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La peau, un modèle d'horloge périphérique

The skin as a peripheral clock model

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Time is life. Life is time.

To my parents

To me in the past, present and future

To Strasbourg

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Abbreviations

ACTH: adrenocorticotropic hormone

AMPK: adenosine monophosphate-activated protein kinase

ARC: arcuate nucleus

AVP: arginin vasopressin

Bmal: brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like

BNST: bed nucleus of the striata terminalis

cAMP: cyclic adenosine monophosphate

CCGs: clock-controlled genes

CIRP: cold-inducible RNA-binding protein

CKI: casein kinase I

CLOCK: circadian locomoter output cycles kaput

CRE: cAMP responsive element

CREB: cAMP response element binding protein

Cry: cryptochrome

DMH: dorsomedial nucleus of the hypothalamus

DSPS: Delayed Sleep Phase Syndrome

FASPS: Familial Advanced Sleep Phase Syndrome

GABA: gamma aminobutyric acid

GHT: geniculohypothalamic tract

GRP: gastrin-releasing peptide

Gsk3β: glycogen synthase kinase 3 beta

IGL: intergeniculate leaflet

ipRGC: intrinsically photosensitive retinal ganglion cell

KO: knockout

LH: lateral hypothalamus

miRNA: micro RNA

NAc: nucleus accumbens

NAD: nicotinamide adenine dinucleotide

NES: nuclear export signals

NLS: nuclear localization signals

NONO: Non-POU domain-containing octamer-binding protein

NPY: neuropeptide Y

OB: olfactory bulb

PACAP: pituitary adenylate cyclase-activating polypeptide

PBS: Phosphate buffer saline

PCR: Polymerase chain reaction

Per: Period

PSF: polypyrimidine tract-binding protein-associated splicing factor

PVN: paraventricular nucleus of the hypothalamus

PVT: paraventricular nucleus of the thalamus

REV-ERB: reverse viral erythroblastis oncogene product

RHT: retinohypothalamic tract

RK: Rhodopspin Kinase

RNAPII: RNA polymerase II

ROR: retinoic acid-related orphan receptor

RORE: REV-ERB/ROR Response element

SCN: suprachiasmatic nucleus of the hypothalamus

SIN3-HDAC: SIN3 histone deacetylase

SIRT: sirtuin

SUMO: small ubiquitin-like modifier

TG: triglycerides

UTR: untranslated regions

VIP: vasoactive intestinal peptide

VMH: ventromedial nucleus of the hypothalamus

WT: wild-type

ZT: zeitgeber time

Résumé du travail de thèse

Les organismes vivants s'adaptent aux changements prévisibles de l'environnement grâce à des rythmes biologiques qui sont intrinsèquement déterminés et modulés par l'alternance des paramètres externes. Une variation environnementale majeure est l'alternance du jour et de la nuit, au cours de laquelle la lumière et la température varient considérablement. Les comportements cycliques et les processus physiologiques (y compris endocriniens) rythmiques sont basés sur des horloges biologiques qui suivent les cycles quotidiens de l'environnement et permettent aux diverses activités de se dérouler au bon moment de la journée. Chez les mammifères, ces mécanismes sont dominés par une horloge centrale située dans une petite structure bilatérale de l'hypothalamus, les noyaux suprachiasmatiques (SCN), et qui génère des rythmes dont la période est d'environ 24 heures. Le rythme de l'horloge endogène des SCN est généré de façon continue et soutenue, même en l'absence de signaux de synchronisation de l'environnement, et est en même temps entraîné par ces signaux ou « zeitgebers ».

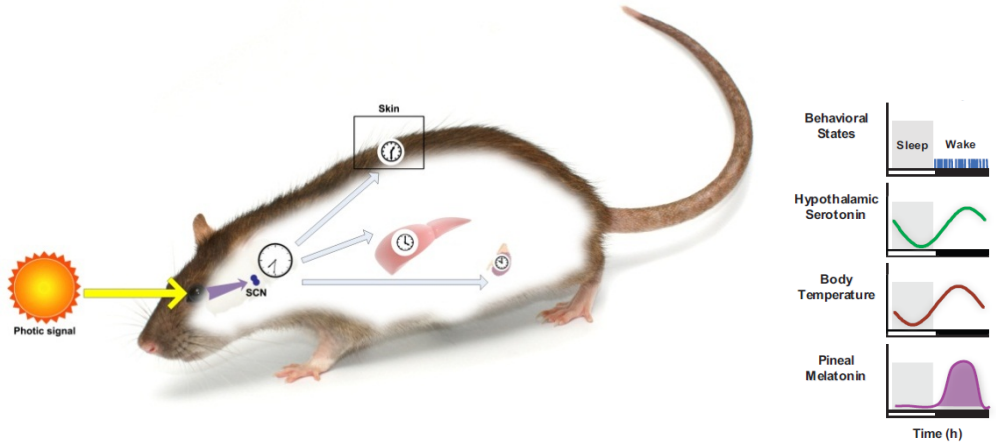


Figure1: Le système circadian chez les mammifères. Chez les rongeurs, une horloge centrale localisée dans les noyaux suprachiasmatiques (SCN) de l'hypothalamus contrôle des horloges secondaires (panneau de gauche). L'horloge centrale est synchronisée par des signaux environnementaux tels que la lumière, perçue par la rétine et synchroniseur le plus puissant. Les horloges périphériques (dans le foie, le rein, la peau…) oscillent selon des patrons tissu-spécifiques mais coordonnés par des signaux émis par les SCN. L'ensemble de ce système régule et organise sur le cycle de 24h, de nombreuses fonctions physiologiques (panneau de droite) telles que la température corporelle ou la production de mélatonine par la glande pinéale. L'horloge présente dans la peau fait l'objet du présent travail de thèse (Adapté de Challet, 2007)

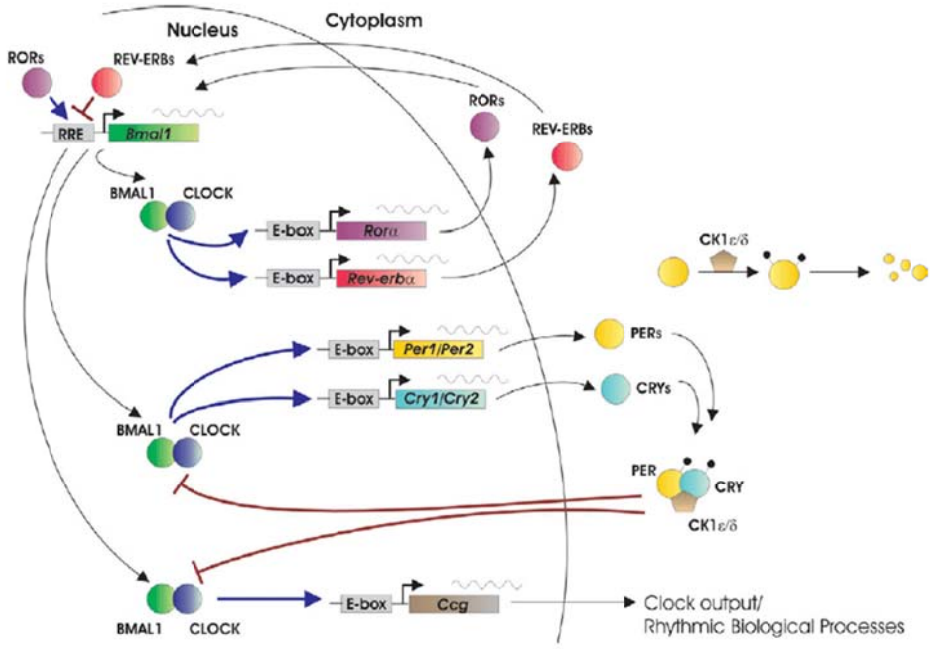


Figure 2: Organisation moléculaire de l'horloge circadienne. Les oscillations circadiennes résultent d'un processus cellulaire mettant en jeu des gènes horloge dont les produits, des facteurs de transcription, se régulent entre eux et entrainent via leurs gènes cibles, les programmes d'expression génique et les fonctions physiologiques sur 24h. (Adapté de Ko *et al.*, 2006)

Outre les SCN, horloge principale, il existe un système d'horloges circadiennes dans beaucoup de structures centrales et périphériques, basées sur la présence d'oscillateurs génétiquement autonomes dans presque toutes les cellules de l'organisme (Fig. 1). Les oscillations circadiennes sont générées par des boucles de rétroaction positives et négatives impliquant des gènes dits « horloge » (les gènes *Period*, *Clock*, *Bmal1*, *Cryptochrome* par exemple) qui s'activent ou se répriment mutuellement de sorte à générer des rythmes d'expression génique avec une période de près de 24 heures (Fig. 2). Ces gènes régulent aussi de nombreux gènes cibles dont l'expression se manifeste en diverses fonctions physiologiques rythmiques encore appelées sorties de l'horloge. Les différents oscillateurs périphériques abritent des horloges moléculaires dont le mécanisme de base peut légèrement différer de celui des SCN. Ceci est probablement dû à la spécificité du tissu, à sa composition cellulaire et aux régulations cellulaires et génétiques locales. L'ensemble du système circadien « multi-oscillant » est entraîné par des stimuli externes tels que la lumière mais aussi l'apport alimentaire ou d'autres donneurs de temps,

et synchronisé par différentes sorties neuronales et humorales des SCN qui agissent de manière tissu-spécifique. Ces éléments font de la recherche sur la synchronisation des horloges périphériques un sujet à la fois passionnant et complexe.

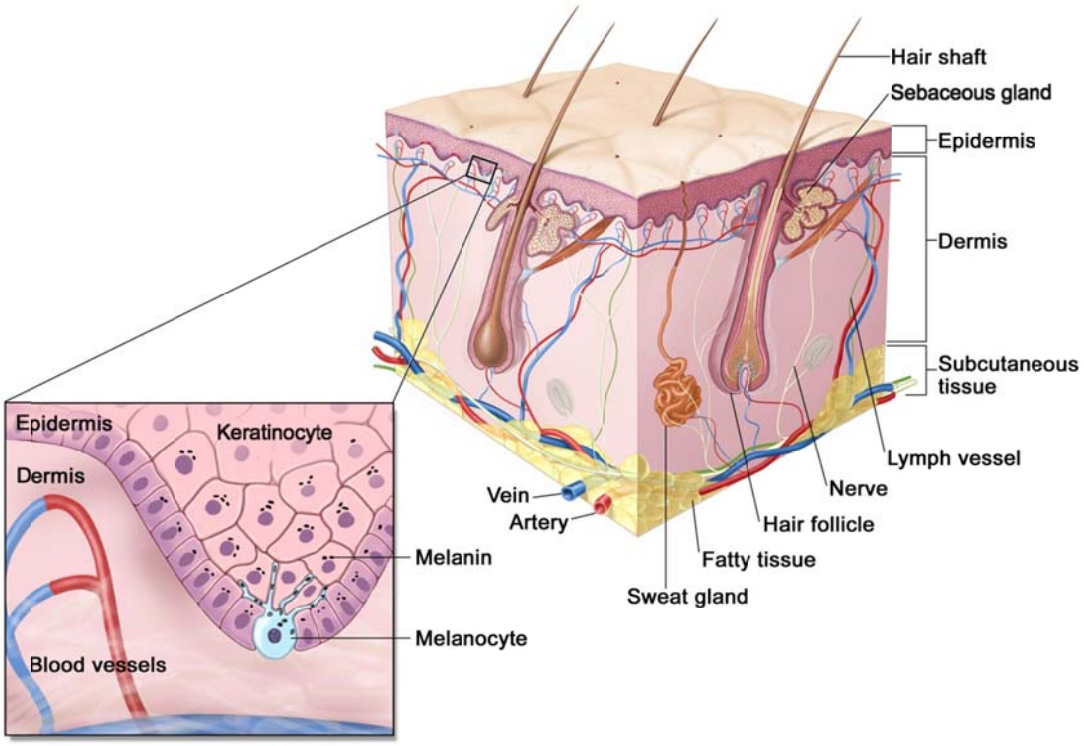


Figure 3: Structure de la peau. La peau est constituée de 2 couches majeures; l'épiderme et le derme. Elle comprend de nombreux types cellulaires tels que les kératinocytes et les mélanocytes dans l'épiderme et les fibroblastes dans le derme, ainsi que plusieurs appendices, l'ensemble formant un organe très complexe. (Adapté de https://visualsonline.cancer.gov/details)

La peau est un oscillateur périphérique qui présente diverses fonctions rythmiques (prolifération cellulaire, hydratation, sécrétion de sébum) incluant l'expression des gènes de l'horloge dans ses différents composants cellulaires : les fibroblastes, les kératinocytes et les mélanocytes (Fig. 3). La localisation de la peau comme tissu d'interface entre l'environnement et l'organisme rend complexe et intéressante sa relation avec le système circadien, mais les données de la littérature indiquent que l'horloge de la peau est principalement synchronisée par des signaux encore inconnus provenant des SCN et qu'elle est peu sensible à la lumière comme zeitgeber. L'expression rythmique du gène *Per1* est une composante essentielle de l'horloge moléculaire et peut être utilisée comme reflet de l'activité de l'horloge dans un tissu ou une cellule. Cette propriété a été mise à

profit pour développer des modèles animaux transgéniques exprimant le gène rapporteur de la luciférase sous le contrôle du promoteur d'un gène horloge comme *Per1* et pour ainsi suivre en temps réel l'expression de ce gène, et donc le fonctionnement de l'horloge.

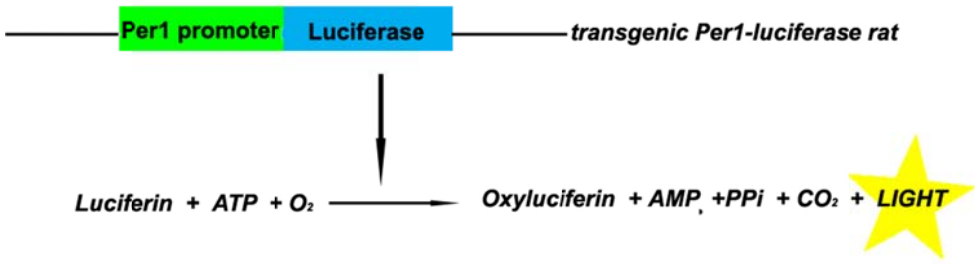


Figure 4: Diagramme représentant la construction rapportrice présente dans le génome des rats transgéniques Per1-luciférase, et la réaction génératrice de lumière, catalysée par la luciférase

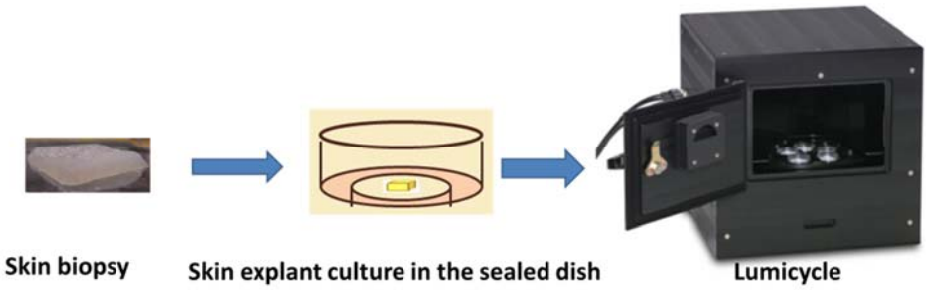


Figure 5: Principe expérimental utilisé pendant la thèse; des explants de peau prélevés chez les rats transgéniques sont coupés en petits carrés puis cultivés sur une membrane semi-perméable Millicell (Millipore) en présence de luciférine. La boîte de culture, scellée, est placée dans le Lumicycle (Actimetrics), une boîte noire équipée de tubes photomultiplicateurs permettant de mesurer en temps réel la bioluminescence émise par l'échantillon.

Dans la première partie de cette thèse, l'horloge circadienne présente dans la peau de rat a été caractérisée de manière systématique grâce à la technique de bioluminescence à l'aide de rats *Per1-luciférase* (Fig. 4 & 5), et étudiée depuis la période postnatale précoce (10 jours : P10) – jusqu'à un âge avancé (animaux âgés de 2 ans) (Fig. 6A). A P10 et P20, l'activité de l'horloge dans des explants de peau n'est pas détectable mais inductible selon les conditions expérimentales, indiquant la présence d'une horloge circadienne non synchronisée. A partir de l'âge adulte, vers un mois, des rythmes circadiens apparaissent dans les explants, avec une amplitude faible et une période instable. Entre 2 et 6 mois les rythmes dans les cultures deviennent de plus en plus robustes avec une amplitude croissante (Fig. 6B). La durée de la période de l'horloge de la peau se rapproche de 24 heures, suggérant que la synchronisation des oscillateurs de la peau est maximale vers 6 mois. Au cours du vieillissement, entre 1 et 2 ans, l'amplitude des rythmes circadiens dans la peau diminue et des cycles anormaux apparaissent. Les différences de phase entre individus sont plus grandes et l'horloge s'amortit plus rapidement. Des fibroblastes primaires que nous avons préparés à partir de la peau des mêmes animaux présentent le même processus de maturation et de vieillissement. Ces mêmes données ont permis d'établir une autre caractéristique intrinsèque des horloges circadiennes, la compensation thermique, pour les oscillateurs présents dans les fibroblastes et dans la peau prise dans son ensemble. De manière générale, nos résultats confirment l'existence d'une horloge circadienne dans la peau, la capacité des oscillateurs qui la composent à se synchroniser entre eux, et son évolution avec l'âge.

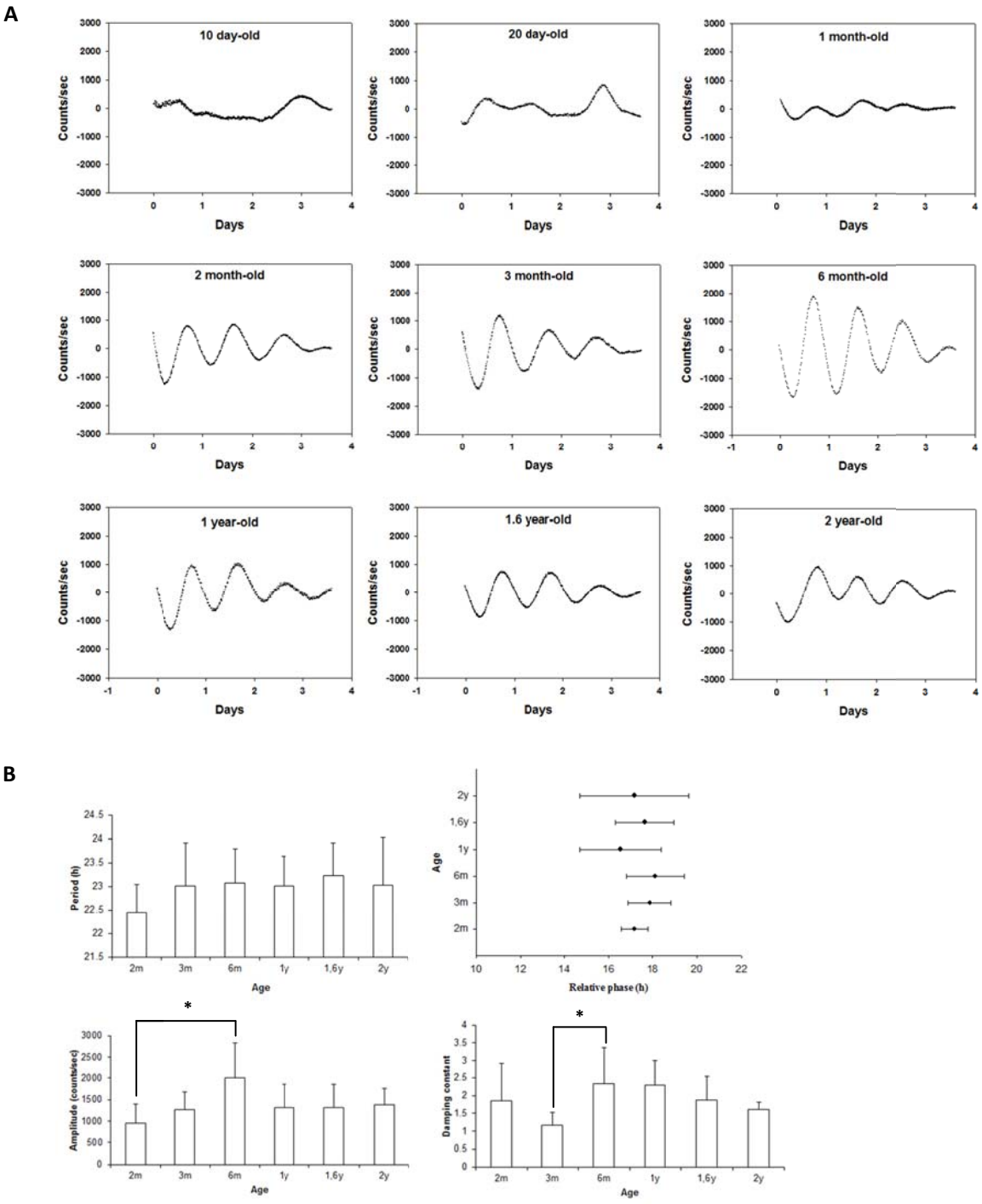


Figure 6: L'activité rythmique de la peau évolue dans la période post-natale et durant le vieillissement chez les rats Per1-luciférase.

A) Enregistrements représentatifs de la bioluminescence (données soustraites) émise par des explants de peau prélevés sur des rats âgés entre 10 jours et 2 ans. **B)** Analyse des paramètres rythmiques déduits des enregistrements. Les périodes, amplitudes et amortissements (damping rate) ont été calculés sur la base des régressions sinusoïdales ajustées aux enregistrements et pour lesquelles les données entre 2 mois et 2 ans présentent des R^2 supérieurs à 0,8, indiquant une bonne rythmicité. Les phases représentent le moment du premier pic observé. Les données montrées sont des valeurs moyennes±écart type (n=7, 10, 9, 6, 7 et 4 pour les groupes 2, 3, 6, 12, 16 et 24 mois, respectivement). * $p<0.05$

Dans la deuxième partie de cette thèse, le rôle de la mélatonine comme un synchroniseur potentiel a été étudiée dans les fibroblastes primaires de la peau. La mélatonine est une production hormonale de la glande pinéale, strictement synthétisée durant la nuit sous le contrôle des SCN. Chez les mammifères à la fois diurnes et nocturnes, la concentration de mélatonine dans le plasma présente un rythme robuste avec un maximum de nuit et elle est donc considérée comme marqueur de phase de l'horloge centrale. La mélatonine présente un effet d'entraînement (chronobiotique) sur l'horloge centrale, dans une fenêtre de temps précise. Des récepteurs de haute affinité pour la mélatonine (MT1 et MT2) sont exprimés dans différents tissus périphériques, indiquant que la mélatonine est un candidat potentiel comme synchroniseur des horloges périphériques, mais cet effet n'a pas été démontré. Les récepteurs MT1 et MT2 sont exprimés dans la peau humaine et chez les rongeurs, sur les différents types cellulaires. Dans notre étude, nous avons démontré un effet de la mélatonine sur les fibroblastes primaires de la peau : elle induit une augmentation de l'amplitude des oscillations du gène *Per1*, uniquement lorsqu'elle est appliquée à une certaine phase, mais ne modifie pas les autres paramètres de l'horloge (Fig. 7). Il s'agit du premier travail montrant un effet de la mélatonine sur une horloge périphérique.

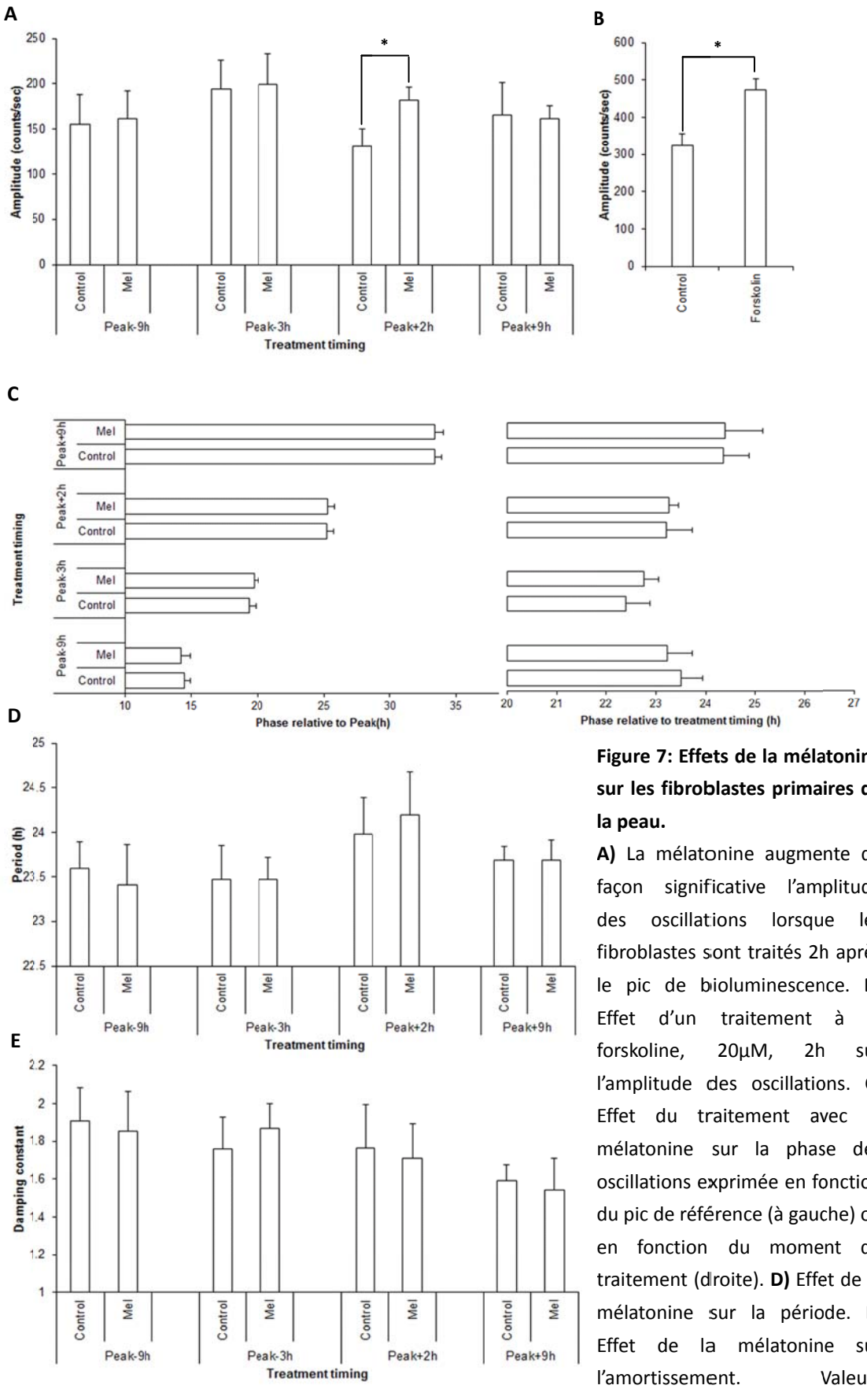


Figure 7: Effets de la mélatonine sur les fibroblastes primaires de la peau.

A) La mélatonine augmente de façon significative l'amplitude des oscillations lorsque les fibroblastes sont traités 2h après le pic de bioluminescence. **B)** Effet d'un traitement à la forskoline, 20µM, 2h sur l'amplitude des oscillations. **C)** Effet du traitement avec la mélatonine sur la phase des oscillations exprimée en fonction du pic de référence (à gauche) ou en fonction du moment du traitement (droite). **D)** Effet de la mélatonine sur la période. **E)** Effet de la mélatonine sur l'amortissement. Valeurs moyennes±écart-type (n=7-8). * $p<0.05$

Notre étude chez le rat s'est limitée aux fibroblastes, seul type cellulaire que nous avons pu isoler et amplifier en culture à partir de peau abdominale chez cet animal. Afin d'étudier l'horloge de la peau dans les autres types cellulaires (kératinocytes, mélanocytes) nous utilisons couramment des cultures préparées à partir de peau humaine obtenue après chirurgie plastique. Afin de pouvoir également étudier ces types cellulaires avec la technique de bioluminescence, nous avons entrepris de construire un vecteur lentiviral exprimant le gène rapporteur luciférase sous le contrôle du promoteur du gène horloge *Bmal1* : c'est ce qui a fait l'objet de la troisième partie de mon travail de thèse (Fig. 8). *Bmal1* est un autre gène horloge requis pour l'expression de rythmes circadiens, et son maximum présente une opposition de phase par rapport à celle de *Per1*. Ce vecteur ainsi construit a été testé et des stratégies pour en améliorer le niveau d'expression et l'amplitude de la rythmicité ont été proposées.

La présence d'oscillateurs cellulaires répandus dans tout l'organisme et l'organisation hiérarchique du système circadien suggèrent l'importance d'une synchronisation des horloges entre le centre et la périphérie. Notre étude démontre que la peau est un oscillateur périphérique complexe et synchronisable, qui peut servir de modèle pour comprendre les mécanismes moléculaires impliqués, voire pour tester d'éventuelles stratégies thérapeutiques vis-à-vis de troubles cliniques liés à l'horloge.

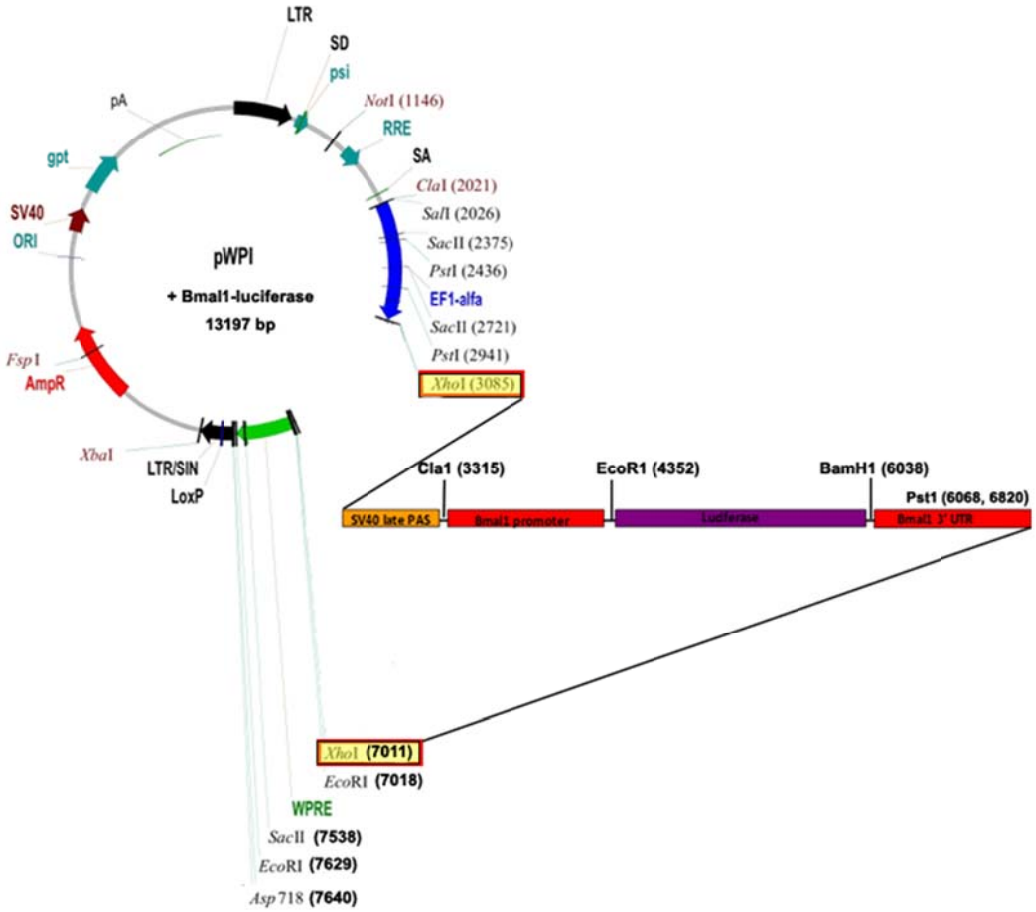


Figure 8: Organisation et carte de restriction partielle du vecteur lentiviral exprimant la luciférase sous le contrôle du promoteur du gène horloge *Bmal1*

Thesis summary

Animals adapt to the predictable changes in the environment through biological rhythms which are intrinsically determined and modulated by the external alternation. A major environmental variation is the day and night cycle, during which light and temperature differ drastically. Virtually, all living organisms develop their circadian rhythms by possessing biological clocks which follow daily cycles and adjust diverse activities to the adequate time of the day. In mammals, this circadian clock mechanism is dominated by the central clock located in a bilateral tiny region of the brain, within the hypothalamus: the suprachiasmatic nucleus (SCN), which controls physiological processes, including behavior and endocrine functions, with a period of approximately 24 hours. The endogenous rhythm of the SCN clock is continuously generated and sustained, in the absence of environment timing cues, and it is at the same time entrained by these zeitgeber (time giving) signals.

Besides the SCN as the master clock in the organisms, a system of circadian clocks in central and peripheral structures exists on the basis of genetically autonomous oscillators present in almost all the cells. Circadian molecular oscillations are produced by positive and negative feedback regulatory loops involving a series of “clock” genes (*Period*, *Clock*, *Bmal1*, *Cryptochrome*, for instance) that activate and repress each other to finally generate rhythms with a period close to 24 hours. These rhythmic gene expressions are transduced into many circadian outputs that drive physiological activities and convey timing signals to other parts of the circadian system. The multioscillatory system is entrained by external stimuli of light, food intake or other time givers and synchronized by various neuronal and humoral outputs of the SCN which act in a tissue- and cell-specific way. The different peripheral oscillators harbor distinct clockworks, although they hold the same genotype as the SCN. This is possibly due to local epigenetic regulation and systemic synchronization as well as the diversity of tissue structure and cell composition. All these contribute to the intriguing and attractive research on the synchronization of peripheral clocks.

As one special peripheral oscillator, the skin exhibits circadian rhythmic functions correlated with the existence of rhythmic clock gene expression in its constitutive cell types, such as keratinocytes, fibroblasts and melanocytes. These cellular oscillators display their own clock phenotypes in vivo and in vitro. To be in concert, the synchronization of the skin clocks is dependent on timing cues emanating from the SCN; thus the distinct role of the skin as an interface tissue between the external and internal environment make it both complicated and interesting among the peripheral clocks in the circadian network. In addition, synchronization of the skin clocks provides an opportunity to understand the mechanisms controlling circadian system of mammals. *Per1* gene is a core component of the molecular clockwork, whose circadian oscillation is required for normal clock functions and reflects the phase of clock activity. Technically it facilitates the study of clock properties by utilizing clock driven bioluminescence of tissue and cells from transgenic animals carrying a luciferase reporter gene controlled by the promoter of a clock gene, such as *Per1* for instance.

Peripheral clocks undergo the processes of early development and ageing during the lifespan. In the first part of this thesis, the skin clock was characterized systematically by using the *Per1-luciferase* transgenic rat, from the early postnatal age 10 day (P10) to 2 years (old animals). At P10 and P20, the skin clock activity was arrhythmic although some oscillations randomly showed up. The synchronization after medium refreshment indicated that the skin clock at early postnatal age was asynchronous, which coincided with the absence of synchronization of circadian system described during early development. Starting at 1 month-old, circadian rhythms appeared with low amplitude and unstable period. From 2 to 6 months, circadian oscillations developed more and more robust with amplitude increasing significantly. The period length of skin clock got closer to 24 hours in adult. These results suggested that the skin clock was better synchronized till the maximum in 6 months. In aged animals, 1 to 2 year-old, circadian oscillations got globally weaker and some abnormal cycles appeared. The phases delayed and variations between individuals turned larger. The clock activity damped faster by aging progress. In elderly, the skin clock was worse synchronized within itself and among individuals. We

also studied primary fibroblasts derived from the skin at the distinct ages and demonstrated a similar pattern of clock activity in maturation and ageing, which was consistent with the synchronization of the skin tissue. Another intrinsic feature of circadian clocks, temperature compensation, was shown the first time in the skin tissue and skin primary fibroblasts. Generally, we corroborate the existence of the skin clock and its synchronization in the development and ageing, and characterized its clock property within skin itself and within the circadian system.

In the second part of this thesis, the role of melatonin as a potential synchronizer was investigated in skin primary fibroblasts. Melatonin is a hormonal output of the SCN pacemaker with robust daily rhythms in plasma level. In both diurnal and nocturnal mammals, melatonin is synthesized primarily in the pineal gland in the night and its plasma rhythm is considered a reliable estimate of the SCN clock. Melatonin has shown entrainment effect on the central clock in a restricted time window at subjective dusk (chronobiotic effect). Since high-affinity melatonin receptors MT1 and MT2 are expressed in various peripheral tissues besides the SCN, melatonin is a potential candidate as a synchronizer of peripheral clocks. However, the effect of melatonin on peripheral clocks has been poorly confirmed. MT1 and MT2 expression has been shown in human and rodent skin tissue and cells, including fibroblasts, keratinocytes and melanocytes. In our study, we demonstrated a phase-dependent effect of melatonin to synchronize primary skin fibroblasts. When applied 2h after the *Per1* peak, the amplitude of oscillations was significantly increased by melatonin to the same extent as for forskolin, a cAMP-mediated synchronizer. The relatively weak effect of melatonin indicated its distinct role on the peripheral clocks. Long term application that induced *Per1* activity might be involved in the melatonin effect on the skin fibroblast clock. Ultimately, our study shows the first time an effect of melatonin on synchronization of peripheral clocks.

In addition, clock gene expression is found in other skin cell-autonomous oscillators such as keratinocytes and melanocytes, which contributes to the complexity of the multioscillatory skin. Fusing luciferase genes to clock gene promoters has arisen as a

convenient method to measure circadian gene expression in tissues and cells by bioluminescence recording. However, right now only fibroblasts can be cultured from dermal tissues of transgenic rodents because of their serum-dependent proliferation. Other skin cells like melanocytes and keratinocytes can be more easily derived and cultured from human skin. Brown and his colleagues have successfully established a lentivirally delivered luciferase reporter to measure clock genes activities in human skin cells. To facilitate our studies on multioscillatory skin, we constructed a *Bmal1-luciferase* lentivirus according to similar design, where *Bmal1* is another core clock component with peak expression almost antiphase to *Per1*. In the third part of thesis, construction of a lentivirus tool delivering *Bmal1-luciferase* reporter was shown although it had not been efficient enough in application. It is worth trying to improve the efficiency of this lentiviral reporter, with which clock activities and synchronization could be elucidated not only in fibroblasts but also in melanocytes and keratinocytes of the multioscillatory skin.

The widespread cell-autonomous oscillators and the hierarchical organization of the circadian system in mammals suggests the importance of synchronization of clocks in center and periphery. The present work demonstrates the synchronization of peripheral clocks in the skin and potential mechanism. Further investigation can enhance our understanding of how peripheral clocks are synchronized and which roles diverse synchronizers do play. The knowledge of these mechanisms in circadian system function and organization might enable to improve health care and clinical treatment of clock-related disorders.

Introduction

Rhythm, a regular recurrence or pattern in time, applies to a wide variety of cyclic natural phenomena, with a period from microseconds to millions of years. Rhythmic units provide an order to the world and reduce the entropy. They even describe the time per se. For instance, the rotation of the earth, by itself and around the sun, is rhythmic enough to determine days and seasons. Earth environment changes in the day length and temperature. To adapt to nature, biological rhythms commonly exist in the living world, covering periods in a wide range. For example, in seasonal cycles, including reproduction, hibernation and migration, animals change their way of life from summer to winter, according to the variations of temperature and food, to organize activity at the appropriate time. Each day, the rising and setting of the sun causes daily environmental changes, and consequently daily behaviors of animals, such as sleep-wake cycle. Hence, to adapt to and to anticipate predictable conditions in next days, animals follow biological rhythms with a period of approximately 24 hours, which are named circadian rhythms. Therefore, animals (and virtually all living organisms) have developed internal systems or endogenous abilities, called circadian clocks. The circadian clocks can drive daily rhythms in behavior and in many physiological, including endocrine, processes. Adapting life style to circadian rhythms is important for the health of animals. In human, life in society can lead to dramatic conditions when resetting of the internal clocks is not done at appropriate time. It may cause some disorders, or even diseases, in conditions like chronic jetlag or shift work.

1. General features about circadian clocks

All biological circadian clocks have several common features: self-sustained, entrained or synchronized by external cues, and temperature compensated. First, circadian clocks can sustain and produce oscillations in the absence of any external cues. This property is due to cell-autonomous persistence determined genetically and based on a molecular clockwork which is still not completely understood yet. Second, circadian

clocks are capable to be entrained or synchronized by time givers, named zeitgebers, to keep harmony with the environment. Light is the chief zeitgeber in nature. Third, temperature compensation can make sure that endogenous periods of circadian clocks will not be altered by changing temperature. All these properties of circadian clocks will be discussed in detail later.

Circadian clocks have been found in virtually all organisms, from bacteria to insects, birds and mammals. In mammals, lesions of specific brain areas confirmed that a small structure in the brain is required for this timekeeping system (Stephan and Zucker, 1972). This master clock is located in the suprachiasmatic nucleus (SCN), a cluster of thousands of neurons located in the anterior hypothalamus. Animals with lesion of the SCN lost their daily rhythms in sleep/wake activity, body temperature and production of several hormones. It is found that many physiological processes are controlled by peripheral oscillators (Fig.1). They form a complex circadian system that organizes an ensemble of structures in periphery. The central clock in the SCN takes charge of this hierarchical network to make sure that physiological functions of the distinct organs take place at the appropriate time of the day.

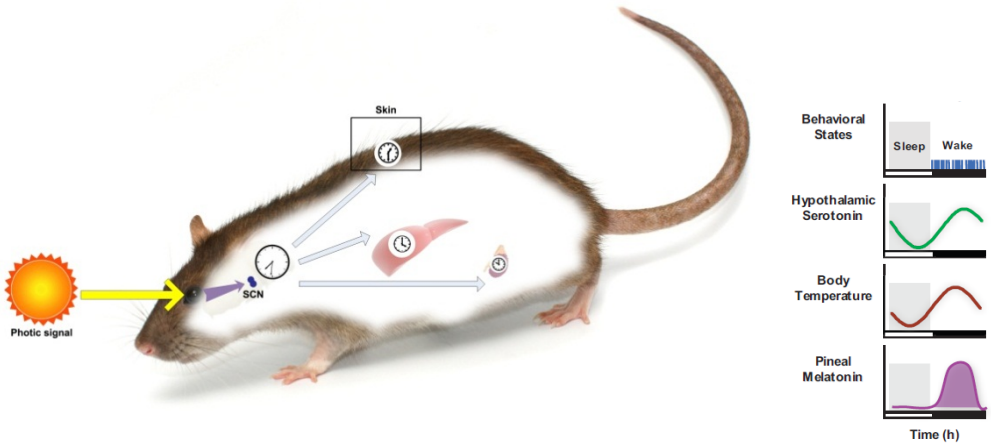


Figure 1: Mammalian circadian system. In mammals, for example in nocturnal rodents, a master clock located in the suprachiasmatic nucleus of the hypothalamus controls peripheral clocks. Environmental cues entrain the SCN and light, which is perceived by retina, is the most important one. Peripheral clocks (in liver, kidney and skin…) oscillate with tissue-specific patterns, which need coordination via the SCN output signals. This circadian system drives many physiological rhythms over 24h (right panel), such as body temperature cycle and melatonin production in pineal. The skin clock is the focus of this manuscript. (Adapted from Challet 2007)

A functional circadian system requires the central clock, with input and output pathways. The central clock oscillates with a period close to 24 hours. On one side, inputs derived from environment convey the timing message to entrain the central clock to varying conditions. One of the most powerful entraining signals is the daily alternation of light and dark, whose period is precisely 24 h. On the other side, circadian clocks are present in almost all the cells in the organisms and outputs from the center allow the clock system to control subsidiary targets in the brain and periphery (Fig. 1). All these peripheral clocks also need to be synchronized within each tissue, to maintain homeostasis required for physiological functions.

Another intrinsic feature of circadian clocks is that they are temperature compensated. Their period lengths remain almost constant across the physiological temperature range (Pittendrigh, 1993). This temperature compensation can be described quantitatively by the temperature coefficient, Q10, which means the change in the rate of a biological reaction when increasing the ambient temperature by 10 °C. This Q10 is 2-3 for most biochemical reactions but only 0.8-1.2 in circadian rhythms (Sweeney and Hastings, 1960). It is unclear but possible that synthesis and degradation rates of core clock components are equally affected by changing temperature. In mammals, temperature compensation has been demonstrated in cell cultures such as fibroblasts and SCN neurons, and tissues like SCN, lung and adrenal gland (Reyes et al., 2008; Dibner et al., 2009; Buhr et al., 2010). This property of resilience to changes in temperature is independent of intercellular coupling. It is crucial for phase coherence of peripheral clocks, for which cellular transcription rates can vary dramatically in different tissues (Dibner et al., 2010). Some interface tissues like skin, especially distal skin, confront largely changing ambient temperatures, even in homeothermic animals. In fact, some oscillators like fibroblasts are even overcompensated (Tsuchiya et al., 2003).

2. Molecular clock mechanisms

Circadian clocks are cell-autonomous processes able to generate persistent oscillations, and which are coordinated in multicellular organisms. The unraveling of the molecular basis of circadian clock began with the discovery of the *tau* mutant hamster in the 1980s and identification of the *Clock* mutant mouse in the 1990s (Ralph and Menaker, 1988; Antoch et al., 1997; King et al., 1997). Then, a set of clock genes were defined as core components whose protein products are necessary for the generation and regulation of circadian rhythms in rest/activity cycles and, later, within individual cells throughout the organisms. *Per1* and *Per2* are induced by light (Albrecht et al., 2001). The photoinduction of the clock gene *Per2* develops later than that of *Per1* (Mateju et al., 2009). Disruptions of *Per1/2* or *Cry1/2* cause immediate behavioral arrhythmicity when double-knockout animals are kept in constant darkness (van der Horst et al., 1999; Zheng et al., 2001). Deletion of *Bmal1* is the only single knockout that abolishes clock function in both the SCN and peripheral tissues (Sun et al., 2006). This chapter will focus on the regulations underlying the molecular clockwork, with particular emphasis on *Per1*, whose promoter was used as a model throughout the thesis work.

2.1. The feedback loops

The molecular mechanism of circadian clocks involves basically a transcription-translation feedback loop, with a time constant of about 24 hours per cycle. This dynamic loop begins with two bHLH-PAS domain transcription factors, BMAl1 (also known as aryl hydrocarbon receptor nuclear translocator-like, ARNTL) and CLOCK. BMAL1 and CLOCK heterodimerize via PAS domains and bind to DNA via bHLH domains. In the nucleus, BMAL1/CLOCK heterodimers bind and activate transcription of genes containing E-box enhancer sequences in promoters, including *Period* (*Per1 and Per2*) and *Cryptochrome* (*Cry1 and Cry2*) genes. This leads to an increase in the levels of PERs and CRYs in the cytoplasm. PER and CRY proteins form heterodimers and, along

with other proteins, constitute a higher order complex and translocate to the nucleus. Subsequently, negative feedback is achieved by the accumulating PER/CRY complex in the nucleus, repressing their own transcription driven by the BMAL1/CLOCK complex. In the cytoplasm, the PER/CRY complex is degraded by proteasomal pathways, which is a critical step for relieving inhibition of BMAL1/CLOCK and initiating a new cycle of transcription: this allows a new cycle of autoregulation to restart. (Fig. 2) Another accessory regulatory loop interconnects with the core loop, mediated by the ROR-biding element (RORE) present in the *Bmal1* promoter. BMAL1/CLOCK activates the transcription of genes encoding two families of cognate nuclear receptors, *Rev-erbα-β* and *Rorα-γ*. Via competing on binding to the RORE, REV-ERBs repress the transcription of *Bmal1*, whereas RORs activate it. These positive and negative regulations of *Bmal1* not only add robustness and stability to the clock mechanisms but also provide additional control and link to other pathways in the cell. (Fig. 2)

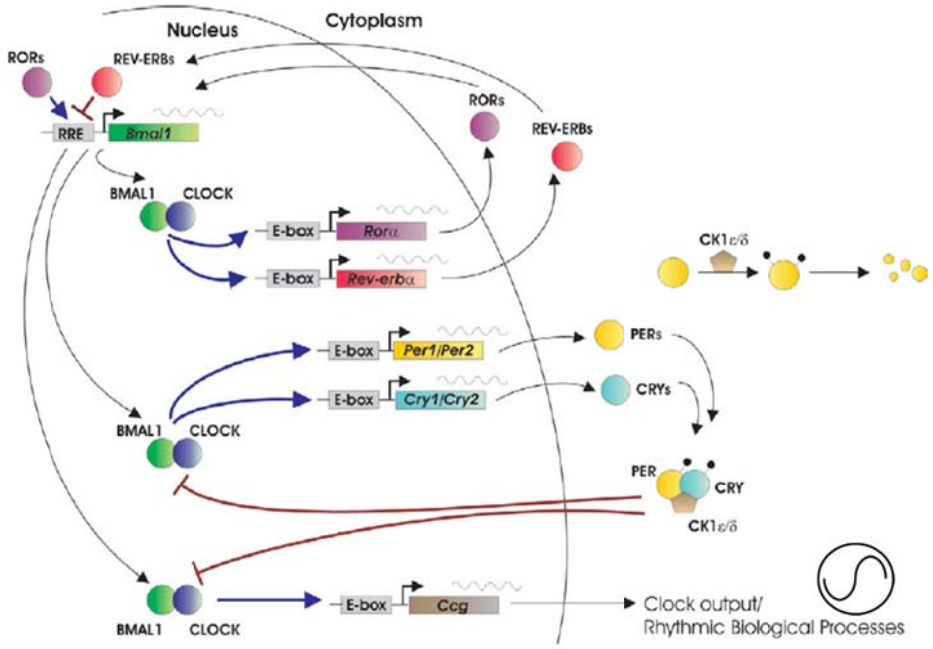


Figure 2: The molecular clockwork of transcription-translation feedback loops. Clock genes in a single cell generate circadian rhythms involving positive and negative feedback loops. Circadian gene transcription drives self-sustained oscillations in clock factor levels and, subsequently, in biological rhythms via the regulation of their target genes. (Adapted from Ko *et al*., 2006)

2.2. Transcriptional activation of *Period* genes by BMAL1/CLOCK

As core clock genes, *Per*1/2 plays an important role in the molecular machinery of dynamic loops. The regulation of *Per1/2* transcription starts from combination and activation of cis-regulatory elements contained in their promoters. Transcription of m*Per1/2* is dependent on the levels of activation of these elements. The conserved five E-box motifs (a consensus CANNTG DNA sequence) are found in the m (mouse) *Per1* promoter. Luciferase reporter assays with deleted/point mutated m*Per1* promoter constructs demonstrate that each identified E-box contributes to transcription of *Per1* activated by CLOCK/BMAL1 (Hida et al., 2000). It was described that CLOCK/BMAL1 constitutively binds to *Per1* E-box sequences over the circadian cycle (Fig. 3A) (Lee et al., 2001). Recent genomic sequence analysis of BMAL1 binding sites in the liver shows that binding is rhythmic and identifies a tandem (E1-E2) of E-box sequences with specific spacing, as mediating cooperative binding of CLOCK/BMAL1 and stronger transcriptional activation (Rey et al., 2011). The circadian transcription of CLOCK/BMAL1 target genes including *Per1/2* and *Cry1* is accompanied by rhythmic chromatin remodeling and circadian binding of RNA polymerase II (RNAPII) at the promoters of these genes (Etchegaray et al., 2003). Circadian histone H3 acetylation and methylation create favorable environments on chromatin for RNAPII recruitment, transcriptional initiation and elongation (Koike et al., 2012). It is the rhythmic RNAPII recruitment at promoters, rather than a rhythmic transition from paused to productive elongation, that executes circadian changes in genes transcription (Le Martelot et al., 2012). The occurrence of various modifications of chromatin, occupancy of DNA by RNAPII and presence of coactivators contribute to the narrow phase of activity for these regulations of circadian transcription (Fig. 3B) (Koike et al., 2012).

Other cis-acting regulatory elements may play a role in shaping the kinetics of transcription that contributes to variability or additional control of clock genes, likely through recruitment and retention of elements of the general transcription machinery (Suter et al., 2011). No typical TATA-box but four conserved CREs (cAMP responsive elements) are identified in *Per1* promoter region, and one CRE and two D-box sequences

in *Per2* promoter (Hida et al., 2000; Albrecht et al., 2007). External stimuli such as light-induced glutamate and PACAP can lead to calcium influx and elevate the level of cAMP in the SCN. This activates several kinase pathways including Protein Kinase A (PKA) and finally causes phosphorylation of CREB, which activates CREs contained in the promoters of *Per1* and *Per2*. CREs have been reported to be responsible for the transcription of other clock-controlled genes such as arginine vasopressin and *c-fos* (Robertson et al., 1995; Iwasaki et al., 1997). *Per* genes, activated by external stimuli without any new proteins synthesis are considered as immediate early genes. CRE element makes the *Per2* promoter much less responsive, in contrast to similar elements in the *Per1* promoter (Travnickova-Bendova et al., 2002). In mice PER oscillation, rather than its absolute quantity, is crucial for both cellular circadian oscillations and behavioral rhythmicity (Chen et al., 2009).

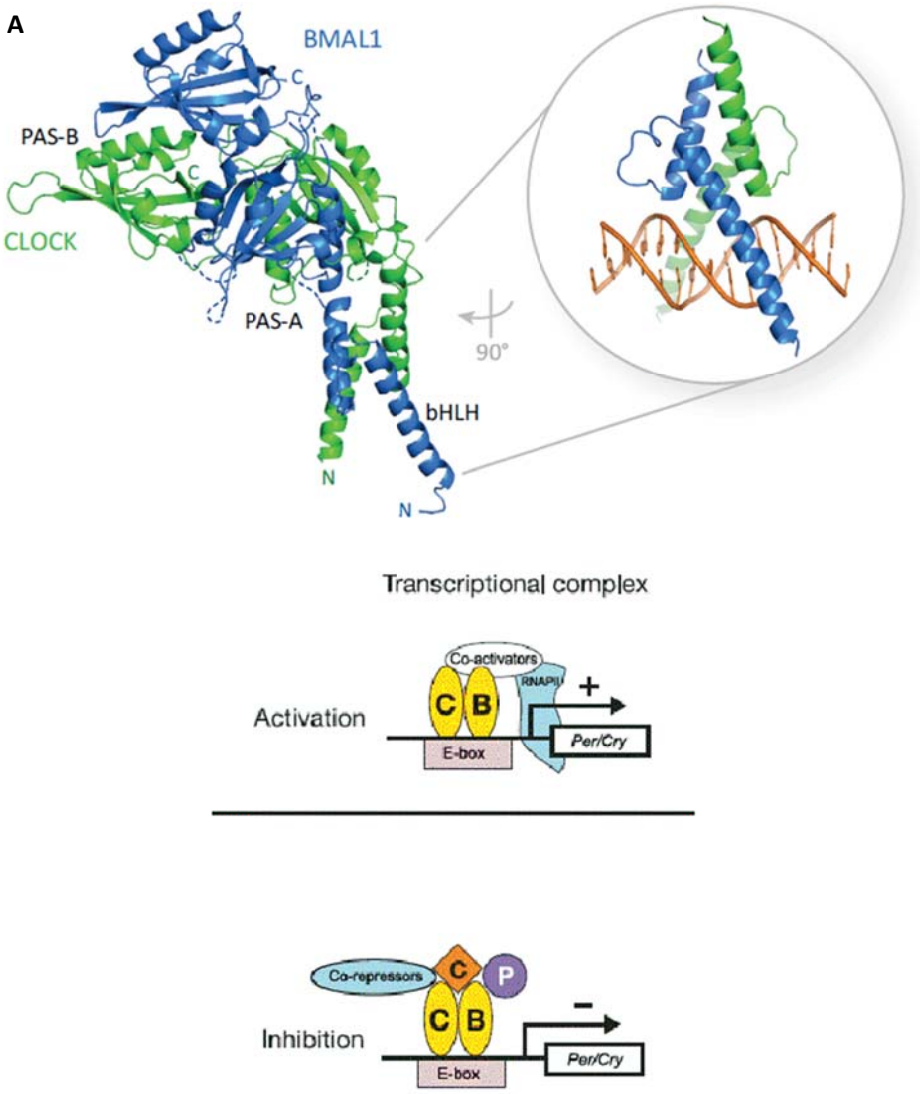


Figure 3: CLOCK/BMAL1 heterodimer and its transcriptional complex

A) Structural diagram of the mouse CLOCK/BMAL1 heterodimer illustrating how the DNA-binding bHLH domain and tandem PAS domains each contribute to complex formation between CLOCK (green) and BMAL1 (blue). This structure lacks the C-terminal regions of each protein (471 of 855 residues in CLOCK and 179 of 626 residues in BMAL1) that lack ordered structure but are required for CLOCK/BMAL1 activity and clock function. Inset, a closer view of the CLOCK/BMAL1 bHLH domain bound to an E-box element in DNA. **B)** CLOCK/BMAL1 heterodimers remain bound to E box forming transcriptional complex over the circadian cycle, according to initial studies in liver. During transcriptional activation, CLOCK (C in yellow) and BMAL1 (B in yellow) are bound to E boxes in the *Per/Cry* promoters, and co-activators and RNA Polymerase II are recruited to activate transcription. During transcriptional inhibition, the PER/CRY complex (P in purple and C in orange) with other recruited co-repressors bind to the CLOCK/BMAL1 heterodimer, and inactivate the transcription. (Adapted from Partch *et al.*, 2013; Reppert & Weaver 2002)

2.3. Post-transcriptional regulations

The general transcription factors are recruited to individual promoters of genes such as *Per1/2* and *Cry1/2* upon CLOCK/BMAL1 binding, and the amount of nascent transcripts appears to peak with a sharp phase about 8 hours after the activation start on individual promoters (Koike et al., 2012). Microarray studies have shown that approximately 10% of all the mammalian transcripts exhibit circadian oscillations, varying among cell types and tissues (Lowrey and Takahashi, 2011). However, only about 30% of genes with rhythmic changes in protein levels also cycle transcriptionally (Deery et al., 2009). Cells treated with a RNA polymerase inhibitor still provide cycling rhythms (Dibner et al., 2009). These data indicate that the majority of rhythmic clock outputs are possibly due to posttranscriptional regulation. Outside core clock genes, post-transcriptional regulation likely helps to provide additional flexibility by generating rhythms in mRNA transcripts in a tissue- or stimulus-dependent manner. Many core clock genes and clock-controlled genes exhibit circadian oscillations in their transcript levels involving post-transcriptional control, including miRNA and RNA binding proteins, such as CIRP (cold-inducible RNA-binding protein) and nocturnin (Fig. 4). Transcribed from noncoding genomic regions, miRNA interact with the 3'untranslated regions (3'UTR) of target transcripts to repress activity of target mRNA, possibly to affect the molecular clock (Bartel, 2009). Two miRNA, miR-219 and miR-132 have been found circadian rhythmic in the SCN and harbor CREs or E-box in their promoters (Cheng et al., 2007). It is proposed that miRNA-132 is involved in chromatin remodeling and translational control in the SCN, mediating clock entrainment by regulating *Per* mRNA decay (Alvarez-Saavedra et al., 2011). In Hela and NIH3T3 cells, miR-192 and miR-194 inhibit *Per* genes containing corresponding target sites in their 3'UTR, which causes a shortened circadian period (Nagel et al., 2009). In addition, several RNA-binding proteins have been identified to regulate clock-related transcripts by distinct mechanisms. CIRP plays an important role in the core clock posttranscriptional regulation through direct interaction with *Clock* mRNA which results in mRNA stabilization in the cytoplasm (Morf et al., 2012). CIRP transcription and translation are rhythmic under the regulation

of systemic signals like body temperature. A reduction of CIRP levels causes low levels of many core circadian components including *Clock*, *Per2* and *Dbp*, leading to low amplitude of molecular circadian rhythms (Morf et al., 2012). Several RNA binding proteins target cis-acting elements located in 3'UTR of RNAs, such as Lark which positively regulates *Per1* transcript (Kojima et al., 2007). Another RNA binding protein Nocturnin is a deadenylase that catalyses the removal of the poly(A) tail from transcripts, followed by mRNAs breakdown, notably of rhythmic genes (Garbarino-Pico et al., 2007). Finally, further investigation of transcriptional and posttranscriptional molecular machinery, as well as how locally controlled clock genes are additionally regulated by systemically controlled genes and systemic cues, will be helpful for a complete understanding of the circadian gene expression in each tissue and cell type.

After their mRNAs have left the nucleus, PER and CRY proteins are produced in the cytoplasm and dimerize through their PAS domains. Then, the PER/CRY dimer is submitted to a shuttling balance between the cytoplasm and the nucleus (Vielhaber et al., 2001). In PER1 and PER2, the presence of nuclear localization signals (NLS) and nuclear export signals (NES) have been characterized for nuclear import and cytoplasmic retention (Yagita et al., 2000; Vielhaber et al., 2001). Whereas nuclear translocation of PER1 and PER2 can occur in the absence of any CRY protein, nuclear localization of PER/CRY proteins is a dynamic process determined by both nuclear import and nuclear export pathways. Nucleocytoplasmic shuttling provides the opportunity for regulation of PER/CRY protein function in the nucleus and degradation in the cytoplasm.

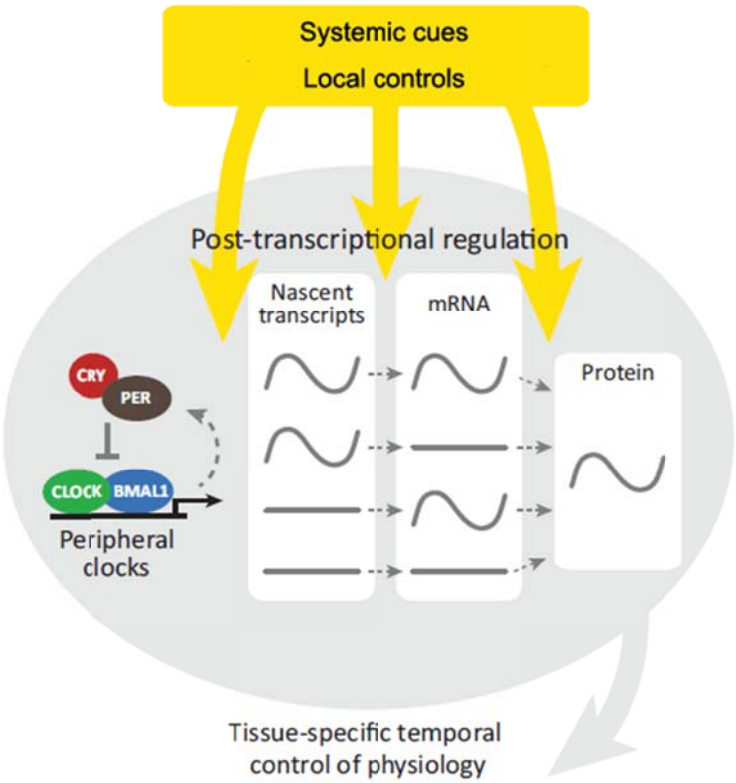


Figure 4: Schematic outline of posttranscriptional regulations contributing to the tissue-specific circadian rhythms. Local control of circadian transcriptional and post-transcriptional mechanisms is integrated with systemic cues from the SCN to generate tissue-specific changes in transcription, mRNA accumulation and protein production. Different patterns of clock-controlled genes are illustrated with either flat lines, constitutive at nascent transcript or mRNA, or sinusoidal lines, circadian regulated with a daily peak. This integrative system sustains the continuous oscillation of the molecular clock in each cell (primarily driven by transcription) while allowing the tissue-specific control of clock-controlled genes necessary for temporal regulation of physiology by the clock network. (Adapted from Partch *et al*., 2013)

In the cytoplasm, PER1 and PER2 are phosphorylated by CK1 (casein kinase 1), which leads to increased ubiquitylation and degradation via the proteasome (Keesler et al., 2000). This phosphorylation is balanced by the action of PP1 phosphatase, resulting in an additional control over the period of the molecular clock (Lee et al., 2011a). Phosphorylation of PER1 can also lead to masking of its NLS and thus impeded heterodimer import (Vielhaber et al., 2001). Nuclear entry of the PER/CRY complex is modulated by the CK1-mediated phosphorylation of PER proteins, with distinct binding sites for CRY and CK1. This trimeric assembly of PER/CRY/CK1 is also required for phosphorylation of CRY proteins by casein kinases. CK1-mediated phosphorylation delays nuclear translocation of PER/CRY complex and repression of

CLOCK/BMAL1-mediated transcriptional activities, contributing to the 24-hour kinetics of the clockwork (Eide et al., 2005). CK1ε mutation in the tau mutant hamster leads to shorter endogenous period. The CK1δ deficient mouse shows longer circadian periods in free-running rhythms and liver tissue rhythms (Etchegaray et al., 2009; Lee et al., 2009). Importantly, posttranslational modifications such as phosphorylation, SUMOylation, deacetylation and ubiquitination can also control degradation of core clock components and, consequently, period, as shown by the effects of proteasome inhibitors (Eide et al., 2005). CK1 mediated phosphorylation of PER proteins promotes their ubiquitination and following degradation via 26S proteasome (Gallego and Virshup, 2007). E3 ubiquitin ligase FBXL3 interacts with CRY and targets it for degradation, following phosphorylation of CRY1 by AMPK and of CRY2 by GSK3β (Harada et al., 2005; Busino et al., 2007; Lamia et al., 2009). SUMO (small ubiquitin-like modifier) modifies BMAL1 and promotes its proteasomal degradation (Lee et al., 2008). NAD^+ dependent histone deacetylase Sirtuin1 (SIRT1) binds to CLOCK/BMAL1 and deacetylates PER2, promoting its degradation (Asher et al., 2008). Interestingly, SIRT1 participates in the regulation of transcription and CLOCK/BMAL1 controls rhythmic NAD^+ in turn (Nakahata et al., 2009; Ramsey et al., 2009). This connection between circadian system and metabolism contributes to the flexibility of the system which helps to confer tissue-specific regulation of physiology (Asher and Schibler, 2011).

In the nucleus, the PER/CRY complex associates with CLOCK/BMAL1 and recruits corepressors, which negatively regulate transcription activity of CLOCK/BMAL1 on core clock genes such as *Per*, *Cry*, *Rev-erb* and *Ror* (Fig. 3B). The rhythmic binding of PER/CRY to CLOCK/BMAL1 generates the rhythm in transcriptional activation. CRY1 can interact with CLOCK/BMAL1 independently of PER proteins, because it is found without PER at CLOCK/BMAL1 bound sites in *Dbp* promoter at CT0 (Ye et al., 2011; Koike et al., 2012). This suggests that CRY1 may act as a molecular gatekeeper to maintain CLOCK/BMAL1 in a poised and repressed state until the proper time for transcriptional activation. This repressed state involves epigenetic regulations. PSF (polypyrimidine tract-binding protein-associated splicing factor) acts as a transcriptional corepressor by rhythmically recruiting the SIN3-HDAC (SIN3 histone deacetylase)

complex to deacetylate histones 3 and 4 at the *Per1* promoter (Duong et al., 2011). It is reported that an RNA- and DNA-binding protein, NONO, associates with PER1/2 to repress the transcription and gates circadian rhythm with cell cycle (Brown et al., 2005a; Kowalska et al., 2013). By contrast, transition to the active state begins with removal of CRY1 from CLOCK/BMAL1 complex and recruitment of coactivator proteins p300 and WDR5 in favor of an active chromatin state, with histone acetylation and methylation (Etchegaray et al., 2003; Brown et al., 2005a; Kiyohara et al., 2006).

Finally, because of these transcription-translation feedback loops and posttranscriptional modulations, the expressions of clock genes show rhythmic profiles creating molecular oscillations that can sustain themselves in the absence of external inputs. Relative phases of clock gene expression, such as antiphase of mRNA level of *Per* and *Bmal1*, are also a property of the molecular clockwork (Nishide et al., 2006). Due to the complicated molecular clockwork, various clock-controlled genes (CCGs) are expressed differently within the 24 hours with peaks occurring at different times of the day, to regulate multiple physiological processes from cell growth to metabolism.

Elimination of one component may be compensated by others. In human, mutations in genes of the molecular clock system are related to some circadian disorders like FASPS (Familial Advanced Sleep Phase Syndrome) and DSPS (Delayed Sleep Phase Syndrome) (Takahashi et al., 2008).

3. The SCN: master clock of the body and a model to understand rhythm generation within tissues

SCN, as the central pacemaker, is capable to be entrained by environmental stimuli. It receives diverse inputs via innervation from several regions of the brain, where three pathways well investigated are from the retina (photic inputs), the intergeniculate leaflet (IGL) and the raphe nuclei (non-photic signals).

3.1. The SCN clock

The suprachiasmatic nucleus (SCN) of the hypothalamus is a bilateral structure sited just above the optic chiasma. The pair of nuclei are located at each side of the third ventricle and comprise approximately 20000 neurons of small size (Moore et al., 2002). All the circadian functions are suppressed by ablating the SCN, not any other part of the brain. Transplantation of fetal SCN tissue into other brain regions like the third ventricle of SCN-lesioned animals can restore circadian behavioral rhythms (Lehman et al., 1987; Silver et al., 1996). The SCN tissue from the *tau* mutant hamster, which shows shorter period in circadian rhythms, could restore rhythms in SCN-lesioned wild-type hamster with a shortened period as in the mutant donor (Ralph et al., 1990). The SCN can self-sustain rhythms with a period of approximately 24 hours without any inputs, for months in slice culture (Abe et al., 2002). Metabolic activity and glucose uptake in the SCN are dependent on time as well as gene expression (Dibner et al., 2010).

3.2. Multioscillatory nature of the SCN

In the SCN, there are mainly neurons which contribute to the pacemaker functions. Other glial cells also exhibit circadian rhythms such as astrocytes (Prolo et al., 2005). Of all the SCN neurons, about 10% contain vasoactive intestinal polypeptide (VIP) and 20% contain arginine vasopressin (AVP) (Welsh et al., 2010). The GABA and GABA receptors are present in most SCN neurons (Moore and Speh, 1993; Belenky et al., 2008).

Within the tissue, the SCN is a heterogeneous structure, with theoretical subdivisions traditionally defined as core and shell in the ventral and dorsal parts (Fig. 5). These regions differ by neuropeptide phenotypes, inputs and even functions. In rodents, the ventral part cells in the SCN express VIP and GRP while the dorsal part cells express AVP and somatostatin. However, the regional expression of neuropeptides varies substantially

among species. For instance, in mice, ventral SCN also possess AVP neurons (Morin et al., 2006). The rodent ventral SCN contains gastrine-releasing peptide (GRP) expressing cells and VIP cells which receive input from the retina (Ibata et al., 1989). Actually the SCN is much more complicated with numerous neurotransmitters and neuropeptides expressed in many cell types. The functions of the cells are not yet clear and cells cannot be referred to specific roles (Webb et al., 2009). They can be grouped depending on the neurotransmitters and neuropeptides, as well as the regions they innervate.

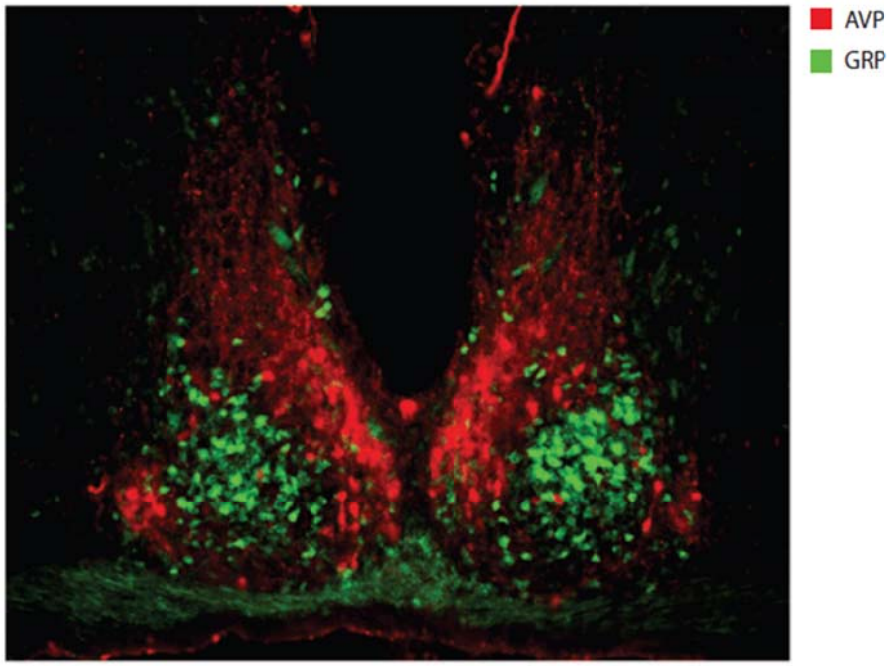


Figure 5: Expression of neuropeptides in a mouse SCN coronal slice. The bilateral nuclei are located directly above the optic chiasm and positioned on either side of the third ventricle. Sections are immunostained to detect expression of the neuropeptides, AVP (red) and GRP (green). AVP expression is observed most prominently in the dorsal shell region of the SCN, whereas GRP expression is strongest in the ventral core SCN. (Welsh *et al*., 2010)

Numerous projections drive from the ventral to the dorsal parts, but fewer in the opposite direction (Leak et al., 1999). It is proposed that the shell SCN represents the circadian pacemaker, with cells expressing AVP (Li et al., 2009). In turn, the core part is entrained by the photic input from the retina, and expresses VIP and GRP (Abrahamson and Moore, 2001; Albus et al., 2005). Ventral neurons relay this information to the dorsal

region using VIP, GABA and GRP. In addition, expression of *Per1* gene seems to start in the dorsal SCN and to progressively spread to the ventral SCN (Yamaguchi et al., 2003). Amplitude of rhythms in gene expression and neural activity in ventral and dorsal neurons are relatively low and high respectively. Low amplitude rhythms may be easier to reset to perturbations, which is supported by mathematical modeling studies (Colwell, 2011). The complexity of the SCN also exists along the other axes, both anatomically and regarding produced peptides (Evans et al., 2011).

Another theory believes that two groups of oscillators in the SCN are identified as activated at dawn and dusk respectively, and named morning and evening oscillators (Daan et al., 2001). Horizontal slices of the hamster SCN show two peaks of neuronal activity, which support this binary oscillator's configuration (Jagota et al., 2000). Exposed to a phase-shifting agent, these two oscillators respond differently, depending on the time of the day. These morning and evening oscillators alter the phase under different day lengths, helping the animals to adapt to seasonal changes. Studies at the molecular and electrophysiological levels show that two or more types of cell populations in the SCN can respond respectively to short and long photoperiods (Meijer et al., 2010). Recently, different periods were observed in two regions of the SCN - a region with periods shorter than 24 h and another with periods longer than 24 h. The short-period region is located in the smaller medial region of the dorsal SCN, whereas the long-period region occupies the remaining larger region(Koinuma et al., 2013). Photoperiod duration and light-dark transitions show region-specific influence on entrainment of *Per1* and *Per2* expression in subdivisions of caudal and rostral SCN (Sosniyenko et al., 2009). However, further studies remain to be performed to trace the different oscillators to specific SCN neurons and to clarify the mechanisms of morning and evening oscillators.

At cellular level, the SCN could be defined as an ensemble of neurons harboring cell autonomous oscillators. Single neurons within the SCN exhibit a wide range of periods (22 h ~ 30 h) and phases in rhythms of firing rate and gene expression (Ko et al., 2010). This heterogeneity is also observed when dissociated SCN cells are dispersed in low density culture (Welsh et al., 1995). Synchrony is shown when dispersed neurons are cultured at a high density (Aton et al., 2005). To maintain synchrony within SCN

neuronal populations, intercellular coupling is crucial towards a robust, precise and stable SCN rhythm. Neurotransmission and gap junctions are important in the intercellular communication (Shirakawa et al., 2000; Rash et al., 2007; Ko et al., 2010). Interestingly, SCN resistance to entrainment by temperature cycles, which is a powerful cue for many species, depends on the intercellular coupling of neurons (Underwood and Calaban, 1987; Yoshida et al., 2009; Abraham et al., 2010; Buhr et al., 2010). Even absence of cell autonomous rhythms does not lead to loss of noise-induced oscillations in SCN explants of *Bmal1* mutant mice, which requires intercellular coupling (Ko et al., 2010). Generally, the mechanisms and dynamics of the coupling network in the SCN still remain largely undefined.

Synapses and gap junctions are two coupling mechanisms that get involved in the synchronization of SCN neurons. Neuronal connections are involved in the coupling within the SCN by neuronal firing and chemical synapses. Blocking action potentials with TTX leads to asynchrony of SCN neurons in slice culture or high-density dispersed culture (Honma et al., 2000; Yamaguchi et al., 2003). Gap junctions could also be involved in SCN coupling because short-term firing synchrony can be found in SCN slices with synaptic transmission blocked by using calcium-free medium (Bouskila and Dudek, 1993). In SCN slices from wild-type mice, the gap junction blockers octanol or halothane destroy circadian rhythms of neuronal firing or neuropeptide release (Shinohara et al., 2000).

3.3. Photic input: light information as a major external signal for the clock

The retina provides the major photic input through the retinohypothalamic tract (RHT) directly into the SCN and indirectly, via the IGL. In the retina, there exists three main types of photoreceptor cells as rods, cones and ipRGCs (intrinsically photosensitive retinal ganglion cells) (Fig. 6). Rods and cones are photoreceptors first discovered to be involved in image-forming vision, whereas the ipRGCs, a subset (~1%) retina ganglion cells, were identified recently (Berson et al., 2002). And ipRGCs contain the

photopigment melanopsin as a G-protein-coupled receptor, which is mostly sensitive to blue light (Bailes and Lucas, 2010). These ipRGCs are able to transduce light information via melanopsin into electrical signals and to directly innervate the SCN by the optic nerve through RHT, involving glutamate and PACAP neurotransmitters (Schmidt et al., 2011). This “non-image forming vision” explains the circadian photoentrainment in some blind individuals and in mice lacking rods and cones (Czeisler et al., 1995; Freedman et al., 1999). Thus, melanopsin expressing ipRGCs support the main photic entrainment of the SCN, though influenced synaptically by intraretinal rods and cones (Berson, 2003).

The phase of the rhythm in electrical activity of the SCN is determined mostly by light. The peak of neuronal activity occurs usually in the middle of the light period, at ZT6 in the 12h/12h light/dark cycle (Gillette, 1986). Shifting the light period or applying a light pulse during the dark can entrain the clock to a new phase, advanced or delayed according to its phase-response curve (Fig. 7) (Khalsa et al., 2003). Injection with the neurotransmitters glutamate and PACAP in vivo or in SCN slices can mimic the entrainment of the SCN by light, inducing phase delays during the early subjective night and phase advances during the late subjective night (Hannibal, 2002; Meijer and Schwartz, 2003). Additionally, exposure to light at night induces *Per1* transcription in the ventral SCN and *Per2* transcription in the whole SCN, and suppresses the melatonin peak, even in blind patients (Czeisler et al., 1995; Dardente et al., 2002). In the SCN, *Per1* is induced within 5–15 min after light pulse via a CRE-mediated pathway, similarly to c-*fos* and other immediate early genes which also have CRE sequences in their promoters (Wollnik et al., 1995). Retina ipGRCs also innervate the IGL, which projects then to the SCN via GHT (geniculohypothalamic tract) and release neuropeptide Y. The GHT tract can modulate the photic and non-photic input pathways (Biello et al., 1997; Prosser, 1998).

Interestingly, circadian responses to light at both behavioral and molecular levels are similar in diurnal and nocturnal species (Fig. 7). For instance, the clock gene *Per1* in the SCN peaks at midday in both species. Phase-response curves show roughly similar pattern in both categories (Challet, 2007).

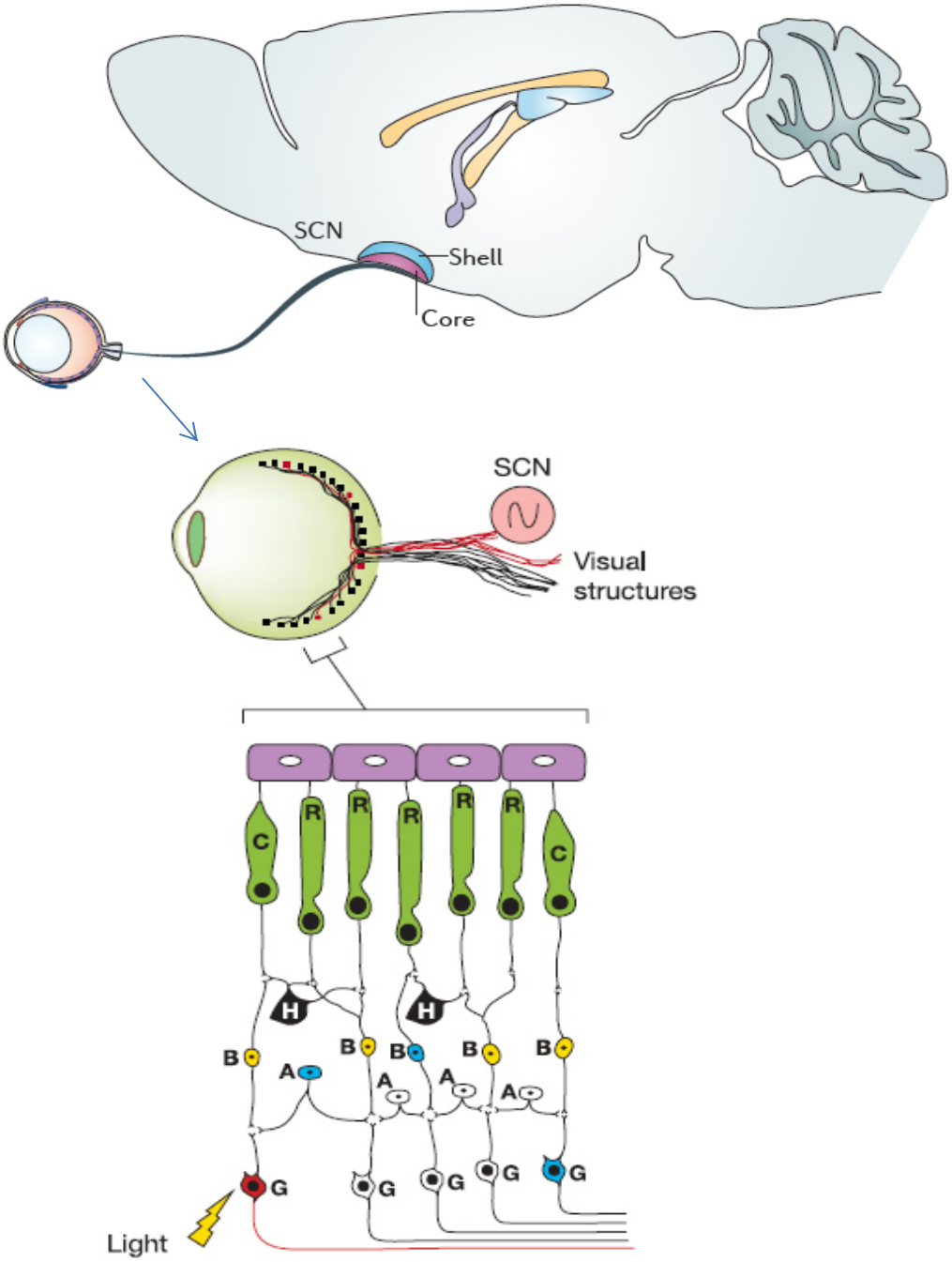


Figure 6: The photic input to the SCN. The melanopsin positive ipRGC cells (red) located in the retina and dispersed among other ganglion cells (G) deliver photic information to the SCN in the hypothalamus via the retinohypothalamic tract (RHT). The SCN can be divided into two anatomical and functional subdivisions: a ventral core and a dorsal shell. SCN core neurons are thought to integrate external inputs, including light from the retina. Rods (R) and cones (C), which are located in the outer part of the retina, also contribute to photoentrainment, via synaptic transmission. (Adapted from Cowell 2011; Reppert and Weaver 2002)

3.4. Non-photic inputs: feeding and social activity

The midbrain raphe nuclei innervate the ventral SCN and IGL via serotoninergic projections (Meyer-Bernstein and Morin, 1999). It mediates a non-photic pathway to synchronize the circadian clock, probably to social interaction.

Serotonin is involved in many physiological functions such as social behavior, sleep, emotion and thermoregulation. Activation of serotoninergic receptors is closely related with the level of arousal. 5-HT agonists are capable to induce behavioral phase-advances only during resting, in both nocturnal and diurnal animals (Mistlberger and Holmes, 2000). And 5-HT stimulation during daytime could reduce the mRNA levels of *Per1* and *Per2* in the SCN of nocturnal rodents (Challet, 2007).

In addition, metabolic signals like feeding also affect the synchronization of the SCN clock. Hypocaloric restriction under the light-dark cycle could phase-advance molecular loops in the SCN, and alters the shift response to light (Challet et al., 1997; Mendoza et al., 2005). Temporal restricted feeding on meal time induces food-anticipatory activity (Stephan et al., 1979).

In mammals, external temperature cycles, a universal entraining signal, are very weak entraining factors for the SCN (Refinetti, 2010), possibly resulting from the fact that homeotherms regulate the core body temperature against environmental fluctuation, even if a limited part of the body, like distal skin, is still sensitive to it.

Non-photic inputs normally have phase advance effects at resting period in diurnal and nocturnal animals (Fig. 7). However, stress does not have non-photic-like synchronizing effects, but leads to photic-like phase delays in both hamsters and mice (Van Reeth et al., 1991).

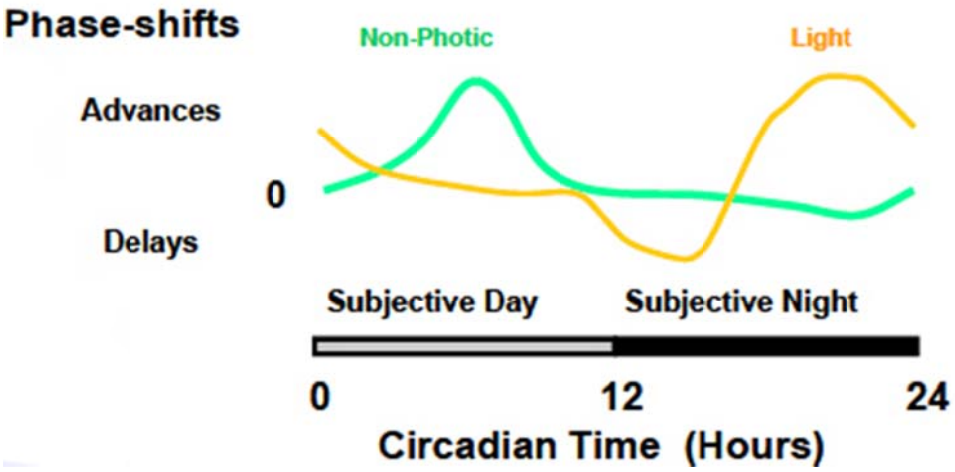


Figure 7: Phase responsive curves to light and non-photic signals. In nocturnal animals, external cues of light and non-photic signals can shift the phase of circadian rhythms in constant darkness. Behaviorally, the phase can be advanced or delayed depending on the occurrence of signals with respect to circadian time. Interestingly, this phase responsive curve is roughly similar between nocturnal and diurnal animals. Light and non-photic signals have these phase shift effects mostly when they are not present in normal conditions. (Adapted from Challet 2011)

3.5. Neuronal outputs from the SCN

The SCN launches both neuronal and hormonal outputs to deliver circadian messages as the intrinsic timekeeping signals (Fig. 9).

Basically, the SCN efferences innervate directly a range of brain regions, a great proportion of which is located in adjacent structures (Fig. 8) (Abrahamson and Moore, 2001). Direct SCN target areas include lateral septum (LS), bed nucleus of the stria terminalis (BNST), subparaventricular zone (sPVZ), paraventricular hypothalamic nucleus, dorsomedial hypothalamus (DMH), as well as paraventricular nucleus (PVN) (Watts and Swanson, 1987; Watts et al., 1987; Kalsbeek et al., 1993; Buijs, 1996; Leak and Moore, 2001; Morin and Allen, 2006). Injecting tracers shows that these projections arise from both ventral and dorsal regions of the SCN, although most fibers from the ventral SCN project to the dorsal part (Leak and Moore, 2001). In order to deliver messages to other parts of the brain, the SCN cells transduce the molecular signals into output signals. The output signals can be a change of firing rate associated with a change of neurotransmitter release.

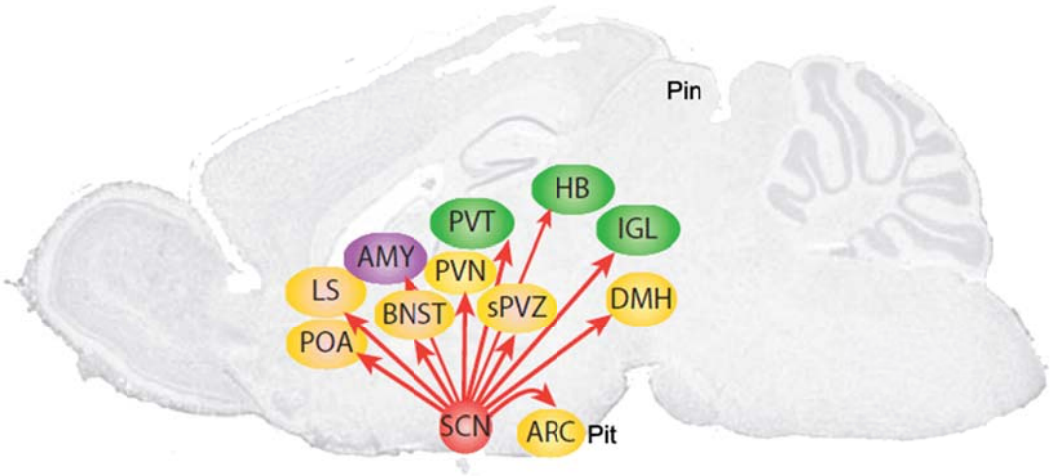


Figure 8: Efferences from the SCN (red) to hypothalamic (yellow) and thalamic (green) brain regions. These regions comprise amygdala (AMY), arcuate nucleus (ARC), bed nucleus of the stria terminalis (BNST), dorsomedial hypothalamus (DMH), habenula (HB), intergeniculate leaflet (IGL), lateral septum (LS), preoptic area (POA), paraventricular nucleus of the hypothalamus (PVN), paraventricular nucleus of the thalamus (PVT) and subparaventricular zone (sPVZ). Pineal gland (Pin) and pituitary gland (Pit) are targeted indirectly to control hormonal outputs. (Adapted from Dibner *et al*., 2010)

Neuronal firing as an SCN output is based on a cell autonomous circadian rhythm in spontaneous action potential frequency (Kuhlman and McMahon, 2006). In vivo the use of extracellular electrodes recordings reveals circadian rhythms in the electrical activity in the SCN, with high activity during the day and low at night (see above) (Inouye and Kawamura, 1979). Interestingly, the firing rate of SCN neurons peaks in the middle of the daytime in both nocturnal and diurnal animals. The rhythms in electrical activity continue in the absence of external cues (Gillette, 1986). The oscillations in the firing rate may be a consequence of a change in the membrane potential and input resistance of SCN neurons (de Jeu et al., 1998). Rhythmic SCN electrical activity with several ion channels is likely a critical component of SCN outputs. Several ion channels have been identified in the SCN. Predominantly, the fast delayed-rectifier potassium channel is rhythmically expressed in the SCN, with its expression down-regulated during the night, which is necessary for the rhythmic activity (Itri et al., 2005). Further, the calcium regulated potassium BK channels are rhythmically expressed in the SCN, and the daily expression of BK channel in the SCN is controlled by the intrinsic circadian clock (Meredith et al., 2006). Both pharmacological blockade of BK current and genetic knockout of the channel

gene blunt, but do not completely block, the circadian rhythm in electrical activity (Meredith et al., 2006). BK channel gene knockout mice display reduced circadian amplitude in multiple behaviors including locomotor activity and core body temperature, although they show normal expression of clock genes such as *Bmal1*. This suggests the role of BK channels in SCN output, rather than in the intrinsic timekeeping mechanism of the master pacemaker (Meredith et al., 2006; Kent and Meredith, 2008).

The axonal connections oppose various transmitters like GABA, glutamate, AVP, PK2 and VIP. For some of these neurochemicals, rhythms of synthesis in the SCN have been observed (Dibner et al., 2010). Some internal influence has also been reported, for instance that glutamatergic afferents can contribute to daily changes in synaptic innervation of VIP neurons in the rat SCN (Girardet et al., 2010). VIP is critical for robust rhythms in clock gene expression in the SCN. The absence of this peptide alters both the amplitude of circadian rhythms as well as the phase relationships between the SCN and peripheral clocks (Loh et al., 2011).

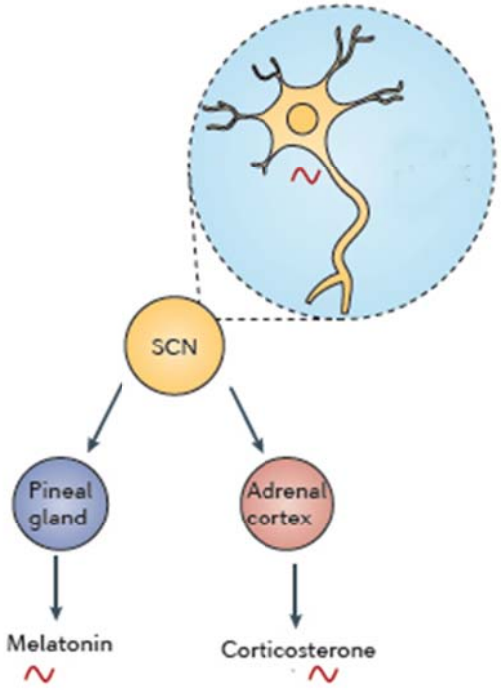


Figure 9: Neuronal and hormonal outputs of the SCN. The central clock in the SCN projects neurons to many other regions in the brain and peripheral tissues. The circadian neuronal signals from the SCN can indirectly regulate other signals like hormone secretion. In the circadian clock system, melatonin and glucocorticoids are two robust rhythmic hormones controlled by the SCN. (Adapted from Masri and Sassone-Corsi 2012)

PK2 mRNA level in the SCN shows circadian oscillation with high level during the day and low or undetectable level at night (Cheng et al., 2002). Studies using PK2 promoter fused to a fluorescence reporter indicate circadian oscillations of a number of PK2-positive neurons in the SCN which project to many known SCN target areas, including the ventral LS, medial POA, sPVZ, PVN, DMH, LT and PVT (Zhang et al., 2009). Double labeling in situ hybridization indicates that more than 50% of the PK2 mRNA-containing neurons coexpress VIP, GRP, or AVP in the SCN (Masumoto et al., 2006). Delivery of PK2 into the lateral ventricle during subjective night, when endogenous PK2 is low, inhibits the nocturnal wheel-running activity of rats (Cheng et al., 2002). The PK2 or PK2 receptors deficient mice show attenuated circadian rhythmicity in a variety of behaviors and physiology, including locomotor activity, sleep/wake cycle, body temperature, hormone levels as well as peripheral clock gene expression (Li et al., 2006; Hu et al., 2007; Prosser et al., 2007). Further, clock gene expression is not altered in the SCN of either PK2 or PKR2 mutant mice which is consistent with the supposed role of PK2 as an output molecule (Li et al., 2006; Prosser et al., 2007). The role of AVP in the circadian regulation of hormone release has been also studied. Circulating plasma corticosterone level displays a circadian rhythm, whereas SCN-lesioned animals display constant, but elevated, levels of plasma corticosterone. AVP is able to suppress elevated levels of corticosterone in SCN-lesioned animals to basal daytime values and infusion of an AVP antagonist induces a sevenfold increase in basal corticosterone levels in intact animals (Kalsbeek et al., 1992; Kalsbeek et al., 1996a; Kalsbeek et al., 1996b). AVP release from the SCN is probably also important for the control of the daily rhythm in hormonal axes, such as the hypothalamic–pituitary–gonadal axis.

3.6. Hormonal “outputs” from the SCN

Through the autonomic nervous system, some parts of the brain also mediate indirect innervation in a multisynaptic pathway, such as SCN-pineal axis controlling melatonin synthesis (Fig. 9). Fibers from the SCN project to the PVN and then innervate the intermediolateral column. Afterwards these neurons project to the superior cervical

ganglions that finally innervate the pineal gland, which releases the melatonin depending on the neuronal activity of the SCN (Fig. 8) (Moore and Klein, 1974; Perreau-Lenz et al., 2003). Another projection of the SCN, via PVN to IML, determines daily changes in sensitivity of the adrenal gland to ACTH, while the SCN affect adrenal hormone secretion by the classical neuroendocrine control via ACTH from the pituitary (Fig. 8) (Buijs et al., 1999).

Moreover, the SCN transplantation can restore circadian behavioral rhythms by implanting SCN into several brain regions (Silver et al., 1996). It has been shown that, though fibers from the dorsal SCN project to the PVN, the PVN rhythmicity dependent on the activity of SCN can be driven without synaptic inputs (Tousson and Meissl, 2004). These results implicate a role of unknown paracrine factors from the SCN tissue instead of nervous transmission. Yet, SCN grafts cannot restore the endocrine rhythms, confirming that rhythms of hormones like melatonin and glucocorticoids are under control of SCN neuronal outputs.

Melatonin is widely distributed in nature with functional activities in unicellular organisms, plants, fungi and animals. In most vertebrates, melatonin is synthesized primarily in the pineal gland at night, independently of opposite locomotor activity pattern in nocturnal and diurnal mammals (Fig. 10). Given the fact that melatonin is not stored in the pineal cells and immediately released into the general circulation, the plasma level of melatonin precisely reflects its pineal synthesis. Melatonin secretion is only influenced by the light/dark cycle, and thus could be regarded as a reliable estimate of the SCN clock functioning. Melatonin can be also extracted from seeds and leaves of a number of plants with concentrations tremendously higher than its night level in human plasma. When chicks are fed melatonin-rich food, serum melatonin level increases (Hattori et al., 1995). Circulating melatonin is metabolized mainly in the liver by cytochrome P450. And melatonin half life is short with a first distribution of 2 min and a second metabolic of 20 min in human (Claustrat et al., 2005). The duration of the nocturnal peak of melatonin secretion also reflects the length of the night. Therefore, nocturnal melatonin is an output signal of the central clock, while melatonin receptors are expressed in many organs including SCN.

Besides melatonin, glucocorticoids are another hormonal output signal driven by SCN via the hypothalamic-pituitary-adrenal axis (Fig. 9). They are synthesized by the adrenal gland and, in contrast with melatonin, secreted mainly around activity onset in diurnal and nocturnal species. For instance, cortisol level of plasma follows circadian rhythms and peaks by the onset of activity. Interestingly, the glucocorticoid nuclear hormone receptors are expressed in virtually all cell types, except in SCN neurons (Balsalobre et al., 2000).

SCN also controls other indirect circadian outputs like body temperature, feeding and rest/activity cycles (Fig. 10). In mammals, external temperature cycles are very weak entraining factors to the SCN that regulates the body temperature, in contrast with the peripheral clocks.

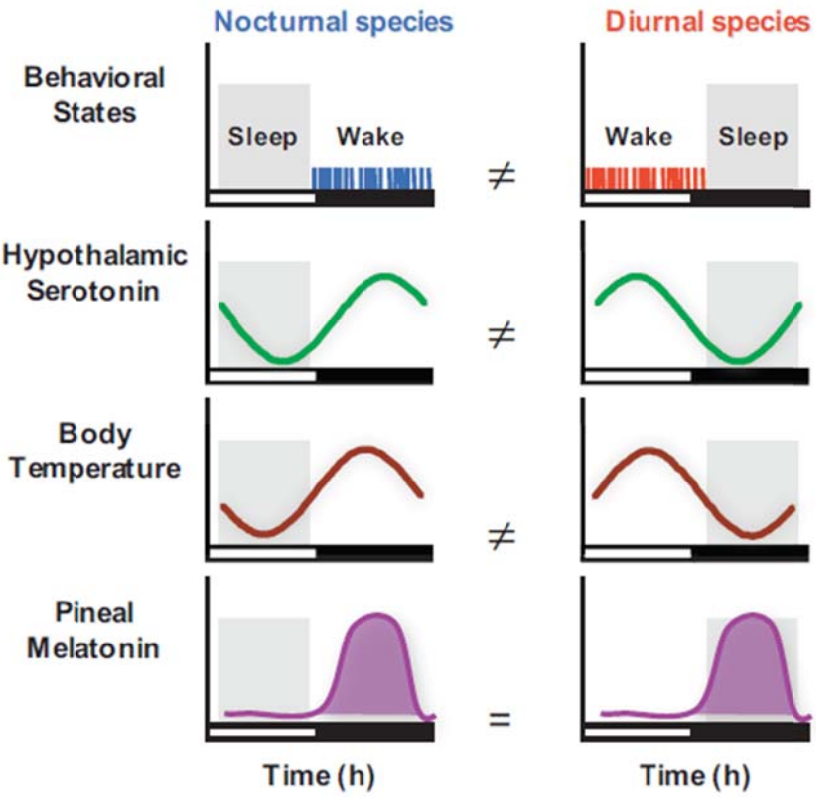


Figure 10: Differential daily rhythms between nocturnal and diurnal mammals. Defined as having their active period in night and day respectively, nocturnal and diurnal mammals exhibit differential daily rhythms, such as the sleep period (shaded areas), body temperature (brown curves) and hypothalamic content of serotonin (green curves) which are in opposite phase between the two categories. Specially, melatonin synthesis by the pineal gland (purple curves) always occurs during nighttime. (Challet 2007)

4. The clock network and its synchronization mechanisms

4.1. Secondary clocks in the central nervous system

Besides the SCN, there have been circadian oscillators shown in a number of structures in the brain (Fig. 11) (Abe et al., 2002). The retina and the olfactory bulb are two neuronal oscillators displaying self-sustained rhythmicity without damping in the absence of the SCN (Vansteensel et al., 2008). Many other regions have been reported to show circadian rhythms in the presence of SCN and disappear after SCN lesion (Tousson and Meissl, 2004).

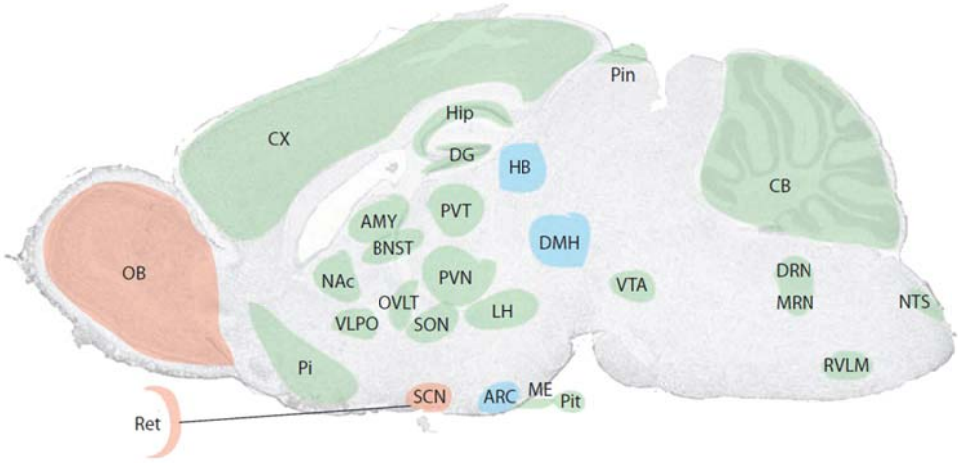
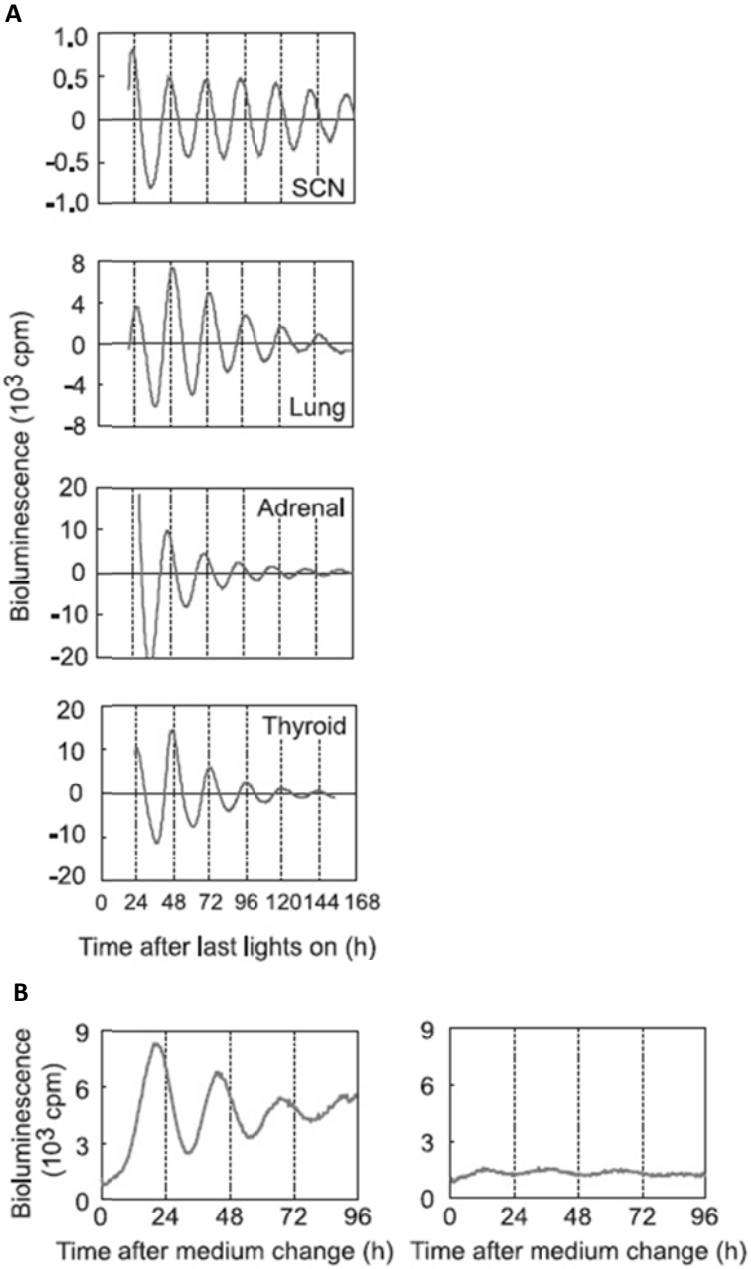


Figure 11: Secondary circadian oscillators in the brain. Besides the SCN, olfactory bulb (OB) and retina (Ret) exhibit self-sustained and long-lasting circadian oscillators (red). Other regions are proposed as semi-autonomous oscillators (blue), including arcuate nucleus (ARC), dorsomedial hypothalamus (DMH) and habenula (HB). Many oscillators in the brain are rhythmic quite depending on the SCN (green), such as amygdala (AMY), bed nucleus of the stria terminalis (BNST), cerebellum (CB), cortex (CX), dentate gyrus (DG), dorsal raphe nucleus (DRN), hippocampus (Hip), lateral hypothalamus (LH), median eminence (ME), median raphe nucleus (MRN), nucleus accumbens (NAc), nucleus of the solitary tract (NTS), vascular organ of the lamina terminalis (OVLT), piriform cortex (Pi), pineal gland (Pin), pituitary gland (Pit), paraventricular nucleus of the hypothalamus (PVN), paraventricular nucleus of the thalamus (PVT), rostral ventrolateral medulla (RVLM), supraoptic nucleus (SON), ventrolateral preoptic area (VLPO) and ventral tegmental area (VTA). (Adapted from Dibner *et al*., 2010)

4.2. Peripheral circadian clocks

With the central clock in the SCN, the circadian timing system is composed of multiple oscillators, in a hierarchical manner. Out of the brain, peripheral oscillators are orchestrated by the SCN to appropriate phases, in order to control the numerous physiological functions, even if they are cell-autonomous and self-sustained. Since the finding that rat-1 immortalized fibroblasts display circadian gene expression after serum shock, many peripheral cells and tissues have been shown to display circadian rhythms in gene expression in mammals (Fig.12) (Balsalobre et al., 1998; Yagita et al., 2001). They possess similar molecular clockwork to that operative in the SCN. However, the clear rhythms quickly fade within a few days in cultures of these peripheral oscillators, contrarily to the SCN and the retina. In addition, single cell recordings of circadian gene expression prove that individual cells are self-sustained oscillators and able to be synchronized (Nagoshi et al., 2004; Welsh et al., 2004). These results indicate that peripheral oscillators harbor cell-autonomous and self-sustained clocks. Furthermore, damped oscillations of cell cultures could be restarted by stimuli like serum shock or medium change (Yamazaki et al., 2000). Given the fact that the peripheral clocks in mice with SCN ablation are no longer well-coordinated and display large phase differences among individuals, fainting of peripheral oscillations is rather due to the loss of synchrony between cell-autonomous clocks in the absence of the SCN (Yoo et al., 2004). This is in the opposition of SCN slices that keep synchrony in cultures. Altogether, this suggests that the SCN-emanating signals are necessary for synchronizing the peripheral clocks, for the phase coherence among cells and tissues.



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4.3. Difference between the SCN and peripheral clocks

Most core components of the molecular clock are expressed in the SCN as well as in peripheral tissues. However, some components manifest tissue-specific expression patterns. For example, *Clock* mRNA cycles in the peripheral tissues, but is expressed

constitutively in the SCN(Lowrey and Takahashi, 2004). The SCN can be reset rapidly by shifts of the light-dark cycle, but resynchronizing peripheral oscillators can take over a week (Yamazaki et al., 2000). Disruption of *Per* and *Cry* genes individually has no effect on the maintenance of circadian rhythmicity, and the observed period of mutant SCN tissue reflects the free-running behavioral period of the corresponding mutant animal (Liu et al., 2007a). By contrast, results obtained with single neurons or isolated or confluent fibroblasts, indicate that not only intracellular molecular mechanisms but also intercellular coupling, make a difference between SCN and peripheral clocks (Fig. 13). It has also been confirmed by the results from *Bmal1* mutant animals (Ko et al., 2010). Weakening coupling between neurons by blocking action potentials or cAMP production increases the range of temperature cycles able to entrain the SCN (Abraham et al., 2010). This cell-to-cell interaction involves both synaptic interactions and humoral mediators, such as nitric oxide (Plano et al., 2010). Coupling of cellular oscillators in the SCN tissue compensates for the dysfunction due to clock gene mutations and desynchronization within and between the regional pacemakers that suppresses the coherent rhythm expression from the SCN. The cell coupling of SCN is advantageous for responding to a wide range of environmental challenges without losing coherent rhythm outputs. A mathematic model supports the idea that the complex and heterogeneous architecture of the SCN decreases the sensitivity of the network to short entrainment perturbations while, at the same time, it improves its adaptation abilities to long term changes (Hafner et al., 2012).

Peripheral oscillators seem not to be coupled through organ-specific or paracrine communications, but require the SCN-derived signals to be phase coherent (Guo et al., 2006). Identifying these signals that might be tissue-specific, and their functions, is an important question in the circadian field. Non-photic cues would be mainly candidates, because those peripheral clocks do not need light to be synchronized, although light pulse could indirectly, for example, induce expression of *Per1* and other immediate early genes in the cortex of the adrenal gland (Ishida et al., 2005).

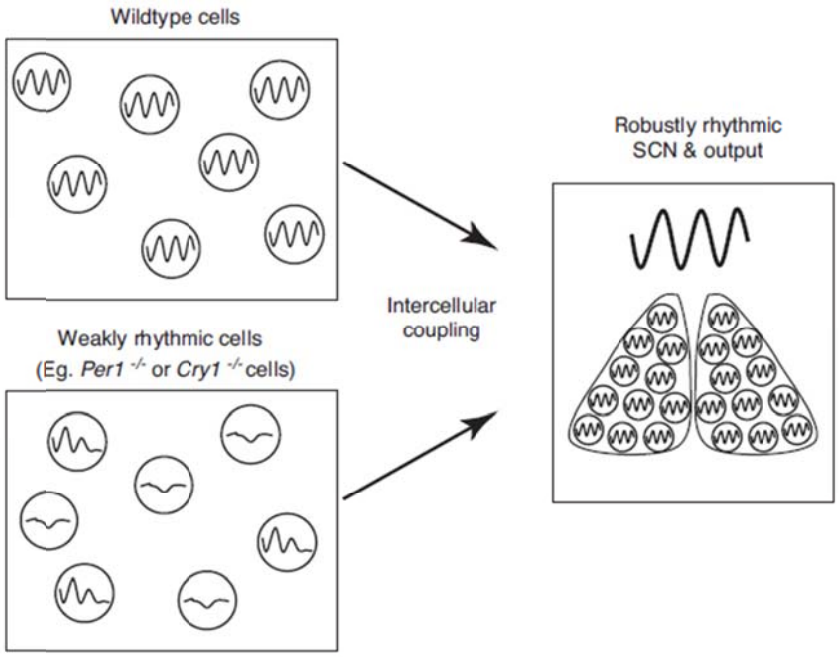


Figure 13: Intercellular coupling in the SCN. The SCN neurons are rhythmic in wild type. Genetically mutant SCN neurons prove weakly rhythmic and display largely variable phases when dispersed in culture. However, in wild type and genetically mutant mice, the SCN explants are rhythmic similar to locomotor activity. Intercellular coupling compensates for the weakly rhythmic cellular oscillations, to preserve the stable rhythmicity in SCN activities. (Adapted from Mohawk and Takahashi 2011)

4.4. Resetting and synchronization: external influence on phase, period and amplitude

Changes of environmental conditions, gradually or rapidly, lead to resetting of clocks to be synchronized to the new timing. Given the multioscillatory nature of tissues, in spite of large differences in coupling strength, the new status could be characterized by different parameters of oscillation. This can be observed in vivo as well as in vitro following treatments, as the phase, period or amplitude of the circadian oscillation will change in comparison with those expected from previous cycles (Fig. 14).

A new phase is usually the most direct result of the resetting or synchronization. In

the actogram of locomotor activity, this parameter is simply shown by the start of the wheel running. In oscillations of cumulative quantities, like firing rate, mRNA level or bioluminescence, intrinsic time of the biological oscillator can be determined by the peak, shifting either advanced or delayed. Since the shift of the peak is due to the majority of the population, oscillators could be classified as simultaneous or not in response to the external cues. For instance, bimodal oscillation in resetting of SCN neurons suggests the existence of a rapid and more efficiently synchronized subpopulation further responsible for shifting the whole pacemaker (Rohling et al., 2011).

Another important parameter is the period of the oscillation, which defines the circadian rhythms. Although it stays close to 24 hours in the presence of external cues in nature, the period of circadian oscillations can significantly differ under constant conditions or other periodical entrainment. The period could be calculated by the distance between two neighboring peaks. Normally, the free-running periods or entrained periods vary in the circadian range, from 20 hours to 28 hours. But it has been reported that some extremely short or long periodic entrainments, like temperature cycles from 10 hours to 30 hours, are capable to synchronize the fibroblasts to oscillate following such environmental periods (Saini et al., 2012).

Besides direct timing parameters such as phase and period, the strength parameter of the amplitude reflects the phenotype of the population, in the precision and integration of the circadian clocks. The amplitude can be determined by the distance between consecutive trough and peak. The bigger the amplitude, the better the oscillators are synchronized to a unique phase. At the same time, the width of the peak could also justify the synchronized level as well as the precision and phase plasticity of intrinsic clocks. In peripheral oscillators, the amplitudes of populations damp rapidly in a few days. Damping rates imply the level of variation in intrinsic periods and the intercellular asynchrony, especially among very weakly-coupled peripheral clocks.

In general, the phase and the period are prone to show the intrinsic properties of clocks, whilst the amplitude rather reflects the synchronization of clocks.

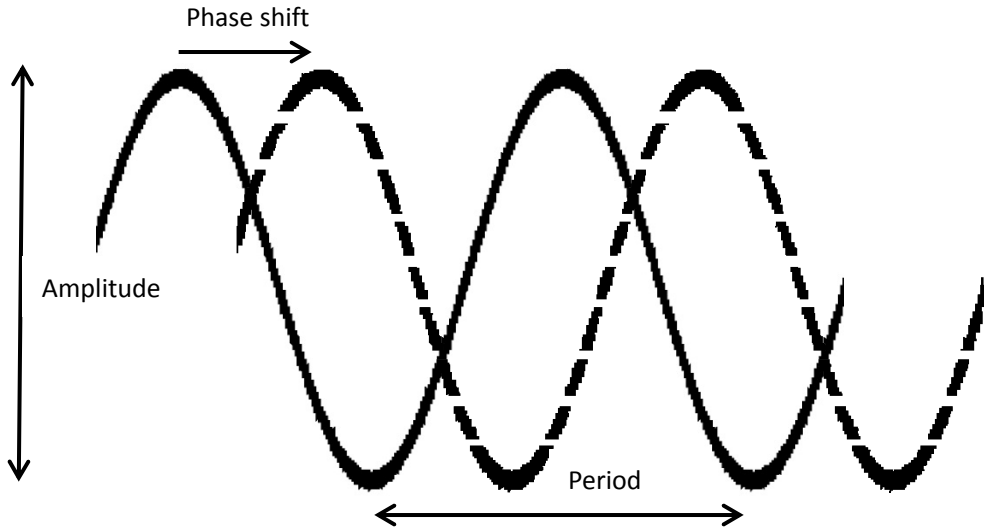


Figure 14: Parameters of circadian oscillations. Circadian rhythms can be reset or synchronized by external cues. In the level of clock gene expressions, parameters such as period, phase and amplitude can reflect the level of resetting or synchronization.

4.5. Potential synchronizers

In the hierarchical architecture of the circadian network, the SCN pacemaker orchestrates the subsidiary clocks in periphery. Given the fact that most circadian transcripts are expressed in a tissue-specific pattern and can accumulate large phase differences within a given tissue, daily resetting cues derived from the SCN are necessary and cooperative for phase coherence of peripheral clocks in the animal. Different tissues might require different combinations of synchronizers. Hence, several candidates have been studied to identify these synchronizers, including hormonal glucocorticoids and melatonin.

Glucocorticoid hormones are strong candidates for blood-borne factors with robust daily oscillations in plasma levels. In the absence of the glucocorticoid hormone or of its receptor, the peripheral clocks get synchronized more rapidly with the altered feeding time (Le Minh et al., 2001). Dexamethasone, a glucocorticoid receptor agonist, acts as a strong synchronizer for rat-1 fibroblasts and peripheral tissues, but not for the SCN (Balsalobre et al., 2000).

Melatonin is another strong output of the SCN pacemaker with robust daily rhythms. Exogenous melatonin can induce behavioral phase-advances when injected at subjective dusk with pharmacological dose (Pitrosky et al., 1999; Slotten et al., 2002). Since high-affinity melatonin receptors MT1 and MT2 are expressed in various peripheral tissues besides the SCN, melatonin is a potential candidate as a synchronizer of peripheral clocks.

Temporal restricted feeding with diurnal meal time in nocturnal mice proves that feeding could be a potent synchronizer of peripheral oscillators, especially in the liver, where even core clock genes such as *Bmal1*, *Per1* and *Per2* are not essential (Damiola et al., 2000; Feillet et al., 2006; Pendergast et al., 2009b).

Another circadian cue regulated by the SCN is the core body temperature with a fluctuation of 1-4 °C (Refinetti and Menaker, 1992). Simulated body temperature fluctuations can entrain the peripheral tissues like lung and cell cultures like rat-1 fibroblasts and NIH3T3 fibroblasts (Brown et al., 2002; Buhr et al., 2010; Saini et al., 2012). And exposure to inverted environmental temperature cycles can reverse circadian gene expression in the liver without affecting the central clock (Brown et al., 2002). This SCN resistance to entrainment by temperature cycles depends on the intercellular coupling of SCN neurons (Abraham et al., 2010; Buhr et al., 2010).

4.6. Circadian network throughout life

4.6.1. Postnatal development

The clock network undergoes remarkable transformations of exogenous and endogenous environments at perinatal periods, in altricial species like humans and rats. Within this course, circadian oscillators may change during development to maintain adaptive phase relationships with important aspects of the drastically changing environments. During the short period of postnatal development, neonates are mostly influenced by the maternal care including feeding, warmth and social contact. After birth,

the SCN clocks are already set to proper phase and develop intensively in the intercellular communication by forming the synapses and cell couplings (Moore and Bernstein, 1989). The amplitudes of the rhythms in clock gene expression in the SCN gradually increase until the adult-like state, P10 in the rats (Sladek et al., 2004; Kovacikova et al., 2006). The circadian activity rhythms of AANAT, the rate-limiting enzyme of melatonin synthesis, in the pineal gland can be observed from P10 accurately reflecting rhythmic output from the developing SCN (Deguchi, 1982; Reppert et al., 1984). Moreover, terminals of the ipRGCs gradually spread throughout the retinal innervation zone of SCN, though both the retina and the SCN are sensitive to the light immediately after birth (Mateju et al., 2009). At the same time, food availability is a key environmental signal from maternal care until weaning. Rodents feed their pups during the day while the mother takes food during the night (Ohta et al., 2003). Restriction of maternal care by depriving the day-time contact could cause reversed phase in the *Per1* and *Per2* expression in the SCN of pups. Many other results also suggest that maternal care entrains the circadian system of pups when it becomes restricted. In this course, nocturnal neonates are fed as the diurnal animals, and appear to be nocturnal with increasing independence regarding maternal lactation during gradual weaning. In the peripheral tissues, molecular clocks seem to mature at different rates and the phase gradually shifts until the adult stage is achieved, while the SCN remains the same phase in this developmental period (Yamazaki et al., 2009). Therefore, it is to be determined with the role of the developing SCN in the synchronization of the peripheral clocks during the early postnatal period, while non-photic maternal cues are important external factors. During this period, low amplitude rhythms may be easier to reset to environmental perturbations.

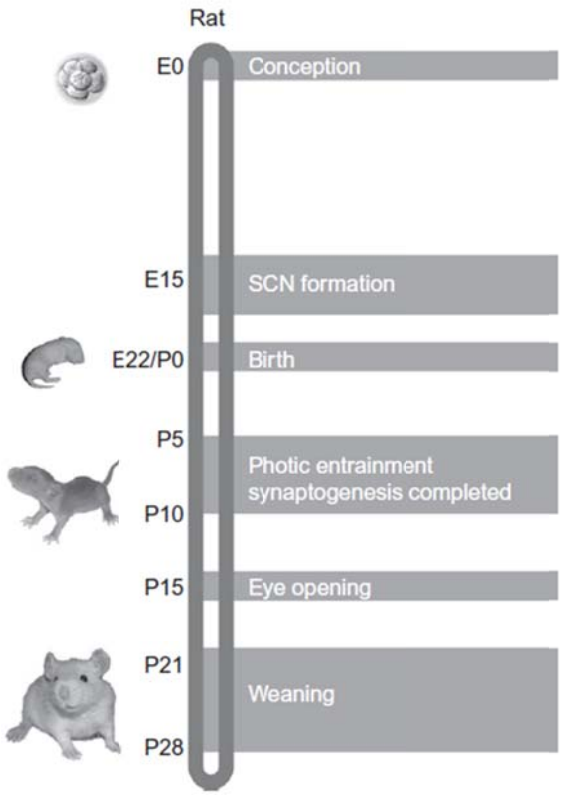


Figure 15: Perinatal development of the rat. Simplified time line represents from embryonic day (E) 0 to postnatal day (P) 28. Clock related factors are developing during this scale, such as SCN, RHT and maternal cues (Sumova *et al*., 2012)

4.6.2. Ageing

In aging animals, it was observed that the pituitary, secondary clock is more dispersed in phase (Yamazaki et al., 2002). Circadian outputs decline at the level of neural activity rhythms in the SCN and dispersed SCN neurons, due to increasing variability (Aujard et al., 2001; Nakamura et al., 2011a). These indicate that synchronization of the peripheral clocks by the SCN is less efficient, probably due to the structural and functional alteration in the clock system. In elderly, reductions in SCN volume and neuron number by the hypertrophy of astrocytes and microglia have been reported (Hofman and Swaab, 2006; Deng et al., 2010). The endogenous period of the SCN is shortened in aged rats, although individual variation seems constant (Yamazaki et al.,

2002). Mouse lemur, a primate, exhibits a shortened free-running period in aged animals compared to adulthood, with altered AVP and VIP rhythms (Aujard et al., 2006). In addition, aged rodents exhibit decline of the SCN presynaptic GABAergic terminals (Palomba et al., 2008). Decreases in neurotransmitter production of the SCN and low amplitude of electrical activity might weaken the ability of the SCN to synchronize peripheral oscillators.

The ageing of the SCN capacity to synchronize the circadian system has been studied first focusing on entrainment alterations. Aged mice are less sensitive to light entrainment than young ones (Wyse and Coogan, 2010). Ageing increases crystalline lens light absorption especially of short wavelengths and decreases pupil area, which results in progressive loss of retinal illumination (Turner and Mainster, 2008). Besides that, the responsiveness of the circadian clock to some neurochemical stimuli like GABA is attenuated(Biello, 2009). In aged hamsters, light-induced *Per1* expression is markedly reduced in the SCN with a significantly longer delay to resynchronization, although in DD *Per1* shows similar rhythms as young rat (Kolker et al., 2003).

Another factor that has been involved in aging is the physiological decline of hormones (Goldman et al., 2011). Aging changes hormone production and corresponding target sensitivity. Melatonin oscillates daily and decreases by aging with advanced phase, which is a putative marker of the decline of the circadian clock (Bondy et al., 2010; Hardeland, 2013). The aged SCN reduces responsiveness to melatonin(von Gall and Weaver, 2008). The adrenal cortex releases less aldosterone and cortisol hormones with aging. Circadian oscillation of cortisol damps with reduced peak levels and increased evening levels in older people (Ferrari et al., 2001b). Interestingly, PER1 increases with morning cortisol in elder women with low cortisol levels rather than young women with higher cortisol levels. This PER1 response to cortisol increase seems to be sensitive at low cortisol levels rather than at higher cortisol levels (Olbrich and Dittmar, 2012). Corticosterone secretion is altered with advancing age due to disruption of the diurnal rhythm of CRH (Cai and Wise, 1996a).

Another phenotype associated with aging is energy metabolism. Reduced appetite and related change of feeding rhythms are known to be important for entraining

peripheral oscillators like liver. Additionally, epigenetic regulations also play a role in ageing of circadian clock system. Different tissues like the stomach and the spleen in older mice exhibit specific alternations of methylation and methylation frequency at the promoters of clock genes (Zhang et al., 2013).

5. Rhythmic properties and clock activity in the skin

5.1. Skin structure and rhythmic functions

As a protective outer covering of vertebrates, skin is an interface tissue between external conditions and the organism. Exposed to daily variations of environmental influences like solar irradiation, humidity and temperature, the skin contributes as a barrier, to the internal homeostasis, which itself results from multiple physiological processes including cyclic ones, such as hormones and nutrition. The skin performs its function at once with continuous cell proliferation and differentiation, forming the corneum and hair, under controlled rate. Many investigations have been directed at the circadian changes of skin functions due to their importance on health care, hair regrowth and drug delivery (Reinberg et al., 1990). In human skin, several physiological functions have been demonstrated to change in a circadian manner, by measuring biophysical parameters of healthy women skin surface (Le Fur et al., 2001). Over 24 h, sebum excretion, skin temperature, pH, capacitance (measurement of stratum corneum hydration) and transepidermal water loss (measurement of stratum corneum barrier function), not only show rhythmic patterns (phase, amplitude), but also differ from one site to another; for instance, skin temperature is found rhythmic on the forearm rather than on cheeks. These regional variations possibly can be explained by differing thickness of stratums, vascularization and distribution of eccrine glands. Forearm skin blood flow and temperature as well as sweat loss play a role in thermoregulation and vary along the day during mild exercise, although the rise of heart rate is not significantly different (Waterhouse et al., 2004). As the largest organ of mammals, skin covers a big area of

surface and consists of two main layers, epidermis and dermis. The outermost epidermis provides toughness and water resistance. It is rich in keratin which originates from differentiated keratinocytes, its major structural cells, whereas melanocytes constitute less than 10% in the human epidermis. The underlying dermis contributes to elasticity and contains numerous appendages of skin like the hair follicles, the sebaceous glands, sweat glands and capillaries, which together comprise many cell types including fibroblasts. All these subunits contribute to the cellular complexity of the skin, and to the complexity of its biological rhythms (Fig. 16).

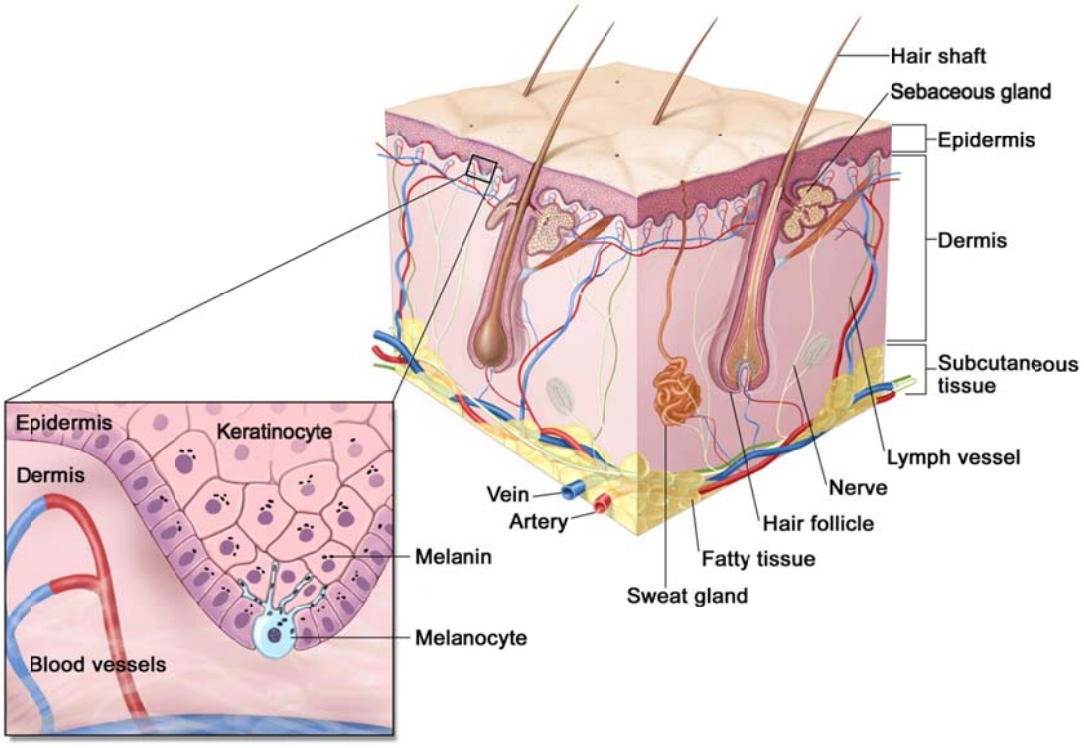


Figure 16: Multiple structures in the skin. Epidermis and dermis are two main layers of skin. The skin comprises many types of cells and appendages together forming a complex organ. (Adapted from https://visualsonline.cancer.gov/details)

5.2. The circadian rhythm of skin cell proliferation

Circadian rhythms in cell proliferation have been noticed since 100 years ago by Droogleever Fortuyn van Leyden (1917). Decades later, Bullough found mitotic activity in mouse epidermal keratinocytes with a 24 hour period. In the 1960s, the

autoradiographic technique made it possible to trace the role of DNA synthesis in cell cycle. Since then, many studies have analyzed circadian rhythms in epidermal cell proliferation in many species regarding S-phase and M-phase (Fig. 17). The rhythms in human epidermis are found in the opposite phase with respect to those of nocturnal rodents like rats and mice (Brown, 1991). In human, most keratinocytes go through the S-phase with a peak in the late afternoon, compared to rodents where it is at night (Brown, 1991; Geyfman et al., 2012). In mouse skin, 5% of the transcriptome is rhythmic in the telogen phase (rest state) of the hair follicle compared to 2% in the anagen phase (growth state). Most circadian outputs related to cell division dominate in the "quiescent" telogen skin suggesting the interfollicular epidermis may have higher amplitude in its circadian rhythm of cell proliferation, than highly proliferative follicles. But in rat-1 immortalized fibroblasts, cell mitosis appears independent of the circadian cycle and similar results have been found in cancer cells (Chen et al., 2005; Winter et al., 2007; Yeom et al., 2010). This indicates that the circadian rhythm/proliferation link is one character of normal cells irrelevant to those abnormal under uncontrolled proliferative rate.

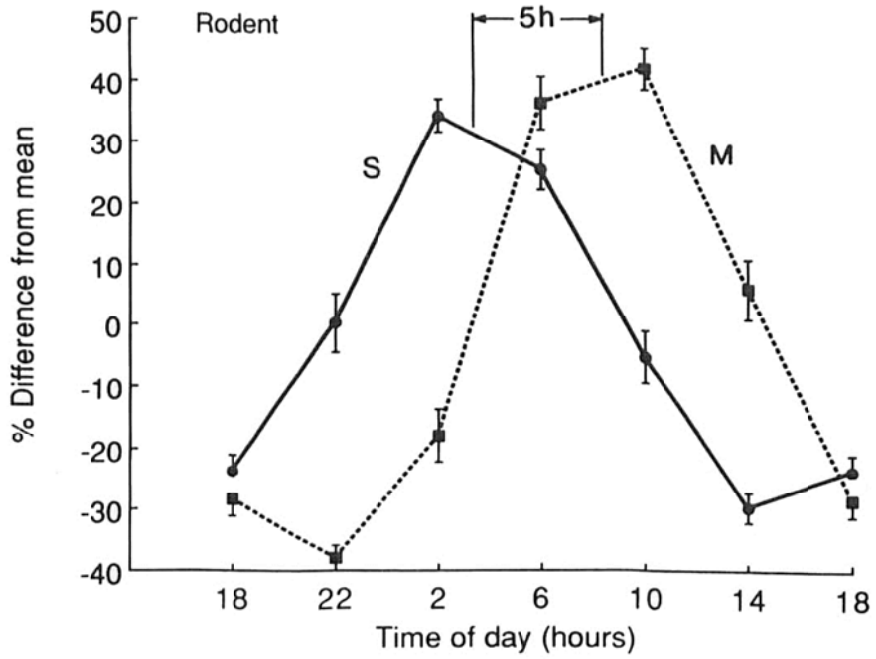
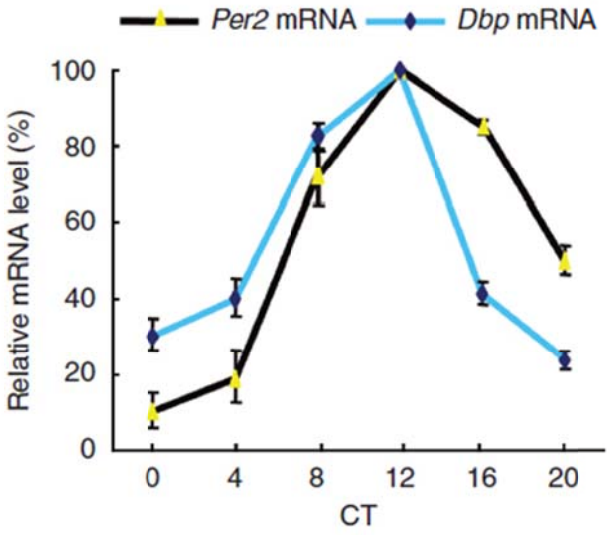


Figure 17: Circadian rhythms of rodent epidermal S-phase and M-phase in proliferation. Cell mitosis (M) peaks in the daytime, which is the resting time of the rodents (rats and mice). S-phase rhythm shows that DNA synthesis is rhythmic for preparing for daily cell growth in the epidermis. (Brown 1991)

5.3. Clock gene activity in the skin

Circadian variations of skin functions and cell proliferation have not been correlated with clock gene activity, until daily oscillations in clock gene expression in skin were first demonstrated both in humans and mice (Bjarnason and Jordan, 2002; Oishi et al., 2002). In the oral mucosa, clock gene expression associates with the circadian rhythms in S-phase activity (Bjarnason et al., 2001). Tanioka clarified that expression of clock and clock-controlled genes sustain circadian rhythmicity in mouse skin under constant dark condition (Fig. 18) (Tanioka et al., 2009). According to mRNA (obtained from distinct animals) analysis, *Cry1/2* knock-out mice completely lose their circadian oscillation in *Per2* and *Dbp*. This is similar to the fact that ablation of the central clock in the SCN leads to arrhythmic expression of *Per1/2*, *Bmal1* and of the clock controlled gene *Dbp*, which is not recovered in mice housed in normal light-dark condition. However, Yoo *et al.* reported that lesion of the SCN does not destroy circadian rhythms in bioluminescence in peripheral tissues ex vivo including cornea, liver, pituitary, kidney and lung from animals showing completely arrhythmic locomotor activity, but instead causes phase asynchrony among the tissues of individual animals and from animal to animal (Yoo et al., 2004). These results suggest indeed that removal of the SCN disrupts synchrony among tissues and individuals, which can lead to obvious arrhythmicity in peripheral clocks, but the behavior of the skin clock in vivo in the SCN-lesion condition remains to be further elucidated.

In the skin, previous studies reported the presence of *Clock* and *Per1* mRNA and protein in human keratinocytes, melanocytes and dermal fibroblasts (Zanello et al., 2000). These multiple particularities of clock or clock-controlled gene expression in the skin suggest the complexity of the skin clock and of the rhythmic processes it controls.



5.4. Rhythms of clock gene expression in skin cells including fibroblasts

Within the circadian clock network, skin, as a peripheral tissue, contains many cell types, representing multiple functional autonomous oscillators (Fig. 19A). Sandu *et al.* elucidated the expression pattern of clock genes in primary keratinocytes, melanocytes and dermal fibroblasts, derived from a human skin biopsy (Fig. 19B) (Sandu et al., 2012). Distinct circadian clock gene activities in each cell type confirm that the skin is itself a multi-oscillatory system. These autonomous oscillators could interact with signals from the SCN to drive together the skin circadian functions. Different periods, amplitudes and phase relationships between clock genes according to cell types indicate the complexity of the skin circadian clockwork and of the regulatory mechanisms occurring within the skin.

In mice, periods measured by bioluminescence in fibroblasts from wild-type inbred strains correlate with, though not perfectly, the period of wheel running behavior and SCN clock gene activity. In human, likely reflecting genetic heterogeneity, periods measured in primary skin fibroblasts were shown to vary widely among 19 individuals but were proportional to the physiological period length determined by salivary melatonin during three consecutive cycles (Brown et al., 2005b). Interestingly, blind individuals present on average the same period length in fibroblasts as sighted ones, although their

physiological periods are significantly longer (Pagani et al., 2010). This could suggest that the differences in circadian properties between sighted and blind people are due to physiological reasons like the synchronization rather than reflecting genetic ones. Therefore period of cultured fibroblasts, rather than period of human physiological functions, could keep independent from prior light history and retinal function and truly reflect genetic particularities.

A

Publication date/ Reference	Sample	Results
2000.07. Zanello et al. (2000)	Keratinocyte- (HaCaT), melanocyte-, fibroblast-, melanoma A375 cell lines	Evidence for expression of *clock* and *per1* mRNAs and proteins in various skin cell types.
2001.02. Bjarnason et al. (2001)	Human skin biopsies	Daily rhythm in the expression of *clock, tim, cry1, bmal1, per1* in vivo.
2002.08. Kawara et al. (2002)	Human keratinocyte cell lines	Low-dose UVB irradiation induces altered expression of *clock, per1, bmal1*.
2008.11. Tanioka et al. (2009)	C57BL6 wild type and $Cry1^{-/-}Cry2^{-/-}$ mice	Oscillation of skin clocks abolished after SCN ablation.
2009.07. Lin et al. (2009)	C57BL6 wild type, $Clock^{-/-}$, and $Bmal1^{-/-}$ mice	CLOCK–regulated genes are modulated in phase with the hair growth cycle. Delay in anagen phase is observed in case of mutant mice. Upregulation of p21 in $Bmal1^{-/-}$ mice.
2010.09. Spörl et al. (2011)	HaCaT keratinocytes	In vitro rhythmic expressions of circadian clock genes and clock controlled genes, involved in epidermal physiology (*Insig2a, c-myc, Ldl receptor, Hmgcr*).
2011.11. Gaddameedhia et al. (2011)	Male outbred SKH-1 mice	The xeroderma pigmentosum group A (XPA) protein expression and the excision repair rate show daily rhythmicity in the skin. Exposure to UV radiation at 4:00 AM caused cca. five fold higher multiplicity of skin cancer (invasive squamous cell carcinoma) than mice exposed at 4:00 PM.
2011.11. Janich et al. (2011)	$Bmal1^{-/-}$/K5-SOS mice	Fewer neoplastic lesions on the skin of $Bmal1^{-/-}$/K5-SOS mice in all stages of cancer development if compared to K5-SOS.
2012.05. Sandu et al. (2012)	Human melanocyte, keratinocyte and fibroblast cultures	In vitro rhythmic expression of the clock genes with different amplitudes of oscillation in each cell types.
2012.05. Spörl et al. (2012)	Human epidermal biopsies from healthy volunteers	Hundreds of genes show daily fluctuations in their mRNA levels in the human epidermis in vivo (microarray results).
2012.12. Lengyel et al. (2012)	Human skin biopsies from melanoma patients (tumor vs. adjacent skin) and healthy volunteers (nonmalignant naevus vs. adjacent skin)	Decreased expression of *per1, per2, clock* in cancerous tissue biopsies vs non-cancerous biopsies of melanoma patients. Elevated expression of *clock* mRNAs and CLOCK proteins in the epidermis of melanoma patients compared to healthy volunteers.

B

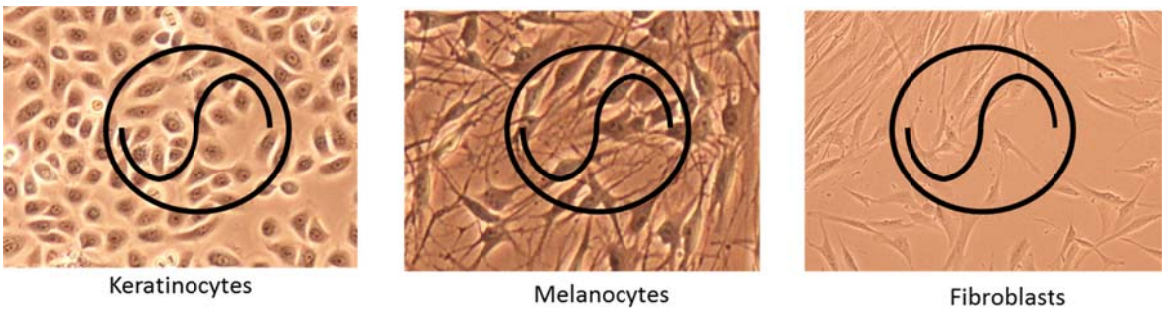


Figure 19: Expression of circadian genes in skin tissue and derived cells in human and rodents.
A) Expression of circadian clock genes in the skin. (Adapted from Lengyel *et al.*, 2013)
B) Circadian gene expression shows rhythmic pattern in these cells in culture, after dexamethasone treatment. (pictures of human primary cell cultures, C. Sandu) Sandu *et al.*, 2012

As independent oscillators, primary skin cells sustain persistent but damping oscillations of clock gene expression measured by bioluminescence for several days in vitro (Brown et al., 2008; Johansson et al., 2011). It is reported that peripheral tissues could oscillate in a self-sustained manner for about 7 cycles, and even over 20 cycles in isolation without media changes (Yamazaki et al., 2000; Yoo et al., 2004). Peripheral tissues and primary cells usually present limited cycles of oscillation when compared to the SCN or retina, which consist of neurons mostly, and it raises the question whether the difference is due to loss of tissue connection in primary cultures or if these cells are just desynchronized. There is no evidence of coupling of cell-autonomous circadian oscillators in fibroblasts but, interestingly, maturing fibroblasts cultured at high density and with decreasing proliferative rates tend to display oscillations with higher amplitude (O'Neill and Hastings, 2008).

5.5. Synchronizing signals to fibroblasts

Leise *et al.* managed to image *Per2* bioluminescence for six weeks on primary fibroblasts dissociated from mice tails and selected for 6 months in vitro (Leise et al., 2012). These fibroblasts sustain cell-autonomous circadian oscillations in a constant environment, at a 100 cells/mm^2 density. Additionally, Noguchi *et al.* demonstrated that tail fibroblasts lose clear *Per2* circadian rhythmicity in low cell density (till 27 cells/mm^2), which could be rescued by increasing the density with cells that were either rhythmic (wild type) or not, or even by adding conditioned medium from high-density fibroblast cultures (Noguchi et al., 2013). This suggests that intercellular factors help to generate robust circadian rhythms and that they are diffusible paracrine signals rather than direct contact with neighboring cells. Similar effects were shown with immortalized SCN2.2 cells able to confer molecular oscillations to cocultured NIH/3T3 fibroblasts via the secretion of unknown diffusible signals (Allen et al., 2001).

Some factors in the culture medium have been shown to affect the expression of clock genes. Glucose down-regulates *Per1/2* mRNA levels in rat-1 fibroblasts (Hirota et

al., 2002). Low extracellular pH (6.7) shortens periods of human primary fibroblasts from 24 h to 22.6 h, increases the amplitude and decreases the damping rate without affecting cell viability (Lee et al., 2011b). Pharmacologically, inhibition of JNK protein kinase (SP600125) increases the period of rat1 fibroblast rhythms, as well as of SCN, pineal gland and lung explants from Per1- and Per2-luciferase mice (Chansard et al., 2007). As predicted, valproic acid, enhancing JNK phosphorylation, shortens the *Per1* period in Rat1 fibroblasts. In contrary, it is reported that valproic acid has no effect on the period of *Per2* expression in primary skin fibroblasts derived from the base of the ear, while showing significant time-dependent phase-shifting effects on the SCN and primary fibroblasts (Johansson et al., 2011). These different effects indicate the complicated clock machinery in cells from distinct origins. For instance, the majority of rhythmic genes in rat-1, NIH3T3 and Rat 3Y1 fibroblasts are different, as well as those found in SCN 2.2 cells and rat SCN (Menger et al., 2007).

As one non-neuronal cell type displaying clock gene expression, immortalized rat-1 fibroblasts is a non-tumorigenic fibroblast cell line. It is derived from embryos of rats and used frequently on studies of synchronizing signals. Isolated fibroblasts, rat-1 and NIH3T3, exhibit cell-autonomous circadian rhythms just as SCN neurons do (Nagoshi et al., 2004; Welsh et al., 2004). A wide variety of signals, including serum shock, forskolin, glucocorticoids, co-cultured adrenal glands and temperature changes, can synchronize rat-1 fibroblasts (Balsalobre et al., 1998; Balsalobre et al., 2000; Yagita and Okamura, 2000; Izumo et al., 2003; Buhr et al., 2010; Noguchi et al., 2012). However, in contrast to SCN neurons, cultures of fibroblasts damp rapidly (Nagoshi et al., 2004). It is explained by loss of synchrony among cells with diverse periods lacking highly efficient communication, unlike SCN neurons (Fig. 20) (Nagoshi et al., 2004; Welsh et al., 2004). In spite of these results, synchronization by the SCN of peripheral clocks located within the skin remains unclear. No evidence has been found for direct inter-peripheral coordination of circadian clock. Thus, how skin gets synchronized remains to be studied.

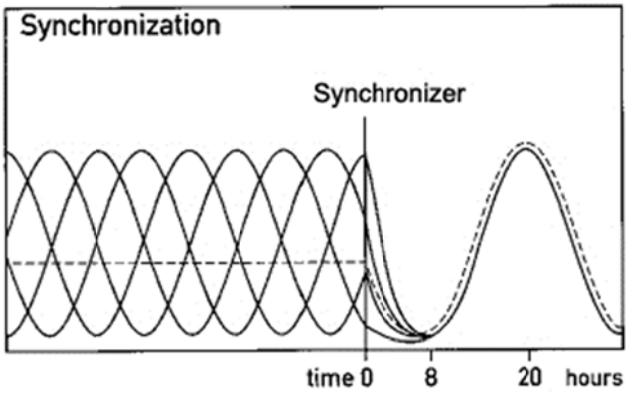


Figure 20: Synchronization model in cell cultures. With variable phases, populations of cellular oscillators show apparent arrhythmicity, even when rhythmic at the individual level. After synchronization, coordinated phases lead to a cumulative curve via the synchronizing stimuli. (Adapted from Balsalobre *et al*., 1998)

5.6. Skin and melatonin

Melatonin, one pleiotropic hormone, was first identified as a chief secretory product of the pineal gland. In mammals, it is secreted into the blood circulation with a significant circadian rhythm to exert as a chemical messenger of light and darkness besides a wide range of bioactivities (Pevet and Challet, 2011). Because of its small size and amphiphilic property, melatonin is easily distributed throughout all the organism and cellular compartments. Besides pineal gland, melatonin synthesis has been found in many central and peripheral tissues including the gastrointestinal tract and the skin (Bubenik, 2002). Physiological levels of melatonin within tissues are quite specific and 10 to 1000-fold higher than plasma melatonin concentration (Tan et al., 1999; Bubenik, 2002; Kobayashi et al., 2005; Fischer et al., 2006b). Of extrapineal sites, only the retina is found to produce melatonin with a circadian rhythm (Hamm and Menaker, 1980; Tosini and Menaker, 1996). Regarding skin, melatonin is known to lighten skin pigmentation in frogs, the way this molecule was discovered (Lerner and Lerner, 1958). Compared to the corresponding

level in the plasma, melatonin exhibits 10- to 100-fold higher concentration in murine skin organ culture, murine vibrissae follicles, and human hair follicles (Kobayashi et al., 2005). As revealed by a series of studies, melatonin production and metabolism involve not only several mammalian skin cells, but also the whole rodent and human skin as a fully functional melatoninergic system (Slominski et al., 2008). Mammalian (human and rodent) skin can also produce serotonin and transform it into melatonin, while receptors for serotonin and melatonin are expressed in keratinocytes, melanocytes and fibroblasts (Slominski et al., 2005b). Melatonin effects to skin function involve either direct actions or are mediated by membrane and cytosolic receptors expressed in the skin cells (Slominski et al., 2008). It has been demonstrated that human and rodent skin cells and tissues express specific high-affinity membrane receptors MT1 and MT2 expressed with variations according to species (Table 1) (Slominski et al., 2005b; Fischer et al., 2006a). Additional low-affinity nuclear melatonin receptor RORα has been identified in human skin cells and melanoma cell lines as well as rodent epidermis and hair follicles but the capacity of RORα to act as a melatonin receptor has not been confirmed lately (Slominski et al., 2005b). Thus, given its numerous biological effects, the locally produced melatonin may serve to protect and modulate the function of skin, whereas its short lived plasmatic version could be useful as a general time-giver for the skin within the circadian clock network (Slominski et al., 2005a).

	MT1	MT2
Epidermis	++ (Stratum Granulosum) + (Stratum Spinosum)	–
Hair follicle	+ (Upper outer root sheath) + (Inner root sheath)	+ (Inner root sheath)
Eccrine glands	+++	+++
Blood vessels	+++ (Endothelium)	++ (Endothelium)

(–) = negative; (+) = weak; (++) = moderate; (+++) = strong.

Table 1: Localization of MT1 and MT2 immunoreactivities in human skin. (Slominski *et al.*, 2005)

Expression of melatonin receptors in skin cells have been found to be modified by environmental factors, especially UVB. Effect of UVB to induce or up/down regulate expression of MT1 and MT2 is defined by genetic background, varying between normal and immortalized cells (Slominski et al., 2005b).

The major layers of the skin, epidermis, dermis and appendages are all targets for melatonin regulation, including numerous effects observed in skin biology and clinical dermatology, from pigmentation to hair regrowth (Fischer et al., 2008). Besides many protective functions as antioxidant, damage repair, and suppressor of pathological changes, melatonin displays some chronobiological impacts on skin, given its photoperiod-associated changes in mammals (Fischer et al., 2006a). One of above evidences exists in the field of hair growth. In wool industry, several kinds of goats fed with melatonin-supplemented diet are found to increase the rate of hair growth in the spring, the period of fur change from the winter to summer (Nixon et al., 1993; Ibraheem et al., 1994). Mink treated with melatonin molts the summer pelage and grows the winter pelage earlier (Rose et al., 1984). In human hair regrowth, topical melatonin application has shown positive effect on the rate of hair found in the anagen (follicle growth) phase in a pilot-randomized clinical study in women suffering from androgenic or diffuse alopecia (Fischer et al., 2004). Another evidence exists in rats after pinealectomy, in which reduction of collagen deposition induced atrophy of epidermis, dermis and hair follicle, similar to the aging phenotype in skin thickness (Drobnik and Dabrowski, 1996). Melatonin administration could reverse collagen fiber damage and mitochondrial swelling in these animals.

In clinics, several skin diseases show seasonal variation in severity (Weiss et al., 2008). Some of these diseases have been reported to exhibit abnormal plasma melatonin level in patients. For example, plasma melatonin levels lose their circadian rhythms in psoriasis, or display greatly reduced rhythmicity in atopic eczema (Mozzanica et al., 1988; Schwarz et al., 1988; Rupprecht et al., 1995). Lower melatonin concentration is found in melanoma patients compared with controls, while there exists a higher prevalence of melanomas in pilots and aircrews with increased jetlag risks as well as office workers exposed to fluorescent lighting (Kvaskoff and Weinstein, 2010). To many specific skin

diseases, skin-related pathological conditions and syndromes, melatonin shows potential application as a skin protectant by topical or oral administration (Fischer et al., 2008).

5.7. Skin and glucocorticoids

Glucocorticoid receptors are widely expressed in skin compartments and cell types, including keratinocytes, melanocytes and fibroblasts (Serres et al., 1996; Karstila et al., 1994). In addition to the strong synchronizing effect of glucocorticoids, these all indicate that glucocorticoids play an important role in synchronization of peripheral clocks in the skin.

Interestingly, it has been further shown that the skin has the intrinsic capacity to produce elements of HPA axis as well as corresponding receptors. CRH is mainly produced in the PVN of the hypothalamus, although CRH gene is widely expressed in the extracranial tissues including skin but at level much lower than the hypothalamus (Slominski et al., 1995). However, CRH gene is only detected in human skin cells but not rodent skin (Ermak and Slominski, 1997; Slominski et al., 1998). Hence it is proposed that CRH is imported into skin via afferent nerves endings which targets local CRH-dependent POMC production compartments. In the skin, POMC synthesis including a-MSH and ACTH is regulated by multiple factors, which is, for example, stimulated by UV and inhibited by glucocorticoids (Slominski et al., 2000). And the cortisol synthesis has been found in human skin hair follicle, keratinocytes, melanocytes and fibroblasts (Ito et al., 2005; Slominski et al., 2005c; Slominski et al., 2006). These findings provide the potential of HPA axis homologue in the skin with respects to the systemic rhythmic glucocorticoids in the synchronization of peripheral clocks, although further investigations are needed.

5.8. Importance of clock genes for normal functions of the skin

Although the synchronization mechanisms of the organized skin oscillators remain unclear, the integrity of the molecular clock in the skin has proven crucial regarding normal skin functions and response to stressors. *Bmal1* deficiency results in premature aging phenotype in mice skin. And *Clock* deficiency increases the age-related dermatitis in mice (Dubrovsky et al., 2010). *Bmal1* has been proven to control the circadian variation in the S-phase of cell cycle in keratinocyte proliferation, because keratinocyte-specific deletion of *Bmal1* demolishes the time-of-day dependent epidermal cell division. Consequently, since cells in S-phase are sensitive to DNA damage, the corresponding time-of-day dependent variation in epidermis susceptibility to DNA damage induced by UVB has also been proven *Bmal1*-dependent in the back skin of shaved mice (Geyfman et al., 2012). This indicates that maximal susceptibility of skin to UVB takes place in the subjective night of nocturnal rodents, whereas it might be during the subjective day in human skin when S-phase peaks and UV intensity is highest. Consistently, Gaddameedhi *et al.* reported daily rhythmicity in mouse skin expression of a rate-limiting subunit of the nucleotide excision repair system, the XPA protein, and associated rhythmicity in the excision repair rate in the mouse skin following UV-induced DNA damage (Gaddameedhi et al., 2011): this repair rate shows a minimum in the subjective night and a maximum in the subjective day, and accordingly, mice exposed to UV in the night display 5 fold increase in occurrence of invasive skin cancers. They have also shown that circadian S-phase is obliterated in *Cry1/2* knockout mice (Gaddameedhi et al., 2011).

Meanwhile it was shown that *Bmal1*-controlled ROS levels correlate to the expression of oxidative phosphorylation genes in the skin and fluctuate in antiphase to the cell cycle S phase (Geyfman et al., 2012). It suggests the molecular clock system in the skin contributes to efficient time dependent protection directed towards stressors of intrinsic or environmental origin and which both display rhythmic levels. Nakamura *et al.* have reported that the *Per2* gene regulates a time-of-day-dependent variation in the cutaneous anaphylactic reaction (Nakamura et al., 2011b). This IgE-mediated

immediate-type skin reaction triggered by the exposure of mast cells to specific environmental allergens shows a diurnal rhythm. Mice with *Per2* mutation lose the circadian variation in a passive cutaneous anaphylactic reaction observed in wild type mice, similar to those seen under adrenalectomy and in aged mice associated with loss of rhythmic secretion of corticosterone. In addition, *Per2* mutant mice exhibit decreased sensitivity to the inhibitory effects of dexamethasone on the passive cutaneous anaphylactic reaction and IgE-mediated mast cell degranulation (Nakamura et al., 2011b). In delayed-type skin allergic reactions, such as contact hypersensitivity (CHS), circadian rhythmicity might be an important regulatory factor via corticosterone cycling level. Indeed, it was shown that *Clock* mutation exacerbates the immune response towards exogenous haptens in mice, similar to the effect of adrenalectomy, likely because corticosterone has a protective effect on CHS (Takita et al., 2013). And it was reported that a number of antigen-presenting cells lose their circadian rhythmicity in antigen-specific delayed-type hypersensitivity and skin inflammatory responses in arrhythmic hamsters (Prendergast et al., 2013). These results strongly suggest that the circadian clock drives and regulates the skin immune system and inflammatory response under both systemic and local control.

Another intensive field of research regarding skin clocks is about hair regrowth. Regenerative cycling of hair follicles not only has big economical perspectives in the pharmaceutical industry, but also offers a great opportunity to explore the role of the circadian clock in physiological tissue repair and renewal. First, Lin *et al.* elucidated that the circadian clock contributes to the regulation of the non-circadian hair growth cycle by controlling the progression of the anagen (proliferatory) phase, which may have evolved to allow seasonal regulation of hair growth (Lin et al., 2009). It was then understood that the clock regulates mitosis in the amplifying cells of the follicle in anagen. In the *Per2*-luciferase mouse, telogen/anagen skin and vibrissae hair follicles show circadian rhythms (Plikus et al., 2013). Some type of transient amplifying cells in anagen hair follicles display prominent daily mitotic rhythms, and as consequence hairs grow differently according to mitotic peak and depression. Subsequently, hair loss induced by radiation is found more dramatic when administrated during mitotic peak. This circadian

radioprotective effect gets lost in *Cry1/2* mutant mice, consistent with asynchronous mitoses in their hair follicle.

Besides the importance of proliferative cells in hair growth, the hair follicle is also quite important because it harbors epidermal stem cells. Impacts of the clock on hair regrowth raised the question of its putative role in the crosstalk between circadian rhythms and differentiation. Janich *et al.* identified two subpopulations of dormant hair-follicle stem cells coexisting in mouse epidermis in opposite clock phases (Janich et al., 2013). *Bmal1* modulates in an oscillatory manner the expression of stem cell regulatory genes such as those linked to Wnt and TGFβ signaling, to drive cell populations into alternate cycles of dormancy and differentiation, responding to repressive or activating stimuli. Disruption of the clock by deleting *Bmal1* results in a higher number of dormant stem cells and fewer proliferative cells in the hair bulge, while *Per1/2* deletion does the opposite. This looks consistent with the molecular clock mechanisms and strengthens the key role of the circadian clock system on regulation of stem cells differentiation. In addition, lacking *Bmal1* leads to epidermis ageing and reduction of skin tumorigenesis. This meaningful study thus demonstrates that the circadian clock machinery controls the fate of epidermal stem cells in hair follicles, which again links the (likely local) circadian clock to skin renewal. Recent study shows that human keratinocyte differentiation induced by calcium shows time-dependent predisposition (Janich et al., 2013). This predisposition time window coincides with the transition from late-night to morning, which protects cells from radiation-induced DNA damage. In addition, circadian arrhythmia established by overexpressing *Per1/2* profoundly affects epidermal stem cell function in culture and in vivo. This might be linked to the fact that cyclic environment-induced perturbations can contribute to ageing and carcinogenesis in the skin (Janich et al., 2013). Accordingly, *Bmal1* mutant mice show a range of premature skin ageing phenotypes including hair regrowth defect (Kondratov et al., 2006).

To understand the human circadian rhythms and clock gene expression, a new approach presents a convenient, reliable and less invasive way to take use of biopsy samples of hair follicle cells from the head or chin (Akashi et al., 2010). This technique

reflects accurately individual clock gene expression and behavioral rhythms, confirming further applications to evaluate time lag for shift workers and so on.

Finally, the skin, and especially the hair follicle, could be an ideal model to study the complexity of circadian clocks in periphery.

5.9. Regulation of the skin temperature

Submitted to a changing temperature every day, thermoregulation requires the coordination of a complex network linked to circadian rhythms in locomotor activity and body temperature. Food intake as an energy supply is also strongly related to the entrainment of the central clock. Heat production and heat loss exhibit a circadian balance. They are modulated by blood circulation and numerous hormones. Sleep normally starts at the circadian phase corresponding to decreasing heat production and increasing heat loss. This occurs by the decrease of the core body temperature. At the same time, the heat loss coincides with the vasodilation in the skin (Tikuisis and Ducharme, 1996). This phasic vasodilation could result from melatonin released from the pineal gland at the same circadian phase, which selectively promotes skin, but not cerebral, vasodilation and blood flow, and subsequently influences skin temperature rhythm (Tong et al., 2000; van der Helm-van Mil et al., 2003). The elevated skin blood flow results in increasing skin temperature further favoring heat loss. And covering with bedding in human or cuddling in animals with fur creates a warm microclimate which helps limit heat loss (Okamoto et al., 1997; Goldman et al., 2011). Interestingly, this temperature range of 34-36 °C for human, is crucial for maintaining a high level of skin blood flow, which decreases steeply towards 33°C (Fagrell and Intaglietta, 1977). Recordings of human skin temperature show different circadian patterns in proximal and distal skin under normal circumstances, with amplitude of the latter being significantly higher than that of the former (Fig. 21) (Van Someren, 2006). Distal skin temperature is high during sleep and low during wakefulness, which is clearly lower and antiphasic to the core temperature fluctuation. And proximal skin temperature stays in between, depending on the experimental

conditions (Van Someren, 2006). This suggests a strong correlation between skin temperature rhythms and the sleep-wake cycle. At the elder age, lower skin temperature at the extremities could be a reason to the decreased stability of sleep (Rasmussen et al., 2001). A series of studies in the group of van Someren show that mild cutaneous warming enhances sleep depth, especially in elderly (Raymann et al., 2005, 2008). Thus, the skin temperature shows the potential to provide the center with information on sleep-permissive and wake-promoting conditions while it changes with environmental heat and cold, as well as posture, environmental light, danger, nutritional status, pain and stress (Romeijn et al., 2012). This effect on the center may thus moderate the clock and homeostasis, which in turn influence the skin clock itself.

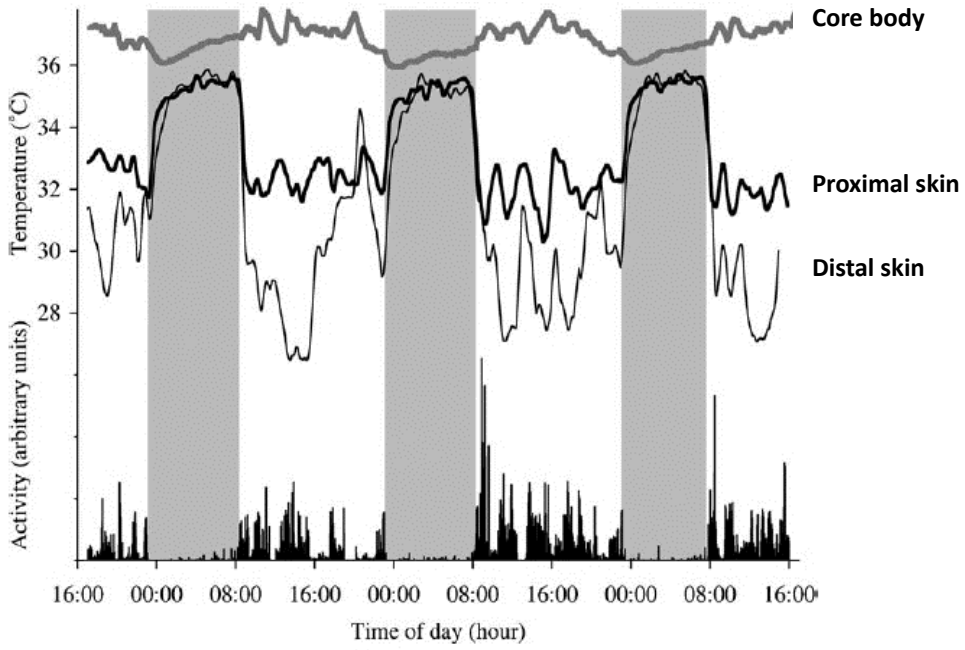


Figure 21: Daily rhythms of core body temperature and skin temperature in human. This profile is measured in the habitual conditions with sleeping microclimate of 34-36 °C. Distal and proximal skins have minimal difference in temperature during sleep. The skin temperature does not exceed 33 °C during the daytime. (Adapted from Van Someren 2006)

On the other hand, simulated body temperature rhythms could reset the circadian rhythms in NIH3T3 fibroblasts over a range of temperature amplitudes and periods (Tsuchiya et al., 2003; Saini et al., 2012). Recently, concerning temperature effects on

peripheral clocks, heat shock response pathways have been shown to have both phase and period influence on the circadian clock, while heat shock factors are also intimately related to the molecular circadian clock (Buhr and Takahashi, 2013). Within the physiological range of temperature, rat-1 and NIH3T3 fibroblasts display the property of temperature compensation (Izumo et al., 2003; Tsuchiya et al., 2003). This characteristic of persistence with a period ≈ 24 h, supports the idea that these oscillators are controlled by a circadian clock (Hastings, 2001). Further studies need to confirm this property in the skin clock *per se*. As one tissue facing dramatic changing temperature in the environment, the skin can keep its stable circadian clock network in balance to hold the scheduled homeostasis, with the advantage of temperature compensation.

Aim of the thesis and Methodology

1. Aim of the thesis

The circadian network is a complex system that involves multiple clocks located throughout the organisms. These self-sustained clocks are at the same time driven by the central circadian pacemaker contained within the SCN. Different cues emanating from the SCN are essential to coordinate the peripheral clocks, while the SCN themselves get entrained predominately by the light/dark cycle. Within each tissue, different mechanisms deliver synchronizing signals to downstream physiological processes. To adapt to the environment, oscillators are meant to interrelate within the circadian system. They are independent as functional units but depend on the synchronizing messages transmitted towards them. As discussed in the introduction, circadian clocks display the following features: to be self-sustained, able to be entrained and temperature compensated. To study the properties of a circadian clock in periphery and to better understand its role in the circadian network requires characterizing the above-mentioned features and the neural or hormonal pathway through which it gets synchronized. To get insight into these aspects we chose as a model of peripheral oscillator the skin which characterization was the subject of the PhD project.

The expression of circadian clock genes in the skin has been reported in mouse and human (Tanioka et al., 2009; Sandu et al., 2012). The major skin cell types were shown to oscillate in a circadian manner, in line with the diverse rhythmic functions they display. Their molecular clockwork indicates that they are cell-autonomous and part of a multioscillatory local network where synchronizing cues are unknown. In order to gain insight into potential signals that might entrain skin clocks, we undertook a study of the skin clock maturation during post-natal life. We extended this study to the effects of aging, which affects the amplitude of the central clock (Nakamura et al., 2011a) and might also affect the clock within the skin. Indeed, *Bmal1* knock out results in a premature aging phenotype in mice skin and *Clock* deficiency promotes age-related dermatitis in mice (Dubrovsky et al., 2010). As an interface tissue the skin is exposed to varying environmental temperature: thus we also analyzed its temperature compensation

properties as part of its global characterization.

We also investigated potential synchronizing signals by studying candidate molecules. A wide variety of signals, including serum shock, forskolin, glucocorticoids and temperature changes, can synchronize Rat-1 fibroblasts, a cellular model which is not derived from the skin (Balsalobre et al., 1998; Balsalobre et al., 2000; Yagita and Okamura, 2000; Izumo et al., 2003; Buhr et al., 2010). Another potential synchronizer is melatonin. Melatonin is a strong output from the SCN pacemaker with robust daily rhythms. Exogenous melatonin has been proven to affect central clocks, due to the fact that melatonin receptors are widely expressed in the organisms including the SCN (Pitrosky et al., 1999; Slotten et al., 2002). It has been demonstrated that human and rodent skin harbors specific high-affinity membrane receptors for melatonin, MT1 and MT2 (Slominski et al., 2003b; Fischer et al., 2006a). Thus, we investigated the potential property of melatonin as a time giver to synchronize the oscillators in the skin and focused essentially on its effect on fibroblasts.

Besides fibroblasts, there are also keratinocytes and melanocytes as main cell types in skin. We did not yet develop proper methods to isolate these cell types from rat skin but, since we are currently studying it in the laboratory, we turned to human skin (plasties) as a potential source of melanocytes and keratinocytes (Sandu et al., 2012). To take advantage of bioluminescence because it is real-time and time-saving, we designed and constructed a lentivirus tool, following the design from a paper published by Brown *et al.* (Brown et al., 2005b). In this vector, luciferase expression is controlled by the *Bmal1* promoter. As reported, this construct was successfully used to assay clock properties in human fibroblasts and keratinocytes.

In the results part afterwards, to follow the aims of the thesis, three main chapters are organized and written as respective small papers, each including abstract, introduction, method, result and discussion. The general discussion is to show the global view of the thesis work. The bibliographies are listed together in the end after the general discussion.

2. Methodological strategy: real-time recording of circadian gene transcription by bioluminescence

Properties of circadian clocks are usually studied by monitoring the activity level of clock gene transcription, because of the stable intrinsic negative feedback loops in the molecular clockwork. An important aspect here is the necessity to monitor the active processes over a period of at least 24 hours, or several times 24 hours. Initially, this measurement was achieved by frequent sampling over at least 24 h and analyzing the mRNA levels over time. Although a large amount of animals and work are required, numerous studies have managed to determine many endogenous clock properties by this approach (Balsalobre et al., 1998; Balsalobre, 2002; Brown et al., 2002). With this technique, several core clock genes can be checked at the same time to compare clock activities such as relative phases (Sandu et al., 2012). However, some details in oscillatory activity like bimodal activity can be easily missed because of unavoidable intervals between sampling times. Another limitation would happen due to inter-individual differences in phases which could lead to neutralization on the populated clocks, especially in SCN-lesioned animals (Tanioka et al., 2009). Since luminescence reporters are widely used to measure gene transcription, this technique has been adapted to study circadian oscillations in cells, cultured tissues and whole organisms (Yamazaki et al., 2000; Izumo et al., 2003; Vallone et al., 2004). Given that it is easy and noninvasive to use luminescence to assay circadian genes, transgenic cells and animals have been developed on the basis of a clock gene promoter fused to the luciferase reporter gene. This reduces greatly the amounts of samples and inter-sample variability. Real-time recording of circadian oscillations of gene expression can be done from the whole animal to cultured tissue and cells (Collaco and Geusz, 2003; Yamazaki et al., 2009). As an example, in *Per1-luciferase* transgenic rats, the *Per1* transcription rhythm in the SCN slices can last for 32 days continuously (Yamazaki et al., 2000). In this long-lasting recording, properties of clocks can be observed through parameters like phase, period and amplitude. The timing of a reference point in the cycle, normally the first peak, is

regarded as the phase. The time interval between phase reference points, for instance two peaks, is referred to the period. And the distance in the level between adjacent trough and peak value reflects the amplitude, which normally damps more or less within days of oscillations.

Tissues and cells derived from the transgenic *Per1luc* rat strain (Fig. 22: a gift from Dr. Menaker, Virginia University, Charlotte Ville, USA) were used in PhD project to study the skin circadian clock properties.

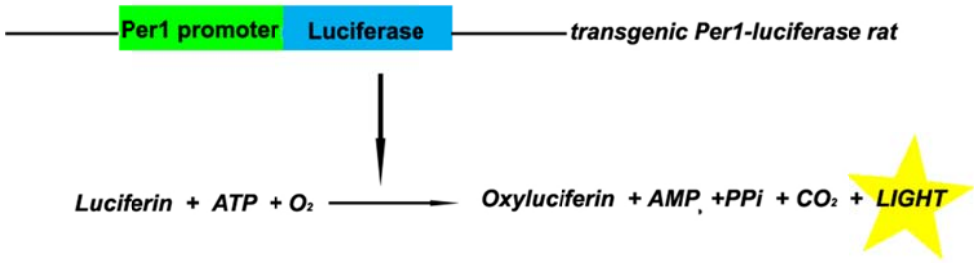


Figure 22: Diagram of the construct carried by the transgenic *Per1-luciferase* rat genome and luciferase reaction.

Practically, in the lab we are using a user-friendly equipment with photomultiplier tubes (PMTs), named LumiCycle machine (Actimetrics) (Fig. 23). This photosensitive device can detect light emission from cultures kept in sealed dishes in the presence of luciferin. Due to the photon counting potency of PMTs, the luminescence signals can be amplified by increasing the density of cells or the size of tissue explants. To be mentioned, all the samples and PMTs need to be kept in a light-tight box.

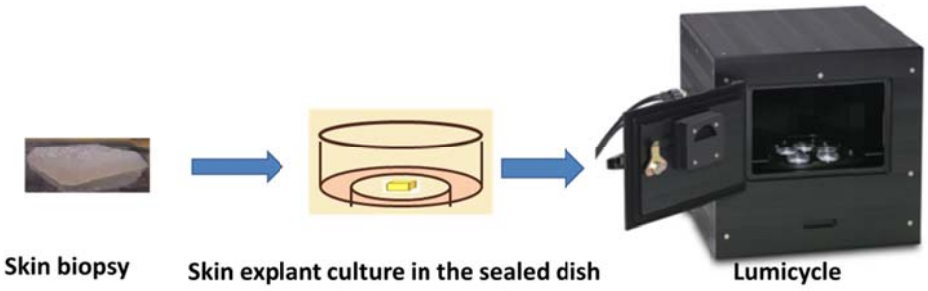


Figure 23: Measurement of bioluminescence by the LumiCyle machine. As an example, a skin biopsy is sampled and cut into a small piece. The skin explant is cultured on the Millicell semi-permeable membrane (Millipore) and medium containing luciferin. The dish is sealed with grease and then transferred into the Lumicycle machine.

To analyze the data from bioluminescence, we usually use the following strategy. We first subtract the baseline by 24 hours running average (Fig. 24). Afterwards, we get rid of the first, unusually high data recording (normally in the first day after start of bioluminescence recording). Then we keep 3-4 full cycles to get a dynamic curve fitting by using the Sigma Plot 12 software: we fit a function based on a damping sinusoid like below.

$$f = y_0+a*\exp(-x/d)*\sin(2*pi*(x-c)/b)$$

The period is obtained by *b*. The amplitude is referred to *a*, as the initial amplitude of the oscillations, before they damp. Constant *c* is a corrector for the phase. And *d* reflects the damping rate, which means the duration of days taken to decrease to 1/e (37%) of the reference level. The y_0 is a corrector of Y axis.

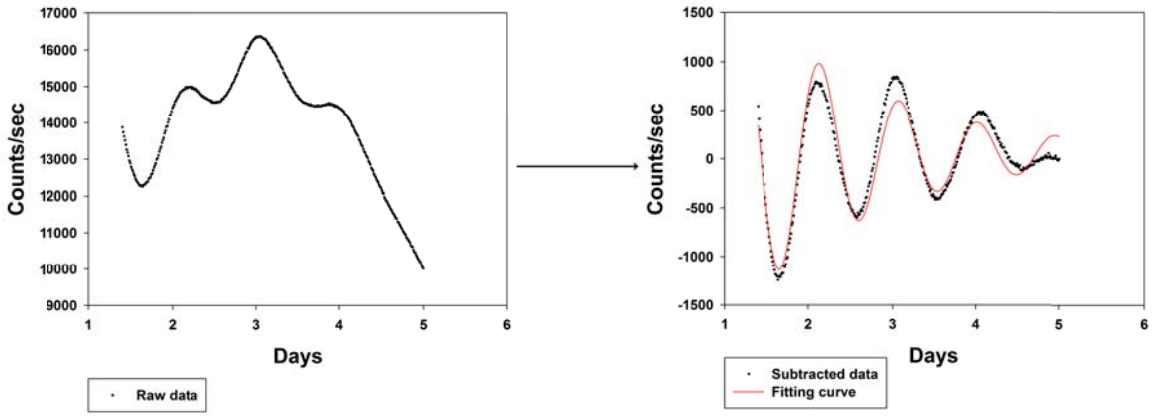


Figure 24: Subtraction of raw data by the 24 hours running average and subsequent regression curve fitting.

Results

Chapter 1

Characterization of the skin clock in postnatal development and ageing

1. Abstract

In mammals, circadian rhythms are generated and controlled by a hierarchical system including a master clock, located in the suprachiasmatic nuclei, the SCN, and multiple oscillators present in central and peripheral tissues such as liver, retina and skin. Due to the types of cell-autonomous oscillators that are involved, circadian clocks exhibit tissue-specific patterns within this network. In the present study, we investigated the clock present in the skin and characterized its properties during postnatal development and aging.

Circadian rhythmicity was examined in abdominal skin explants from 10 day- to 2 year-old *Per1-Luciferase* transgenic rats, using the real-time bioluminescence recording method. We found that the *Per1* rhythmic expression shows up at the age of 1 month, when rats get adult. Starting at 1 month, the skin clock gets more mature and synchronized with higher amplitude oscillations, reaching the highest at 6 months. In 1 to 2 year-old rats, skin circadian rhythms show decreased amplitude with fast damping and phases are more dispersed between individuals. Our study shows the level of synchronization in the skin tops at the age of 6 months, whereas it is decreasing during ageing. In addition, we characterized the skin as a temperature compensated oscillator which might be related to its distinct role in the circadian clock network.

With this study we functionally confirm the presence of a circadian clock within the skin and demonstrate that it is a valuable experimental model to study peripheral clock properties and synchronization mechanisms.

2. Introduction

The circadian clock network is comprised of multiple oscillators that generate daily rhythms in physiology and behavior. In this system, a central clock in the SCN can be entrained by photic and non-photic environmental time cues. The light is the prominent zeitgeber (time giver), which delivers time information through intrinsically photosensitive retina ganglion cells and the retinohypothalamic tract (RHT) (Berson et al., 2002). These external signals entrain the clock system to adapt to cyclic environment. The SCN drive rhythmic oscillations in neuronal activities and hormonal secretions. These outputs are essential for other oscillators in periphery to function integrally. Peripheral oscillators harbor cell-autonomous clocks, based on an intracellular molecular mechanism. Genetically, the molecular clockwork involves interlocking transcriptional-translational feedback loops (Ko and Takahashi, 2006). Several transcription factors are core components to regulate rhythmic gene expressions, with a period of about 24 hours. Transcription of Period (*Per*) and Cryptochrome (*Cry*) genes are activated by heterodimers of BMAL1 and CLOCK. PER and CRY proteins form a complex in the cytoplasm and translocate to the nucleus, repressing their transcription driven by the BMAL1/CLOCK complex. Additional regulatory loops involve *Bmal1* transcriptional regulation by REV-ERB and ROR factors. BMAL1/CLOCK activates the transcription of *Rev-erb* and *Ror* genes, which products respectively repress and activate the transcription of *Bmal1*. These negative and positive regulation loops provide original gene expression rhythms. Posttranscriptional and posttranslational pathways also regulate the clock factors and contribute ultimately to the mechanisms of rhythms.

Under the coordination of the SCN, peripheral clocks set appropriate phase and period to behave in an integrated manner for many physiological rhythms. Skin is an interesting example of peripheral clock, which displays daily rhythmic variations in functions like sebum secretion and temperature (Le Fur et al., 2001). At the interface between the body and external environment, skin plays a key role on the maintenance of body homeostasis, exposed to daily variations of solar irradiation, humidity and temperature. Previous studies reported expression of CLOCK and PER1 proteins in

human keratinocytes, melanocytes and dermal fibroblasts (Zanello et al., 2000). Rhythmic expressions of clock genes have been reported in the skin in mouse and human (Tanioka et al., 2009; Sandu et al., 2012). Several types of cells in the skin are shown to oscillate in a circadian manner, which indicates that they are cell-autonomous. Together, these results suggest the complexity of the skin clock and present it as a potential model to study the peripheral clocks.

In rodents, *Bmal1* deficiency results in premature aging phenotype in mice skin. And *Clock* deficiency increases the age-related dermatitis in mice (Dubrovsky et al., 2010). Old rats showed age-related shortening of the free-running period and dispersed phase in some secondary clocks like the pituitary (Yamazaki et al., 2002). Here we characterized the skin clock by real-time recording of skin explants from *Per1-luciferase* rats from the postnatal period to 2 years. Since skin is a superficial tissue exposed to varying environmental temperature, we studied the temperature compensation property of the skin and of primary cells derived from it.

3. Materials and Methods

3.1. Animals

Per1-luciferase transgenic rats (Yamazaki et al. 2000) were used in this study. All rats were raised and maintained in our animal care facilities (Animal resource and experimentation platform, Chronobiotron, UMS 3415, France). *Per1-luciferase* transgenic rats were housed under a 12:12 h light-dark cycle (lights - 300 lux - on at 07:00 A.M.). The infant rats were kept with maternal care until the beginning of experiment. Male rats were used in all the experiments. All experiments were performed in accordance with the rules from the French Department of Agriculture (license no 67-67-298) and the 86/609/EEC European Committee Council Directive.

3.2. Tissue culture

Per1-luciferase transgenic rats aged from 10 days to 2 years were used. Animals were anesthetized and euthanized with CO_2 (20% in an air tight box) (usually in the afternoon). Right after sacrifice, abdominal fur was shaved with a single-edged razor blade and the skin tissue was excised as much as possible from the belly. Other peripheral tissues like eyeball and lung were removed at the same time, when necessary. Harvested tissues were immediately placed in cold Hank's balanced saline solution (HBSS, Invitrogen) on ice. The skin biopsies were then dissected under the binocular lens to remove subcutaneous tissue, mainly fat, and washed at least three times. A rectangular piece of explant with an area of about 20 mm^2 was isolated, flattened, placed (dermis side down) on a Millicell semi-permeable membrane (PICMORG50, Millipore) and cultured in 35 mm dishes (Nunc) with 1 ml DMEM supplemented with 10% Fetal Bovine Serum (FBS: Biowest) and 0.1 mM luciferin (Promega). For lung and eyeball explants FBS was replaced with 2% B27 (Invitrogen). For culture medium, 1 L of DMEM was regularly prepared from powder (Sigma, D9202) and supplemented with 10 mM HEPES (Sigma H0887), 25 mM D(+) glucose (Sigma G7021), 4.2 mM sodium bicarbonate (7.5%, Sigma S8761), 2 mM L-Glutamine and antibiotics (penicillin and streptomycin) until use. Dishes were sealed with vacuum grease (Dow Corning) and placed into the Lumicycle set at 37°C (usually by the end of the afternoon). Remaining parts of the skin biopsies were kept temporarily in ice-cold HBSS for subsequent fibroblast culture. Light emission from cultured tissues was measured immediately and without interruption for about 7 days: each plate was counted for 1 min and 48 sec, every 15 min.

3.3. Fibroblasts culture

To prepare primary fibroblasts from rat skin, biopsies handled as described above were washed in PBS and placed on a 10 cm culture dish with dermis side downwards. Then the explant was cut into small squares with sharp surgical blades, to create edges for

fibroblast outgrowth. Afterwards, these skin pieces were placed in the culture dish left to slightly dry for about 5 min until no visible PBS is left. Then warm complete DMEM medium (20% FBS) was added to the dish to cover the skin pieces. Medium was changed to complete DMEM (10% FBS) after fibroblasts grew out of skin pieces and cultures were maintained for 2-3 weeks until fibroblasts reached confluence. Fibroblasts were passaged and cultures were seeded at a density of 50,000 cells per 35 mm dish. Bioluminescence recording of fibroblasts started when cultures reached confluence. Condition of recordings was the same as for explants and time window for analysis started at the first trough.

3.4. Data analysis and statistics

Acquisition of bioluminescence data reflecting *Per1* activity was made with Lumicycle Analysis software (Actimetrics). This software was also used to subtract the baseline drift from the raw data: the 24 hours running average method was efficient in this process. To further investigate *Per1-luciferase* rhythms, subtracted data were analyzed by using Sigma Plot 12 software. A damped sinusoid function (see below) was used to fit the subtracted curves. The unusual data, normally in the first day after bioluminescence starting were removed. We kept 3-4 full cycles of recordings to get a dynamic fitting by Sigma Plot 12 software. The window of analysis for skin explants started at a common time point (slightly ahead of the first trough) corresponding to 9:30 A.M. of the second day of culture. In the case of fibroblasts, for which cultures were started more randomly, the window of analysis started precisely at the first trough.

$$f = y_0+a*\exp(-x/d)*\sin(2*pi*(x-c)/b)$$

The best-fit solutions were validated based on the fact that they passed the normality test (Shapiro-Wilk test ($p = <0.0001$)) and passed constant variance test. With a linear drift of y_0, the standard damped sinusoid allowed to determine the period of the oscillations by constant *b*. The value of *a* referred to the initial amplitude when *x* equaled to zero ahead of damping (therefore, the time scale was shifted for the analysis, so that 0

corresponded to the beginning of the studied time window). Constant *c* was a corrector for the phase corresponding to the middle of trough and subsequent peak but in our results we considered as phase value the time of the first peak. And *d* reflected the damping rate, which meant the duration (days) taken to decrease to 37 % (1/e) of its reference level. The y_0 represented a Y axis corrector. Curves were considered rhythmic when consecutive circadian cycles showed up. Samples with only one visible peak of *Per1-luc* were designated as arrhythmic.

Data are presented as mean values ±SD. One-way and two-way ANOVA followed by *post hoc* comparisons with the LSD Fisher test were used to compare groups. All statistics were performed using Sigma Plot 12 software.

4. Results

4.1. Skin clock at different locations

Since the skin covers a large surface with different rhythmic functions, circadian clock gene expressions have been checked in distinct locations like the dorsal, flank and vibrissae skin in the mouse (Le Fur et al., 2001; Tanioka et al., 2009). The molecular makeup found in all these locations indicates the presence of cell-autonomous systems there in. Prior to our age-study, we compared in vitro clock activity in explants derived from distinct parts of the body (Fig. 1) including the back, thigh and forehead in addition to the abdomen. Rhythmicity proved the most robust in the abdomen and forehead skin. But because of the amount of tissue that could be sampled, we finally chose the abdomen to conduct the whole study.

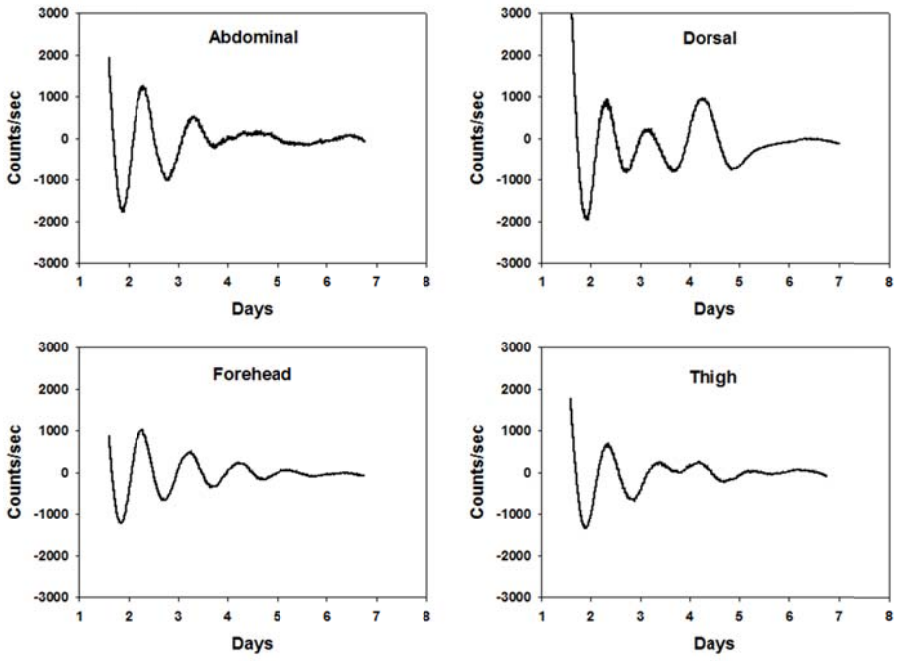


Figure 1: Representative profiles of substracted bioluminescence recordings from skin explants sampled in different locations. Skin biopsies were sampled from more proximal or more distal body locations of Per1-luciferase rats (2.5 month-old): abdominal, dorsal, forehead and thigh (n=3).

4.2. Maturation and aging of the skin clock

To understand the skin characteristics as a peripheral clock during development and aging, we first analyzed *Per1-luc* expression in skin explants from rats aged between 10 days and 2 years. Three stages were found (Fig. 2A).

First stage was from 10 days to 1 month, where young rats were still kept in litter with maternal care. No clock rhythmicity could be clearly seen at 10 and 20 days (P10 and P20). *Per1* activity in skin exhibited arrhythmic pattern with random peaks and troughs. At P10, variations in *Per1* activity levels showed up only in the first day or 2-3 days later. At P20, bioluminescence showed non-circadian fluctuations with 2-3 continuous peaks but with low robustness. At 1 month, unstable circadian rhythms could be found in skin clock activity, with low amplitude. However, the most robust peak did not show up in the first cycle but mostly in the second one. Given the fact that the activities from 10 days to 1month were not in the classic rhythmic way, this course was not analyzed by curve fitting.

The second stage started at 2 months: bioluminescence recordings in adult rats between 2 and 6 months showed circadian oscillation that appeared more and more robust with amplitudes increasing significantly (Fig. 2B). The period at 2 months was 22.4±0.5 h which was shorter in tendency than 23 h averagely observed at other adult ages. The phases got progressively delayed and more variable. The amplitude of clocks in skin damped significantly faster at 3 months. The largest amplitude combined with slowest damping occurred at the 6 month age (amplitudes $p=0.019$, one-way ANOVA; damping constant $p=0.032$, one-way ANOVA).

Third stage was the aging part, from 6 months to 2 years. Circadian oscillations got globally weaker and some abnormal cycles appeared. The phase variations among animals were much larger than in earlier adult ages. The amplitude of circadian oscillations damped faster upon aging.

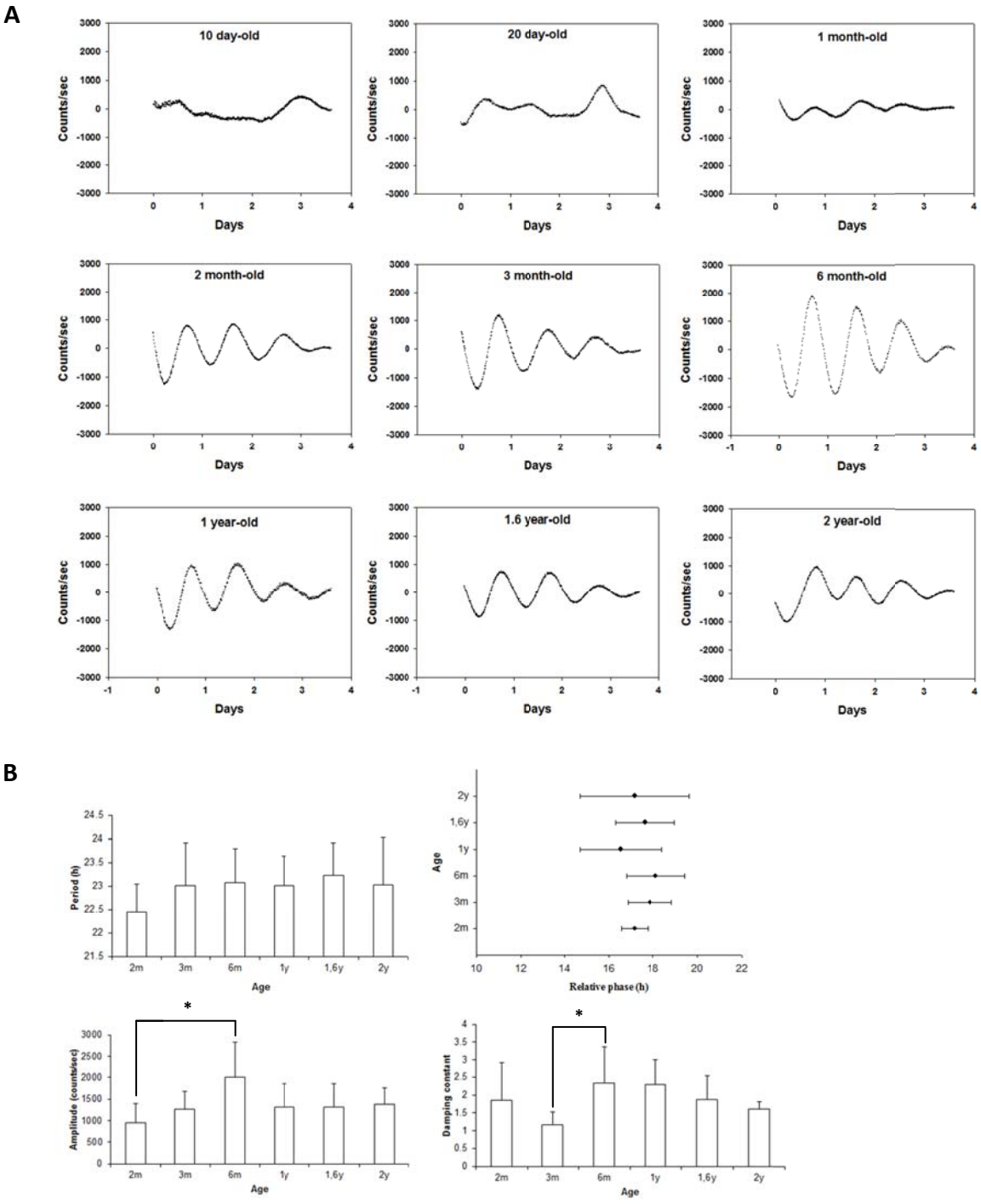


Figure 2: Circadian clock activity in the skin evolves during life in *Per1-luciferase* rat.

A) Representative profiles of bioluminescence recordings (subtracted data) from skin explants prepared from rats aged between 10 days and 2 years. The zero on X-axis means the beginning of the 3-4 day window of analysis. **B)** Summary of circadian clock parameters from subtracted bioluminescence recordings (A). The period, amplitude and damping rate are calculated from dynamic curve fitting. Data from 2 month- to 2 year-old rats show relatively high goodness of fit (R^2 >0.8 generally), representing standard circadian rhythmicity. Relative phase is determined by the first peak of *Per1* bioluminescence rhythms. Mean value±SD are shown (n=7, 10, 9, 6, 7 and 4 for rats from 2, 3, 6 months and 1, 1.6 and 2 years respectively). * $p<0.05$

4.3. Maturation and aging in skin fibroblasts

Besides the skin tissue explants, we also checked the clock activity in primary fibroblasts from rat skin. Several ages were chosen similarly, to cover postnatal development. Fibroblasts from donors of these ages showed distinct oscillations in comparison with the skin explants (Fig. 3A). Fibroblasts cultured from 10 day-old donors, showed no rhythmic clock activity, but a peak at the first day, and circadian oscillations appeared and got more rhythmic in cell populations from the age of 2 months on. When donors were as old as 2 years, circadian rhythms still existed in the fibroblast populations, though some abnormal peaks and bimodal activity appeared in the activities (Fig. 3A). Since the activities at 2 months did not conform to classical rhythmic patterns and their goodness of fit was below 0.8, this age was not included in the curve fitting results. During the ages between 3 months and 1.6 years, robust oscillations occurred for several days with fast damping. The period was 26.0±1.5 h at 3 months and decreased till 22.5±0.5 h at 2 years ($p<0.001$ one-way ANOVA, Fig. 3B). The amplitude of fibroblast oscillations decreased as the donors got elder ($p=0.002$ one-way ANOVA), while the damping rate was slower at older age ($p<0.001$ one-way ANOVA), which was contrary to the skin explants. Significant differences are determined by $p < 0.05$, post-hoc Fisher LSD test.

4.4. Medium change induces the oscillation of skin oscillators in the early postnatal age

In early postnatal ages (P10 and P20), *Per1* activities in the skin exhibited arrhythmic patterns with random peaks. Half of the samples showed an unusual cycle with high amplitude around the 4th day after sacrifice (Fig. 4A). As a synchronizing signal reported before, medium change induced clear circadian oscillations in the skin and in skin fibroblasts which were not rhythmic in the beginning (Fig. 4B). The induced rhythms in fibroblasts were not as robust as in the skin. These results indicated that autonomous oscillators are present but not yet synchronized.

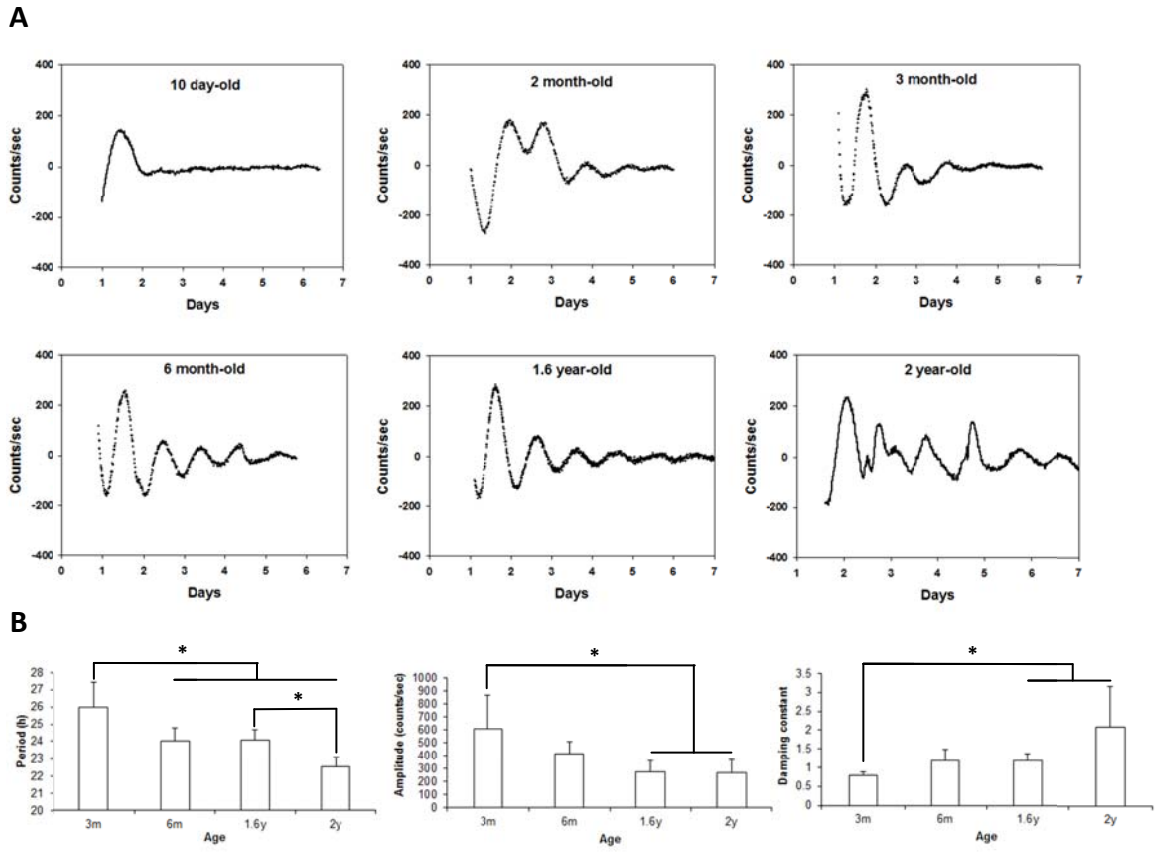


Figure 3: Clock activity in primary fibroblasts derived from *Per1-luciferase* rats during development. **A)** Representative oscillations of primary fibroblasts cultured from rats aged from 10 days to 2 years. Cells were cultured until reaching confluence and recording started after medium change. The time is shown on the x axis since the day at which bioluminescence recording of fibroblasts culture began. **B)** Summary of circadian clock parameters from subtracted bioluminescence recordings (A). The period, amplitude and damping rate are calculated from dynamic curve fitting. Data from 3 month- to 2 year-old rats are shown for relatively high goodness of fit (R^2 >0.8 generally), representing standard circadian rhythmicity. Mean value±SD are shown (n=7, 3, 10 and 4 for 3 month- to 2 year-old rat respectively). * $p<0.05$

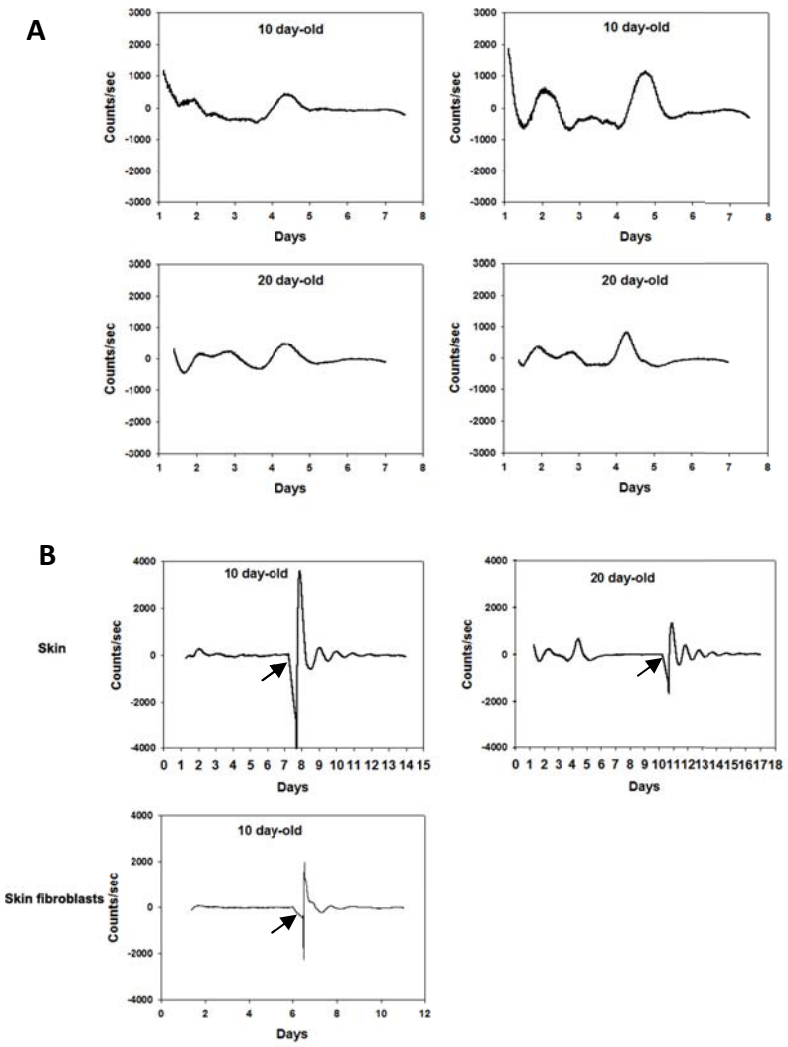


Figure 4: Medium change induces oscillations in skin explants and fibroblasts from young rats.
A) Examples of *Per1* activity in skin explants from rats aged 10 days (upper panels) and 20 days (lower panels). No rhythmic clock activity was found. Half of samples showed a strong noise cycle at around 4 days after sacrifice. **B)** Medium change induces circadian oscillations in skin from P10 and P20 rats (upper panels) and fibroblasts derived from P10 rats (lower panel). Arrows: medium change.

4.5. Comparison of the skin with other peripheral clocks

To further characterize the skin clock, we compared it with other peripheral clocks like the lung or the eyeball which were sampled after sacrifice, together with abdominal skin. 3 months was the age chosen according to the maturation of circadian activity in the skin (Fig. 2) for the comparison of the skin with the lung and the eye ball. The circadian oscillations from skin were similar to the eyeball, and significantly higher than in the lung

(Fig. 5A). In detail, the skin had fewer cycles than the eyeball did. The first peak phase of skin clock was similar to lung (around projected ZT21), and delayed about 3 hours with respect to the eyeball (Fig. 5B). According to the study done by our colleagues in the laboratory, the SCN has its first peak during the day, about 12 hours before the first peak of skin, in agreement with what is reported regarding phase relationships between central and peripheral clocks.

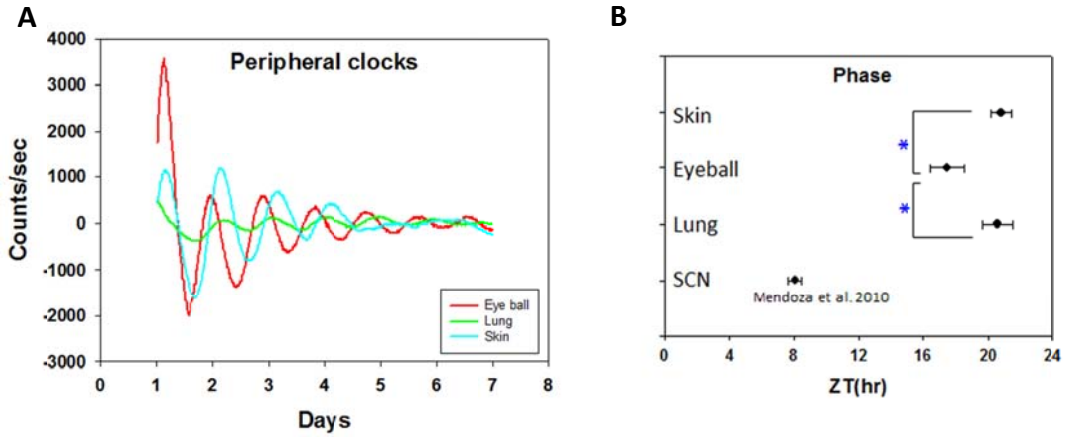


Figure 5: Peripheral clocks compared with the skin.

A) Representative bioluminescence recordings from skin, eye ball and lung explants taken from a 3 month-old rat. **B)** The phase (first peak, expressed on a projected ZT scale) of the skin clock was the same as that of the lung and delayed with respect to the eye ball and the SCN central clock (determined in an independent study from the laboratory, conducted with the same rat strain). Mean values±SD are shown (n=4 for the skin, eyeball and lung; n=8 for the SCN from Mendoza et al. 2010). * $p<0.05$

To further study the aging of peripheral clocks, we compared the lung clock in young and old rats, which were sampled after sacrifice, together with abdominal skin. Clock activity in the lung from aged animals showed similar characteristics to skin (abnormal cycles, faster damping) when compared to young animals (Fig. 6). Indeed, the lung oscillations in 2 year-old rats became damped and arrhythmic soon after the first cycle.

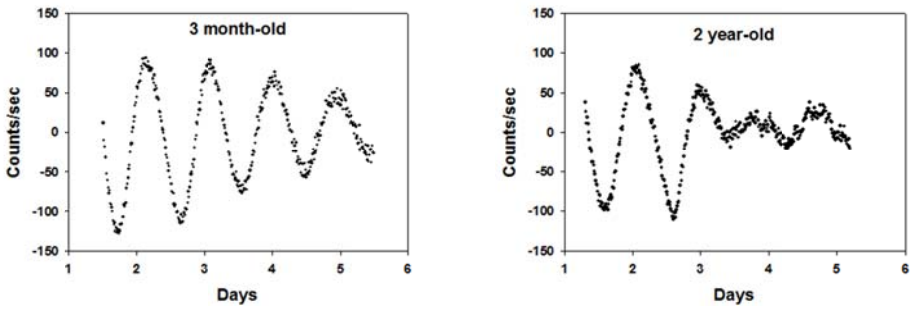
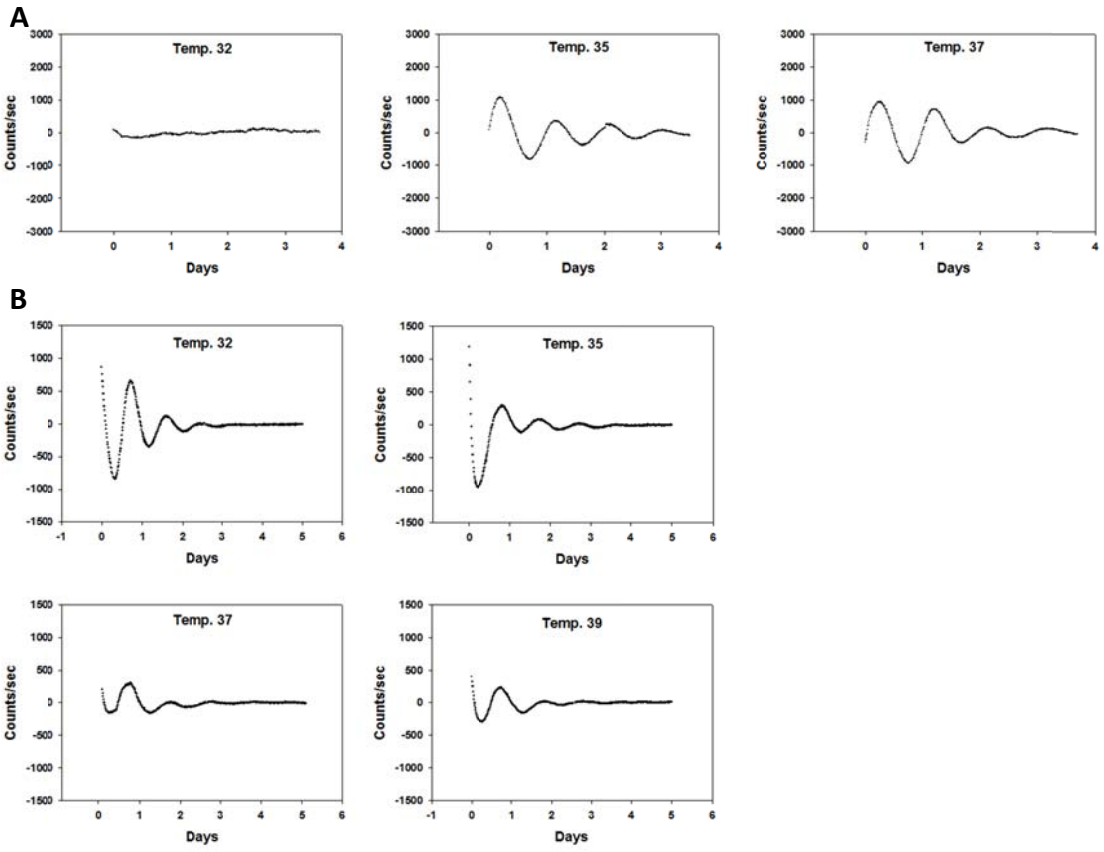


Figure 6: Clock activity in the lung from young and old *Per1-luciferase* rats. Representative profiles of the lung bioluminescence from *Per1-luciferase* rats aged 3 months (left) and 2 years (right) (n=4).

4.6. Temperature compensation of the skin clock

Clock activity in skin and skin primary fibroblasts were checked at different temperatures across the physiological range. Skin explants were sampled from 2.5 month-old rats and the fibroblasts were cultured from 3 month-old rats. The skin clock showed well-rhythmic activities at temperatures of 35 and 37 °C, but not at 32 °C (Fig. 7A). In contrast, skin fibroblasts exhibited circadian oscillations at temperatures ranging from 32 to 39 °C (Fig. 7B). The amplitude in the first cycle changed drastically according to temperatures ($p=0.013$ one-way ANOVA, Fig. 7D). At 32°C, the trough and the peak in first cycle were both large, while only the first though was large at 35 °C. At 37 and 39 degree, oscillations had quite low amplitudes in the first cycle and damped like the others in following cycles. The period lengths of skin showed at tendency to increase at higher temperature and in fibroblasts they were clearly increasing ($p<0.001$ one-way ANOVA), reaching more than 26 h at 37°C, as seen above (Fig. 7C and 7D). But the periods were still in the circadian range. These results were in consistence with the circadian clock property of temperature compensation. To further characterize this property in skin, we calculated the Q10 by roughly estimating the biochemical reaction rate from frequency of the rhythms (Fig. 7E). Q10 means the changing frequency when increasing the temperature by 10 °C. The value of Q10 was 0.77 in the skin fibroblasts. Since only 2 temperatures allowed skin explants to display oscillations, we could not trace the same trend line but noticed that the frequencies obtained in these cases (Q10 calculated as 0.78), were in agreement with the trend obtained with fibroblasts (Fig. 7E).


A
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B
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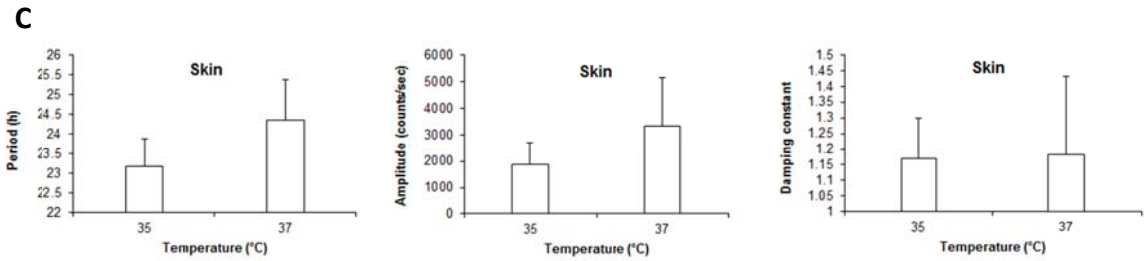
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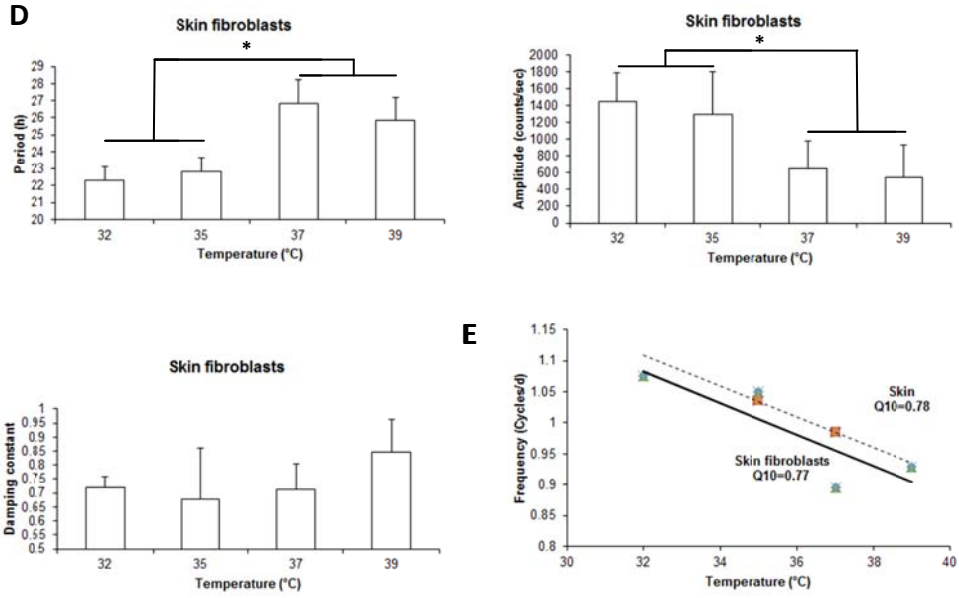
D
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Damping constant
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Frequency (Cycles/d)
Skin
Q10=0.78
Skin fibroblasts
Q10=0.77
Temperature (°C)

Figure 7: Effects of temperature on the circadian clocks in the skin and primary fibroblasts.
A) Representative bioluminescence recordings from skin explants at 32, 35 and 37 °C. The skin biopsies were sampled from 2.5 month-old rats. **B)** Representative bioluminescence recordings from skin primary fibroblasts at 32, 35, 37 and 39 °C. Skin fibroblasts were derived from 3 month-old rats. **C D)** Summary of circadian clock parameters from subtracted bioluminescence recordings in skin (C) and skin fibroblasts (D). The period, amplitude and damping rate were calculated from dynamic curve fitting. Data are shown for relatively high goodness of fit (R^2 >0.8 generally), representing standard circadian rhythmicity. Mean values±SD are shown (number of donors, n=3 for skin and n=4 for skin fibroblasts). * $p<0.05$
E) Frequency estimates of circadian rhythms by the average of period values in C and D. The Q10 is calculated by the changing frequency when increasing the temperature by 10°C. Red: skin; blue: skin fibroblasts

5. Discussion

The present study describes a new in vitro model for analyzing skin rhythmic properties based on real-time bioluminescence recording in skin explants or primary fibroblasts derived from Per1-luciferase rats. This study is the first to show that whole skin explants are able to show high amplitude oscillations in vitro, supporting the idea that this experimental system allows to analyze synchronization within the skin tissue as a whole. Our work also addressed the question of the functionality of the circadian clock in the skin and skin fibroblasts during postnatal development and ageing, an aspect of skin rhythmic properties that has not been examined previously. The temperature study confirmed that the clock present in skin fibroblasts conforms to the rule of temperature compensation and that the whole skin might behave likewise.

We found clear changes in the parameters of the skin clock activity between early life and aging. During this long life span, the internal (including circadian system) and external environment around the skin change a lot. Involved in a clock network coordinated by the SCN master clock, the phenotype of the skin clock might reflect the quality of its synchronization by direct and indirect signals. Tanioka *et al.* showed that circadian oscillations in the skin depend on the integrity of the SCN, although the results of such studies conducted with several arrhythmic animals are difficult to interpret. Our results showed that skin explants from rats over 1 month, devoid of any synchronizing signal display high amplitude oscillations, suggesting efficient synchronization of

individual oscillators present in the tissue. By contrast, explants from 10-20 day rats showed no circadian activity. The synchronizing signals from the SCN also change during the lifetime and this potentially leads to variable clock activities in the periphery.

In the circadian system, the role of the SCN as a central pacemaker evolves with age (Sumova et al., 2012). At P10, the amplitudes of the rhythms in clock gene expression in the rat SCN get adult-like (Sladek et al., 2004; Kovacikova et al., 2006). The circadian activity rhythms of AANAT, the rate-limiting enzyme of melatonin synthesis in the pineal gland, can be observed from P10, which accurately reflects rhythmic outputs from the developing SCN (Deguchi, 1982; Reppert et al., 1984). Peripheral clocks like liver and lung have been found rhythmic at P11 and P20 respectively, and their phase gradually shifts until the adult stage, while the SCN keeps the same phase during this developmental period (Yamazaki et al., 2009). Meanwhile, the circadian clockwork in the rat liver develops gradually and is roughly completed by 30 days after birth (Sladek et al., 2007). We showed that in the early postnatal days, skin oscillators could be synchronized by medium change which induced sustained oscillations in the explants (Fig. 4B). This suggested that autonomous oscillators existed already in the P10-P20 skin. Arrhythmicity first observed in these explants possibly reflects their desynchronisation due to the lack of synchronizing signals. Compared with adults, neonates before weaning are exposed to non-photic maternal cues including feeding, warmth and social contact but these might not be efficient towards the skin clocks. Alternatively, they might require a more mature circadian system to be efficient. Indeed, circadian activity is present early in the liver, likely directly driven by the feeding rhythm.

Low amplitude rhythms at young age can be meaningful because it might be easier to reset them according to environmental perturbations, an hypothesis which is supported by mathematical modeling studies (Colwell, 2011). In the adult, we observed that periods of skin clock activity get closer to 24 hours and that their amplitude increases. This indicates the skin clock, until 6 months, gets more mature and better synchronized. After 1 year, skin clock activity displays decreased amplitude and faster damping, which indicates decreasing synchronization efficiency. Accordingly, the phases of the skin clocks get much dispersed among individuals in aged animals. It has been reported that other

secondary clocks like pituitary are more dispersed in phase at old age (Yamazaki et al., 2002). Middle-aged mice (12 to 14-months-old) have shown reduced day-night difference in SCN neural activity (Nakamura et al., 2011a). Thus, based on the hypothesis that skin gets entraining cues from external environment and synchronization by the SCN, our results indicate that these processes are less efficient in the elder age but the mechanisms involved remain to be characterized. Reversely, *Bmal1* deficiency results in premature aging phenotype in mice skin. And *Clock* deficiency increases the age-related dermatitis in mice (Dubrovsky et al., 2010). These data suggest that circadian clocks are less functional in elderly and correlated with the skin physiology. Through the development and ageing, the changing amplitude may also result from changes in the composition of the skin, since skin cells show cell-specific robustness in clock activities (Sandu et al., 2012). For example, with aging, the outer skin layer (epidermis) thins, even though the number of cell layers remains unchanged. The number of melanocytes decreases, but the remaining melanocytes increase in size.

Fibroblasts cultures in our study showed circadian profiles similar to skin during postnatal development. The opposite tendency of damping rate in skin fibroblasts and skin explants suggested that whole skin oscillations not only reflect clock activity in fibroblasts. Given the fact that the medium change in the beginning constitutes a synchronizing signal, it may indicate that the response capacity of fibroblasts evolves during the development and that this is likely determined endogenously. Unlike neuronal tissues such as the SCN and the retina, apparent damping observed in the skin circadian oscillation can be explained by a reduction of medium nutrition or luciferin–luciferase reaction in the cells due to impoverishment of the medium conditions, although the amount of luciferin is quite enough in the medium. In skin explants and primary fibroblasts, refreshment of culture medium reinitiates circadian rhythms of Per1-luciferase supporting the idea that peripheral rhythms are also self-sustained, as is the case of the SCN circadian rhythms (Yoo et al., 2004).

Since skin is an interface tissue exposed to varying environmental temperatures, the biological reaction underlying its clock might be influenced by these changes. At very low temperature like 32 °C, abdominal skin lost its rhythmicity. However, in skin

fibroblasts, circadian rhythms still existed at 32°C, although the oscillations damped fast. In rat-1 fibroblasts, the circadian rhythm of *Per1* gene keeps its pace in low temperature even at 28°C and Q10 in rat-1 fibroblasts is 0.85-0.88 (Izumo et al., 2003). Taken together with those from rat-1 cells, our data indicate that the apparent desynchronization observed at low temperature may be due to the loss of phase coherence rather than the absence of individual rhythms. Moreover, in fibroblasts, low temperatures of 32 and 35 °C induced a large amplitude cycle in the first day. This might be correlated with the over compensation at these temperatures: enzymatic reactions get faster at lower temperature and lead to the high amplitude in the *Per1* gene transcription. Overall, our results indicate that clocks in the skin are temperature compensated in the physiological range of temperatures, which probably helps to keep its own rhythmic pace in the changing environment. It is also one fundamental property intrinsic to circadian clocks, which is confirmed in skin by our study.

Our data allow concluding that the skin clock undergoes maturation and ageing, with different levels of synchronization, along the life span. Mechanisms underlying these observations need to be further determined. We confirm that the skin is a self-sustained oscillator, capable to be synchronized and temperature compensated. In the clock network, skin has distinct phase and oscillation pattern as compared with other peripheral clocks. It represents a valuable experiment model to study peripheral clock properties and synchronization mechanisms.

Chapter 2

Synchronizing effects of melatonin on skin clock

1. Abstract

In the mammalian circadian clock system, the central pacemaker in the suprachiasmatic nucleus (SCN) influences numerous physiological activities by launching both neuronal and hormonal signals to deliver circadian messages as endogenous timekeeping cues. Several SCN-controlled signals such as glucocorticoids and temperature changes have been evidenced to have synchronizing effect on peripheral clocks, especially fibroblasts, widely used to model peripheral synchronization.

As one significant output from the SCN, melatonin has shown entraining effect on the central clock in a restricted time window. In our study, we demonstrated a phase-dependent effect of melatonin to synchronize primary skin fibroblasts. Following melatonin application at 2 h after the oscillation peak, the amplitude of *Per1* activity was significantly increased in the same range as following treatment by forskolin, another well-studied synchronizer. Our study shows for the first time an effect of melatonin on synchronization of peripheral clocks.

2. Introduction

In the circadian timing system, the SCN pacemaker orchestrates the subsidiary clocks in periphery. Throughout the organisms, circadian oscillators locate in almost all cells of organs and tissues. This multioscillatory network is modulated by the daily resetting cues derived from the SCN through neuronal and humoral pathways. Among humoral pathways, it has been reported that serum shock can synchronize rat-1 fibroblasts and glucocorticoids can synchronize peripheral oscillators including liver, kidney, cornea and rat-1 fibroblasts (Balsalobre et al., 1998; Balsalobre et al., 2000; Pezuk et al., 2012). Other synchronizing candidates have been proposed including melatonin whose synthesis is controlled by the SCN-pineal axis.

Melatonin is a strong output from the SCN pacemaker with robust daily rhythms. In most vertebrates, melatonin is synthesized primarily in the pineal gland at night, independently of locomotor activity pattern. Given the fact that melatonin is not stored in the pineal cells and immediately released into the general circulation, the plasma levels of melatonin precisely reflect its pineal synthesis. Melatonin secretion is only influenced by the light/dark cycle, thus could be regarded as a reliable estimate of the functioning of the SCN clock. Melatonin can induce behavioral phase-advance when injected at subjective dusk with pharmacological doses (Pitrosky et al., 1999; Slotten et al., 2002). Since high-affinity melatonin receptors MT1 and MT2 are expressed in various peripheral tissues besides the SCN, melatonin is a potential candidate as a synchronizer of peripheral clocks.

Besides pineal gland, melatonin synthesis has been found to occur in many central and peripheral tissues including the skin (Conti et al., 2000; Bubenik, 2002; Liu et al., 2004; Slominski et al., 2005b). Melatonin exhibits 10- to 100-fold levels in mouse skin, vibrissae follicles, and human hair follicle with respect to plasma (Kobayashi et al., 2005). Given the numerous known biological effects of melatonin, it may serve in the skin to protect and modulate its function, where its short plasma half-life suggests it could be useful as an internal time-giver for the skin in the circadian clock network.

Melatonin effects on skin function involve actions mediated by membrane receptors

expressed in skin cells. It has been demonstrated that human and rodent skin cells and tissues express high-affinity membrane receptors MT1 and MT2 with variations among species(Slominski et al., 2005b; Fischer et al., 2006a). Expression of melatonin receptors in several skin cells including normal and malignant fibroblasts, keratinocytes and melanocytes, indicates the potential role of melatonin as a time giver to synchronize all the oscillators within the skin.

Expression of MT1 and MT2 receptors in skin cells has been found to be affected by environmental factors, especially UVB, which act differentially according to cell type and genetic background (Slominski et al., 2005b).

The major layers of the skin, epidermis, dermis and adnexa are all targets for melatonin regulation, including numerous effects observed in skin biology and clinical dermatology, from pigmentation to hair regrowth (Fischer et al., 2008). In rats after pinealectomy, a reduction of collagen deposition was observed, which induces atrophy of the epidermis, dermis and hair follicle, similar to the aging phenotype in skin thickness, whereas melatonin administration could reverse collagen fiber damage and mitochondrial swelling in these animals (Drobnik and Dabrowski, 1996, 1999; Esrefoglu et al., 2005).

In clinics, several skin diseases have been correlated with abnormal plasma melatonin levels in patients. For example, plasma melatonin levels lose their circadian rhythms in psoriasis, and their rhythms exhibit greatly reduced robustness in atopic eczema (Mozzanica et al., 1988; Schwarz et al., 1988; Rupprecht et al., 1995). To many specific skin diseases, skin-related pathologic conditions and syndromes, melatonin shows potential application as a skin protectant by topical or oral administration (Fischer et al., 2008).

To investigate the effect of melatonin on the skin clock, we performed acute melatonin treatment at different times of the circadian cycle and measured the real-time bioluminescence of skin primary fibroblasts derived from *Per1-luciferase* transgenic rats.

3. Materials and Methods

3.1. Animals

Per1-luciferase transgenic rats (Yamazaki et al. 2000) were used in this study. All rats were raised and maintained in our animal care facilities (Animal resource and experimentation platform, Chronobiotron, UMS 3415, France). *Per1-luciferase* transgenic rats were housed under a 12:12 h light-dark cycle (lights - 300 lux - on at 07:00 A.M.). The infant rats were kept with maternal care until the beginning of experiment. Male rats were used in all the experiments. All experiments were performed in accordance with the rules from the French Department of Agriculture (license no 67-67-298) and the 86/609/EEC European Committee Council Directive.

3.2. Cell culture

Per1-luciferase transgenic rats aged 3 months were used. Animals were anesthetized and euthanized with CO_2 (20% in an air tight box) (usually in the afternoon). Right after sacrifice, abdominal fur was shaved with a single-edged razor blade and the skin tissue was excised as much as possible from the belly. Harvested tissues were immediately placed in cold Hank's balanced saline solution (HBSS, Invitrogen) on ice. The skin biopsies were then dissected under the binocular lens to remove subcutaneous tissue, mainly fat, and washed at least three times. To prepare primary fibroblasts from rat skin, biopsies were washed in PBS and placed on a 10 cm culture dish with dermis side downwards. Then the explant was cut into small squares with sharp surgical blades, to create edges for fibroblast outgrowth. Afterwards, these skin pieces were placed in the culture dish left to slightly dry for about 5 min until no visible PBS is left. Then warm complete DMEM medium (20% FBS) was added to the dish to cover the skin pieces. Medium was changed to complete DMEM (10% FBS) after fibroblasts grew out of skin pieces and cultures were maintained for 2-3 weeks until fibroblasts reached confluence.

Fibroblasts were passaged and cultures were seeded at a density of 50,000 cells per 35 mm dish (Nunc). Bioluminescence recording of fibroblasts started when cultures reached confluence. Every culture in 35 mm dish with 1 ml DMEM supplemented with 10% Fetal Bovine Serum (FBS: Biowest) and 0.1 mM luciferin (Promega). For culture medium, 1 L of DMEM was regularly prepared from powder (Sigma, D9202) and supplemented with 10 mM HEPES (Sigma H0887), 25 mM D(+) glucose (Sigma G7021), 4.2 mM sodium bicarbonate (7.5%, Sigma S8761), 2 mM L-Glutamine and antibiotics (penicillin and streptomycin) until use. Dishes were sealed with vacuum grease (Dow Corning) and placed into the Lumicycle set at 37°C. Light emission from cultured cells was measured immediately and without interruption for several days: each plate was counted for 1 min and 48 sec, every 15 min.

3.3. Drug application

All drug applications were done in darkness: medium was replaced with serum-free DMEM containing the drug and the culture was placed back into the incubator for the indicated time Then, medium was again replaced with DMEM supplemented with 10% FBS and 0.1 mM luciferin. Dishes were sealed with vacuum grease and transferred to LumiCycle. The concentration of drugs was as follows: 50 µM melatonin (control: ethanol with final concentration 0.1% in the medium), 20 µM forskolin (control: ethanol with final concentration 0.1% in the medium).

3.4. Data analysis and statistics

Acquisition of bioluminescence data reflecting *Per1* activity was made with Lumicycle Analysis software (Actimetrics). This software was also used to subtract the baseline drift from the raw data: the 24 hours running average method was efficient in this process. To further investigate *Per1-luciferase* rhythms, subtracted data were analyzed by

using Sigma Plot 12 software. A damped sinusoid function (see below) was used to fit the subtracted curves. The unusual data, normally in the first day after drug treatment, were removed. We kept 3-4 full cycles of recordings to get a dynamic fitting by Sigma Plot 12 software. The window of analysis for skin fibroblasts started at a common time point (slightly ahead of the first trough).

$$f = y_0+a*\exp(-x/d)*\sin(2*pi*(x-c)/b)$$

The best-fit solutions were validated based on the fact that they passed the normality test (Shapiro-Wilk test ($p = <0.0001$)) and passed constant variance test. With a linear drift of y_0, the standard damped sinusoid allowed to determine the period of the oscillations by constant *b*. The value of *a* referred to the initial amplitude when *x* equaled to zero ahead of damping (therefore, the time scale was shifted for the analysis, so that 0 corresponded to the beginning of the studied time window). Constant *c* was a corrector for the phase corresponding to the middle of trough and subsequent peak which was used as phase value. And *d* reflected the damping rate, which meant the duration (days) taken to decrease to 37 % (1/e) of its reference level. The y_0 was a corrector of Y axis. Curves were considered rhythmic when consecutive circadian cycles showed up. Samples with only one visible peak of *Per1-luc* were designated as arrhythmic and removed from the study.

Data were presented as mean values ±SD. One-way and two-way ANOVA followed by *post hoc* comparisons with the LSD Fisher test were used to compare groups. All statistics were performed using Sigma Plot 12 software.

4. Results

4.1. Phase-dependent melatonin effect on skin fibroblasts

Skin tissues and skin fibroblasts both show circadian rhythm in *Per1-luciferase* activity. According to the variations of clock profiles with age (results from Chapter 1), we used material derived from 3 month-old rats to investigate the effects of melatonin on skin rhythmicity. We performed preliminary experiments on whole skin explants, but we could not draw clear conclusions about melatonin effect, due to the extensive phase dispersion among individuals following several days in vitro. Dexamethasone, a glucocorticoid receptor agonist acting as a strong synchronizer, also did not display any clear effect on skin explants.

To simplify the experimental system and to reduce the problem of phase dispersion among samples, we used skin primary fibroblasts derived from one unique animal and designed a specific strategy for treatment (Fig. 1). To determine a clear phase reference for drug application, we monitored bioluminescence from a series of identical fibroblast cultures during 2-3 days. After 2 cycles had been observed, we anticipated the time of the third PEAK, based on the moments of the first two peaks and on the period between them. Then we chose 4 time points in the next anticipated cycle before and after the anticipated PEAK taken as a time reference: these time points (n=7-8 dishes per time point) for melatonin treatment (50µM for 30 min) were Peak-9h, Peak-2h, Peak+2h, Peak+9h. Control dishes were treated at similar time points (n=7-8 per time point). The data obtained after drug application were subtracted and fitted with a damped sinusoid function, as described in methods. Parameters like period, amplitude, phase and damping were compared between melatonin groups and corresponding control groups.

In the group treated at Peak+2h, melatonin showed a significant positive effect on the amplitude of circadian oscillations which increased by 30% ($p=0.0057$, one-way ANOVA) (Fig. 2A). This effect was similar to the one obtained after forskolin treatment done at random moment of the cycle (47 %, $p=0.020$) (Fig. 2B), which was in agreement

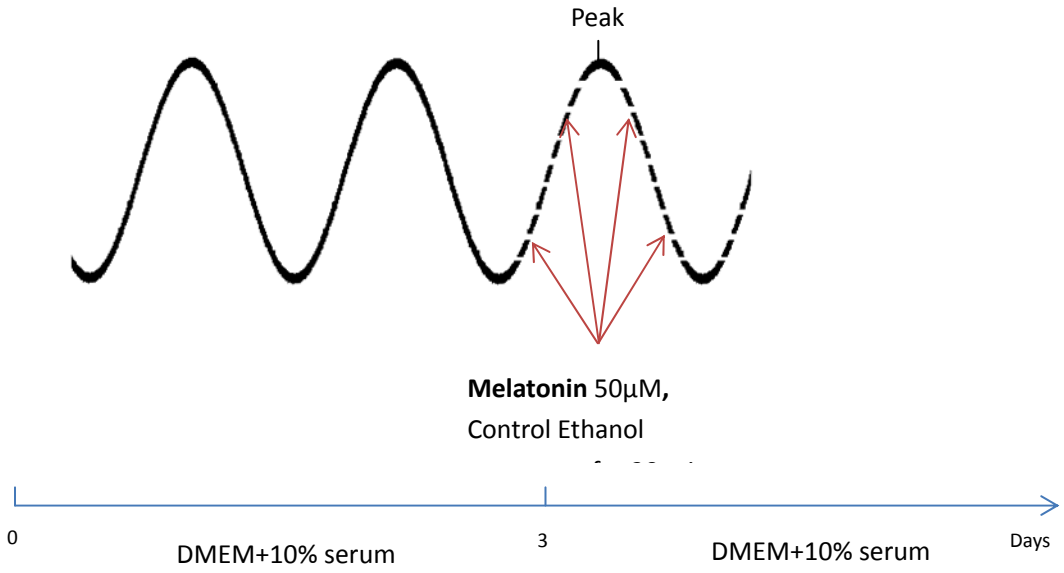

Figure 1: Strategy for phase-dependent melatonin treatment at Peak-9h, Peak-2h, Peak+2h, Peak+9h. Replicate dishes of fibroblasts from the same 3 month rat were recorded for *Per1* bioluminescence for 2-3 days. After 2 cycles had fully showed up, we anticipated the third Peak based on the first two peaks and the duration between them. Then we chose 4 time points in the next anticipated cycle with the reference of the anticipated Peak, as Peak-9h, Peak-2h, Peak+2h, Peak+9h, to apply melatonin (50µM for 30 min, or control medium). Afterwards, media were renewed and bioluminescence recorded for the next 7 days.

with previous reports with fibroblasts (Yagita and Okamura, 2000). With respect to the ratio of each amplitude in treated group on the mean value from control group, Peak+2h was significantly higher than other time points ($p=0.003$ one-way ANOVA, data not shown). No effect was observed at the other time points of melatonin application and no effect of melatonin was seen on any of the other circadian parameters like period and damping. Although melatonin showed no effect directly on the phase of fibroblast culture oscillations between control and melatonin treated dishes from the same group, we observed that the resulting phases (expressed with respect to treatment time point) were similar between groups (less than 2 h difference) (Fig. 2C). This might result from the synchronizing effect of changing the medium or simply manipulation of dishes. Indeed, we found no significant difference in the phase of the following oscillations between control and treated samples, indicating that medium change induces phase resetting of the culture, regardless of which part of the circadian cycle cultures were at the moment of treatment.

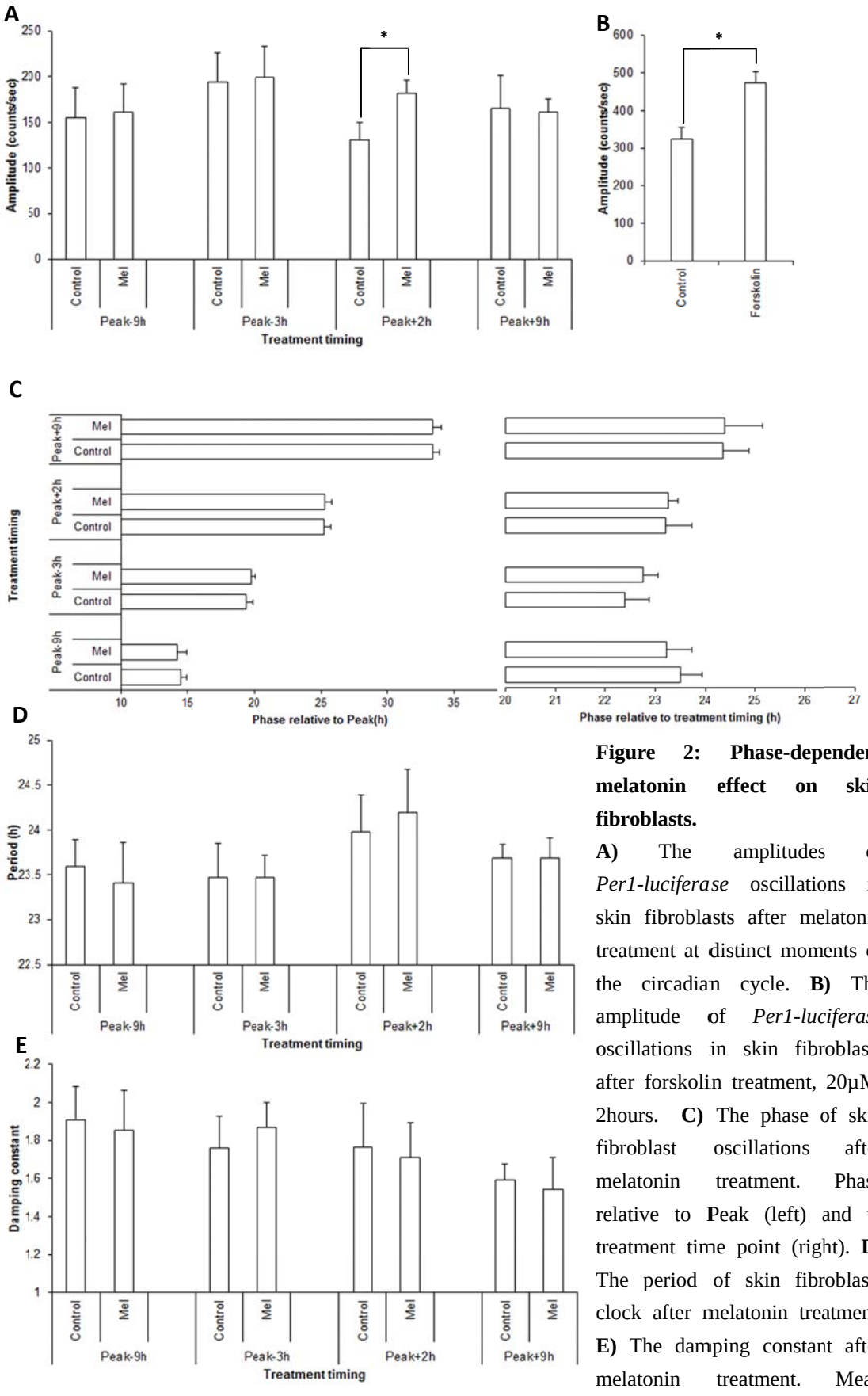

Figure 2: Phase-dependent melatonin effect on skin fibroblasts.

A) The amplitudes of *Per1-luciferase* oscillations in skin fibroblasts after melatonin treatment at distinct moments of the circadian cycle. **B)** The amplitude of *Per1-luciferase* oscillations in skin fibroblasts after forskolin treatment, 20µM, 2hours. **C)** The phase of skin fibroblast oscillations after melatonin treatment. Phase relative to Peak (left) and to treatment time point (right). **D)** The period of skin fibroblasts clock after melatonin treatment. **E)** The damping constant after melatonin treatment. Mean value±SD are shown (n=7-8). * $p<0.05$

4.2. Effect of constant melatonin on the transcriptional level of skin fibroblast clock

To further investigate the effects of melatonin on the fibroblast clock, we also chronically treated cultures with 50 µM melatonin without renewing the medium. We did not detect any effect on circadian parameters (data not shown) but observed that the baseline of bioluminescence recording was increased, indicating the level of *Per1* transcriptional activity in skin fibroblasts was augmented in the presence of melatonin (Fig. 3). These results however need to be investigated further since the subtracted data in both melatonin and control (ethanol) group were not as smooth as those cultures in normal medium. This might be due to the solvent of ethanol even though its low concentration of 0.1% in the medium.

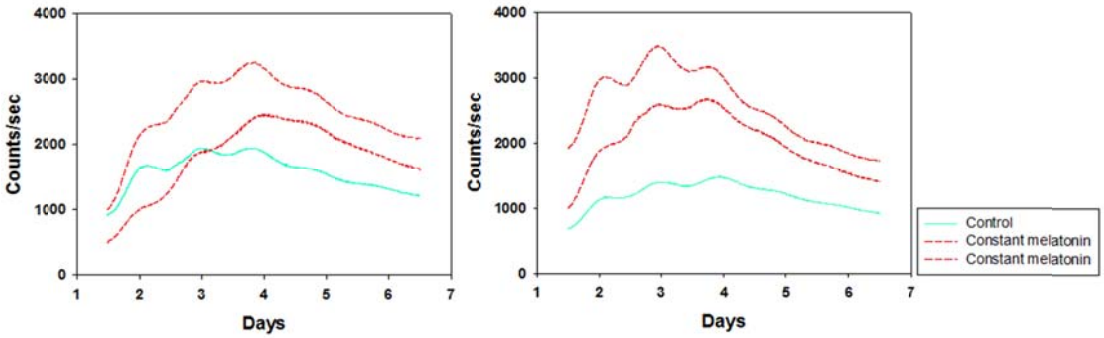

Figure 3: Effect of constant melatonin on the skin fibroblasts clock.
With melatonin (50µM) in the medium starting right after dissection and beginning of recordings, the raw data of bioluminescence in skin fibroblasts showed an increased level of luciferase activity. In each panel, fibroblasts were replicates from the same donor of 3 month-old *Per1-luciferase* rat. Two experiments with distinct rats are shown

5. Discussion

In the present study, we show the first evidence that melatonin has an effect on peripheral clocks. We demonstrate a phase-dependent effect of melatonin increasing the amplitude of the skin fibroblast clock. The amplitude is largely determined by the dispersion of phases of the present individual oscillators. The larger the amplitude, the more the oscillators are synchronized to a unique phase (Balsalobre et al., 1998). In

addition, oscillations damped at same rate at all time points (Fig. 2E). It is known that, in contrast to cultures of SCN neurons, cultures of fibroblasts damp rapidly (Nagoshi et al., 2004). This is explained by prompt loss of synchrony among cells lacking highly efficient communication as exists among SCN neurons. The skin fibroblasts possibly lack this kind of coupling as well. This is in agreement with the idea that peripheral oscillators seem not to be coupled through organ-specific or paracrine communications, but require the SCN derived signals to be phase coherent (Guo et al., 2006).

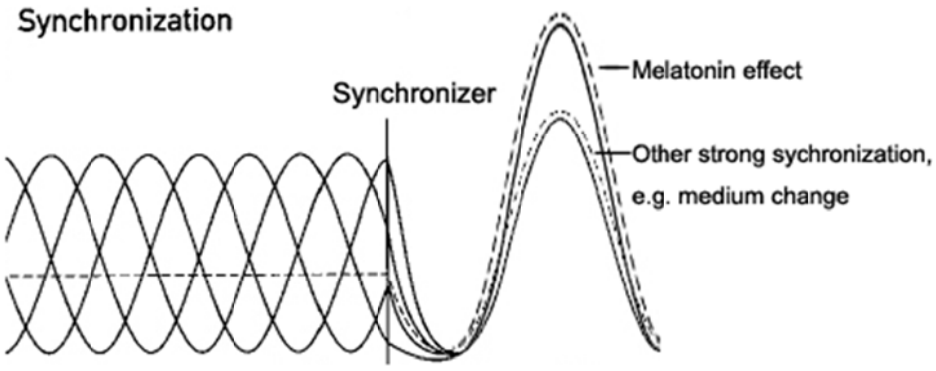

Figure 4: Synchronization model of melatonin effect. (Adapted from Balsalobre *et al*. 1998)

The amplitude of melatonin treatment in the Peak+2h group was increased similarly to what was obtained after forskolin treatment. This synchronizing effect could involve the effect of the medium change, because of its strength as a synchronizer, which in addition appears phase-independent (Fig. 2C) (Yamazaki et al., 2000). The melatonin effect can be regarded as an additive synchronization which is dependent on the moment when it was applied (Fig. 4). Based on results from our lab, it is known that levels of pineal melatonin release are increased following acute phase-dependent subcutaneous melatonin treatment, which is due to an effect on the central clock. Bothorel et al,. 2002 And this phase coincides with the pineal release at night. In vivo, pharmacological levels of melatonin phase-advanced the clock behavioral output, in a restricted time window, which is generally in the late subjective day in nocturnal and diurnal animals (Pitrosky et al., 1999). During this time window, it is reported that melatonin leads to phase-advance of *Rev-erbα* expression profile and up-regulates the expression of *Rorβ* during the subjective night, without any acute effect on *Per1* and *Per2* in nocturnal animals (Agez et

al., 2007). This phase-dependent effect of melatonin is consistent with the existence of strong rhythms in plasma melatonin levels and with the restricted window in which melatonin affects the central clock. Whether the timing that we observed is consistent with the nocturnal release of melatonin is to be determined. Also, since predominantly the MT1 receptors are expressed in the skin fibroblasts, their role involved in this phase-dependent effect needs to be determined (Slominski et al., 2003a; Fischer et al., 2006a).

Due to the complexity of the skin, there are many types of oscillators with different clock properties. These oscillators control numerous rhythmic physiological activities. In the skin, various cells function in the local compartments and influence each other. Several signal transduction receptors are coupled to classic intracellular second messenger pathways, like cAMP mediated response. As investigated in the pituitary, melatonin could modulate the cAMP signal pathway by inhibiting the phosphorylation of cAMP response element binding protein (CREB) (McNulty et al., 1994). Neurons in the SCN show oscillating activation of MAPK cascade and CREB, while efferences from the SCN depend on the cAMP pathway and daily CREB mediated activities (Zawilska et al., 2009). It was found that in NIH3T3 cells transfected with human MT2, high concentration of melatonin inhibits cAMP synthesis (Reppert et al., 1995). In comparison, forskolin elevates the cAMP level and enhances the phosphorylation and activation of CREB. CREB has also been involved in enhancing circadian clock rhythms with the acute induction of the *Per1* mRNA in rat-1 fibroblasts (Yagita and Okamura, 2000). In addition, melatonin has been shown to be an inhibitor of SIRT1-HDAC activity, as well as of SIRT1 expression, indicating that melatonin plays a role in the regulation of circadian rhythms through SIRT1 (Jung-Hynes et al., 2011). Further studies are necessary to clarify the molecular mechanisms of melatonin effect.

The medium change effect on the circadian clock has been observed commonly, and serum within the medium is necessary for initiation of the rhythmicity and for long term culture (Yamazaki et al., 2000; Izumo et al., 2003; Ko and Takahashi, 2006). Serum shock has been reported to synchronize rat-1 fibroblasts and *Per1/2* plays an immediate role in the process as being transcriptionally induced (Balsalobre et al., 1998). Serum shock

induces the phosphorylation of CREB in rat-1 cells as well as forskolin (Yagita and Okamura, 2000). The melatonin effect could be masked by the strong synchronization of medium change, which could indicate that melatonin is relatively weaker than medium change or serum effect. To eliminate the influence of medium change from melatonin effect, the flow-through system might be a promising method with fewer disturbances due to manual manipulation, although it might be difficult to make the treatments in the absence of serum in that case. It should be noted that all new circadian phases were nearly identical irrespective of whether fibroblasts were treated with control medium or with melatonin and, when considered with respect to the time of treatment, there were no substantial differences. The corresponding phase response curve (PRC) conforms to type 0 PRC with large phase shifts, whereas normal photic or non-photic stimulus acts on circadian behavior with gradual phase shifts, which is called type I PRC. The “restored” circadian rhythms after medium change all begin with the ascending phase of circadian cycle, which is unlikely to be due to the restart of the circadian rhythm. A switch from a slow-resetting type I PRC to a fast-resetting type 0 PRC has been reported in circadian clocks under increasing stimuli strength such as light intensity and drug concentration or changing the sensitivity of the clock to the administered stimuli (Johnson, 1999). Mammals like rodents tend to have gradually shifts and it requires large amounts of light energy to make the switch from type I to type 0 PRC. It was found that circadian rhythms in NIH3T3 fibroblasts reset by dexamethasone treatment demonstrate a type 0 PRC (Nagoshi et al., 2004). Cell oscillations are instantaneously reset by a strong synchronizer like dexamethasone or medium change in our study at whatever circadian phases they used to be. By contrast, circadian oscillations in cultured liver tissues or in pup SCN are phase reset depending on the timing of medium change and do not show type 0 PRC to refreshment of medium (Nishide et al., 2006; Nishide et al., 2008). However, a type 0 PRC for medium change is found in the SCN of adult *Clock* mutant mice whereas the SCN of wild-type mice does not respond to medium change at any phase (Vitaterna et al., 2006). These results could be due to tissue architecture preserved in the liver slices and the SCN. Cell types and cell communication may introduce variability in the response of the circadian system to zeitgebers. In this context, instantaneous resetting of “peripheral

clocks” with strong synchronizers as observed in NIH3T3 cells or in our study, indicates a possible mechanism of phase adjustment of peripheral rhythms by the SCN. The glucocorticoid potency of dexamethasone is much higher than that of physiological glucocorticoids like cortisol (Adrenal Cortical Steroids, Drug Facts and Comparisons, 5th ed. St. Louis, Facts and Comparisons, Inc.:122-128, 1997). In addition, in pharmacological treatment, concentration of dexamethasone or melatonin is quite higher than physiological levels. SCN can be reset rapidly by shifts of light-dark cycle, but resynchrony of peripheral oscillators can take over a week, which is regulated by hormones like adrenal glucocorticoid (Yamazaki et al., 2000; Kiessling et al., 2010). And it often takes more days of gradual phase advances than of phase delays. A relatively weak synchronizer like melatonin is possibly involved in the synchronization of peripheral clocks, which might be cell-specific, due also to local particularities in the molecular clockwork. Given the fact that many peripheral cells are proliferative and need to “pass” time to daughter cells, the use of a weak synchronizer that induces gradual phase resetting might be the best compromise to maintain molecular clock phases in the body (Nagoshi et al., 2004).

In the multioscillatory skin system, it is not easy to determine whether other oscillators, in addition to fibroblasts, could be synchronized by melatonin The other cells like keratinocytes are not as highly proliferative as fibroblasts, although keratinocytes follow circadian rhythms in mitosis cycle (Brown, 1991; Geyfman et al., 2012). In cell cultures, cell density is crucial for the synchronization among oscillators, in which intercellular factors help to generate robust circadian rhythms (Noguchi et al., 2013). Fibroblasts express connexins and are connected through gap junctions in high-density cultures (Goodenough et al., 1996). However, rather than intercellular couplings, paracrine pathways appear to play a more important role on helping synchronization among cells (Noguchi et al., 2013). Existence of paracrine diffusible signals have been shown in immortalized SCN2.2 cells which were able to confer molecular oscillations to cocultured NIH/3T3 fibroblasts (Allen et al., 2001). These kinds of unknown diffusible signals have also been found in the SCN-lesioned animals in which SCN transplantation into distinct brain regions can restore circadian behavioral rhythms (Silver et al., 1996).

The cell to cell communication may play an important role in the response of peripheral clocks to synchronizer. Other paracrine factor like fibroblasts growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factor (IGF) may also get involved.

In conclusion, our study shows for the first time evidence that melatonin has a phase-dependent synchronizing effect on peripheral clocks. Whether this effect is restricted to increasing molecular clock activity within cells or synchronization by concentrating the phases, is to be elucidated further. The synchronization of other cells in the skin could permit the opportunity to understand more about the synchronization mechanisms in a multioscillatory system.

Chapter 3

Construct of lentivirus of Bmal1 luciferase reporter

1. Abstract

In circadian oscillatory system, cell-autonomous clocks exist in almost all the tissues and cells through the organisms. Molecular clockwork involves clock gene activities and drives clock-controlled outputs in single cells. Monitoring molecular clock activity leads to further insight into circadian rhythms and their mechanisms in tissues and cells. In the skin tissue, clock gene oscillations are found in fibroblasts, keratinocytes and melanocytes as well as cells constituting hair follicles, including stem cells.

In the present work, we constructed a lentivirus tool delivering *Bmal1-luciferase* reporter to study clock activities in human skin cells. Following the design from a paper published by Brown and his colleagues (PLOS Biology 2005), we constructed this tool to take advantage of bioluminescence, especially for human samples or samples from species for which transgenic tools do not exist. Within this tool, luciferase expression was controlled by the *Bmal1* promoter which could be integrated into DNA of host cells via lentiviral infection. This kind of lentiviral tools were successfully used to assay clock properties in human fibroblasts and keratinocytes. We did not develop yet an appropriate method to isolate and culture rat melanocytes and keratinocytes from skin but currently use these cells derived from human biopsies. This lentiviral tool would help to investigate clock activities not only in fibroblasts but also in melanocytes and keratinocytes of the multioscillatory skin.

2. Introduction

The mammalian circadian system is composed of a network of circadian clocks located throughout the organisms. A central clock residing in the suprachiasmatic nucleus (SCN) of the brain synchronizes the other secondary oscillators present in most cells of the body. Peripheral oscillators harbor cell-autonomous clocks, based on an intracellular molecular mechanism. Genetically, molecular clockwork involves interlocking transcriptional-translational feedback loops (Ko and Takahashi, 2006). Transcription of Period (*Per*) and Cryptochrome (*Cry*) genes are activated by heterodimers of BMAL1 and CLOCK factors. PER and CRY proteins forms a complex in the cytoplasm and translocate to the nucleus, repressing their transcription driven by BMAL1/CLOCK. Additional regulatory loops involve the regulation of *Bmal1* transcription by REV-ERB and ROR clock proteins. BMAL1/CLOCK activates the transcription of *Rev-erb* and *Ror*, which products respectively repress and activate the transcription of *Bmal1*. These negative and positive regulation loops provide original gene expression rhythms.

Circadian clock gene expressions in the skin have been reported in mouse and human (Tanioka et al., 2009). Several types of skin cells, such as fibroblasts, keratinocytes and melanocytes, display circadian oscillations in clock gene expression indicating that the skin clocks are cell-autonomous (Sandu et al., 2012). Since transgenic animals have been established by fusing *luciferase* genes to clock gene promoters, it has become possible to measure circadian gene expression in different tissues and cells by luminescence recording. Skin biopsies from transgenic rodents can be used for preparing primary cell cultures but melanocytes and keratinocytes, unlike fibroblasts are difficult to isolate and maintain in culture. Brown and his colleagues have successfully established a lentivirally delivered luciferase reporter to measure clock gene activities in human skin cells (Brown et al., 2005b). To facilitate our studies on multioscillatory skin, we constructed a *Bmal1-luciferase* lentivirus according to Brown's design, in order to study circadian oscillations within human keratinocytes and melanocytes.

3. Materials and methods (Fig. 1)

3.1. PCR

To amplify the different fragments meant to constitute the luciferase cassette of the vector, (SV40 late Poly Adenylation Signal (PAS), *Bmal1* promoter, firefly *luciferase* and *Bmal1* 3'UTR), we used the Polymerase Chain Reaction (PCR) technique with corresponding primers designed specifically. Phusion polymerase (Thermo Scientific) was used because of its high proofreading capacity allowing minor errors during amplification. When Phusion polymerase was ineffective, Pfu polymerase (Euromedex) was used. The PCR programs were as follows: for Phusion, initial heating at 98 °C for 2 minutes, followed by 35 cycles of denaturation at 98 °C for 10 seconds and annealing at 71 °C for 20 seconds and elongation at 72 °C for 30 seconds, final elongation at 72 °C for 5 min. For Taq polymerase and Pfu: initial heating at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 1 min and annealing at 56 °C for 1 min and elongation at 72 °C for 2 min, final elongation at 72 °C for 5 min. Genomic DNA was prepared from tail biopsies of C57Bl6 mice. The fragments of SV40 late PAS and firefly luciferase were amplified from pGL3 plasmid (Promega). SV40 late PAS, *Bmal1* promoter, luciferase and *Bmal1* 3'UTR fragments were purified, enzymatically digested and eventually ligated into pBluescript SK+ vector (Stratagene). Primers were designed to contain restriction enzyme sites for further oriented cloning into the cassette, like below: SV40 late PAS (forward 5'GCC TCG AGC AGA CAT GAT AAG3', reverse 5'AAG GAG CTG ACT GGG TTG AAG3'), *luciferase* (forward 5'TGG AAT TCC GGT ACT GTT GG3', reverse 5'CCG GAT CCT AGA ATT ACA CGG3'), *Bmal1* promoter (forward 5' GAT CGA TAG GGC TAC AAC AGA ACA ACT AA C3', reverse 5' TCT GAA TTC CCT AAA TGT CGC ACC AC3'), *Bmal1* 3'UTR (forward 5'CGG ATC CAC ACT ACA TTT GCT TTG GC3', reverse 5'TCT CGA GTA GGA GCA TAC CTG TAG GG3').

3.2. Subcloning

Specific restriction enzyme sites at 5’ and 3’ ends of PCR fragments were introduced by primers to facilitate further subcloning of these fragments into pBluescript SK+ which contains multiple cloning sites and was used as an intermediate vector. The fragments of *Bmal1* promoter and *Bmal1* 3’UTR were amplified from the genomic DNA of mouse with Pfu polymerase and polyA 3’ ends were added with Taq polymerase at 72 °C for 30 min. These fragments were then ligated into another intermediate vector specifically designed for the easy cloning of A-tailed PCR fragments: PCR2.1 (Invitrogen). The fragments of SV40 late PAS and luciferase were amplified from pGL3 plasmid, digested with appropriate restriction enzymes and directly ligated into pBluescript SK+ vector. The fragments of *Bmal1* promoter and *Bmal1* 3’UTR were digested from the pCR2.1 subclones and then subcloned into pBluescript SK+ vector. Each fragment with two cloning sites was digested by one restriction enzyme at 37 °C for 1 hour followed by the purification by gel electrophoresis and then the other digestion was run in the appropriate buffer. Ligation with T4 DNA ligase was performed at 14 °C for 6 h and products were then transformed into NovaBlue competent cells (Millipore). Positive clones were amplified and selected based on digestion with restriction enzymes. Individual positive clones were sequenced to check for the absence of mutations in the target PCR fragment, by LGC Genomic service.

Once the cassette comprising the 4 fragments was constituted inside the pBluescript SK+ vector, the whole fragment was excised by XhoI digestion and cloned into the pWPI lentiviral vector (Addgene). The pWPI vector was first linearized by Xho1 digestion (this removed the GFP marker gene from the vector), followed by treatment with shrimp alkaline phosphatase (SAP) at 37°C for 30 min (inactivation 10 min at 65°C) to remove the phosphate groups on the 5' ends and prevent self-ligation of the linearized pPWI. The final pWPI-Bmal1-luciferase construct was checked for the right orientation by digestion with multiple appropriate restriction enzymes.

4. Results

4.1. Strategy of lentivirus construction

We designed the construction of lentiviral *Bmal1-luciferase* tool according to the publication of Steven Brown and his colleagues in PLOS Biology 2005 (Fig.1). The presence of the late SV40 polyadelylation signal aims at putting a stop to the transcriptional unit located just upstream of the cassette, and which is driven by the promoter of the EF1α gene, already present in the pWPI vector. This should also attenuate the influence of transcription driven by the viral Long Terminal Repeat sequence located at the 5' end of the integrated cassette. Regarding the *Bmal1* promoter fragment, we chose to amplify the -968 / +67 region, that was meant to contain most regulatory elements and transcription start site (based on genome annotation at the time of primer design). Regarding 3'UTR fragment, we chose a 970 bp region starting right at the stop codon. The fragments of SV40 late PAS and luciferase would be amplified from the plasmid pGL3, while *Bmal1* promoter and 3'UTR from mouse genomic DNA.

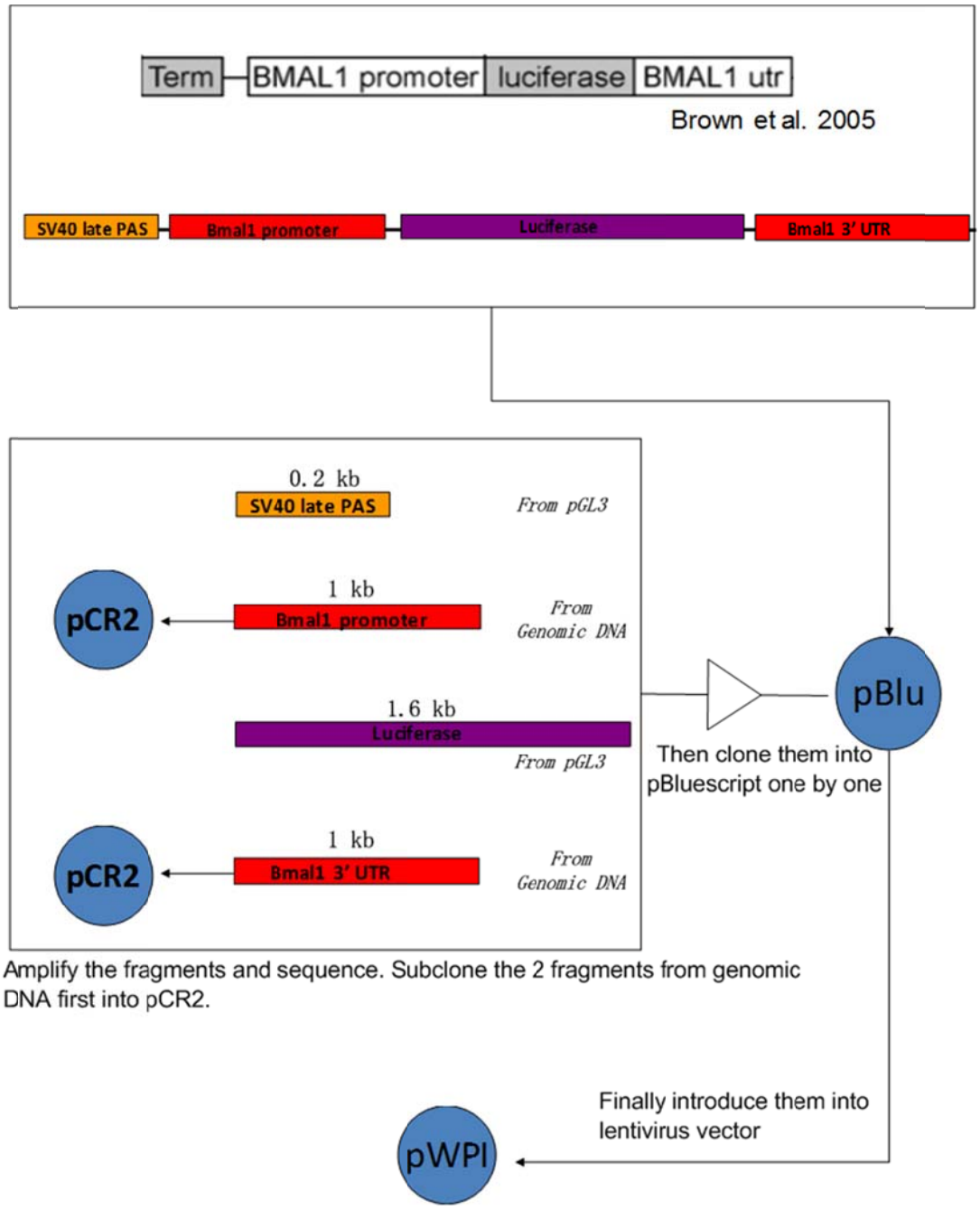

Figure 1: Strategy for the construction of the lentiviral pWPI-Bmal1-luciferase vector. pBlu: pBluescript SK+

4.2. Construction of the pWPI-Bmal1-luciferase

We cloned pWPI-Bmal1-luciferase as designed before (Fig. 2) and checked it was in the right orientation by multiple enzymatic digestions (Fig. 3). The final size of pWPI-Bmal1-luciferase is 13197 bp with the size of the Bmal1-luciferase insert being 3932 bp.

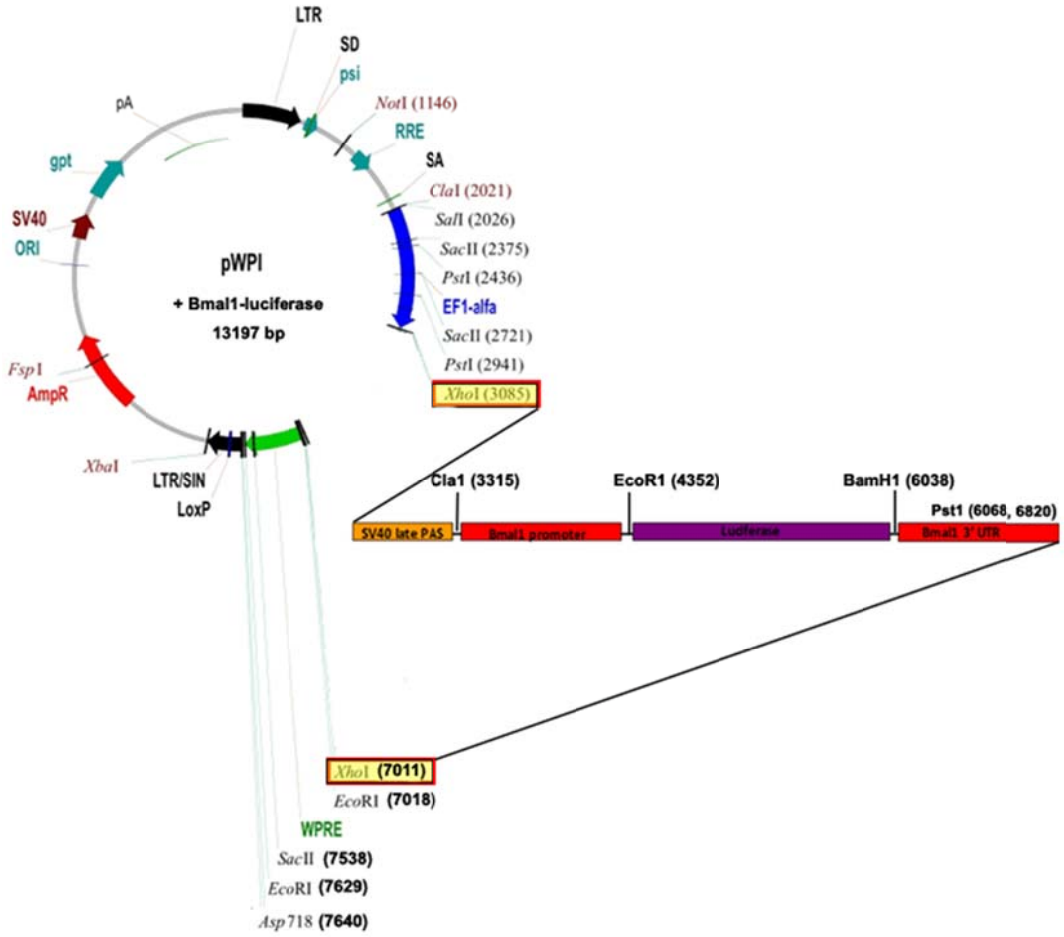

Figure 2: Construction of the pWPI-Bmal1-luciferase vector

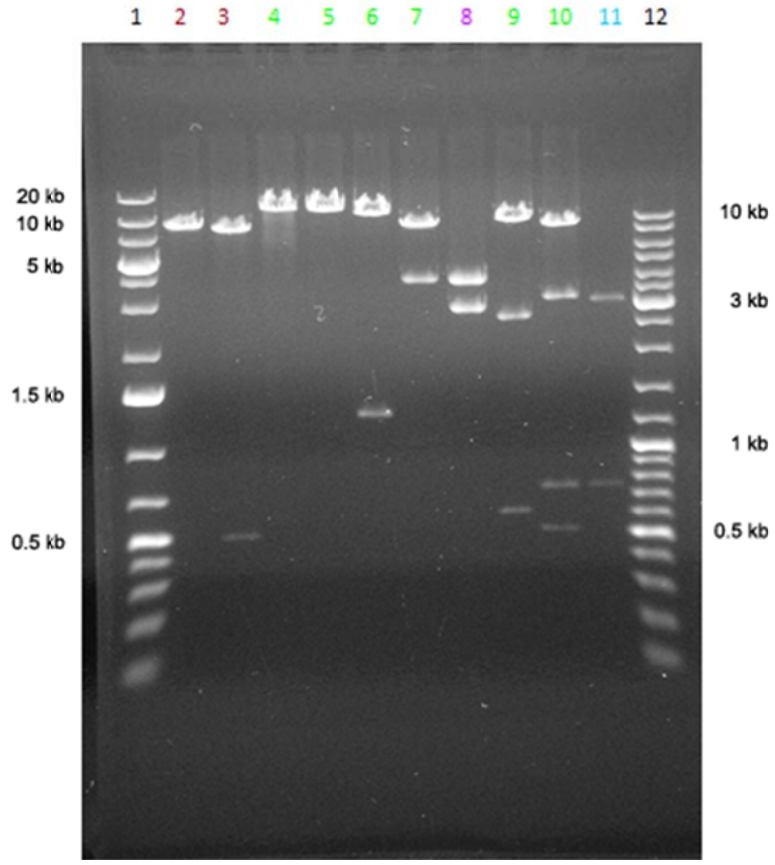

Figure 3: Restriction enzyme digestions of pWPI-Bmal1-luciferase and self-ligated vector for final validation

1. 20 kb DNA ladder
2. pWPI vector without XhoI-XhoI fragment (self-ligated vector): Xho1
3. pWPI vector without XhoI-XhoI fragment: Pst1
4. pWPI-Bmal1-luciferase: Not1
5. pWPI-Bmal1-luciferase :BamH1
6. pWPI-Bmal1-luciferase: Cla1
7. pWPI-Bmal1-luciferase: Xho1
8. pBluescript-Bmal1-luciferase: Xho**1**
9. pWPI-Bmal1-luciferase: EcoR1
10. pWPI-Bmal1-luciferase: Pst1
11. insert Bmal1-luciferase: Pst1
12. 10kb DNA ladder

4.3. Bmal1-luciferase lentiviral transduction in human skin cells.

The lentivirus was produced by transfecting the pWPI-Bmal1-luciferase vector into 293T cells together with packaging vectors psPax2 and pMD2.g (Addgene): this was performed at the Plateforme de Vectorologie, Ecole Normale Supérieure de Lyon. The resulting virus was used as crude culture supernatant and tested on human skin primary fibroblasts and was compared with the one originated from the lab of Steven Brown, which plasmid we obtained in between and which was produced in parallel. The bioluminescence recordings showed oscillations with limited amplitude with respect to the construct from Brown's laboratory (Fig. 4), indicating that its efficiency needed to be improved.

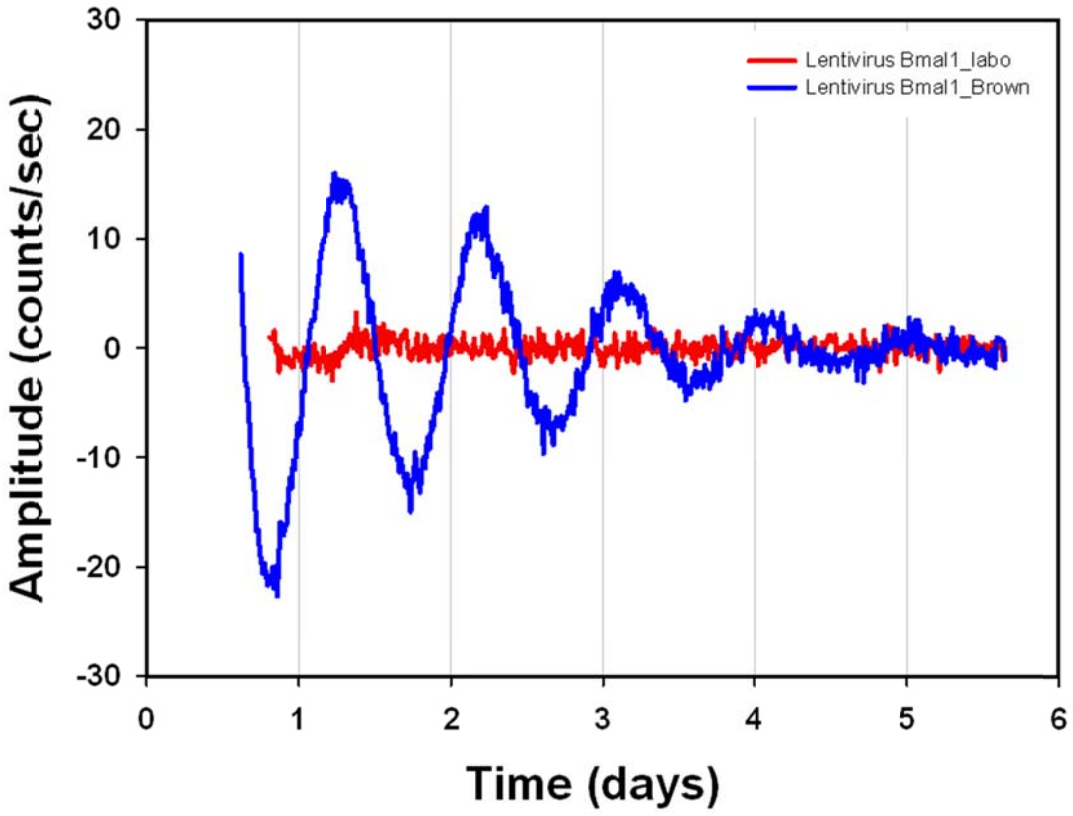

Figure 4: Bioluminescence of Bmal1-luciferase lentivirally transduced human skin fibroblasts.
The human skin fibroblasts were infected by lentiviral Bmal1-luciferase tool that originated from our laboratory (red) and from the laboratory of Steven Brown (blue).

5. Discussion

Core clock gene regulatory elements have been used separately in the context of luciferase fusion constructs, to provide bioluminescence to measure circadian clock activity, both in transfected cells and in explanted tissues from transgenic animals (Yamazaki et al., 2000; Yoo et al., 2004). Many constructs of luciferase reporters driven by clock gene promoters such as *Per1* promoter, *Per2* promoter and *Bmal1* promoter have been successfully used to monitor clock gene activities in a numerous types of samples (Yamazaki et al., 2000; Nagoshi et al., 2004; Welsh et al., 2004; Yoo et al., 2004; Brown et al., 2005b; Brown et al., 2008; Yeom et al., 2010; Noguchi et al., 2012; Saini et al., 2012).

Besides tissues, it is convenient to measure clock-driven bioluminescence in cells cultured from transgenic animals. In cell lines such as rat-1 fibroblasts and NIH3T3 fibroblasts, or primary cells derived from human, introduction of luciferase reporters is required and have widely taken advantage of delivery via chemical and viral approaches. Plasmids are easily used to transfect cell lines though exogenous DNA is not integrated into the nuclear genome. Dilution or degradation of the introduced DNA through mitosis following transfection can be avoided by a selection process (e.g. antibiotic resistance encoded by the plasmid) so that a stable transfection can be accomplished with the characteristics of good luciferase activity and good clock gene oscillations. However, with human cells, a usually limited amount of primary cells are available and not easy to transfect. In addition, given the small number of cells, selection is difficult to perform and so is the stable transfection. Lentiviral infection has advantages over other transfection methods including infection of dividing and non-dividing cells, with high copy number, long term stable expression of a transgene and low immunogenicity. Thus, lentivirus is a good alternative for human primary cells. In addition, since the provided titer is usually high enough, it is quite possible to get a high percentage of transduced cells.

With the published Bmal1-luciferase construct, most reports did not explain plasmid construction in detail. However, the exact content in *Bmal1* promoter part probably plays an important role. It is proved that the core promoter of *Bmal1* gene is located upstream

of the transcription start site between -146 and -17 bp, and that important cis-acting regulatory elements are present in the region from -428 to -146 bp (Yu et al., 2002). Several elements such as three SP1, one AP1, and eight NF-Y binding sites are found within the -428 to -17 bp (411 bp) region. Two E-boxes are also found in the 411 bp region, although they do not perfectly match the CACGTG consensus sequence (Yu et al., 2002). Furthermore, REV-ERB and ROR regulate the transcription of *Bmal1* by binding to RORE sequences. Two adjacent ROREs are found in the 1st exon of *Bmal1* gene (+36 and +72) and they are required to recruit co-activators or co-repressors in close proximity to ensure transcriptional regulation (Preitner et al., 2002). In the *Bmal1* promoter, the proximal and distal ROREs are separated by 25 bp, and both are required to recruit co-regulators to modulate *Bmal1* transcriptional activity mediated by REV-ERB and ROR (Yin and Lazar, 2005; Liu et al., 2007b).

In our Bmal1-luciferase construct, the *Bmal1* promoter part covers the region between -968 and +67, while it has been used in transfection plasmid between -816 and +99 successfully before (Yeom et al., 2010; Noguchi et al., 2012). The loss of second RORE perhaps contributes to the low amplitude rhythmicity and transcription level of our lentiviral Bmal1-luciferase reporter. It would be worth trying a construct that includes it.

Alternatively, the design of the lentiviral construct *per se* might also be involved in the low efficiency of the vector. Another lentiviral circadian reporter also containing Bmal1 promoter-luciferase-Bmal1 UTR has proven efficient in the study of human primary dermal fibroblasts (Brown et al., 2008). In this construct, two insulators (derived from chicken beta-globin gene) were inserted at both ends of the reporter cassette and this might improve its efficiency because it blocks the interference of other enhancers with the *Bmal1* promoter. These insulators can help to determine the set of genes that *Bmal1* promoter can influence and it is critical that the inducing or repressing mechanisms of *Bmal1* do not interfere with neighboring gene. In this way, the addition of insulators could help improve the Bmal1-luciferase activity in lentiviral transfected human primary fibroblasts.

General Discussion

In the last decades, the circadian system has been demonstrated as a hierarchical network that comprises numerous oscillators regulated by different zeitgebers. Within this network, the SCN displays an indispensable function that controls many other structures, directly or indirectly (Kalsbeek et al., 2006). These structures are regulated and coordinated with each other and within themselves. In spite of the diversity of tissues and cells with respect to physiological processes they are in charge of, all the paces are set at appropriate time of the day and evolve during the life time. To keep this coherent circadian network, many kinds of clocks in periphery possess specific patterns and responses to various time cues. How are peripheral clocks synchronized, from the generation of circadian molecular rhythms to the coherence among oscillators? Since the skin expresses circadian clock genes and plays multiple roles in the organism (Le Fur et al., 2001; Tanioka et al., 2009; Sandu et al., 2012), our goal in this thesis was to investigate peripheral clock properties and synchronization in the skin.

As one peripheral clock, the skin displays several properties, such as being multicellular, proliferative and multiple-influenced from endogenous and exogenous environment, so that it constitutes an interesting model to study synchronization in the circadian system, including through development and ageing process. Technically, the real-time bioluminescence recordings driven by *Per1* transcriptional activities constitute a useful tool to study the clock phenotypes. The skin exhibits its distinct role in the clock network and faces numerous zeitgebers. We showed the potential time-giver role of melatonin which has synchronizing effects on skin clocks, at least on the skin fibroblasts, suggesting new discussion in the field of the entrainment/synchronization of the circadian system.

1. Circadian clock in the skin

Skin has its distinct structure and function in the mammalian organisms, with many physiological activities regulated by a circadian clock. It is commonly considered that biological circadian clocks have several features such as self-sustained, able to be entrained or synchronized and temperature compensation. To clarify the circadian clocks in the skin, all these properties of circadian clocks will be discussed in detail, based on our results and those from the literature.

First, circadian clocks can sustain and produce oscillations in the absence of any external cues. This property is due to cell-autonomous oscillators which are genetically determined. The molecular transcriptional-translational feedback loops drive circadian paces endogenously. In whole mouse skin, ex vivo analysis showed that expression of core clock genes *Bmal1*, *Cry1*, *Per1/2*, *Rev-erbα* and *Dbp* sustain circadian rhythms under constant dark condition (Tanioka et al., 2009). Relative phases of gene expression patterns and that 4-8 hours phase delay between *Per2* mRNA and protein accumulation, are both consistent with the fundamental molecular feedback loops described to generate circadian oscillations at the level of transcription (Ko and Takahashi, 2006). In human skin, our lab has demonstrated rhythmic pattern of core clock genes transcriptions in the human keratinocytes, melanocytes and dermal fibroblasts (Zanello et al., 2000; Sandu et al., 2012). Thus, skin clock activity in the whole skin results from the circadian clocks present in multiple individual cells.

These molecular-driven cell-autonomous oscillations pass through the cell division, which may introduce a phase shift in the daughter cells because of various amounts of core clock proteins transmitted through division (Nagoshi et al., 2004). Skin is a self-renewing tissue in which multiple skin cells go through controlled and gated proliferation throughout the lifespan. Several mechanisms are involved in the regulation. For example, miRNAs regulate skin keratinocytes differentiation and other physiological process in elderly cellular protein expression (Schneider, 2012). A clock transcriptional regulator NONO has been reported to gate cell cycle in the skin (Kowalska et al., 2013).

Telomere shortening acts as a mitotic gate that prevents aberrant proliferation such as cancer (Buckingham and Klingelhutz, 2011). Among transcripts regulated in a daytime-dependent manner, Krüppel-like factor 9 (Klf9) was identified in human epidermal keratinocytes as a circadian transcriptional regulator with an expression highly sensitive to cortisol and with its protein bearing a strong anti-proliferative potential (Sporl et al., 2012). In epidermis, putative Klf9 target genes including proliferation/differentiation markers also show rhythmic expression in vivo (Sporl et al., 2012). Given the fact that many skin cells keep organized growth and daily proliferation in a controlled rate, the clock activity is quite possibly involved in this process of daily mitosis and stem cells differentiation (Janich et al., 2011; Janich et al., 2013). Resilience to cell division is essential in many skin physiological processes, such as terminal differentiation of epidermal keratinocytes, activation of hair follicle. The daily mitotic rhythmicity is one key aspect discriminating normal cells and immortalized (cancer) cells. As proliferative cells, skin fibroblasts are modulated by the internal clocks to keep the pace on the proliferation, which is also one factor that may cause intercellular desynchronization in vivo and in vitro. In vitro, this fibroblasts proliferation is serum dependent and might shift when fibroblasts are kept in the lumicycle with limited serum for several days.

In the central clock SCN, neurons normally do not divide, whereas in the periphery cell proliferation and differentiation is much more common for self-renew and self-repair. And these cellular activities are often gated by local clocks. Given the fact that many cells keep organized growth and proliferation which may introduce phase shift in divided cells, the synchronization of clock activity is quite possibly involved in this process. Rhythmic pattern can be passed through mitosis from mother to daughter cells (Nagoshi et al., 2004). In skin, keratinocytes differentiation is regulated by circadian clock and arrhythmia leads to disturbed tissue growth (Janich et al., 2011; Janich et al., 2013). In vivo, as proliferative cells, skin fibroblasts are modulated by the synchronized internal clocks to keep the pace on the proliferation. In vitro, loss of synchronization may cause intercellular asynchrony in cultures. Cultured human fibroblasts lose replicative capacity after approximately 50 population doublings, commonly termed the Hayflick limit, with a

half-life of only approximately 8 doublings. The relevance of either figure to survival of cells in the body is questionable, given that stem cells in some renewing tissues undergo >1,000 divisions in a lifetime with no morphological sign of senescence (Rubin, 2002). In another way, this could provide a easily arrhythmic model to study synchronization, for which skin primary fibroblasts proliferation is serum dependent and might phase shift when kept in the lumicycle with limited serum for several days.

Abnormality in the skin clock is intimately related to pathological phenotype. Melanoma, the least common form of skin cancer, is often lethal compared with non-melanoma skin cancers (Desotelle et al., 2012). Expression levels of clock gene mRNAs and proteins is reduced in human skin melanoma, which significantly associates with clinical characteristics like Breslow thickness and is likely caused by altered cellular composition (Lengyel et al., 2013).

Understanding the molecular basis of cell-autonomous oscillations provides a good insight into the skin circadian system. In fact, conventional methods to examine the circadian clocks in the skin can hardly reflect continuously the functionality of autonomous oscillators in the skin. In our study, the real-time bioluminescence recordings of *Per1* gene activity within the skin allowed the monitoring of the clock gene rhythmicity in the global view of skin multioscillatory structure. Among different biochemical methods used to measure circadian rhythms in vivo and in vitro, only bioluminescence recordings employed allow long-term and continuous monitoring of individual tissue samples, which greatly reduce the problems caused by variability among individuals. This long lasting recording is quite meaningful for peripheral oscillators like lung and liver which can be monitored during more cycles (Yoo et al., 2004). Subsequently, this method provides an insight into the rhythmic pattern of constituent cells without disturbing intercellular communication. It allowed us to study the synchronization between living cells. In the molecular clockwork, the *Per1* gene *per se* reflects the general activities of circadian clocks. The *Per1* deficient embryonic fibroblasts showed rhythms with short period in the culture (Pando et al., 2002). And *Per1* and *Per2* have been reported several times as immediate early regulators in the synchronization of circadian clocks in the SCN and rat-1 fibroblasts (Balsalobre et al.,

1998; Farre et al., 2005). In this respect, our *Bmal1-luciferase* lentivirus construct will offer an opportunity to investigate the clock of human skin in another view.

Circadian clocks are capable to be entrained or synchronized by daily external cues to keep harmony among themselves and with the environment. Light is the most chief zeitgeber in nature. Although it does not have direct influence on the skin clock, UVB has been reported to alter clock gene oscillation in human keratinocytes (Kawara et al., 2002). Not surprisingly, light exposure (3 h, broad spectrum fluorescent white light, 13000 lux) on abdomen and chest skin shows no effect on the circadian rhythms of serum melatonin, cortisol and thyrotropin in six subjects, which does not support the existence of extraocular photic regulation of the circadian rhythms in humans (Lindblom et al., 2000). The phase of the skin clock is delayed with respect to that of the SCN, as is the case for other peripheral clocks, which might offer the duration for synchronizing signals like neuronal or humoral to affect the skin clocks (Result Chapter 1 Fig. 5B) (Shigeyoshi et al., 1997; Yamazaki et al., 2002). In the SCN-lesioned animals, it was not possible to assess whether peripheral tissues did lose the circadian rhythms because of desynchronization between individuals (Tanioka et al., 2009). This indicated that SCN controlled signals were necessary to synchronize the skin clock between individuals. Our data (Result Chapter 1 Fig. 4B) proved that the oscillators of the skin from 10 day-old donor can been synchronized, which suggests that functional cell-autonomous oscillators exist and can be entrained in the complex of the skin.

An intrinsic feature of circadian clocks is temperature compensation which ensures that endogenous periods of circadian clocks are not altered by changing temperature across the physiological temperature range (Pittendrigh, 1993). The temperature coefficient Q10 is 2-3 for most biochemical reactions, but 0.8-1.2 in circadian rhythms (Sweeney and Hastings, 1960). It is unclear but possible that synthesis and degradation rates of core clock components are equally affected by changing temperature. Posttranslational pathways may get involved, for instance that enzymatic activity of CK1 toward circadian substrates such as PER2 is temperature insensitive, but temperature

sensitive toward non-circadian substrates (Isojima et al., 2009). In mammals, temperature compensation has been demonstrated in cell cultures like fibroblasts and SCN neurons, and tissues like SCN, lung and adrenal gland (Reyes et al., 2008; Dibner et al., 2009; Buhr et al., 2010). This property is crucial for phase coherence of peripheral clocks, for which cellular transcription rates can vary dramatically according to tissue (Dibner et al., 2010). Some interface tissues like skin, especially distal skin, are submitted to largely changing ambient temperatures, even in homoeothermic animals. In contrary to the distal skin, proximal skin gets close to the core body and has less difference in daily rhythms. At very low temperature like 32 °C which does not exist in physiological condition for the proximal skin, abdominal skin lost its rhythmicity. Even the temperature of the distal skin is not lower than 33 °C. However, in skin fibroblasts, circadian rhythms still exist at 32 °C, although the oscillations damp rapidly. In rat-1 fibroblasts, the circadian rhythm of *Per1* gene keeps its pace at low temperature, even at 28 °C (Izumo et al., 2003). And Q10 in rat-1 fibroblasts is 0.85-0.88, indicating that fibroblasts are even overcompensated (Tsuchiya et al., 2003). These data suggested that the loss of obvious rhythmicity at low temperature may be due to the loss of phase coherence rather than to the absence of individual rhythms. In fibroblasts, low temperature of 32 and 35 °C induced a large amplitude cycle in the first day. This might be correlated with the overcompensation reported at these temperatures. It might be that the enzymatic reactions involved in the quite conserved transcriptional-translational loops, get faster at lower temperature and lead to the high amplitude observed in the *Per1* gene transcription. This would indicate that skin clocks are temperature compensated in the physiological range of temperature, which helps keep its own rhythmic pace in the environment and that cellular oscillators are resilient to changes in temperature, independent of intercellular coupling.

2. Synchronization of peripheral clocks during the development and ageing

During the life span, the functioning of circadian clocks change since the young age to elderly. In human, babies spend a lot of time sleeping and get sleep early. It is found that the phases of sleep/wake cycles shift delayed at adolescence, perhaps due to modern life style and hormonal changes. Based on simple questionnaires, from adolescence onward, human circadian clocks shift advanced upon ageing. Between the ages of 20 and 80 years, the timing of sleep shifts an average of 2 h (Roenneberg et al., 2007). Behavioral and sleeping pattern changes are reported in subjects aged 50–60 years (Hofman and Swaab, 2006). In addition, the total amount of slow-wave sleep decreases as well as the period and cycles of rapid-eye-movement sleep (Espiritu, 2008). Disturbed sleep is an explanation for advanced phase of circadian clocks in elderly. In both of center and periphery, circadian clock gene activities and related functions have been described as a changing process due to maturation and ageing in entrainment and synchronization.

Skin *per se*, during postnatal development, gets mature and aged with large differences in its structure and physiology. Infant skin has thinner stratum corneum and smaller keratinocytes. During this long span of time, the internal and external environment around the skin change a lot together with the endogenous clockwork. In elderly, the skin does not only undergo aging because of endogenous factors, but also due to exogenous ones like direct contact with several environmental factors like UV irradiation. UVB exposure induces the increase in dermal collagen fibril diameter and elastogenesis (Carneiro et al., 2007). As a consequence, aged skin shows typical characteristics including wrinkles, dryness and loss of elasticity (Makrantonaki and Zouboulis, 2007). In addition, UVB alters clock gene oscillation in human cultured keratinocytes in addition to its effect on the expression of melatonin receptors (Kawara et al., 2002; Slominski et al., 2005b). Generally, “endogenous” skin aging includes decrease in proliferative capacity, DNA repair capacity and telomeres which are common in cellular senescence. Importantly, as a member of the multioscillatory network coordinated

by the SCN, the skin clock is under the influence of functional synchronization by direct and indirect signals (Tanioka et al., 2009). The entrainment of the SCN and the kinetics of its outputs acting as synchronizing signals gradually change during the lifetime and this potentially leads to changes in clock activities in periphery, including the skin.

First, in the postnatal age, development of the molecular clockwork may contribute to the ontogenesis of clock system. Developmental activation of transcription factor expression is involved in the specification and maturation of the SCN necessary for circadian behavior in the mouse (VanDunk et al., 2011). Epigenetic regulations such as DNA methylation on promoters of *mPer1* might contribute to turning on expression of clock genes during perinatal development (Ji et al., 2010).

Besides the skin, multiple rat tissues demonstrate that the various circadian clock activities change in phase and amplitude in early postnatal development. Peripheral clocks like liver and lung have been found rhythmic at P11 and P20, and their phases gradually shift until the adult stage is achieved, while the SCN remains in the same phase in this developmental period (Yamazaki et al., 2009). Such age-dependent and tissue-specific changes in the phase relationship among circadian clocks are quite possibly due to entrainment to tissue-specific time cues at different developmental stages (Nishide et al., 2013). In the circadian system, the SCN shows developing maturation of its role as a central pacemaker (Sumova et al., 2012). Photoperiodic entrainment of the molecular core clockwork in the rat SCN gets more and more effective in early postnatal development (Kovacikova et al., 2005). The rat retina is light-sensitive immediately after birth and gradually matures during early postnatal development before the circadian clock starts to control the response (Mateju et al., 2009; Mateju et al., 2010). At P10, the amplitudes of the rhythms in clock gene expression in the rat SCN get “adult-like” (Sladek et al., 2004; Kovacikova et al., 2006). The circadian activity rhythms of *Per1* and AANAT, the rate-limiting enzyme of melatonin synthesis in the pineal gland, can be observed as early as P4 and are gradually increased until puberty, which accurately reflects rhythmic output from the developing SCN (Deguchi, 1982; Reppert et al., 1984; Wongchitrat et al., 2012). Additionally, the nocturnal changes of *Per1* and *Aanat* mRNA levels in the rat pineal gland from P4 to adults are strongly correlated (Wongchitrat et al.,

2012). In the rat retina, the clock system begins to regulate the circadian AANAT expression after P14, whereas the light/dark cycle is able to induce its rhythmic expression quite earlier (Sakamoto et al., 2002).

Interestingly, diffusible paracrine signals play an important role in the relation between SCN and other nuclei, especially in early postnatal age when the SCN is still immature. Bidirectional projections among the SCN, arousal-related dorsomedial hypothalamus (DMH) and locus coeruleus (LC) are nearly absent at P2 but present at P8 (Gall et al., 2012). The SCN modulates behavior at the early postnatal age (P2) via humoral factors despite the relative lack of SCN connectivity with downstream structures. This humoral action diminishes at P8, which suggests an age-related decline in the SCN's humoral influence on sleep-wake behavior that coincides with the emergence of bidirectional connectivity among the SCN, DMH, and LC (Gall et al., 2012). Responsiveness of cultured SCN to medium change is much larger in pups than in adult mice, which may be due to immaturity of the structural organization in the pup SCN (Nishide et al., 2008).

Compared with adults, neonates are more strongly influenced by non-photic maternal cues including feeding, warmth and social contact before weaning. Maternal master clock and plasma melatonin rhythms play a master role on the fetal circadian systems possibly via fetal corticosterone rhythmic signaling (Mendez et al., 2012). Early postnatal maternal deprivation results in a decrease in total sleep and REM sleep, as well as significantly reduced melatonin levels in adult rats (Feng et al., 2012). Some adulthood effects of maternal deprivation in rat pups depend on time of day, length and ambient temperature during maternal deprivation (Yamazaki et al., 2005). Wild-type offsprings, fostered by *Clock* mutant mothers with fragmented patterns of nursing behavior during the light period, exhibit increased anxiety-related behavior in adulthood, which is coupled with reduced levels of brain serotonin at P 14 (Koinuma et al., 2013). The SCN of previsual newborn rabbits is entrained by nursing, where the rabbit is blind at birth and only visited by the mother to be nursed once every 24 h for about 3 min without much maternal interference (Caldelas et al., 2009). Given that the diurnal pattern of clock gene expression develops earlier in the olfactory bulb (OB) than in the SCN of newborn rabbits,

it is possible that the OB plays an important role in temporal regulation during pre-visual life in rabbits (Montufar-Chaveznava et al., 2012). This early entrainment system works in infants before the maturation or activation of the synchronization mechanism in adult.

Besides maternal care, external environment including light and ambient temperature, during early postnatal development has long term influence on the circadian clock system. Postnatal light experience can affect clock function and clock output in the adult mouse, as well as the SCN subpopulation like astrocytes (Canal et al., 2009; Smith and Canal, 2009). Adult CBA/J mice, which exhibit loss of rod and cone photoreceptors and blindness by weaning age, have higher numbers of ipRGCs, VIP positive cells in the SCN and enhanced light responses with a greater degree of light-induced FOS expression compared with CBA/N control mice (Ruggiero et al., 2010). However, these mice exhibit attenuated phase shifting behaviors, which indicate an auxiliary role for rods and cones in early postnatal development of the circadian system. Neonatal rats, exposed to low ambient temperatures (10, 20 or 30 °C) during 6 h of maternal separation in the early light phase from P1 to P5, show strongly delayed circadian clock in the SCN by about 12 hour in 10 °C group and moderately delayed by about 4 hour in 20 and 30 °C groups (Yoshikawa et al., 2013). Other factors yet unidentified might be also involved in maternal entrainment.

At young age, low amplitude rhythms might be meaningful because it is easier to reset to environmental perturbations, which is supported by mathematical modeling studies (Abraham et al., 2010; Colwell, 2011). Oscillators with lower amplitude typically shift more easily to a given stimulus. In elderly, it is been reported that a peripheral clock like the pituitary has a more dispersed phase, which is similar to our observations in the skin (Yamazaki et al., 2002). This indicated that the entrainment from external environment is less efficient and/or that synchronization of the peripheral clocks by the SCN is much less efficient in the elder age. It might be due to the structural and functional alteration in the SCN in elderly. In human, reductions in SCN volume and neuron number begin at 80 years of age (Hofman and Swaab, 2006). Hypertrophy of astrocytes and microglia in the aged SCN has also been reported (Deng et al., 2010). Aging is associated with reduced expression of specific clock genes in peripheral clocks,

but not systematically in the SCN (Yamazaki et al., 2002; Kolker et al., 2004). The endogenous period of the SCN is shortened in aged rat, although individual variation seems constant (Yamazaki et al., 2002). Mouse lemur, a primate, exhibits a shortened free-running period in aged animals compared to adulthood, with altered AVP and VIP rhythms (Aujard et al., 2006). In mice, circadian output declines at the level of neural activity rhythms in the SCN by aging (Nakamura et al., 2011a). In addition, aging led to decreased amplitude of firing-rate in dispersed SCN neurons and increased variability in the circadian waveform (Aujard et al., 2001). It has been reported that VIP mRNA levels in the SCN displayed a daily rhythm in young female rats which was absent in middle-aged animals (Kawakami et al., 1997). Ageing SCN also shows alterations in the expression AVP (Roozendaal et al., 1987). In addition, aged rodents exhibit decline of the SCN presynaptic GABAergic terminals (Palomba et al., 2008). In elder human post mortem SCN, the number and density of AVP/VIP-expressing neurons do not change but the number and density of MT1-expressing neurons do (Wu et al., 2007). Decreases in neurotransmitter production by the SCN and low amplitude of electrical activity might weaken the ability of the SCN to synchronize peripheral oscillators. Reversely, *Bmal1* deficiency results in premature aging phenotype in mice skin and *Clock* deficiency increase the age-related dermatitis in mice (Dubrovsky et al., 2010).

Abnormal clock functioning in elderly could be result from worse entrainment/synchronization, or more easily disturbed system, or both. Altered SCN functionality affects its ability to reset peripheral oscillators and drive damped downstream oscillators. A 6-h advance or delay in the light schedule leads to a disrupted resynchronization of the liver of two year-old Per1-luciferase rats compared with young rats (Davidson et al., 2008). Two year-old Per1-luciferase rats also showed altered rhythms in peripheral tissues (Yamazaki et al., 2002). Interestingly, transplantation of a fetal SCN into aged animals restored diurnal rhythm of corticotropin-releasing hormone (CRH) and locomotor activity(Li and Satinoff, 1995; Cai et al., 1997). These results confirm that the SCN plays a crucial role in aging and longevity.

Aging in the circadian system was first studied focused on alterations in the entrainment of the SCN. Aged mice are less sensitive to light entrainment than young

ones (Wyse and Coogan, 2010). Although aged mice do not exhibit alteration in retinal innervation of the SCN even with a small decrease (8-50%) in lens transmittance, they are 20 times less sensitive to the entraining light compared with young animals, which likely occurs within the SCN and/or photoreceptors (Zhang et al., 1996; Zhang et al., 1998). Some of the age-related changes in the response of the clock to a phase-shift stimulus can be reversed by implanting old animals with fetal SCN tissue (Van Reeth et al., 1993; Van Reeth et al., 1994; Turek et al., 1995). In aged rats, light-stimulation leads to decreased response of the immediate early genes in the SCN (Sutin et al., 1993). Ageing increases light absorption by lens crystallines especially of short wavelengths, and decreases pupil area, which results in progressive loss of retinal illumination (Turner and Mainster, 2008). A 10 year-old child has circadian photoreception 10-fold greater than a 95 year-old phakic adult, while a 45 year-old adult retains only half the daily photoreception of early youth (Turner and Mainster, 2008). Besides that, the responsiveness of the circadian clock to some neurochemical stimuli like GABA is attenuated with ageing (Biello, 2009). In the aged hamster, light-induced *Per1* expression is markedly reduced in the SCN, with a significantly longer delay to resynchronization, although in DD *Per1* shows similar rhythms as a young rat (Kolker et al., 2003). Still under discussion, the expression of *Per2* is impaired in the SCN of aged mice, while adverse evidence also exists (Asai et al., 2001; Weinert et al., 2001). These findings suggest that disruption of circadian rhythms in elderly may result, in part, from reductions in the sensitivity of the SCN to entraining signals.

Downstream circulating factors might contribute to the aged chronotype. Indeed, it was reported that the same skin primary fibroblasts exhibit shortened period length and advanced phase of cellular circadian rhythms, in the presence of human serum from older donors compared with young ones, which is caused by a thermolabile factor present in the serum of older individuals (Pagani et al., 2011). During aging, a major factor that has been implicated is the physiological decline of hormones (Goldman et al., 2011). Aging changes the amounts of hormone production and the corresponding target sensitivity. Melatonin oscillates daily and its amplitude decreases under aging with an earlier phase, which is considered a putative marker of the decline of the circadian clock (Bondy et al.,

2010). The aged SCN shows reduced responsiveness to melatonin (von Gall and Weaver, 2008). And melatonin may play a role in the altered regulation of SIRT1 and metabolism due to ageing (Hardeland, 2013). The adrenal cortex releases less aldosterone and cortisol hormones with aging. Circadian oscillation of cortisol dampened with reduced peak levels and increased evening levels in older people (Ferrari et al., 2001a). Interestingly, *Per1* expression in the oral mucosa increases with morning cortisol in elder women with low cortisol levels rather than young women with higher cortisol levels. This *Per1* response to cortisol increase seems to be sensitive at low cortisol levels rather than at higher cortisol levels (Olbrich and Dittmar, 2012). Hormones show multiple influences on skin physiology although potential hormone replacement therapy against skin ageing still remains controversial (Zouboulis and Makrantonaki, 2012).

Another robust phenotype associated with aging is energy metabolism. Appetite and feeding rhythms are known to be important for entraining peripheral oscillators like liver. Increases in body fat mass as observed in rats subcutaneous adipose tissue and increased insulin resistance are associated with aging. The nocturnal rise in circulating leptin levels of younger animals is attenuated in older animals, as well as in the primate rhesus monkey (Downs and Urbanski, 2006). The rhythms of hormones associated with metabolic function, such as insulin, corticosterone and prolactin, are disrupted in obese aged rodents. Interestingly, administration of these hormones at specific times of day mimicking the rhythms of the younger phenotype leads to metabolic characteristics of younger animals (Cincotta et al., 1993). Corticosterone secretion is altered with advancing age due to disruption of the diurnal rhythm in CRH (Cai and Wise, 1996b). Sustainable endogenous period of ~24 h in alpha MUPA transgenic mice (overproduce in many brain sites the urokinase-type plasminogen activator, uPA), which spontaneously eat less and live longer compared with their wild-type control mice, may contribute to their prolonged life span (Gutman et al., 2011). Due to masking effect of the environment, it is argued that circadian rhythm of body temperature varies with age, where, in comparison with adults, it is poorly developed in the neonate and deteriorates in the aged subject (Weinert and Waterhouse, 2007).

Finally, different tissues exhibit specific patterns of clock gene promoter methylation

and methylation frequency decreases significantly in older mice at the *Per1* promoter in the stomach and increased significantly in older mice at the *Cry1*, *Bmal2*, and *Npas2* promoters in the spleen (Zhang et al., 2013). This indicates that epigenetic regulation also play a role in ageing of circadian clock system.

3. Synchronization of the multioscillatory circadian system

In the mammalian circadian timing system, many oscillators are coordinated by the SCN, to adapt to external environment. There are many potential zeitgebers delivering the timing message to the entire clock network. Many signals, mostly pharmacological, including serum shock, forskolin, glucocorticoids, medium change and temperature changes have been shown to act as a synchronizer to the peripheral clocks(Balsalobre et al., 1998; Balsalobre et al., 2000; Yagita and Okamura, 2000; Buhr et al., 2010). Although some of them are controlled directly by the SCN, the signaling pathways are not completely clarified yet.

With the central clock in the SCN, the circadian timing system is composed of multiple oscillators organized in a hierarchical manner. Resetting stimuli can phase-shift individual cellular clocks. But individual cells need the coordination of an integral network that synchronizes to produce coherent rhythms (Shirakawa et al., 2000). In the SCN, the cell coupling network property is responsible for the precision of the output signals. Out of the brain, peripheral oscillators receive multiple signals via neuronal and humoral outputs from the SCN. To identify this signals in the peripheral target can server to specialize the synchronizing pathways and functions. And tracing the synchronization during development and ageing and comparing with the changing profile of signals and pathways, help understand how synchronization is done under various circumstances.

Among numerous peripheral oscillators, the skin is a good experimental model with the cyclic expression of clock genes. Regions of skin have been associated to different circadian functions, such as hair follicle for hair growth and keratinocytes for proliferation. Similar to many other peripheral cells and tissues, the skin and skin cells

possess molecular clockwork similar to that operative in the SCN. However, clock gene oscillations in the skin damp rapidly, even if they are cell-autonomous and self-sustained. The amplitudes quickly fade within a few days in skin and skin fibroblast cultures, contrarily to the SCN and retina. Furthermore, damped oscillations in cell cultures could be restarted by signals like medium change (Izumo et al., 2003). Given the fact that the skin in mice with SCN ablation is no longer well-coordinated and display large phase variation among individuals, fainting of skin oscillations is rather due to the loss of synchrony between cell-autonomous clocks in the absence of the SCN (Tanioka et al., 2009). This is in the opposition to the case of SCN slices that keep synchrony in culture. Generally, SCN-emanating signals are suggested to be necessary to synchronize the skin clocks, as well as other peripheral clocks, for the phase coherence among cells and tissues.

It is important to keep in mind that the synchronization does not force the circadian oscillation. Instead, it modulates the clocks which in turn drive all the other biological phenomena. Synchronization is a process similar to resetting delayed or advanced clocks every day, to keep it ticking reasonably well in coherence, which normally agrees to type 1 phase responsive curves. The synchronization of targets is usually dependent on the strength of the stimulus, such as the dose of drugs, amplitude of temperature cycles and intensity of light (Johnson, 1999). In addition, the efficiency of synchronizing strength is relatively determined by sensitivity and rigidity of the oscillatory system. Apart from cell-autonomous rhythmicity, coupling among oscillators affects both qualities, which discriminate clocks in the SCN and in the periphery. This suggests that coupling-induced rigidity in the SCN filters exogenous and endogenous noise to sustain a robust circadian oscillation. Medium change can show different effects: in cultured fibroblasts and *Clock* mutant SCN , type 0 PRC, in cultured liver tissue and pup SCN, type I PRC, and in adult wild-type SCN, no response (Nagoshi et al., 2004; Nishide et al., 2006; Vitaterna et al., 2006; Nishide et al., 2008). Distinct responses of SCN confirm that the magnitude of cell-autonomous rigidity is more crucial to that of cell-coupling rigidity to the synchronization.

In the circadian network, the SCN can be reset rapidly by shifts of light-dark cycle,

but resynchrony of peripheral oscillators can take over a week (Yamazaki et al., 2000; Kiessling et al., 2010). Contrary rhythms phenotypes in single neurons and populated/single fibroblasts, indicate, not only intracellular molecular mechanisms, but also intercellular coupling, make a difference between SCN and peripheral clocks, as well as among peripheral clocks. Differences in tissue structures and cell compositions cannot be ignored in the tissue-specific pattern of circadian clocks regarding synchronization. Molecular mechanisms inherent to the clock, such as posttranscriptional regulation and epigenetic regulation, doubtless play an essential role in the tissue-specific circadian clocks and synchronization. Cell properties, especially differentiation and proliferation, are involved in the complex of circadian system and synchronization, for introducing multiple variations and noises into the clocks. Inversely, circadian clock would control this cell growth process, by regulating the activity and expression of several critical cell cycle and cell cycle check-point-related proteins. Out of balance may lead to a series of pathologies including arrhythmia and carcinogenesis (Khapre et al., 2010).

Among numerous potential zeitgeber, melatonin is the one that we first demonstrate as a synchronizer to peripheral clocks. To get synchronized by melatonin, whose secretion precisely refers to environmental time, skin fibroblasts need to express its receptors, which is indeed the case for MT1 mainly (Sandu unpublished) (Slominski et al., 2005b). This phase-dependent effect of melatonin is consistent with the large amplitude rhythms of plasma melatonin levels and with the restricted window of melatonin effect on central clock (Pevet and Challet, 2011). However, melatonin shows no effect on the SCN slices in vitro, as compared to the skin fibroblasts (non-published result from the laboratory). The increased amplitudes that we observe at a specific phase suggest that interrelation between different oscillators is more intensified and that there is appropriate coherence of the fibroblast phase and the melatonin treatment timing. Whether this timing is consistent with the nocturnal release of melatonin is to be determined.

This melatonin effect as a synchronizer is relatively weaker than that of medium change, serum shock or glucocorticoid treatment but could yet be involved in the synchronization of peripheral clocks. Given the fact that a majority of peripheral cells are

proliferative, gradual phase resetting with relatively weak synchronizers is easier to adapt to and less risky regarding potential change in the molecular clock phase (Nagoshi et al., 2004). The glucocorticoid potency of dexamethasone is much higher than that of physiological glucocorticoids like cortisol (Adrenal Cortical Steroids, Drug Facts and Comparisons, 5th ed. St. Louis, Facts and Comparisons, Inc.:122-128, 1997). In addition in these treatments, pharmacological concentrations of dexamethasone or melatonin are enormously higher than physiological levels. Given that strength of time givers is closely related to their effect on types of oscillators, further studies are needed to demonstrate this relationship between the stimulus strength like dose of melatonin and targets like different cells and tissues.

In laboratory, several strong synchronizers are used to induce oscillations to phase-dispersed oscillators. They function via different pathways. Dexamethasone, a glucocorticoid receptor agonist, acts as a strong synchronizer to rat-1 fibroblasts and peripheral tissues, but not to the SCN (Balsalobre et al., 2000). Forkolin, which elevates cAMP level and enhance the activation of CRE elements, can induce circadian gene expression in rat-1 fibroblasts (Yagita and Okamura, 2000). Melatonin has been reported to inhibit cAMP synthesis, cAMP signal transduction cascade and CREB phosphorylation (McNulty et al., 1994; Brydon et al., 1999; Jones et al., 2000). Although they lead to different effect on the cAMP signaling, melatonin and foskolin have similar effects with respect to the increased level of oscillation amplitude.

Many synchronizing pathways are dependent on each other for similar rhythmic pattern: for example of feeding-dependent body temperature. Feeding rhythms could be a potent synchronizer of peripheral oscillators, especially in the liver, where even *Bmal1*, *Per1* and *Per2* are not essential (Damiola et al., 2000; Feillet et al., 2006; Pendergast et al., 2009a). Core body temperature is regulated by the SCN and simulated body temperature fluctuations can entrain the peripheral tissues like lung and cell cultures like rat-1 fibroblasts and NIH3T3 fibroblasts (Brown et al., 2002; Buhr et al., 2010; Saini et al., 2012). And exposure to inverted environmental temperature cycles of day and night, can reverse the circadian gene expression in the liver without affecting the central clock SCN (Brown et al., 2002). Along one pathway of synchronization, many factors can be

involved. Meal feeding interval influences the phase of mouse peripheral circadian clocks (Kuroda et al., 2012). The food, palatable or high-fat, has an influence on the sensitivity of the master circadian clock to changes in motivational states related to palatability of the food (Mendoza et al., 2008; Mendoza et al., 2010). These results suggest that the complexity of synchronization pathways may involve not only the diversity of signals but also intersections of transmitting routes.

As a whole circadian system, the entrainment or synchronization of the clocks to the external environment are achieved not only by the periodicity of physical light/dark signals but also by the periodicity of social signals involving perceptions with eyes, ears, nose and skin (Reinberg and Touitou, 1996). Many peripheral clocks are involved in responses to different non-photic time cues, such as odors (olfactory bulb), feeding-fasting cycle (liver), sound (ear) and immunological cues (white blood cells) (Amir et al., 1999; Goel, 2005; Berger, 2008; Vollmers et al., 2009). And the skin might also be involved via temperature, touching or hormone production like vitamin D. For example, in mice, scheduled exercise phase shifts the circadian clock in skeletal muscle and lung but not in the SCN (Wolff and Esser, 2012). Although details of physiological pathways that transduce environmental time information to the clock system may differ among various cues through various tissues in various species, the essential properties of entrainment are thought to be the same for all of them. Properties of time cues such as strength (e.g. light intensity) and spectral composition (e.g. wavelength spectrum) and their functional phases (e.g. morning or afternoon) have strong influences on the entrainment.

Given the fact that circadian system develops during maturation and ageing, the synchronizing signals, both exogenous and endogenous, may differ accordingly. For example, the hormone level during the life has changed largely in addition to the responsive capacities in corresponding targets. Most hormonal receptors are expressed in a tissue-specific pattern and can lead to large differences in responsive levels and downstream messages. Within a given individual, daily resetting cues can cooperate to deliver combined information for phase coherence among phase differentiated oscillators.

Different individuals at different stages might require different combinations of synchronizers.

In research, distinct biological models in different conditions have provided benefits regarding the study of synchronization by specific factors. For instance, rabbit pups ingest milk once a day and are a natural model of feeding synchronization. During nursing, several sensory systems receive information about properties of the food including the olfactory system, where olfactory bulb is synchronized during milk ingestion to anticipate feeding, but during fasting its oscillations perhaps are modulated by the SCN (Nolasco et al., 2012). Some animals live in colonies, such as bees and drosophila, with highly developed social structures requiring temporal synchronization, where social cues may thus be critical to the adaptive function of the circadian system (Bloch et al., 2013).

Regarding the present work, it needs to be noted that the used in vitro experimental models have some limits as models for the skin in vivo, notably concerning its physiological synchronization. First, the Per1 luciferase rat model may represent the clock system in nocturnal animals but not diurnal case like human. In addition, skin explants lose the physiological environment: both internal such as central clock and corresponding synchronizing signals, and external like UV. Finally, the primary fibroblasts culture, as a simplified model to study skin oscillators, undergo many influences in vitro such as medium conditions and manipulation of medium change. Thus, an improvement to the use of these experimental models could be to study skin clock and its synchronization with the help of the promising flow through system in the future.

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Annexes

Article in preparation:

Characterization of circadian rhythms in rat skin and derived dermal fibroblasts
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Characterization of circadian rhythms in rat skin and derived dermal fibroblasts

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Abstract

As a peripheral tissue localised at the interface between internal and external environment, skin performs functions which are critical for the preservation of body homeostasis, in coordination with environmental changes. Some of these functions undergo daily variations, such as temperature or water loss, suggesting the presence of time-keeping mechanisms. Rhythmic functions are controlled by a network of circadian oscillators present virtually in every cell and coordinated by the central clock located in the suprachiasmatic nuclei. At the molecular level, circadian rhythms are generated by conserved transcriptional-translational feedback loops involving several clock genes, among which *Per1* and *Per2* play a central role.

Here we characterized the postnatal development of clock activity in skin of the transgenic *Per1-luciferase* rat by real time recording of bioluminescence in explants and primary dermal fibroblasts, and found that skin contains an internal clock undergoing age-dependant transformation from early life to aging. By using primary dermal fibroblast cultures we also provide evidence that melatonin treatment displays phase-dependent synchronizing effect and that ambient temperature impacts on the oscillation pattern and the period with slight overcompensation.

Introduction

In mammals, numerous physiological and behavioural processes exhibit circadian rhythms that are controlled by an orchestra of molecular clocks in which the master clock of the suprachiasmatic nuclei (SCN), synchronised to astronomical time, sets the phase coherence between and within the peripheral clocks. At the molecular level, rhythmic gene expression is controlled by interconnecting transcriptional/translational feedback loops involving clock genes such as *Bmal1*, *Clock*, *Per1-3*, *Cry1-2*, *RevErbα*, *Rorα* and *Rorβ* (Ko et al., 2006).

Rhythmic clock gene expression and functions have been described in skin and derived cells types. Generally, rodent clock transcripts (nocturnal) are in anti-phase to the human clock transcripts (diurnal). For example, *Per1* and *Per2* have a peak at day-to-night transition in mouse or at night-to-day transition in human skin and oscillate in anti-phase with *Bmal1* (Tanioka et al., 2009; Sporl et al., 2012).

In 1991, Brown reported circadian rhythms in M-phase and S-phase of skin cell proliferation. In rodents, M and S-phase have a maximum in the morning and late night, respectively, while in human epidermis these rhythms are in opposite phase, the M-phase peaking in the early night and S-phase after midday. A more recent study showed that the temporal fate of murine and human epidermal stem cells is controlled by the molecular clock (Janich et al., 2011; Janich et al., 2013). Thus, the oscillatory expression of the core clock regulatory genes is disrupted when *Bmal1* is deleted. Circadian molecular clock was shown also to be involved in the regulation of the hair follicle cycle, a non-diurnal cyclic process (Lin et al., 2009; Plikus et al., 2013). It was also reported that the DNA repair rate in mouse skin exhibit daily rhythmicity, with a minimum in the morning and a maximum in the evening, indicating the control of the UVB-induced skin cancer by the circadian rhythm (Gaddameedhi et al., 2011) and suggesting that humans may also exhibit a daily rhythm in the susceptibility to develop UVB-induced skin cancer Gaddameedhi et al., 2011; Geyfman et al., 2012).

The autonomous molecular clocks are resilient to temperature changes (Pittendrigh et al., 1993). An interface tissue such as skin, localised between the external environment and internal milieu, is submitted to changes in ambient temperatures. It was described that in fibroblasts the temperature is overcompensated (Tsuchiya et al., 2003) which may be related to the synthesis and degradation rates of core clock components.

Synchronisation of circadian rhythms in peripheral tissues as well as in primary cell cultures (e.g. fibroblasts) was shown to be mediated via glucocorticoid signalling in a phase resetting manner (Balsalobre et al., 2000; Dickmeis, 2009; Pezük et al., 2012). Melatonin, the night hormone, can also act on specific receptors that are widely distributed in peripheral tissues, indicating that melatonin may modulate a variety of physiological processes including temporal organisation (Slominski et al., 2012). Chronobiotic effects of exogenous melatonin were observed when it was applied before the synthesis peak of endogenous melatonin, at day to night transition. (Slotten et al., 2002).

Molecular clocks might undergo maturation during development. SCN becomes rhythmic at embryonic (E) day 19 to 20 and seems to keep the same phase during postnatal development (Reppert and Schwarz, 1984, Ohta et al., 2002), while in peripheral tissues the molecular clock shows gradual maturation rates and phases until the adult stage (Yamazaki et al. 2009).

Using the in vitro real-time bioluminescence recordings, we studied the ontogeny of autonomous circadian rhythms in skin explants and primary dermal fibroblasts from *Per1-luciferase* rats. We also investigated the effect of temperature and melatonin treatment on dermal fibroblasts derived from young adult *Per1-luciferase* rats. Our data show robust circadian oscillations in skin and dermal fibroblasts from young adult to aged animals. We also observed a temperature effect on the period and amplitude of *Per1* oscillations and a phase specific effect of melatonin treatment on the amplitude of *Per1* oscillations. This work

provides a functional demonstration that the skin and derived dermal fibroblasts contain an intrinsic circadian clock able generate rhythmic activity when analysed *in vitro*.

Materials and methods

Animals

All experiments in this study were performed on *Per1-luciferase* transgenic rats (Yamazaki et al., 2000) provided by Dr M. Menaker. Males from ten day to two year old, born and reared in the Chronobiotron animal facility UMS 3415, Strasbourg, were housed in standard cages under 12h:12h light-dark cycles (light at 300 lux) with free access to food and water. Rats were handled according to the European Communities Council Directive of 24 November 1986 (86⁄609⁄EEC).

Skin explant cultures

Animals were euthanized with CO_2 (20%) in an air-tight box. The abdominal area was cleaned with 70% ethanol, making sure that the fur is soaked with 70% ethanol, and the fur was shaved. An abdominal skin sample (1-4 cm^2) was dissected carefully using sterile scissors and forceps, to avoid cutting the fat layer, and immediately placed in ice cold HBSS medium (Sigma Aldrich) supplemented with 40 µg/ml penicillin - 40 U/ml streptomycin, 4.2 mM $NaHCO_3$ and 10 mM HEPES until processing. After sampling, the skin biopsy was placed into a sterile 10 cm Petri dish with the epidermis side down and the hypodermis was removed from dermis using a sharp scalpel. Subsequently, the skin biopsy was rinsed 3 times with sterile HBSS medium. For the bioluminescence study, a 3 x 3 mm skin explants were cut using a sharp scalpel and immediately placed with dermis side down onto a semipermeable PTFE membrane (Millipore) into a 35 mm plastic dish (Nunclon) containing 1 ml of pre-warmed 10% FBS (Biowest)/bioluminescence recording medium [DMEM without phenol red (Sigma D2902) supplemented with 2 mM L-glutamine, 25 µg/ml penicillin - 25 U/ml streptomycin, 4.2 mM $NaHCO_3$, 10 mM HEPES, 25 mM D(+) glucose and 0.1 mM luciferin

(Promega)]. The remaining skin biopsy was placed into fresh ice cold HBSS medium until further processing.

Primary fibroblast cultures

From the remaining cleaned skin biopsy, a number of 20-25 explants of 2 x 2 mm were cut and placed with epidermis down onto a sterile 10 cm plastic dish. They were allowed 5 minutes to attach to the bottom of the dish and subsequently incubated with 4 ml of pre-warmed DMEM medium (Gibco) supplemented with 20% FBS, 2 mM L-glutamine, 100 µg/ml penicillin - 100 U/ml streptomycin in a humidified 37°C/5%CO_2 incubator. Medium was changed every third day to allow proliferation of the fibroblasts from the explants. After two weeks, explants were removed from the dish. The fibroblasts were eventually incubated with 10% FBS/DMEM medium until confluence and then split. For the bioluminescence study, $5x10^5$ cells of the obtained cell suspension were seeded into a 35 mm Nunclon plastic dish in 10% FBS/DMEM medium and allowed to grow until the confluent cell monolayer was formed. The remaining cell suspension was cryopreserved in DMEM medium containing 50% FBS and 10% DMSO until further experiment.

Bioluminescence recordings

Bioluminescence recordings were performed as described by Yamazaki and Takahashi, 2005. Bioluminescence was monitored using a Lumicycle luminometer (Actimetrics) equipped with 4 photomultiplier tubes (PMT) which allows simultaneous assessment of 32 cultures. The photon counts were integrated every 15 minutes for 112 seconds. Skin explant dishes prepared as above were sealed with vacuum grease (Dow Corning) immediately after preparation of the culture, placed into the Lumicycle and bioluminescence was recorded over several days. Confluent primary fibroblast cultures were incubated with pre-warmed 10% FBS/bioluminescence recording medium and sealed before being placed into the Lumicycle.

To ensure a constant temperature during the recordings, the Lumicycle luminometer was placed in an incubator. All recordings were performed at 37°C unless specified as in the study of the temperature effect at 32, 35, and 39°C.

Bioluminescence data analysis

Using the Lumicycle analysis software, the 24 h running average was subtracted from the raw bioluminescence data (3 to 4 cycles starting from the first trough which became t=0 in the graphical representations; Figure 1A) in order to remove the baseline drift. Detrended data were analyzed by non-linear least square regression with the help of SigmaPlot V12 (Systat Software Inc., San Jose, CA) and software R (www.r-project.org) (Sandu et al., 2012). Data for each recording were fitted to a cosinor derived sine wave function f = y0 + a*exp (-x/d)* sin [2*pi*(x-c)/b] were a is the peak-to-trough amplitude (of the first oscillation), b is the period (h), c is the phase (h) and d is the damping. Regressions were validated when all of the following conditions were simultaneously fulfilled: non-significant Shapiro-Wilk's test (homogeneity of residuals) and Bartlett's test (homogeneity of variances), significant P-value (F-test) for the global regression, significant P-values (t-test) for each of the parameters.

Differences in bioluminescence parameters between the groups were analyzed by using one-way analysis of variance (ANOVA) or repeated measures ANOVA (temperature study) and post hoc comparisons with the LSD Fisher test (Sigmaplot V 12).

Results are presented as mean ± SEM.

Melatonin treatment of primary fibroblasts

Confluent fibroblast primary cultures were submitted to bioluminescence recording for several days. After two complete cycles, the period of the 2 initial oscillations was calculated using the Lumicycle analysis software (Actimetrics) in order to predict: 1. the peak of the third oscillation (P) and 2. the time of four melatonin treatments during the third cycle: 9

hours before the putative peak (P-9h), 3 hours before peak (P-3h), 2 hours after peak (P+2h) and 9 hours after peak (P+9h) (Figure 1B). At the predicted treatment time, fibroblast cultures were removed from the Lumicycle and incubated at 37° with pre-warmed serum free bioluminescence recording medium in the presence of 50 μM melatonin (Sigma Aldrich). After 30 minutes, the medium was changed to pre-warmed 10% FBS/bioluminescence recording medium and the sealed culture dishes were transferred back to Lumicycle for further recording (at least 3-4 complete cycles). Control cultures (1/1000x ethanol) were treated similarly for each melatonin treated group. Cultures removed from the Lumicycle were carefully manipulated under dim red light.

Data obtained after melatonin treatment were subtracted (24 h running average) and analyzed by non-linear least square regression as described above. Period, phase, amplitude, and damping were calculated as ratios between the treated samples to the mean of the control samples within each treatment group. Statistical significance of the differences in bioluminescence parameters between the groups were analyzed by using one-way analysis of variance (ANOVA) followed by post hoc comparisons with the LSD Fisher test (Sigmaplot V 12).

Results

Our study aimed at understanding how the characteristics of the skin clock change during development and aging. We performed *in vitro* bioluminescence recordings of *Per1-luciferase* on rat skin explants and primary dermal fibroblast cultures. We also studied the effect of temperature and melatonin on *Per1* expression in fibroblast cultures.

Ontogeny of *Per1* circadian rhythms in rat skin explants

We established explant cultures in order to analyse the global *Per1-luciferase* expression at different ages in the rat skin at 37° in vitro. Males at three different ontogeny stages were used: 1. pups of 10 days (n=8), 20 days (n=16) and 1 month (immediately after weaning) (n=9); 2. young adults of 2 months (n=7), 3 months (n=10) and 6 months (n=9); and 3. aged animals at 12 months (n=6), 18 months (n=7) and 24 months (n=4).

Recordings corresponding to the first stage of development did not show consistent oscillations. The fitted regressions at 10 days, 20 days and 1 month did not meet the analysis conditions and could not be further analyzed (Figure 2A, first line of plots). Starting with the age of 2 months until 24 months, sustained oscillations of *Per1* were recorded during several days (Figure 2A, second and third lines of plots).

We further determined the differences in parameters between the age groups (Figure 2B). Calculated periods were in the circadian range, between 22.44±0.59 h and 23±0.69 h, with no significant difference between the groups ($p=0.518$, one-way ANOVA). Similarly, no significant difference was observed for the phase (maximum of the first oscillation) ($p=0.370$, one-way ANOVA). A significant difference was determined by one-way ANOVA in the case of amplitude ($p=0.019$, one-way ANOVA). A highest value of 2001.81±275.25 counts/sec was obtained for the 6 months group, 2.1 fold higher than 2 month group and 1.45- 1.6 fold higher than the other age groups.

Regarding the damping parameter, a significant difference ($p=0.015$, one-way ANOVA) was obtained only between the 3 month (0.17±0.11 days) and 12 month (2.29±0.28 days) groups.

Per1 circadian rhythms in dermal fibroblasts

Rodent and human fibroblasts were shown to display rhythmic expression of clock genes as they harbour a circadian oscillator (Balsalobre et al., 1998; Brown et al., 2005). We established primary fibroblast cultures derived from rat skin explants in order to analyse the *Per1* expression during: 1. adult stage: 3 months (n=7) and 6 months (n=3); and 2. aged stage: 18 months (n=10) and 24 months (n=4). We obtained rhythmic bioluminescence recordings at 37°C for all age groups, showing robust oscillations (Figure 3A) with significantly different circadian periods ($p<0.001$, one-way ANOVA) which values were between 25.99±0.55 h at 3 months to 22.5±0.27 h at 24 months, with intermediate ~ 24 h periods at 6 and 18 months (Figure 3B).

Analysis of amplitude showed a highest level at 3 months 610.63±98.43 counts/sec ($p=0.002$, one-way ANOVA), which declined progressively during aging by 2.3 fold (Figure 3B). An opposite effect was observed for the damping parameter which significantly ($p<0.001$, one-way ANOVA) increased from 0.78±0.01 days at 3 months to 2.08±0.27 days at 24 months (Figure 3B).

Temperature effect

Fibroblasts obtained from the abdominal dermis of 3 month old rats (n=4) were submitted to real-time bioluminescence recording at four different temperatures - 32, 35, 37 and 39°C - in order to study the effect of environmental temperature on circadian expression of *Per1-luciferase*. Dermal fibroblasts exhibited circadian *Per1-luciferase* oscillations at all tested temperatures (Figure 4A). A significant variation in the means of the calculated periods was observed between the groups by repeated measures ANOVA ($p<0.001$), with lower values at

32°C (22.33±0.40 h) and 35°C (22.85±0.41 h) and higher values at 37°C (26.81±0.70 h) and 39°C (25.84±0.67 h) (Figure 4B). A statistically significant difference (p= 0.013, repeated measures ANOVA) was observed in the case of the amplitude parameter; the amplitudes of the faster cycling groups (32 and 35°C), were 2 to 2.7 fold higher than those of the slower cycling groups (37 and 39°C) (Figure 4B). There was no statistically significant difference between the temperature groups in the case of the damping parameter (p=0.349, repeated measure ANOVA) (Figure 4B).

Melatonin effect on *Per1* expression in primary fibroblast cultures

It was shown that melatonin can act via melatonin receptors in skin (Slominsky et al., 2005). Generally, the synchronizing effect of pharmacological melatonin is restricted to a specific time window corresponding to the day to night transition interval in diurnal and nocturnal rodents (Pitrosky et al., 1999). We compared the effect on *Per1-luciferase* expression of melatonin application at 4 different time points, P-9h, P-3h, P+2h and P+9h, during the third cycle of real-time bioluminescence recording in dermal fibroblast cultures (n=7-8 in treated and control groups). Period ratios (treated/control mean) showed values between 0.99 ± 0.007 to 1.01 ± 0.008, with no significant difference between the groups (p=0.246, one-way ANOVA). Similar non-significant differences were obtained for phase (p=0.173, one-way ANOVA) and damping (p=0.242, one-way ANOVA) ratios. In contrast, strong statistically significant difference was observed in the case of amplitude ratios (p=0.003, one-way ANOVA, the amplitude of the P+2h melatonin treated group increasing approximately 20-34% compared with the other three treatment groups.

Discussion

The aim of this study was to characterize the skin clock during postnatal development and aging in *Per1-luciferase* transgenic rats and to understand the effects of temperature and melatonin treatment on dermal fibroblast cultures.

Ontogeny in skin and fibroblasts

No circadian oscillations could be observed *in vitro* in the skin explants from rats at 10 to 20 days. *Per1* expression profiles had arrhythmic patterns, indicating that, although *Per1* gene is expressed at early ages, the molecular makeup of the clock is not fully developed or that the synchronization of individual oscillators is not yet achieved at the tissue level. However, we could observe a restoration of rhythmic expression of *Per1* by medium change after 7-10 days *in vitro* (data not shown). This might be explained by the presence of strong synchronizing signals in the serum that are able to act on the immature molecular clock of skin explants adapted one week in culture. Our results are in agreement with the literature: while intrinsic rhythms may appear in the rat SCN at E19-E21 (Reppert and Schwartz, 1984) and the master clock is fully developed at P10 (Weinert, 2005), peripheral clocks (pineal, heart, liver, lung, adrenal, thyroid) may undergo maturation during postnatal development (Sakamoto et al., 2002; Sladek et al., 2007; Ansari et al., 2009; Yamazaki et al., 2009; Christ et al., 2012; Polidarova et al., 2013) being entrained mainly by non-photic maternal cues until weaning (Seron-Ferre et al., 2007). We observed robust circadian oscillations in cultured skin explants starting at 2 month age, with a progressive increase in amplitude until the age of 6 months. These data reflect the sum of all functional oscillators present in the different skin cells types, keratinocytes, fibroblasts and melanocytes (Sandu et al., 2012) that act in a coordinated manner. Although the amplitude decreased and was eventually maintained at constant level, we recorded also autonomous oscillations in skin explants from aged rats (12-24 months).

Similar bioluminescence results were previously reported in other peripheral tissues of aged *Per1-luciferase* rats (Yamazaki et al., 2002). We observed also a faster damping starting at the age of 6 months. The decline of circadian rhythms was reported in central clock in aging mammals (Aujard et al., 2001; Nakamura et al., 2011) and, reciprocally, premature aging was promoted in clock gene deficient mice, such as *Bmal1* KO (Dubrovsky et al., 2010; for review see Yu et al., 2011).

After analysing the global *Per1* expression in skin explants, we further studied its circadian expression in primary dermal fibroblasts in order to understand their contribution to the temporal organization in *Per1-luciferase* rat skin. Our results indicate that intrinsic rhythmicity occurs in fibroblasts *in vitro* at all studied ages. While in skin explants *Per1* oscillated with a period of around 23 h and did not show significant differences between groups, dissociated fibroblast cultures showed slightly different rhythmic characteristics, with a long period of approximately 26 h in the 3 month group and shorter periods (22.5-24 h) in the 6, 18 and 24 month animals. The 3 month group fibroblasts showed also the highest amplitude and the fastest damping. This feature might be related to the degree of intercellular synchronization and the robustness of the molecular clock at different ages. Recent bioluminescence studies using Per2::Luciferase mice (Yoo et al., 2004) showed that rhythms in cultured fibroblasts become more coherent as they mature (O'Neill and Hastings, 2008) and that dissociated fibroblasts display periods that range between 22.5 and 27 h (Leise et al., 2012). It is not yet clear how robust rhythms are generated or why rhythms decline during aging. Noguchi et al., 2013 showed that diffusible paracrine signals rather than direct contact with neighbouring cells help to restore robust circadian rhythms in low density Per2::Luciferase tail fibroblasts cultured in the presence of conditioned medium from high density cultures. Pagani et al., 2011 demonstrated that the absence of yet unidentified

thermolabile serum factors (distinct from cortisol and melatonin) rather than alteration of molecular machinery are responsible for the desynchronization of rhythms during aging.

Effect of temperature

One important feature of circadian rhythms is the temperature compensation, the ability to maintain a relatively stable period of oscillation over a wide range of temperatures. This property indicates also the robustness of a circadian clock in varying environmental conditions. We studied the effect of temperature in the dermal fibroblast cultures maintained at 32, 35, 37 and 39°C and we observed sustained oscillations at all tested temperatures. However, despite the fact that calculated periods were in the circadian range in all temperature groups, we observed significantly longer periods at higher temperatures, 37 and 39°C, compared to low temperatures, 32 and 35°C, which may suggest that synthesis and degradation rates of core clock components might be different at some temperatures and do not behave exactly like NIH3T3 or tail fibroblasts in this respect (Izumo et al., 2003; Tsuchiya et al., 2003, Saini et al., 2013). We also observed that, while the damping rates were similar between the groups, the amplitude of the first oscillation at low temperatures was significantly higher, in agreement with the overcompensation-like mechanism observed in the case of periods. Altogether, our data indicate that dermal fibroblasts obtained from *Per1-luciferase* rats show a particular response to temperature *in vitro*. This might reflect particular properties regarding clock control with respect to the strong temperature fluctuations to which skin is exposed.

Effect of melatonin

Fibroblasts were the first non-neuronal cells shown to exhibit circadian rhythms when synchronized *in vitro* by a serum shock or dexamethasone (Balsalobre et al., 1999; Balsalobre et al., 2000) or by forskolin (Izumo et al., 2003). The hormone melatonin, synthesized and

secreted by pineal gland during the night both in diurnal and nocturnal species, is a strong output of the SCN. Plasma melatonin profile is a valuable marker of circadian phase. In mammals, melatonin acts via MT1 and MT2 receptors which are expressed in various peripheral tissues including skin (Slominski et al., 2005), representing a potential synchronizer of the local rhythms in skin cells. Here we investigated the effect of melatonin on *Per1* expression in dermal fibroblast cultures when administered at four different phases of a complete cycle. The reference time point in the experimental design was the peak of the third *Per1* cycle. *In vivo*, the maximum of *Per1* transcript expression coincides with the day-to-night transition (Tanioka et al., 2009). We found a significant 20% increase of *Per1* amplitude when fibroblasts were treated with melatonin 2 hours after the peak of *Per1*. This was in agreement with previous finding showing the chronobiotic effect of melatonin when administered at dusk (Pitrosky et al., 1999). Melatonin could thus modulate the amplitude of circadian rhythms in cultured fibroblasts. This is an important finding suggesting that melatonin might be used as a skin clock synchronizer by oral or topical administration in different pathologies reported to be associated with abnormal circadian rhythms of melatonin such as atopic eczema and psoriazis (Schwarz et al., 1988; Mozzanica et al., 1988).

Based on our results on *in vitro* expression of *Per1* in skin explants and cultured fibroblasts, we demonstrate that rat skin harbours an autonomous circadian clock that is fully functional starting at 2 months until 24 months and which period can be modulated by temperature. We show also for the first time that the amplitude of *Per1* rhythm and melatonin can be modulated by melatonin.

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Figure legends

Figure 1

Bioluminescence analysis of Per1-Luciferase recordings in skin and fibroblasts cultures.

A. Detrended bioluminescence recording (24 h moving average). Dashed arrow indicates the first trough of the detrended curve as the start of analysis.

B. Detrended bioluminescence recording representative for melatonin treated fibroblast cultures. Arrows indicate the time of the melatonin administration.

Figure 2

Per1 bioluminescence recordings in rat skin explants

A. Representative bioluminescence recordings over 4 days *in vitro* at different ages (d : day; m: month).

B. Variation of circadian parameters : period, phase, amplitude and damping during postnatal development and aging. Mean values ± SEM are shown (* = $p<0.05$).

Figure 3

Per1 bioluminescence recordings in fibroblast cultures derived from abdominal rat dermis

A. Representative bioluminescence recordings over 4 days *in vitro* at different ages (m: month).

B. Variation of circadian parameters : period, amplitude and damping in dermal fibroblasts from adult and aged animals. Mean values ± SEM are shown (* = $p<0.05$).

Figure 4

Effect of temperature on *Per1* bioluminescence recordings in fibroblast cultures derived from the dermis of 3 month old rats.

A. Representative bioluminescence recordings over 4 days *in vitro* at 32, 35, 37 and 39°C.

B. Variation of circadian parameters : period, amplitude and damping at different temperatures. Mean values ± SEM are shown (* = $p<0.05$).

Figure 5

Effect of melatonin treatment on *Per1* bioluminescence recordings in fibroblast cultures

Variation of circadian parameters : period, phase, amplitude and damping represented as treatment/control mean ratios at different phases of melatonin treatment (P : peak) Mean values ± SEM are shown (* = $p<0.05$).

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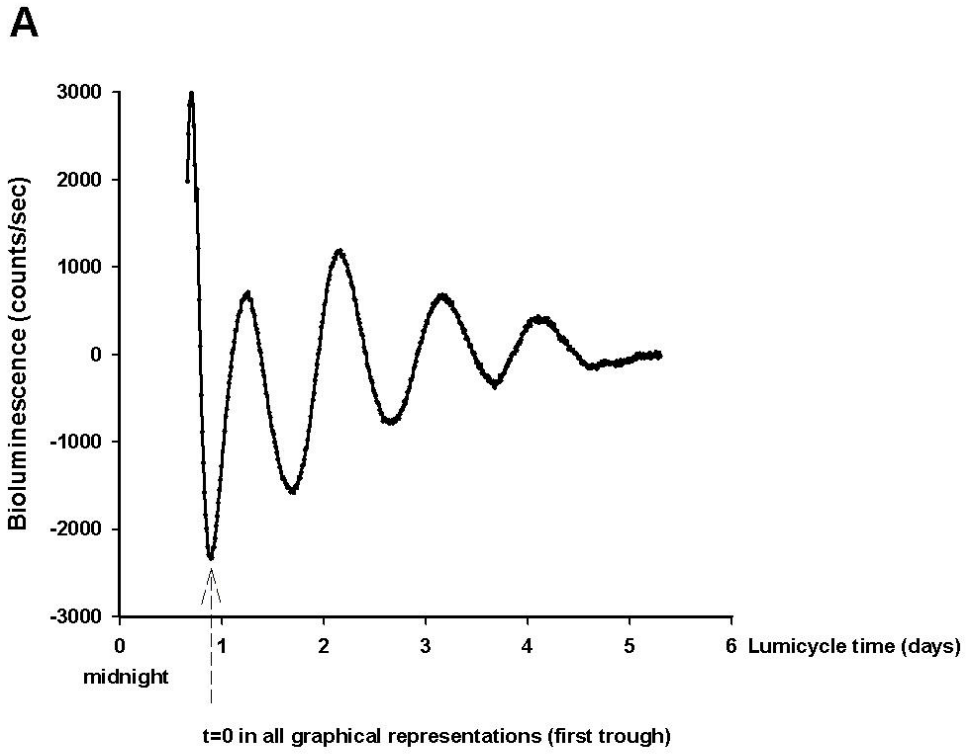
Figures

Figure 1



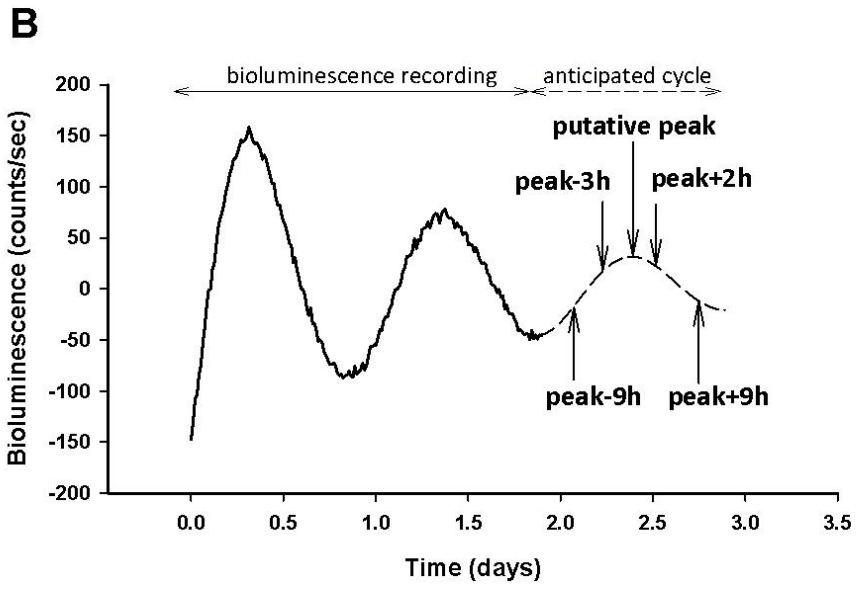


Figure 2

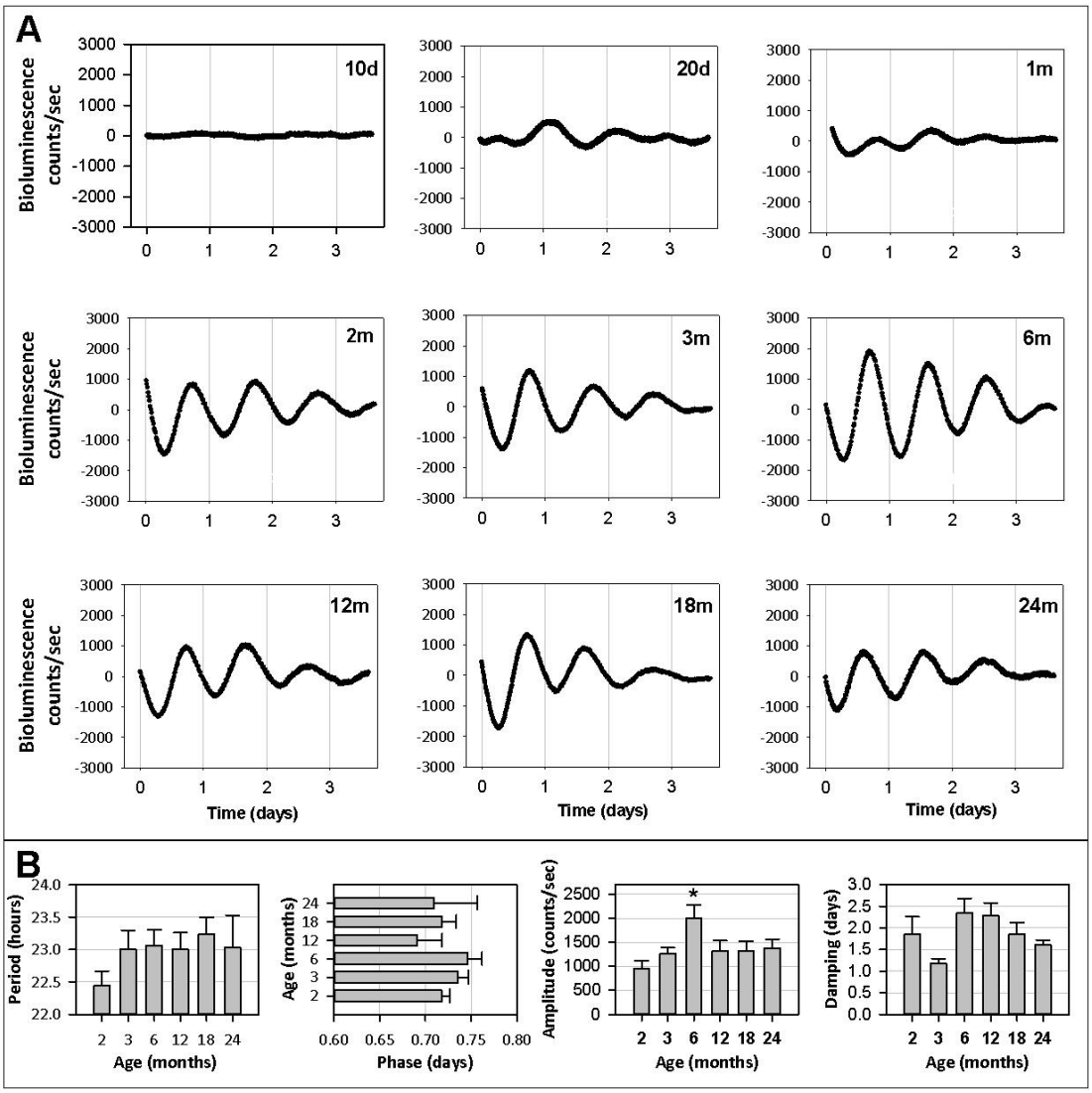

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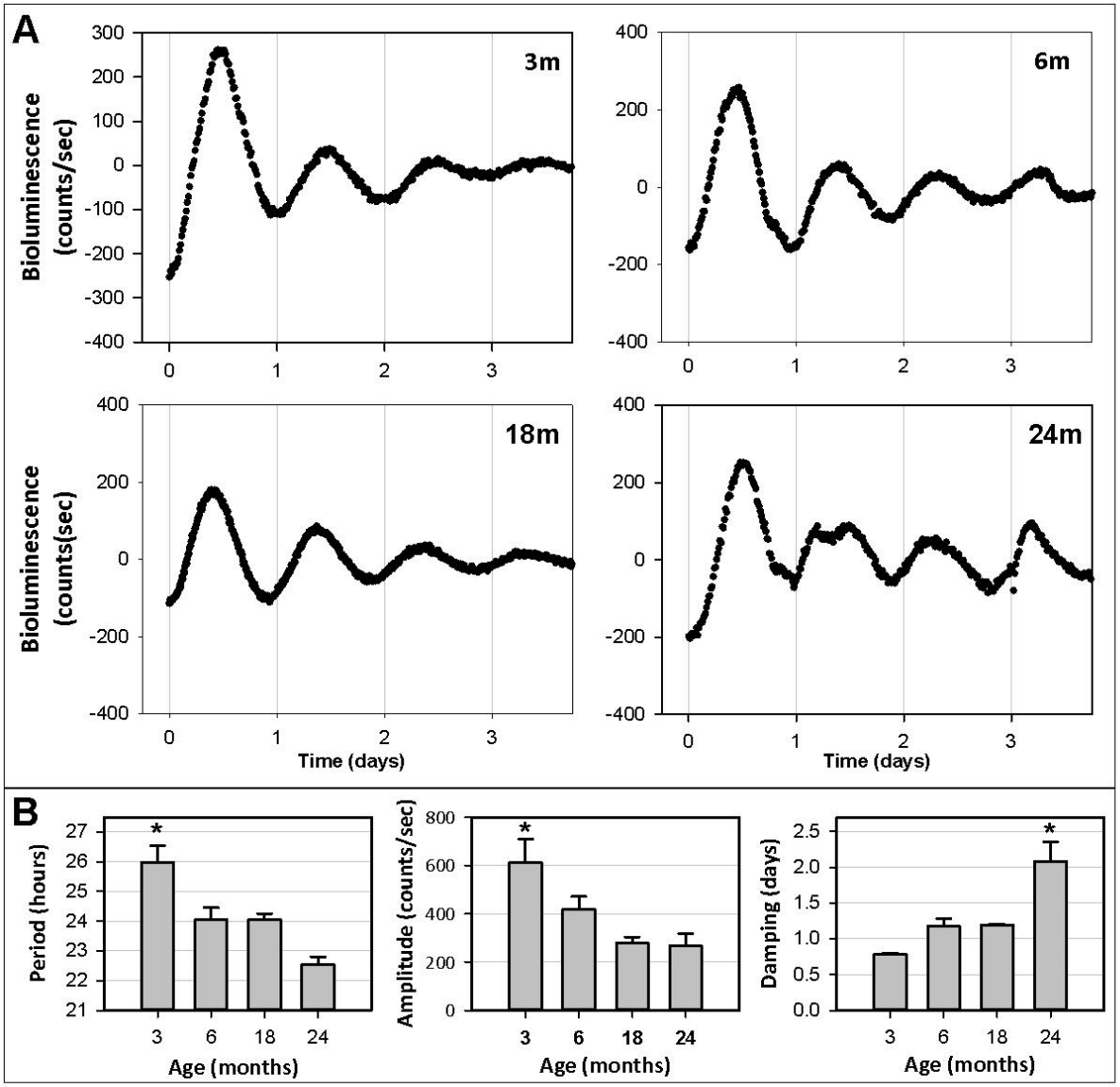
Figure 3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24

Figure 4

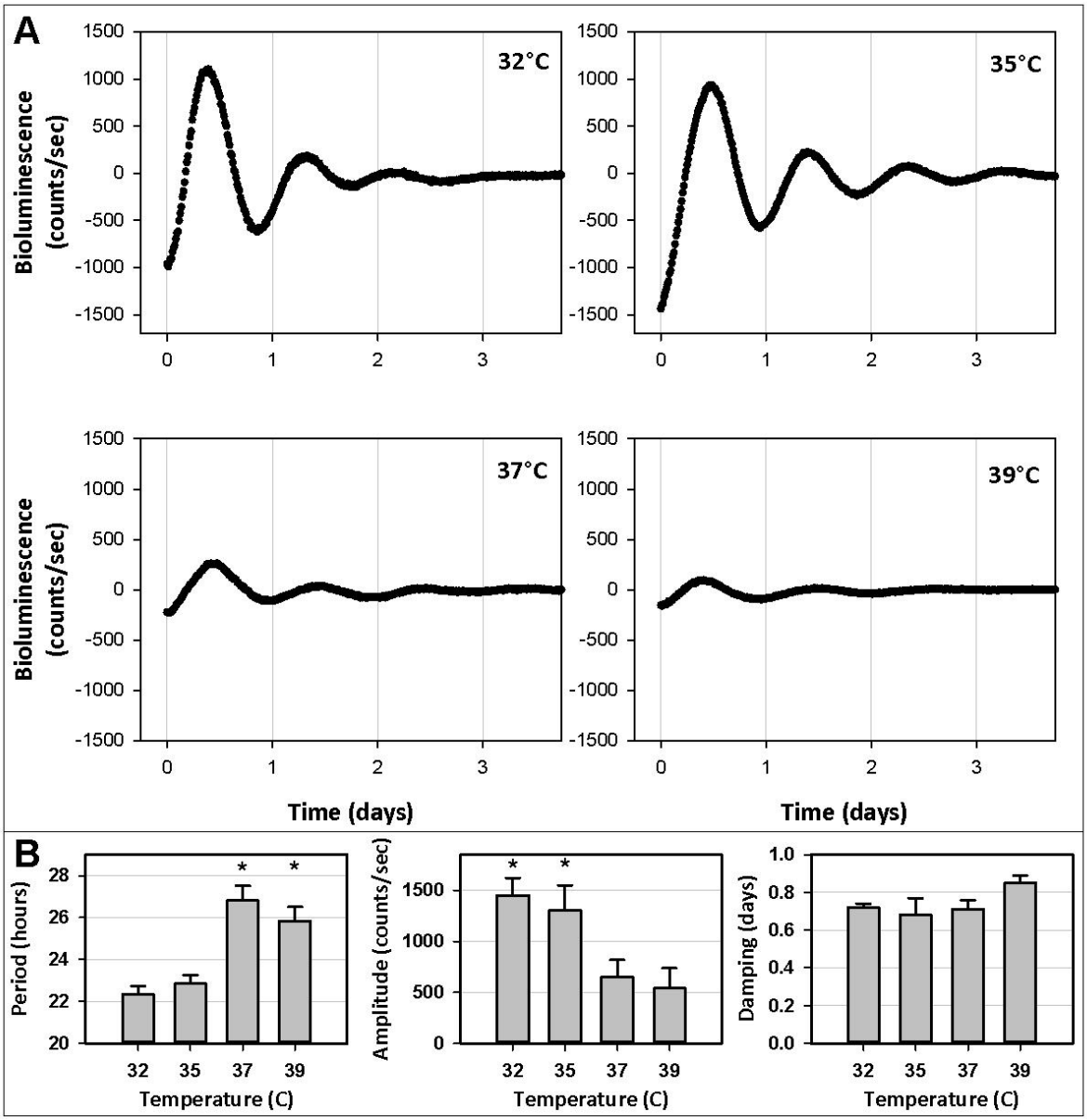
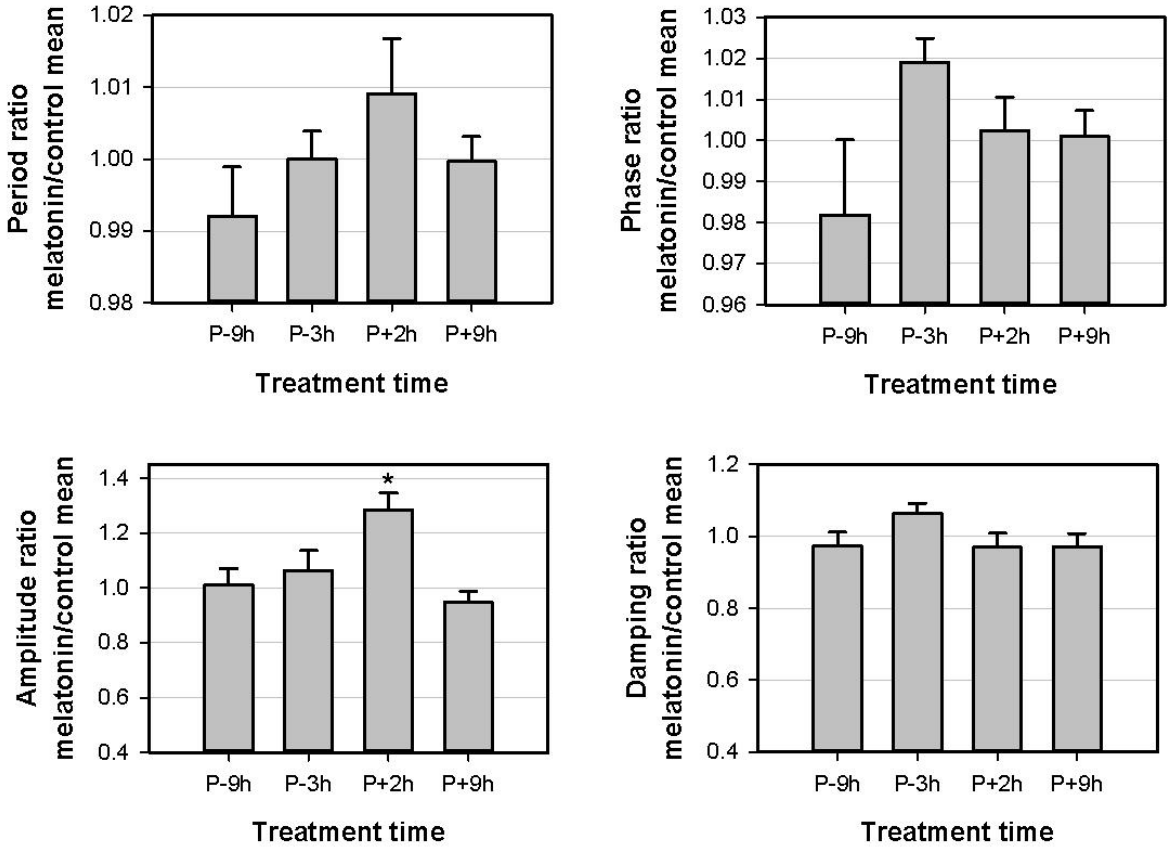

A
32°C
35°C
37°C
39°C
Bioluminescence (counts/sec)
Time (days)
B
Period (hours)
Amplitude (counts/sec)
Damping (days)
Temperature (C)
32
35
37
39

Figure 5


Period ratio melatonin/control mean
1.02
1.01
1.00
0.99
0.98
P-9h
P-3h
P+2h
P+9h
Treatment time
Phase ratio melatonin/control mean
1.03
1.02
1.01
1.00
0.99
0.98
0.97
0.96
P-9h
P-3h
P+2h
P+9h
Treatment time
Amplitude ratio melatonin/control mean
1.4
1.2
1.0
0.8
0.6
0.4
*
P-9h
P-3h
P+2h
P+9h
Treatment time
Damping ratio melatonin/control mean
1.2
1.0
0.8
0.6
0.4
P-9h
P-3h
P+2h
P+9h
Treatment time

LIU Taole

La peau, un modèle d'horloge périphérique

Résumé

Ce travail avait pour objet d'étudier les propriétés d'horloge et de synchronisation de la peau, un modèle potentiel d'horloge périphérique. L'activité rythmique a été analysée par bioluminescence en temps réel, sur des explants de peau abdominale et des fibroblastes dermiques primaires, isolés à partir de rats transgéniques *Per1-luciférase*.

Nous avons montré que des explants de peau présentent une activité rythmique soutenue en culture, indiquant une importante synchronisation interne dans le tissu. Cette synchronisation se manifeste au cours du développement post-natal à partir de 1 mois et augmente jusqu'à 6 mois, avant de décroître, laissant place à des rythmes altérés à l'âge de 2 ans. Nous avons aussi établi que les fibroblastes dermiques présentent la propriété de compensation thermique commune à toutes les horloges circadiennes, et qu'ils sont potentiellement synchronisables par la mélatonine puisque celle-ci augmente leur amplitude en culture. Nous avons aussi préparé un vecteur lentiviral exprimant le gène rapporteur luciférase sous le contrôle du promoteur du gène horloge *Bmal1*, un nouvel outil pour compléter l'étude des rythmes dans les cellules de la peau.

Mots-clés: *Per1*; *Bmal1*; Horloge périphérique; Rythmes circadiens; Synchronisation; Peau; Fibroblastes; Bioluminescence; Développement postnatal; Vieillissement; Compensation thermique; Mélatonine; Lentivirus

Résumé en anglais

This work aimed to investigate the skin as a potential model of peripheral clock by characterizing its rhythmic and synchronization properties. Circadian activity was examined in abdominal skin explants and fibroblasts derived from *Per1-Luciferase* transgenic rats by real-time recording of bioluminescence.

First, the skin clock was characterized from early postnatal to old age. Low amplitude oscillations appeared at 1 month only and their robustness increased until 6 months. In 1-2 year-old rats, skin circadian rhythms showed decreasing amplitude and abnormal cycles. Primary fibroblasts derived from the skin at the same ages demonstrated similar pattern of clock activity. Temperature compensation, an intrinsic clock feature, was shown the first time in skin and primary fibroblasts. Secondly, we demonstrated a phase-dependent effect of melatonin to increase the amplitude of oscillations in skin primary fibroblasts, indicating it displays a synchronising role in the circadian system. Finally, to facilitate our studies on the multioscillatory skin tissue, we constructed a lentivirus carrying a Bmal1-luciferase reporter, to measure clock genes activities in human skin cells.

Keywords: *Per1*; *Bmal1*; Peripheral clock; Circadian rhythms; Synchronization; Skin; Fibroblasts; Bioluminescence; Postnatal development; Ageing; Temperature compensation; Melatonin; Lentivirus