., H. Duez, P. Gervois, C. Fievet, J. C. Fruchart, S. Besnard, J. Mariani, A. Tedgui and B. Staels (2001). "Transcriptional regulation of apolipoprotein C-III gene expression by the orphan nuclear receptor RORalpha." J Biol Chem **276**(4): 2865-71.

Raspe, E., H. Duez, A. Mansen, C. Fontaine, C. Fievet, J. C. Fruchart, B. Vennstrom and B. Staels (2002). "Identification of Rev-erbalpha as a physiological repressor of apoC-III gene transcription." J Lipid Res **43**(12): 2172-9.

Raspe, E., G. Mautino, C. Duval, C. Fontaine, H. Duez, O. Barbier, D. Monte, J. Fruchart, J. C. Fruchart and B. Staels (2002). "Transcriptional regulation of human Rev-erbalpha gene expression by the orphan nuclear receptor retinoic acid-related orphan receptor alpha." J Biol Chem **277**(51): 49275-81.

Rea, M. A. (1989). "Light increases Fos-related protein immunoreactivity in the rat suprachiasmatic nuclei." Brain Res Bull **23**(6): 577-81.

Reddy, A. B., N. A. Karp, E. S. Maywood, E. A. Sage, M. Deery, J. S. O'Neill, G. K. Wong, J. Chesham, M. Odell, K. S. Lilley, C. P. Kyriacou and M. H. Hastings (2006). "Circadian orchestration of the hepatic proteome." Curr Biol **16**(11): 1107-15.

Reick, M., J. A. Garcia, C. Dudley and S. L. McKnight (2001). "NPAS2: an analog of clock operative in the mammalian forebrain." Science **293**(5529): 506-9.

Renner, M. (1960). "The contribution of the honey bee to the study of time-sense and astronomical orientation." Cold Spring Harb Symp Quant Biol **25**: 361-7.

Ribeiro, A. C., E. Sawa, I. Carren-LeSauter, J. LeSauter, R. Silver and D. W. Pfaff (2007). "Two forces for arousal: Pitting hunger versus circadian influences and identifying neurons responsible for changes in behavioral arousal." Proc Natl Acad Sci U S A **104**(50): 20078-83.

Richter, C.P. (1922). A behavioristic study of the activity of the rat. Comp. Psychol. Monogr. **1,** 1–55.

Richter, C. P. (1967). "Sleep and activity: their relation to the 24-hour clock." Res Publ Assoc Res Nerv Ment Dis **45**: 8-29.

Roberts, A. J., J. S. McDonald, C. J. Heyser, B. L. Kieffer, H. W. Matthes, G. F. Koob and L. H. Gold (2000). "mu-Opioid receptor knockout mice do not self-administer alcohol." J Pharmacol Exp Ther **293**(3): 1002-8.

Rodgers, J. T., C. Lerin, W. Haas, S. P. Gygi, B. M. Spiegelman and P. Puigserver (2005). "Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1." Nature **434**(7029): 113-8.

Rosenwasser, A. M., J. Schulkin and N. T. Adler (1985). "Circadian wheel-running activity of rats under schedules of limited daily access to salt." Chronobiol Int **2**(2): 115-9.

Ross, S. E., N. Hemati, K. A. Longo, C. N. Bennett, P. C. Lucas, R. L. Erickson and O. A. MacDougald (2000). "Inhibition of adipogenesis by Wnt signaling." Science **289**(5481): 950-3.

Roybal, K., D. Theobold, A. Graham, J. A. DiNieri, S. J. Russo, V. Krishnan, S. Chakravarty, J. Peevey, N. Oehrlein, S. Birnbaum, M. H. Vitaterna, P. Orsulak, J. S. Takahashi, E. J. Nestler, W. A. Carlezon and C. A. McClung (2007). "Mania-like behavior induced by disruption of CLOCK." Proceedings of the National Academy of Sciences of the United States of America **104**(15): 6406-6411.

Rudic, R. D., P. McNamara, A. M. Curtis, R. C. Boston, S. Panda, J. B. Hogenesch and G. A. Fitzgerald (2004). "BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis." PLoS Biol **2**(11): e377.

Rutter, J., M. Reick, L. C. Wu and S. L. McKnight (2001). "Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors." Science **293**(5529): 510-4.

Sadacca, L. A., K. A. Lamia, A. S. deLemos, B. Blum and C. J. Weitz (2011). "An intrinsic circadian clock of the pancreas is required for normal insulin release and glucose homeostasis in mice." Diabetologia **54**(1): 120-4.

Sage, D., J. Ganem, F. Guillaumond, G. Laforge-Anglade, A. M. Francois-Bellan, O. Bosler and D. Becquet (2004). "Influence of the corticosterone rhythm on photic entrainment of locomotor activity in rats." J Biol Rhythms **19**(2): 144-56.

Salgado-Delgado, R., M. Angeles-Castellanos, M. R. Buijs and C. Escobar (2008). "Internal desynchronization in a model of night-work by forced activity in rats." Neuroscience **154**(3): 922-31.

Salgado-Delgado, R., M. Angeles-Castellanos, N. Saderi, R. M. Buijs and C. Escobar (2010). "Food intake during the normal activity phase prevents obesity and circadian desynchrony in a rat model of night work." Endocrinology **151**(3): 1019-29.

Sans-Fuentes, M. A., A. Diez-Noguera and T. Cambras (2010). "Light responses of the circadian system in leptin deficient mice." Physiol Behav **99**(4): 487-94.

Saper, C. B., J. Lu, T. C. Chou and J. Gooley (2005). "The hypothalamic integrator for circadian rhythms." Trends Neurosci **28**(3): 152-7.

Saunders, D. S. (1977). "Insect Clocks." Comparative Biochemistry and Physiology a-Physiology **56**(1): 1-5.

Scheer, F. A., M. F. Hilton, C. S. Mantzoros and S. A. Shea (2009). "Adverse metabolic and cardiovascular consequences of circadian misalignment." Proc Natl Acad Sci U S A **106**(11): 4453-8.

Scheving, L. E., T. H. Tsai and L. A. Scheving (1983). "Chronobiology of the Intestinal-Tract of the Mouse." American Journal of Anatomy **168**(4): 433-465.

Schibler, U., J. A. Ripperger and S. A. Brown (2001). "Circadian rhythms. Chronobiology--reducing time." Science **293**(5529): 437-8.

Schibler, U. and P. Sassone-Corsi (2002). "A web of circadian pacemakers." Cell **111**(7): 919-22.

Schmutz, I., J. A. Ripperger, S. Baeriswyl-Aebischer and U. Albrecht (2010). "The mammalian clock component PERIOD2 coordinates circadian output by interaction with nuclear receptors." Genes Dev **24**(4): 345-57.

Schoonjans, K., L. Gelman, C. Haby, M. Briggs and J. Auwerx (2000). "Induction of LPL gene expression by sterols is mediated by a sterol regulatory element and is independent of the presence of multiple E boxes." J Mol Biol **304**(3): 323-34.

Schoonjans, K., J. Peinado-Onsurbe, A. M. Lefebvre, R. A. Heyman, M. Briggs, S. Deeb, B. Staels and J. Auwerx (1996). "PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene." EMBO J **15**(19): 5336-48.

Sears, I. B., M. A. MacGinnitie, L. G. Kovacs and R. A. Graves (1996). "Differentiation-dependent expression of the brown adipocyte uncoupling protein gene: regulation by peroxisome proliferator-activated receptor gamma." Mol Cell Biol **16**(7): 3410-9.

Shen, X., C. Purser, L. T. Tien, C. T. Chiu, I. A. Paul, R. Baker, H. H. Loh, I. K. Ho and T. Ma (2010). "mu-Opioid receptor knockout mice are insensitive to methamphetamine-induced behavioral sensitization." J Neurosci Res **88**(10): 2294-302.

Shi, S. Q., A. Hida, O. P. McGuinness, D. H. Wasserman, S. Yamazaki and C. H. Johnson (2010). "Circadian Clock Gene Bmal1 Is Not Essential; Functional Replacement with its Paralog, Bmal2." Current Biology **20**(4): 316-321.

Shibata, S. (2004). "Neural regulation of the hepatic circadian rhythm." Anat Rec A Discov Mol Cell Evol Biol **280**(1): 901-9.

Shibata, S., Y. Minamoto, M. Ono and S. Watanabe (1994). "Aging impairs methamphetamine-induced free-running and anticipatory locomotor activity rhythms in rats." Neurosci Lett **172**(1-2): 107-10.

Shimba, S., N. Ishii, Y. Ohta, T. Ohno, Y. Watabe, M. Hayashi, T. Wada, T. Aoyagi and M. Tezuka (2005). "Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis." Proc Natl Acad Sci U S A **102**(34): 12071-6.

Shimba, S., T. Ogawa, S. Hitosugi, Y. Ichihashi, Y. Nakadaira, M. Kobayashi, M. Tezuka, Y. Kosuge, K. Ishige, Y. Ito, K. Komiyama, Y. Okamatsu-Ogura, K. Kimura and M. Saito (2011). "Deficient of a Clock Gene, Brain and Muscle Arnt-Like Protein-1 (BMAL1), Induces Dyslipidemia and Ectopic Fat Formation." PLoS One **6**(9).

Shirakawa, T., S. Honma, Y. Katsuno, H. Oguchi and K. I. Honma (2000). "Synchronization of circadian firing rhythms in cultured rat suprachiasmatic neurons." Eur J Neurosci **12**(8): 2833-8.

Siepka, S. M., S. H. Yoo, J. Park, W. M. Song, V. Kumar, Y. N. Hu, C. Lee and J. S. Takahashi (2007). "Circadian mutant overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression." Cell **129**(5): 1011-1023.

Silver, R., P. D. Balsam, M. P. Butler and J. LeSauter (2011). "Food anticipation depends on oscillators and memories in both body and brain." Physiology & Behavior **104**(4): 562-571.

Silver, R., J. LeSauter, P. A. Tresco and M. N. Lehman (1996). "A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms." Nature **382**(6594): 810-3.

Solt, L. A., Y. J. Wang, S. Banerjee, T. Hughes, D. J. Kojetin, T. Lundasen, Y. Shin, J. Liu, M. D. Cameron, R. Noel, S. H. Yoo, J. S. Takahashi, A. A. Butler, T. M. Kamenecka and T. P. Burris (2012). "Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists." Nature **485**(7396): 62-68.

Spruijt, B. M., R. van den Bos and F. T. Pijlman (2001). "A concept of welfare based on reward evaluating mechanisms in the brain: anticipatory behaviour as an indicator for the state of reward systems." Appl Anim Behav Sci **72**(2): 145-171.

Stephan, F. K. (1981). "Limits of Entrainment to Periodic Feeding in Rats with Suprachiasmatic Lesions." Journal of Comparative Physiology **143**(4): 401-410.

Stephan, F. K. (1983). "Circadian-Rhythm Dissociation Induced by Periodic Feeding in Rats with Suprachiasmatic Lesions." Behavioural Brain Research **7**(1): 81-98.

Stephan, F. K. (1984). "Phase shifts of circadian rhythms in activity entrained to food access." Physiol Behav **32**(4): 663-71.

Stephan, F. K. (1992). "Resetting of a feeding-entrainable circadian clock in the rat." Physiol Behav **52**(5): 985-95.

Stephan, F. K. (1997). "Calories affect zeitgeber properties of the feeding entrained circadian oscillator." Physiol Behav **62**(5): 995-1002.

Stephan, F. K. and A. J. Davidson (1998). "Glucose, but not fat, phase shifts the feeding-entrained circadian clock." Physiol Behav **65**(2): 277-88.

Stephan, F. K., J. M. Swann and C. L. Sisk (1979). "Anticipation of 24-hr feeding schedules in rats with lesions of the suprachiasmatic nucleus." Behav Neural Biol **25**(3): 346-63.

Stephan, F. K., J. M. Swann and C. L. Sisk (1979). "Entrainment of circadian rhythms by feeding schedules in rats with suprachiasmatic lesions." Behav Neural Biol **25**(4): 545-54.

Stephan, F. K. and I. Zucker (1972). "Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions." Proc Natl Acad Sci U S A **69**(6): 1583-6.

Sternson, S. M. (2011). "METABOLISM Let them eat fat." Nature **477**(7363): 166-167.

Stokkan, K. A., S. Yamazaki, H. Tei, Y. Sakaki and M. Menaker (2001). "Entrainment of the circadian clock in the liver by feeding." Science **291**(5503): 490-3.

Storch, K. F. and C. J. Weitz (2009). "Daily rhythms of food-anticipatory behavioral activity do not require the known circadian clock." Proceedings of the National Academy of Sciences of the United States of America **106**(16): 6808-6813.

Stratmann, M. and U. Schibler (2006). "Properties, entrainment, and physiological functions of mammalian peripheral oscillators." J Biol Rhythms **21**(6): 494-506.

Sutton, G. M., D. Perez-Tilve, R. Nogueiras, J. Fang, J. K. Kim, R. D. Cone, J. M. Gimble, M. H. Tschop and A. A. Butler (2008). "The melanocortin-3 receptor is required for entrainment to meal intake." J Neurosci **28**(48): 12946-55.

Sweeney, B. M. and M. B. Borgese (1989). "A Circadian-Rhythm in Cell-Division in a Prokaryote, the Cyanobacterium Synechococcus Wh7803." Journal of Phycology **25**(1): 183-186.

Tahara, Y., A. Hirao, T. Moriya, T. Kudo and S. Shibata (2010). "Effects of medial hypothalamic lesions on feeding-induced entrainment of locomotor activity and liver Per2 expression in Per2::luc mice." J Biol Rhythms **25**(1): 9-18.

Tahara, Y., H. Kuroda, K. Saito, Y. Nakajima, Y. Kubo, N. Ohnishi, Y. Seo, M. Otsuka, Y. Fuse, Y. Ohura, T. Komatsu, Y. Moriya, S. Okada, N. Furutani, A. Hirao, K. Horikawa, T. Kudo and S. Shibata (2012). "In Vivo Monitoring of Peripheral Circadian Clocks in the Mouse." Curr Biol.

Tataroglu, O., A. J. Davidson, L. J. Benvenuto and M. Menaker (2006). "The methamphetamine-sensitive circadian oscillator (MASCO) in mice." J Biol Rhythms **21**(3): 185-94.

Teboul, M., F. Guillaumond, A. Grechez-Cassiau and F. Delaunay (2008). "The nuclear hormone receptor family round the clock." Mol Endocrinol **22**(12): 2573-82.

Tei, H., H. Okamura, Y. Shigeyoshi, C. Fukuhara, R. Ozawa, M. Hirose and Y. Sakaki (1997). "Circadian oscillation of a mammalian homologue of the Drosophila period gene." Nature **389**(6650): 512-6.

Tepperman, J. and H. M. Tepperman (1965). "Adaptive hyperlipogenesis--late 1964 model." Ann N Y Acad Sci **131**(1): 404-11.

Tosini, G. and M. Menaker (1996). "Circadian rhythms in cultured mammalian retina." Science **272**(5260): 419-21.

Tosini, G. and M. Menaker (1998). "The tau mutation affects temperature compensation of hamster retinal circadian oscillators." Neuroreport **9**(6): 1001-5.

Tousson, E. and H. Meissl (2004). "Suprachiasmatic nuclei grafts restore the circadian rhythm in the paraventricular nucleus of the hypothalamus." J Neurosci **24**(12): 2983-8.

Travnickova-Bendova, Z., N. Cermakian, S. M. Reppert and P. Sassone-Corsi (2002). "Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity." Proc Natl Acad Sci U S A **99**(11): 7728-33.

Tsai, L. L., Y. C. Tsai, K. Hwang, Y. W. Huang and J. E. Tzeng (2005). "Repeated light-dark shifts speed up body weight gain in male F344 rats." Am J Physiol Endocrinol Metab **289**(2): E212-7.

Tsiftsoglou, A. S., A. I. Tsamadou and L. C. Papadopoulou (2006). "Heme as key regulator of major mammalian cellular functions: molecular, cellular, and pharmacological aspects." Pharmacol Ther **111**(2): 327-45.

Turek, F. W. (1989). "Effects of stimulated physical activity on the circadian pacemaker of vertebrates." J Biol Rhythms **4**(2): 135-47.

Turek, F. W., C. Joshu, A. Kohsaka, E. Lin, G. Ivanova, E. McDearmon, A. Laposky, S. Losee-Olson, A. Easton, D. R. Jensen, R. H. Eckel, J. S. Takahashi and J. Bass (2005). "Obesity and metabolic syndrome in circadian Clock mutant mice." Science **308**(5724): 1043-5.

Ueda, H. R., W. Chen, A. Adachi, H. Wakamatsu, S. Hayashi, T. Takasugi, M. Nagano, K. Nakahama, Y. Suzuki, S. Sugano, M. Iino, Y. Shigeyoshi and S. Hashimoto (2002). "A transcription factor response element for gene expression during circadian night." Nature **418**(6897): 534-9.

Um, J. H., J. S. Pendergast, D. A. Springer, M. Foretz, B. Viollet, A. Brown, M. K. Kim, S. Yamazaki and J. H. Chung (2011). "AMPK regulates circadian rhythms in a tissue- and isoform-specific manner." PLoS One **6**(3): e18450.

Um, J. H., S. Yang, S. Yamazaki, H. Kang, B. Viollet, M. Foretz and J. H. Chung (2007). "Activation of 5'-AMP-activated kinase with diabetes drug metformin induces casein kinase Iepsilon (CKIepsilon)-dependent degradation of clock protein mPer2." J Biol Chem **282**(29): 20794-8.

Unger, J., T. H. McNeill, R. T. Moxley, 3rd, M. White, A. Moss and J. N. Livingston (1989). "Distribution of insulin receptor-like immunoreactivity in the rat forebrain." Neuroscience **31**(1): 143-57.

Valnegri, P., M. Khelfaoui, O. Dorseuil, S. Bassani, C. Lagneaux, A. Gianfelice, R. Benfante, J. Chelly, P. Billuart, C. Sala and M. Passafaro (2011). "A circadian clock in hippocampus is regulated by interaction between oligophrenin-1 and Rev-erbalpha." Nat Neurosci **14**(10): 1293-301.

van der Horst, G. T., M. Muijtjens, K. Kobayashi, R. Takano, S. Kanno, M. Takao, J. de Wit, A. Verkerk, A. P. Eker, D. van Leenen, R. Buijs, D. Bootsma, J. H. Hoeijmakers and A. Yasui (1999). "Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms." Nature **398**(6728): 627-30.

van der Veen, D. R., D. J. Saaltink and M. P. Gerkema (2011). "Behavioral Responses to Combinations of Timed Light, Food Availability, and Ultradian Rhythms in the Common Vole (Microtus arvalis)." Chronobiology International **28**(7): 563-571.

Van der Zee, E. A., R. Havekes, R. P. Barf, R. A. Hut, I. M. Nijholt, E. H. Jacobs and M. P. Gerkema (2008). "Circadian time-place learning in mice depends on Cry genes." Curr Biol **18**(11): 844-8.

van Esseveldt, K. E., M. N. Lehman and G. J. Boer (2000). "The suprachiasmatic nucleus and the circadian time-keeping system revisited." Brain Res Brain Res Rev **33**(1): 34-77.

Vansteensel, M. J., S. Michel and J. H. Meijer (2008). "Organization of cell and tissue circadian pacemakers: a comparison among species." Brain Res Rev **58**(1): 18-47.

Velasco, A., T. G. Cachero and T. G. Granda (1994). "Entrainment of Circadian-Rhythms of Cholesterol-Ldl, Corticosterone and Motor-Activity by Feeding Schedules in Rats." Biological Rhythm Research **25**(2): 190-198.

Verhagen, L. A. W., M. C. M. Luijendijk, J. W. de Groot, L. P. G. van Dommelen, A. G. Klimstra, R. A. H. Adan and T. A. P. Roeling (2011). "Anticipation of meals during restricted feeding increases activity in the hypothalamus in rats." European Journal of Neuroscience **34**(9): 1485-1491.

Vieira, E., L. Marroqui, T. M. Batista, E. Caballero-Garrido, E. M. Carneiro, A. C. Boschero, A. Nadal and I. Quesada (2011). "The Clock Gene Rev-erbalpha Regulates Pancreatic beta-Cell Function: Modulation by Leptin and High-Fat Diet." Endocrinology.

Vieira, E., E. C. Nilsson, A. Nerstedt, M. Ormestad, Y. C. Long, P. M. Garcia-Roves, J. R. Zierath and M. Mahlapuu (2008). "Relationship between AMPK and the transcriptional balance of clock-related genes in skeletal muscle." Am J Physiol Endocrinol Metab **295**(5): E1032-7.

Vilaro, S., M. Llobera, G. Bengtssonolivecrona and T. Olivecrona (1988). "Lipoprotein-Lipase Uptake by the Liver - Localization, Turnover, and Metabolic Role." American Journal of Physiology **254**(5): G711-G722.

Vilaro, S., M. Reina, I. Ramirez and M. Llobera (1986). "Intralipid Administration Induces a Lipoprotein Lipase-Like Activity in the Livers of Starved Adult-Rats." Biochemical Journal **236**(1): 273-278.

Villaret, A., J. Galitzky, P. Decaunes, D. Esteve, M. A. Marques, C. Sengenes, P. Chiotasso, T. Tchkonia, M. Lafontan, J. L. Kirkland and A. Bouloumie (2010). "Adipose tissue endothelial cells from obese human subjects: differences among depots in angiogenic, metabolic, and inflammatory gene expression and cellular senescence." Diabetes **59**(11): 2755-63.

Vitaterna, M. H., D. P. King, A. M. Chang, J. M. Kornhauser, P. L. Lowrey, J. D. McDonald, W. F. Dove, L. H. Pinto, F. W. Turek and J. S. Takahashi (1994). "Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior." Science **264**(5159): 719-25.

Voshol, P. J., M. C. Jong, V. E. Dahlmans, D. Kratky, S. Levak-Frank, R. Zechner, J. A. Romijn and L. M. Havekes (2001). "In muscle-specific lipoprotein lipase-overexpressing mice, muscle triglyceride content is increased without inhibition of insulin-stimulated whole-body and muscle-specific glucose uptake." Diabetes **50**(11): 2585-90.

Vu-Dac, N., S. Chopin-Delannoy, P. Gervois, E. Bonnelye, G. Martin, J. C. Fruchart, V. Laudet and B. Staels (1998). "The nuclear receptors peroxisome proliferator-activated receptor alpha and Rev-erbalpha mediate the species-specific regulation of apolipoprotein A-I expression by fibrates." J Biol Chem **273**(40): 25713-20.

Vu-Dac, N., P. Gervois, T. Grotzinger, P. De Vos, K. Schoonjans, J. C. Fruchart, J. Auwerx, J. Mariani, A. Tedgui and B. Staels (1997). "Transcriptional regulation of apolipoprotein A-I gene expression by the nuclear receptor RORalpha." J Biol Chem **272**(36): 22401-4.

Waddington Lamont, E., V. L. Harbour, J. Barry-Shaw, L. Renteria Diaz, B. Robinson, J. Stewart and S. Amir (2007). "Restricted access to food, but not sucrose, saccharine, or salt, synchronizes the expression of Period2 protein in the limbic forebrain." Neuroscience **144**(2): 402-11.

Wakamatsu, H., Y. Yoshinobu, R. Aida, T. Moriya, M. Akiyama and S. Shibata (2001). "Restricted-feeding-induced anticipatory activity rhythm is associated with a phase-shift of the expression of mPer1 and mPer2 mRNA in the cerebral cortex and hippocampus but not in the suprachiasmatic nucleus of mice." European Journal of Neuroscience **13**(6): 1190-1196.

Wang, H., G. Astarita, M. D. Taussig, K. G. Bharadwaj, N. V. DiPatrizio, K. A. Nave, D. Piomelli, I. J. Goldberg and R. H. Eckel (2011). "Deficiency of lipoprotein lipase in neurons modifies the regulation of energy balance and leads to obesity." Cell Metab **13**(1): 105-13.

Wang, J. and M. A. Lazar (2008). "Bifunctional role of Rev-erbalpha in adipocyte differentiation." Mol Cell Biol **28**(7): 2213-20.

Warnecke, M., H. Oster, J. P. Revelli, G. Alvarez-Bolado and G. Eichele (2005). "Abnormal development of the locus coeruleus in Ear2(Nr2f6)-deficient mice impairs the functionality of the forebrain clock and affects nociception." Genes Dev **19**(5): 614-25.

Watts, A. G. and L. W. Swanson (1987). "Efferent projections of the suprachiasmatic nucleus: II. Studies using retrograde transport of fluorescent dyes and simultaneous peptide immunohistochemistry in the rat." J Comp Neurol **258**(2): 230-52.

Watts, A. G., L. W. Swanson and G. Sanchez-Watts (1987). "Efferent projections of the suprachiasmatic nucleus: I. Studies using anterograde transport of Phaseolus vulgaris leucoagglutinin in the rat." J Comp Neurol **258**(2): 204-29.

Wells, A. S., N. W. Read, K. Uvnas-Moberg and P. Alster (1997). "Influences of fat and carbohydrate on postprandial sleepiness, mood, and hormones." Physiol Behav **61**(5): 679-86.

Welsh, D. K., D. E. Logothetis, M. Meister and S. M. Reppert (1995). "Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms." Neuron **14**(4): 697-706.

West, D. B. and B. York (1998). "Dietary fat, genetic predisposition, and obesity: lessons from animal models." Am J Clin Nutr **67**(3 Suppl): 505S-512S.

Westerbacka, J., M. Kolak, T. Kiviluoto, P. Arkkila, J. Siren, A. Hamsten, R. M. Fisher and H. Yki-Jarvinen (2007). "Genes involved in fatty acid partitioning and binding, lipolysis, monocyte/macrophage recruitment, and inflammation are overexpressed in the human fatty liver of insulin-resistant subjects." Diabetes **56**(11): 2759-2765.

Wilkinson, C. W., J. Shinsako and M. F. Dallman (1979). "Daily rhythms in adrenal responsiveness to adrenocorticotropin are determined primarily by the time of feeding in the rat." Endocrinology **104**(2): 350-9.

Willard L. Koukkari, Robert B. Sothern (2006). Introducing biological rhythms: a primer on the temporal organization of life, with implications for health, society, reproduction and the natural environment. New York: Springer.

Williams, D. L., H. J. Grill, D. E. Cummings and J. M. Kaplan (2003). "Vagotomy dissociates short- and long-term controls of circulating ghrelin." Endocrinology **144**(12): 5184-5187.

Williamson, D. H., P. Lund and H. A. Krebs (1967). "The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver." Biochem J **103**(2): 514-27.

Yamamoto, H., K. Nagai and H. Nakagawa (1987). "Role of SCN in daily rhythms of plasma glucose, FFA, insulin and glucagon." Chronobiol Int **4**(4): 483-91.

Yamazaki, S., R. Numano, M. Abe, A. Hida, R. Takahashi, M. Ueda, G. D. Block, Y. Sakaki, M. Menaker and H. Tei (2000). "Resetting central and peripheral circadian oscillators in transgenic rats." Science **288**(5466): 682-5.

Yang, S., A. Liu, A. Weidenhammer, R. C. Cooksey, D. McClain, M. K. Kim, G. Aguilera, E. D. Abel and J. H. Chung (2009). "The role of mPer2 clock gene in glucocorticoid and feeding rhythms." Endocrinology **150**(5): 2153-60.

Yang, X., M. Downes, R. T. Yu, A. L. Bookout, W. He, M. Straume, D. J. Mangelsdorf and R. M. Evans (2006). "Nuclear receptor expression links the circadian clock to metabolism." Cell **126**(4): 801-10.

Yin, L. and M. A. Lazar (2005). "The orphan nuclear receptor Rev-erbalpha recruits the N-CoR/histone deacetylase 3 corepressor to regulate the circadian Bmal1 gene." Mol Endocrinol **19**(6): 1452-9.

Yin, L., N. Wu, J. C. Curtin, M. Qatanani, N. R. Szwergold, R. A. Reid, G. M. Waitt, D. J. Parks, K. H. Pearce, G. B. Wisely and M. A. Lazar (2007). "Rev-erbalpha, a heme sensor that coordinates metabolic and circadian pathways." Science **318**(5857): 1786-9.

Yoo, S. H., S. Yamazaki, P. L. Lowrey, K. Shimomura, C. H. Ko, E. D. Buhr, S. M. Siepka, H. K. Hong, W. J. Oh, O. J. Yoo, M. Menaker and J. S. Takahashi (2004). "PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues." Proc Natl Acad Sci U S A **101**(15): 5339-46.

Yoon, M. (2009). "The role of PPARalpha in lipid metabolism and obesity: focusing on the effects of estrogen on PPARalpha actions." Pharmacol Res **60**(3): 151-9.

Yoshihara, T., S. Honma and K. Honma (1996). "Effects of restricted daily feeding on neuropeptide Y release in the rat paraventricular nucleus." Am J Physiol **270**(4 Pt 1): E589-95.

Yu, J. and J. Auwerx (2009). "The role of sirtuins in the control of metabolic homeostasis." Ann N Y Acad Sci **1173 Suppl 1**: E10-9.

Zhao, H. and B. Rusak (2005). "Circadian firing-rate rhythms and light responses of rat habenular nucleus neurons in vivo and in vitro." Neuroscience **132**(2): 519-28.

Zheng, B., U. Albrecht, K. Kaasik, M. Sage, W. Lu, S. Vaishnav, Q. Li, Z. S. Sun, G. Eichele, A. Bradley and C. C. Lee (2001). "Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock." Cell **105**(5): 683-94.

Zheng, B., D. W. Larkin, U. Albrecht, Z. S. Sun, M. Sage, G. Eichele, C. C. Lee and A. Bradley (1999). "The mPer2 gene encodes a functional component of the mammalian circadian clock." Nature **400**(6740): 169-73.

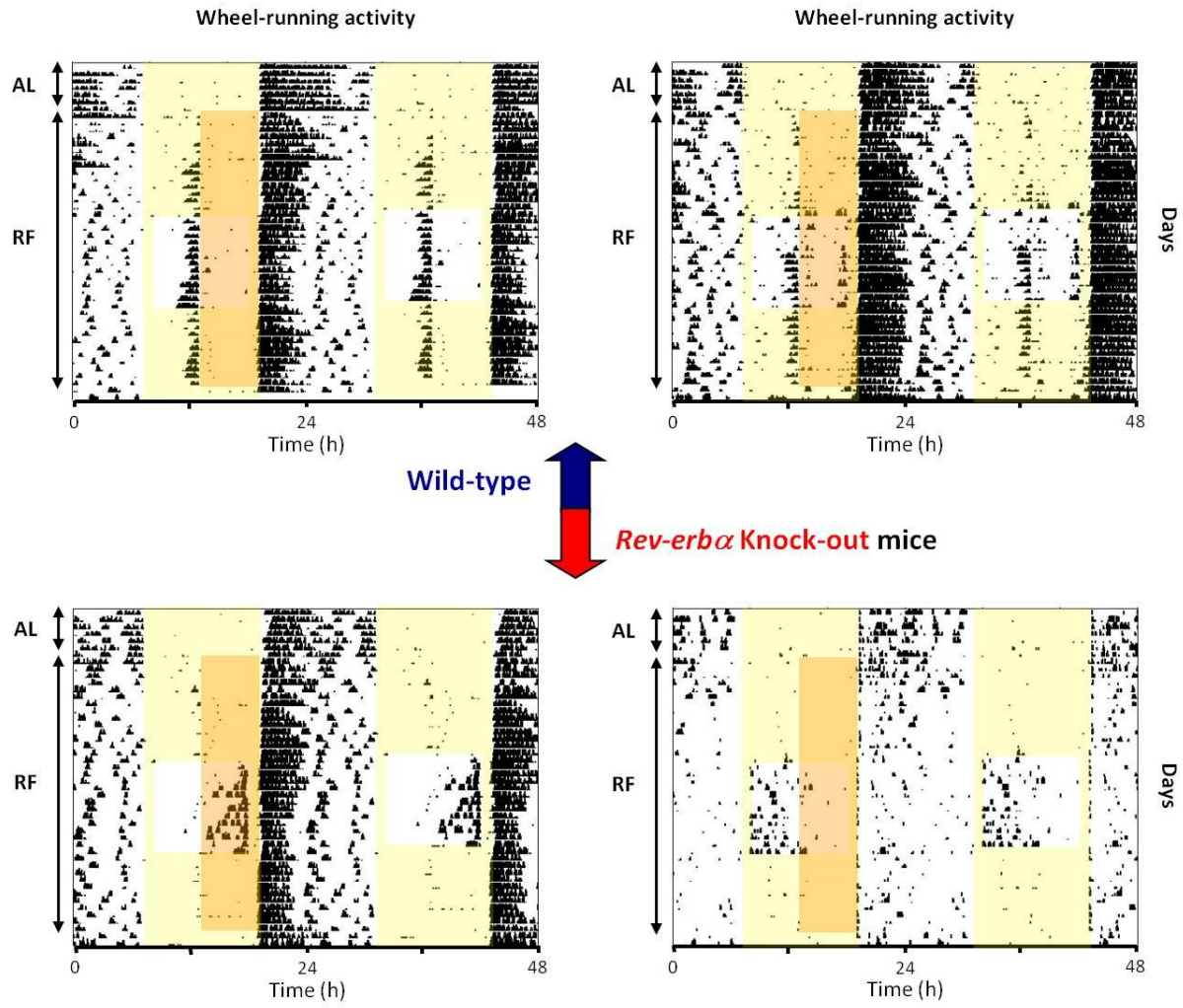
Zhou, D., Z. Shen, A. M. Strack, D. J. Marsh and L. P. Shearman (2005). "Enhanced running wheel activity of both Mch1r- and Pmch-deficient mice." Regul Pept **124**(1-3): 53-63.

Zigman, J. M., J. E. Jones, C. E. Lee, C. B. Saper and J. K. Elmquist (2006). "Expression of ghrelin receptor mRNA in the rat and the mouse brain." J Comp Neurol **494**(3): 528-48.

1. Food-anticipatory activity in a skeleton photoperiod

Below are depicted double-plotted actograms of wheel-running activity from two wild-type and two *Rev-erbα*$^{-/-}$ mice.

Note that the 12-h light phase or the two 1-h light pulses are indicated by yellow rectangles. A few days of *ad libitum* (AL) feeding conditions are illustrated. During restricted feeding (RF), food access is indicated by the orange rectangle.

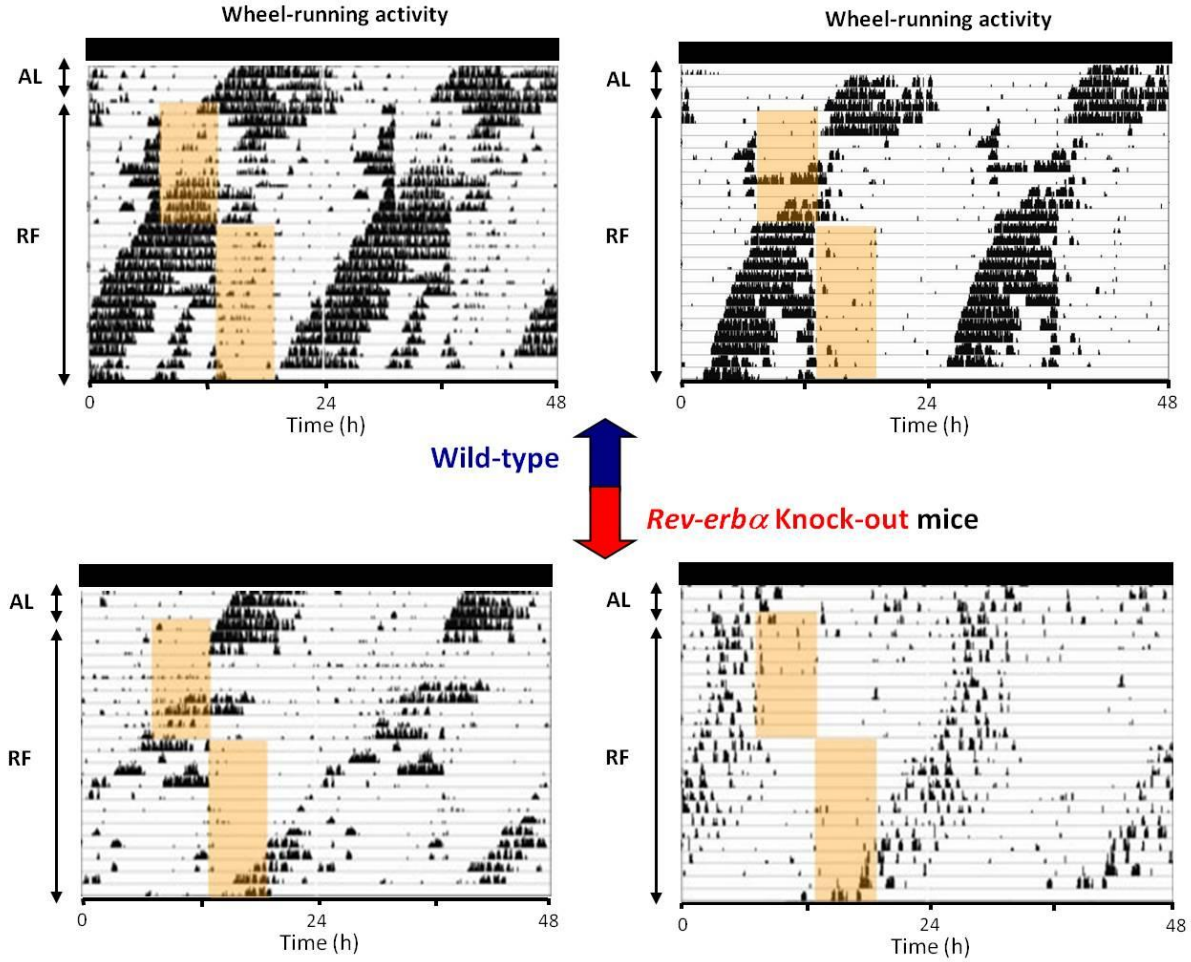


When wild-type mice are challenged with a 6-h restricted feeding schedule, they showed robust food-anticipatory activity, regardless of the lighting conditions. Wheel-running activity levels in anticipation of mealtime, however, were higher during the skeleton photoperiod. This indicates that light can potentially affect the behavioral output of the food-entrainable oscillator. Interestingly, irrespective of the lighting conditions, *Rev-erbα*$^{-/-}$ mice did not exhibit food-anticipatory activity.

2. Food-anticipatory activity in constant darkness following mealtime jet-lag test

Below are depicted double-plotted actograms of wheel-running activity from two wild-type and two *Rev-erbα*$^{-/-}$ mice.

Animals are in constant dark conditions (indicated by the above dark bar). A few days in *ad libitum* (AL) feeding conditions are illustrated. During restricted feeding (RF), food access is indicated by the orange rectangle. After a first period of food restriction at a specific circadian phase, mealtime was 6-h phase-advanced (i.e., jet-lag test).



During the first restricted feeding period, wild-type mice showed food-anticipatory activity, which could be resynchronized after a 6-h phase advance of mealtime. In *Rev-erbα*$^{-/-}$ mice, food anticipation was not observed at the two feeding times.

To assess anxiety- or depressive-like behaviors, 32 age-matched mice (including 12 wild-type, 10 *Rev-erbα*$^{+/-}$ and 10 *Rev-erbα*$^{-/-}$ mice) were used (sex ratio 1:1).

All mice were exposed to a 12 h/12 h LD schedule. Note that animals were all tested around the onset of the active period.

Tests were separated by several days and conducted in the following order: 1) Elevated plus maze; 2) Light-dark box test; 3) Forced swimming test. Prior to the first test, animals were equally accommodated to being handled by the experimenter, who was blinded to the genotype.

Animals were tested in their housing room, except for the Light-dark test for which mice were moved to an adjacent room.

1. Elevated plus maze

a) Protocol

The elevated plus maze is a method to evaluate anxiety responses in rodents (i.e, conflict between their preference for protected closed areas and their motivation to explore a novel environment). For more information, see Walf and Frye 2007.

Briefly, 3-month-old mice were placed in the maze (Fig. 1) at the junction of the open and closed arms, facing the same open arm and a timer was immediately set for a 5-min testing period. During this time lapse, with the help of a PC-based software, the observer recorded the number of entries made by the mouse onto the open and closed arms (i.e. when all four paws entered an arm) and the program automatically evaluated the time spent in each arms.

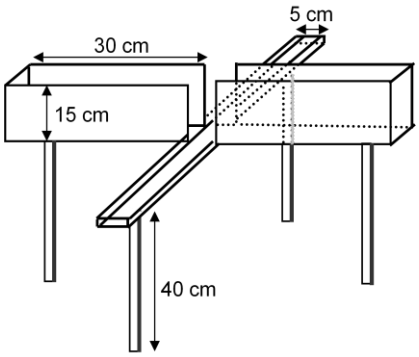


Figure 1. Elevated plus maze

b) Results

Data were analyzed with analyses of variance. We did not find any significant differences between genotypes regarding the time spent inside or the number of entries in the open arms (Fig. 2). Thus, the absence of a reduction in both the time spent and the number of entries in *Rev-erbα*$^{-/-}$ mice does not reveal any anxiety-like behavior.

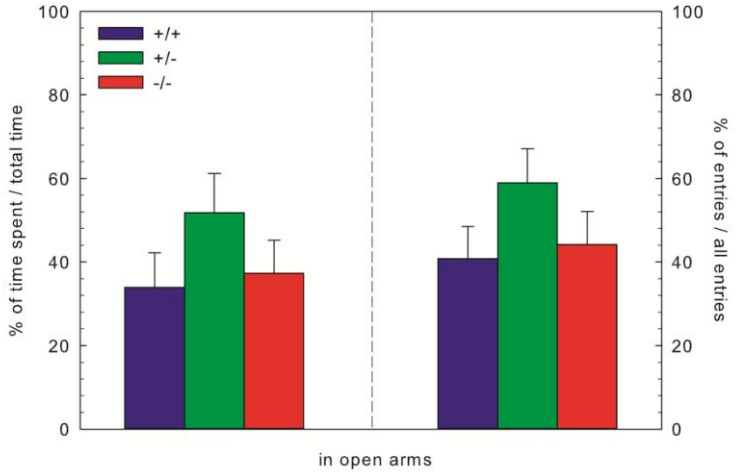


Figure 2. Evaluation of anxiety responses in *Rev-erbα*$^{+/-}$ and *Rev-erbα*$^{-/-}$ mice

2. Light-dark test box

a) Protocol

The light-dark test box is a method to evaluate anxiety responses in rodents (i.e, conflict between their aversion to light and their motivation to explore a novel environment). For more information, see Bourin and Hascoët 2003.

Briefly, mice were moved from their housing room in their individual cage to an adjacent behavioral testing room under dim-light. They were then placed in the box for a 5 min session (Fig. 3). The entry latency to the lit compartment as well the number of entries and the time spent in the light box were evaluated.

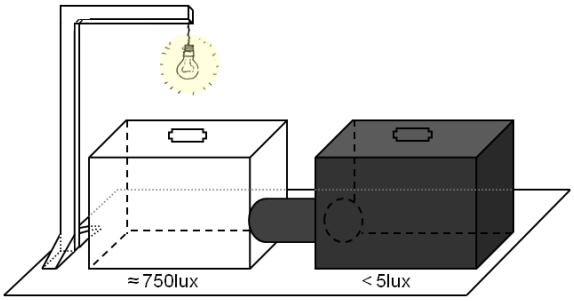


Figure 3. Light-dark box test

b) Results

Data were analyzed with analyses of variance, followed by the Unequal N HSD test, as appropriate (i.e., when *P*-value of main effect of genotypes was less than 0.05). *Rev-erbα*$^{-/-}$ mice displayed a significant decrease in the time spent in the lit compartment compared to the other groups. However, the latency to enter the lit compartment and the total number of entries were not significantly different between genotypes (Fig. 4). Thus, *Rev-erbα*$^{-/-}$ mice demonstrated an aversion to a brightly illuminated area; however, in view of our results in the Elevated plus maze, this aversion may be related to other causes than enhanced “anxiety”.

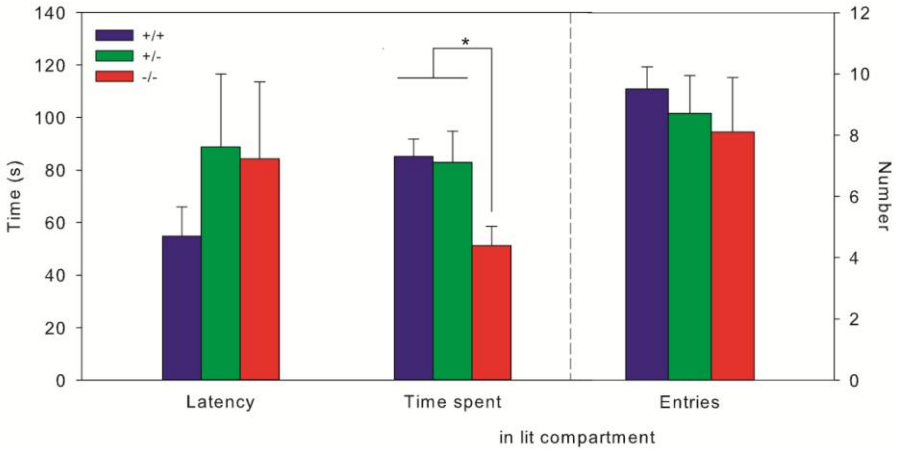


Figure 4. Evaluation of anxiety responses in *Rev-erbα*$^{+/-}$ and *Rev-erbα*$^{-/-}$ mice
Rev-erbα-/- mice spent significantly less time in the light side of the light-dark box ($P < 0.05$) compared to wild-type and *Rev-erbα+/-* mice.

3. Forced swimming test

a) Protocol

The forced swimming test is a method for screening antidepressants drugs. For more information, see Petit-Demoulire et al. 2004.

In a first attempt to evaluate a depression-like behavior consecutive to *Rev-erbα* deletion, we measured baseline levels of immobility (i.e. without any antidepressant drug injection) in each genotype (as in Roybal et al. 2007 and Hampp et al. 2008).

Briefly, mice were placed in a transparent cylinder filled with water (25 ± 0.5°C) to a depth of 10 cm for 6 min (Fig. 5). The water was changed between each mouse. A video camera recorded the testing session for later analysis. Immobility was defined as the absence of active behaviors such as swimming, limb movements, etc. The first two minutes were excluded from the analysis.

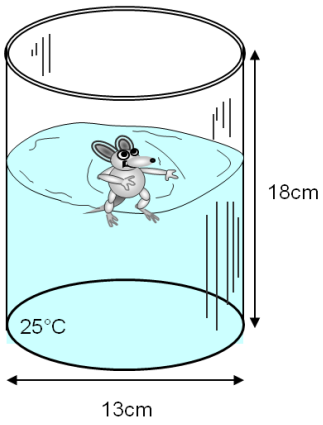


Figure 5. Forced swimming test

b) Results

Data were analyzed with analyses of variance. Immobility time did not differ between genotypes (Fig. 6).

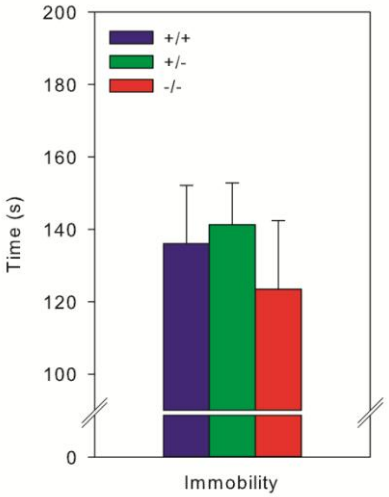


Figure 6. Immobility time in the forced swimming test.

Julien DELEZIE

Rôle du récepteur nucléaire *Rev-erbα* dans les mécanismes d'anticipation des repas et le métabolisme

Résumé

La première partie de mon travail de thèse a été de définir le rôle joué par le récepteur nucléaire *Rev-erbα* dans les mécanismes de synchronisation par la nourriture d'une horloge circadienne putative, non encore localisée, appelée « horloge alimentaire ». La seconde partie de mon travail a consisté à étudier la participation de *Rev-erbα* dans les régulations des métabolismes glucidique et lipidique.

L'ensemble de nos données indique que le répresseur transcriptionnel *Rev-erbα* joue un rôle charnière dans les fonctions circadiennes ainsi que dans le métabolisme. En effet, d'un point de vue circadien, l'absence de *Rev-erbα* altère la synchronisation à l'heure des repas – démontré par une réduction des sorties comportementales et physiologiques de l'horloge alimentaire, ainsi que par l'absence d'ajustement du rythme de la protéine d'horloge PER2 dans l'oscillateur cérébelleux. Sur le plan métabolique, la délétion de ce gène modifie notamment le métabolisme des lipides – démontré par une accumulation excessive de tissu adipeux, une utilisation préférentielle des acides gras, ainsi qu'une perte de contrôle de l'expression de la *Lipoprotéine lipase*.

Mots-clés : Rev-erb alpha, gène d'horloge, horloge alimentaire, métabolisme lipidique et glucidique, lipoprotéine lipase, obésité, anticipation de l'heure des repas, rythme circadien

Résumé en anglais

The work performed during this PhD thesis aimed at investigating the role of the transcriptional silencer *Rev-erbα* in both the circadian clockwork of the food-entrainable oscillator and metabolic regulations. Firstly, by evaluating food-anticipatory components in animals fed once a day at the same time, we showed that mice lacking *Rev-erbα* display a reduction in locomotor activity prior to food access compared to littermate controls. Accordingly, the rises in body temperature and corticosterone that anticipate mealtime are also diminished. Interestingly, daily p-ERK expression in hypothalamic regions and daily PER2 expression in the cerebellum of *Rev-erbα* KO mice are not phase-adjusted to feeding time. These results indicate that *Rev-erbα* participates in the integration of feeding signals and in food-seeking behaviors. Secondly, by investigating energy balance in fasted, normal chow or high-fat fed animals, we revealed that *Rev-erbα* KO mice exhibit greater reliance on lipid fuels as energy substrates, contributing to a mild hyperglycemic state. We also found that *Lipoprotein lipase (Lpl)* expression, is strongly up-regulated in peripheral tissues of *Rev-erbα* KO mice, predisposing mice to obesity. In this regard, we uncovered a new molecular pathway that ties clock-driven *Lpl* expression to energy homeostasis. These findings highlight the significance of daily *Rev-erbα* oscillations to prevent the appearance of the metabolic syndrome.

In conclusion, we provide evidence that REV-ERBα may be a part of the food-entrainable oscillator clockwork that triggers food-anticipatory components, and represents a pivotal player to link the core clock machinery to metabolic pathways.

Keywords: Rev-erb alpha; Food-entrainable oscillator; Lipid metabolism; Obesity; Lipoprotein lipase; Respiratory quotient; Clock gene; Food-anticipatory activity, Circadian rhythm