INTRODUCTION TO CHRONOBIOLOGY

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Введение в хронобиологию

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The International Committee on Nomenclature of the International Society for Chronobiology (ISC) in 1977 formally adopted the definition «chronobiology» as «Chronobiology: science objectively quantifying and investigating mechanisms of biologic time structure, including rhythmic manifestations of life». It also adopted the definition of «circadian» as «Circadian: relating to biologic variations or rhythms with a frequency of 1 cycle in 24±4 hours; circa (about, approximately) and dies (day or 24 hours). Note: term describes rhythms with an about 24-hour cycle length, whether they are frequency-synchronized with (acceptable) environmental schedules (24-hour periodic or other) or are desynchronized or free-running from the local environmental time scale, with periods of slightly yet consistently different from 24 hours» [Chronobiologia 1977; 4 (Suppl. 1), 189 pp.]. The beginnings of chronobiology as a discipline in its own right are recounted, with emphasis on the critical role played by Franz Halberg to provide all the needed ingredients for the new science to develop and strive, from gathering a critical mass of data in cooperation with colleagues worldwide to developing inferential statistical methods for their analysis and interpretation. By unveiling lawful variations within the physiological range, Halberg's clear vision that they have far-reaching implications for health and disease has been vindicated, now that a molecular mechanism of circadian rhythms has been documented and the role played by the suprachiasmatic nuclei and clock genes in the periphery continues to be better understood. Chronobiology, however, encompasses more than just circadian rhythms. Evidence is presented herein for the endogenicity of the about-weekly (circaseptan) rhythm, documented in unicells and early in human life. How they can be used to further optimize treatment timing is illustrated in a few examples, notably in relation to cancer.

Keywords: chronobiology, circadian rhythms, clock genes, suprachiasmatic nuclei, circaseptan rhythms

Международный комитет по номенклатуре Международного общества хронобиологии (ISC) в 1977 году официально принял определение «хронобиология» как «Хронобиология: наука, объективно количественно оценивающая и исследующая механизмы биологической структуры времени, включая ритмические проявления жизни». Она также приняла определение термина «циркадный» как «Циркадный: относящийся к биологическим вариациям или ритмам с частотой 1 цикл в 24±4 часа; circa (около, приблизительно) и dies (день или 24 часа)». Примечание: термин описывает ритмы с длиной цикла около 24 часов, независимо от того, являются ли они частотно-синхронизированными с (приемлемыми) экологическими расписаниями (24-часовыми периодическими или другими) или десинхронизированными или свободно протекающими от местной экологической шкалы времени, с периодами, слегка, но последовательно отличающимися от 24 часов» [Chronobiologia 1977; 4 (Suppl. 1), 189 pp.]. Рассказывается о зарождении хронобиологии как самостоятельной дисциплины с акцентом на ведущую роль Франца Хальберга, который обеспечил все необходимые компоненты для развития новой науки, начиная со сбора критической массы данных в сотрудничестве с коллегами по всему миру и заканчивая разработкой инференциальных статистических методов для их анализа и интерпретации. Открыв закономерные колебания в пределах физиологического диапазона, Хальберг ясно представил, что они имеют далеко идущие последствия для здоровья и болезни, и теперь, когда молекулярный механизм циркадных ритмов задокументирован, а роль, которую играют супрахиазматические ядра и гены часов на периферии, тщательно исследована. Хронобиология, однако, включает в себя не только циркадные ритмы. В обзоре представлены доказательства эндогенности околонедельного (циркасептанного) ритма, зафиксированного у одноклеточных и на ранних стадиях жизни человека. Как их можно использовать для дальнейшей оптимизации сроков лечения, показано на нескольких примерах, в частности, в отношении лечения рака.

Ключевые слова: хронобиология, циркадные ритмы, гены синхронизации, супрахиазматические ядра, циркасептанные ритмы

Introduction

Many years have passed since the time when circadian rhythms were referred to as Halberg's paranoia by his department head. Even before a molecular basis responsible for their manifestation could be unraveled, the study of circadian rhythms and chronobiology more generally had already made seminal inroads to document their critical importance at the organismic level, not only in medicine, but also in veterinarian sciences

and agriculture as well. Now that cellular and molecular mechanisms are studied in earnest in relation to all physiological systems, the bidirectional relationship between the circadian system and clinical health can no longer be disputed. To a large extent, the implications of circadian rhythms in health and disease stem from crosstalk between clock genes and molecular pathways related to metabolism and other important physiological functions. It is now time to integrate knowledge gained at the cellular and molecular levels to reap the benefits at the whole-body level, in terms of screening, diagnosis, and timed treatment, also aimed at primary and secondary prevention.

This review offers a glimpse at the development of the field from a historical perspective. It outlines some of the major contributions made by Franz Halberg at a critical time in the history of chronobiology. After all, «chronobiology» and «circadian» are terms coined by Halberg that would define the field. We briefly review the current state of knowledge about the suprachiasmatic nuclei, responsible for the orchestration of circadian rhythms. We also briefly review how clock genes, components of the circadian «clock», interact with each other in an intricate manner generating oscillations of gene expression. We explore the genetic basis of periodicities beyond circadian rhythms and ask whether the suprachiasmatic nuclei may be implicated in about-weekly (circaseptan) rhythms. Evidence is offered for the partly endogenous nature of circaseptan rhythms. Like in the case of circadian rhythms, there are spontaneous as well as response circaseptan rhythms that account for the hours (or days) of changing resistance, the foundation of chronotherapy. To conclude, some clinical applications illustrating the benefit that can be derived from scheduling treatment in accordance with both circadian and circaseptan rhythms are documented based on work accomplished with Franz Halberg.

Historical perspective

Since antiquity, keen observers have reported cycles in biology. Already in 650 BC, the Greek poet Archilochus of Paros wrote «Recognize which rhythms govern man» [1]. During the fourth century, Androsthenes of Thasos described the opening by day and closing by night of the leaves of the tamarind tree as the «nyktitropic movement» [2]. His book, which is apparently lost but was cited by Theophrastus [3], first reported on the fact that plants are capable of movement, a characteristic previously thought to be an attribute only of the animal world. Androsthenes believed that the daily changes from light to darkness rather than changes in environmental temperature were responsible for the leaf movements [2].

In examining the occurrence of critical days determining a crisis or lysis in the course of illness, Avicenna (980–1037) found, as Hippocrates and Galen did before him [1], that the week was an important unit of biological time, usually elapsing between the start and resolution of disease [4]. When physiological monitoring started in the seventeenth century, William Harvey (1578–1657) noted that «The movement of blood occurs constantly in a circular manner and is the result of the beating of the heart» [5]. It is noteworthy that not only about-daily cycles were reported this early on, cycles with periods longer as well as shorter than a day were also described by then.

Using instruments of precision to record his bodily functions over 30 years, Santorio Santorio (1561–1636) introduced quantitative experimental procedure into his medical research on basal metabolism [6]. Thomas Sydenham (1624–1689) deserves credit for advocating a specific timing in the evening to administer «Peruvian bark», which active ingredient is quinine, to alleviate pain [7]. Julien-Joseph Virey (1775–1846) was first to write a doctoral thesis in medicine devoted to biological rhythms [8]. Jean-Jacques d'Ortous de Mairan (1678–1771) reported that the «sensitive» heliotrope plant still opened its leaves during the day and folded them during the night after he moved it to a place where sunlight could not reach it [9], a finding suggesting the persistence of what we now call circadian rhythms [10].

According to Cambrosio and Keating [11, 12], the study of biological rhythms started in earnest in the 1920s, as a critical mass of integrated contributions from several investigators then appeared. The mere description of periodicities became complemented by analyses of their structure and the emergence of possible underlying mechanisms that recognized the partly endogenous nature of rhythms. Erwin Bünning deserves credit for his interpretation of de Mairan's finding from a «clock» viewpoint [13].

The study of rhythms also became institutionalized around that time. The Internationale Gesellschaft für biologische Rhythmusforschung, which was initiated in 1937 in Ronneby, Sweden, later became known as the Society for Biological Rhythms (SBR). Arthur Jores, who was one of the organizers of the SBR foundation meeting, and who later became president of the Society, was a physician interested primarily in practical aspects of rhythms from a therapeutic perspective. While he still viewed rhythms as being exogenous, the endogenous nature of rhythms, essential for the development of a scientific discipline in its own right, was only considered by the two biologists present at the meeting. The tenth conference of the Society was held in 1971, for the first time in North America, in Little Rock (Arkansas, USA), when it officially became the International Society for Chronobiology (ISC).

Until 1960, the SBR remained primarily European with a strong interest in medicine. The year 1960 is considered a turning point in the history of

chronobiology in view of the Symposium on Biological Clocks held in Cold Spring Harbor [14], organized by Pittendrigh with Aschoff, Bünning, and Bruce. It was the culmination of growing research on rhythms in the USA starting in the 1950s essentially centered on circadian rhythms in a biological rather than medical perspective.

On the medical side, Franz Halberg's participation at the meeting emphasized the temporal coordination of physiologic functions. He was among the first American researchers who attended the fourth SBR meeting held in Basel in 1953. He was to play a critical role in the transformation of the SBR into the ISC. In 1967, Jores had named Halberg as his successor to the presidency of the SBR. At the next meeting in 1971, the now ISC explicitly adopted a disciplinary approach to the study of rhythms, and in 1979, at the 14th Congress, the statement was formally made that «chronobiology should become an academic discipline in its own right», and voted on as part of the Society's constitution.

In the years following Cold Spring Harbor (1960), the dichotomy between the medical and biological approaches sharpened. Pittendrigh considered that rhythms should be studied within the scope of their original scientific discipline, focusing exclusively on biological aspects of rhythms and their «clock» mechanism modeled by self-sustaining oscillators. By contrast, Halberg took a multidisciplinary approach, including problems of growth, development, and senescence of interest to pediatricians and gerontologists within the scope of chronobiology, which he viewed as an integrated discipline like genetics.

By 1950, Halberg had uncovered the circadian adrenal cycle and identified the adrenal as an anatomical entity, with known biochemical factors, capable of certain effects of a physiological nature, namely the depression of blood eosinophil counts [15]. He introduced quantitative methods to study rhythms from a statistical viewpoint. His cosinor method [16] made possible the microscopic analysis of rhythmic phenomena with broad applications in all fields of medicine and biology more generally. Franz Halberg is regarded by many scientists as the father of modern chronobiology and as its undisputed leader, particularly as it related to biomedical research and therapy [17]. He served for more than 10 years as president of the SBR, and for another 15 years as president of the ISC. During his tenure and thereafter, numerous scientists trained in his laboratory in Minnesota. He served as editor-in-chief of Chronobiologia, the official journal of the ISC published between 1974 and 1994.

Additional information related to the historical development of chronobiologiy and its later extension to chronomics can be found in two autobiographical records [18, 19] and in a review of his contributions to the chronobiology of nutrition [20].

Early Studies of Franz Halberg That Led to The Discipline of Chronobiology

As noted earlier in this journal [21], Franz Halberg was assigned to work at Harvard's Peter Bent Brigham Hospital with George W. Thorn when he immigrated from post WW II Europe in 1948 on a fellowship from the World Health Organization. His task was to assess the validity of the epinephrine test by injecting mice with various compounds to evaluate possible corticoid activity based on their ability to cause a drop in eosinophil counts. Epinephrine presumably failed to cause the usual drop in blood eosinophil counts in patients with Addison's disease or other conditions of adrenal insufficiency due to deficient secretion of cortisone-like hormones from the adrenal cortex. The task was difficult and the results confusing because eosinophil counts varied too much. As a result, Halberg's fellowship was not renewed. At his farewell from Harvard in 1949, Thorn told him that he admired Halberg's sticking to his guns, yet it could not be that he was right while everyone else in the department was wrong. A year later, as he joined the University of Minnesota, however, Franz was proved right. He found that the pattern of variability in eosinophil counts was predictable, dropping from high counts in the morning to low counts in the evening [22, 23], the about-daily variation also present in humans [24]. By 1950, hormones from the adrenal such as cortisol were known to lower blood eosinophil counts.

In patients without working adrenal glands, blood eosinophil counts did not change predictably in a 24-hour cycle, indicating that adrenal hormones accounted for the cyclic change in eosinophil counts. The adrenal cycle had thus been found and later confirmed [25].

Halberg went on to show that environmental changes in the lighting regimen synchronized the about 24-hour rhythm in blood eosinophil counts (and other physiological variables), and that the light information was transmitted to the hypothalamus through the eyes. Rectal temperature measured around the clock in mice that were born anophthalmic and in blinded mice cycled with a period that was slightly but statistically significantly shorter than 24 hours, whereas it was 24-hour synchronized in control sham-operated mice [22, 25]. These seminal experiments showing the persistence of an about 24-hour rhythm in blinded mice demonstrated that the cycles were not just patterns of predictable change, they were internally run rhythms of the body, kept in step by environmental cycles. They constitute a key turning point when Halberg's interest shifted from the study of hormones to the study of the cycles themselves. They mark the beginning of chronobiology as a discipline in its own right, for which Halberg provided new concepts, methods,

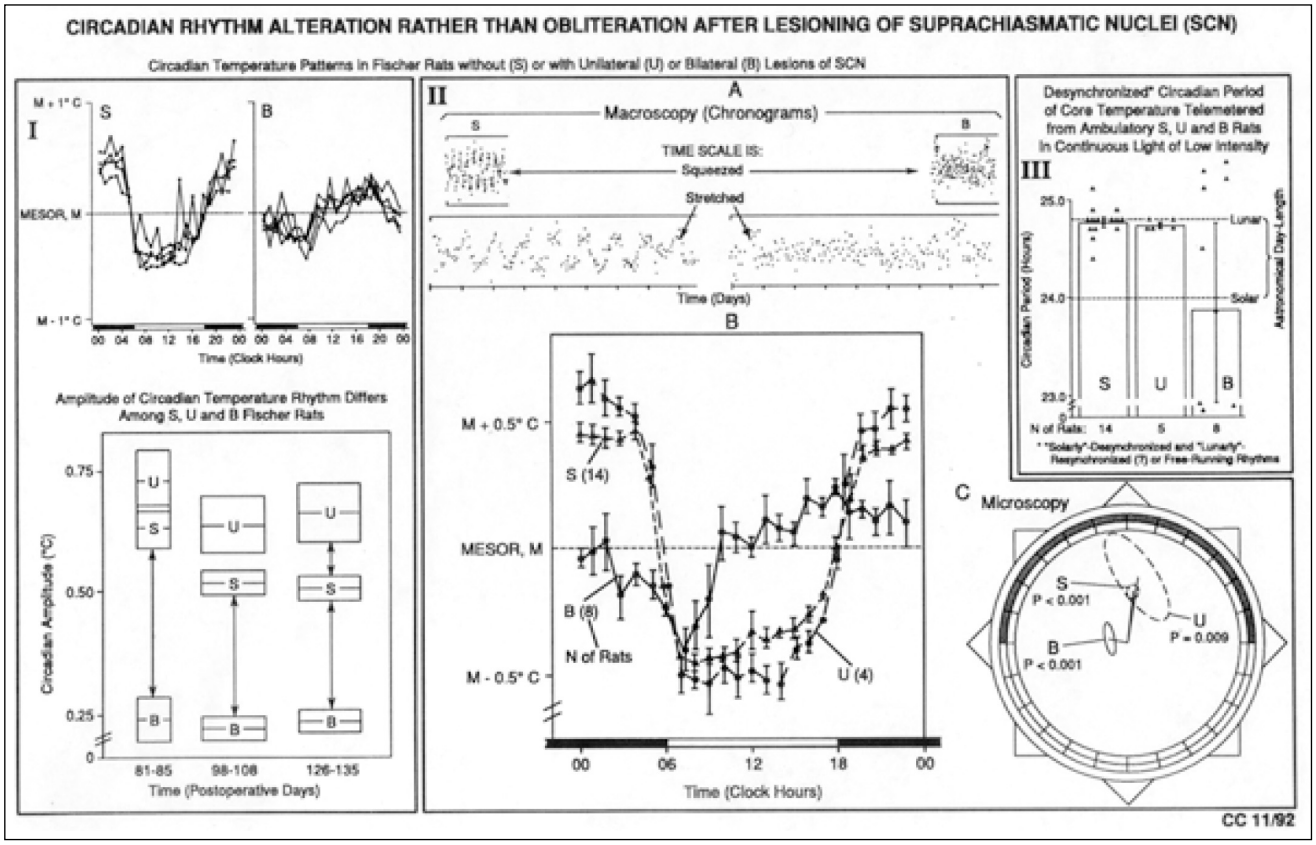

Figure 1. **Persisting circadian rhythmicity after removal of the SCN. Circadian pattern of core temperature of Fischer rats after unilateral (U) or bilateral (B) SCN lesioning, or sham operation (S). I. Data stacked over idealized 24-hour day illustrates rhythm with reduced amplitude and phase advance. II. Illustrative record indicating that rhythm is not readily discernable to the naked eye after bilateral lesioning of the SCN. Note amplification of circadian rhythm in unilateral SCN lesioned rats. Summary by cosinor (polar plot) for all animals documents statistical significance of circadian rhythm in all three groups. III. Free-running period in continuous dim light averages about 24.8 hours in S or U rats, but varies greatly in B rats, averaging about 24 hours. © *Halberg Chronobiology Center***

facts, mechanisms and applications, thereby replacing the limiting view of homeostasis by that of a partly built-in spectral structure in health. The terms chronobiology (from Greek «chronos», time, «bios», life, and «logos», science) and circadian (from Latin «circa», about, and «dies», day) were coined by him [15, 26].

The Suprachiasmatic Nuclei

Evidence accumulated over the past five decades indicates that the suprachiasmatic nuclei (SCN) of the anterior hypothalamus are the site of the circadian pacemaker in mammals. The SCN, situated directly above the optic chiasm, contain an autonomous circadian clock that maintains rhythmicity when isolated *in vivo*, when isolated *in vitro*, or when used in transplantation [27]. Early lesion studies showed ascending monoamine pathways from the brain stem to the limbic system [28, 29]. Follow-up work on pineal monoamine metabolism led to visual pathways controlling pineal biosynthetic activity [30–32]. The retinohypothalamic tract (RHT) was identified as a novel bilateral projection to the hypothalamus in rodents by injecting ^{3}H-amino acids into the eye [33, 34]. Studies aimed at understanding how light and the circadian clock influenced neuroendocrine function also led to visual pathways to the hypothalamus [35]. The perception of light by the circadian system through intrinsically photosensitive retinal ganglion cells, however, is independent of the visual pathways responsible for visually-guided behavior [27]. These studies suggested that lesioning of the SCN would lead to free-running circadian rhythms. The fact that arrhythmicity was observed led to the conclusion that the SCN (or a nucleus near it) were a necessary component of the central circadian oscillator [36, 37]. The large-amplitude circadian rhythm of melatonin production is now used most extensively as a reliable marker (output) of the circadian clock that is generated by the SCN [27].

Mathematical analysis of activity and water drinking data from SCN-lesioned hamsters showed the presence of about 12-hour and 8-hour periodicities, suggesting that the SCN may be composed of populations of oscillators to coordinate the activity of a variety of independent oscillators [38]. Around the same time,

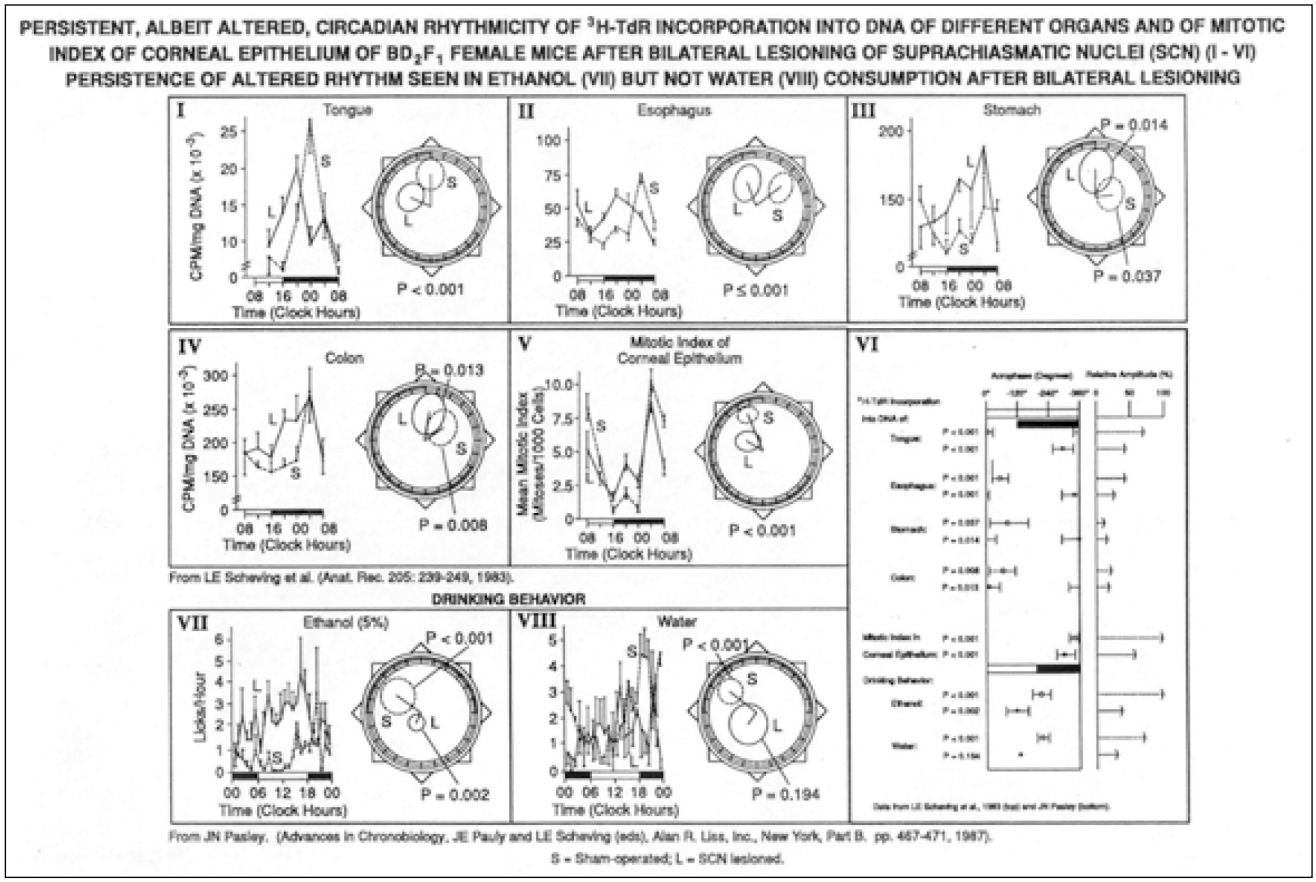

Figure 2. **Persistence of circadian rhythm of ^{3}H-TdR incorporation into DNA of different organs and of mitotic index of corneal epithelium of BD_2F_1 female mice after bilateral lesioning of the SCN (I-VI). While a circadian rhythm cannot be validated for water drinking in these animals, it is statistically significant for the drinking of 5% ethanol (VII and VIII). © *Halberg Chronobiology Center***

bilateral lesioning of the SCN in inbred Fischer rats, carried out at Halberg's laboratory at the University of Minnesota, showed that the circadian rhythm in telemetered core body temperature persisted in LD12:12, albeit with a small amplitude and an earlier phase [39–41], Figure 1. Persistence of the circadian rhythmicity of the ^{3}H-TdR incorporation into DNA of different organs (tongue, esophagus, gastric stomach, and colon) and of the mitotic index of the corneal epithelium of BD_2F_1 female mice after bilateral lesion of the SCN was further demonstrated [42], Figure 2. The most consistent result was a phase advance in the rhythms in cell proliferation in the tongue, esophagus, gastric stomach, colon, and corneal epithelium, and a reduction in the circadian amplitude detected in the tongue, esophagus, and corneal epithelium. Water drinking was the only variable for which a circadian rhythm could not be demonstrated [43]. Later, bilateral lesioning of the SCN was reported to eliminate the group circadian rhythm of systolic blood pressure but not of heart rate in rats [44].

Using *in vitro* hypothalamic slice preparations, neurophysiology studies showed rhythms in the firing rate of SCN neurons in culture [45, 46], thereby providing evidence for the autonomy of the SCN circadian pacemaker [27]. Further evidence was provided by showing in hamsters that transplantation of fetal hypothalamic tissue containing the SCN can restore behavioral rhythmicity in adults previously made arrhythmic by destruction of the SCN [47, 48]. It is now known that individual SCN neurons in culture can maintain independent free-running rhythms of firing rate [49].

The SCN contains multiple circadian oscillators, which synchronize with each other via several neurotransmitters. The SCN is a network structure composed of multiple types of gamma-amino butyric acid (GABA)-ergic neurons and glial cells. Although individual SCN neurons have intracellular molecular machinery of circadian clock and the ability to oscillate cell-autonomously, inter-neuronal communications among these neurons are essential for the circadian pacemaking of the SCN [50]. Gamma-amino butyric acid (GABA), an inhibitory neurotransmitter, is expressed in almost all SCN neurons. Excitatory and inhibitory effects of GABA may depend on intracellular Cl- concentration, in which several factors such as day length,

time of day, development, and region in the SCN may be involved [51]. It has been suggested that AVP neurons may play a critical role in the network mechanism of the central circadian pacemaker of the SCN [52].

Interestingly, in SCN lesioning studies of cell proliferation in the gut [42], the circadian amplitude of ^{3}H-TdR incorporation into DNA of the stomach, colon, and serum corticosterone was increased rather than decreased, suggesting that the SCN may represent but one cog in the overall 'clock' mechanism, however important it may be. The transcriptional/posttranscriptional delayed feedback loop reportedly cannot account for all circadian rhythms in cells [53]. A complementary non-transcriptional-transcriptional coordination mechanism may interact with the classical transcriptional-transcriptional one, involving NAD in their interaction [54]. The intact food anticipatory activity in SCN-ablated rodents or those lacking functional circadian oscillator genes [55] points to yet unidentified genes and circuits in eating pattern determination. Multiple lesion studies at the level of hypothalamic, corticolimbic, and brainstem structures and adrenals suggest that circadian coordination may be achieved by means of a distributed, decentralized system of oscillators, with contribution in gain setting by the metabolic hormones ghrelin and leptin [56]. The relative independence of the gut from the SCN deserves further investigation.

The SCN may also be involved in the coordination of other-than-circadian rhythms, as suggested by a circaseptan (about-weekly) amplification in dentin accretion after ablation of the SCN in Wistar rats [57]. Another interesting observation in Halberg's studies is the amplification of the circadian rhythm in telemetered core temperature of inbred Fischer rats after unilateral lesioning of the SCN, as compared to sham-operated controls [58], Figure 1. This finding was later corroborated in retrospective analyses of data on hamsters [59].

Clock Genes

Once the endogenicity of circadian rhythms became evident from their persistence in constant conditions, when they assumed a period that differed slightly but statistically significantly from exactly 24 hours, it was time to unravel their molecular and cellular bases. Evidence for a genetic basis of circadian rhythms at the molecular level in higher eukaryotes started when mutation screening in *Drosophila melanogaster* led to the discovery in 1971 that alterations of the *period (Per)* gene changed the periodicity of locomotor activity [60]. Mutant flies with atypically short or long periods of their circadian behavior led to the subsequent cloning of the *Period* gene as the molecular target of these mutations. It was then realized that changes in the encoded proteins could make the clock run faster, or slower or not at all. It also became apparent that the key action of the encoded proteins was to inhibit the expression of their cognate genes. Further studies elucidated more genes that altered the timing of behavior, and homologues in mammals were subsequently discovered [61–64]. These studies led to a model consisting of positive and negative autoregulatory feedback loops of transcription and translation. To cycle across the different stages, from gene activation and protein synthesis to intracellular transport and protein degradation takes approximately 24 hours. Even though the molecular components may differ across species, the basic principles underlying the mechanism is conserved [65].

One important feature is the so-called PAS (Per-Arnt-Sim) interaction domains of *Per* and other clock genes. A PAS domain is a protein domain found in all kingdoms of life that acts as a molecular sensor, whereby small molecules and other proteins associate via binding of the PAS domain. This sensing capability of the PAS domain is a key structural motif involved in protein-protein interactions of the circadian clock. In mammals, the circadian clock begins when light activates BMAL1 and CLOCK to bind via their PAS domains. That activator complex regulates Per1, Per2, and Per3, which all have PAS domains that are used to bind to cryptochromes 1 and 2 (CRY 1,2 family) [65].

The development of real-time reporter genes in which circadian regulatory sequences are coupled to bioluminescent or fluorescent proteins led to major advances in analyzing circadian gene expression, and allowed the study of the SCN clock mechanism as it progresses through real time. Applied to peripheral tissues and organs, it was found that circadian genes are not only expressed in such cultures, their expression is also circadian periodic, indicating that the transcriptional clock is active not only in the SCN, but in almost every cell of the organism. Circadian gene expression is sustained at the single cell level, but in the absence of external synchronization (*in vitro*), the phases of individual cells gradually disperse and the population rhythm weakens. The role of the SCN, therefore, is not to impose rhythms upon the rest of the brain and internal organs. Rather, it is to coordinate the activity of the intrinsic transcriptional/post-translational clocks distributed across innumerable cells in all of the major organs and tissues [65].

It was later discovered that some positive factors are rhythmically expressed due to the influence of their targets. *Rev-Erbα* was identified as a highly rhythmic circadian output gene driven by CLOCK/BMAL1 that encodes an orphan nuclear receptor that, in turn, inhibits Bmal1 expression via its retinoic acid receptor-related orphan receptors response elements (RORE) regulatory sequences, thus showing how output of the «core» loop becomes its input [66]. A second circadian-controlled gene, *Rora*, was found to act as a positive factor to *Bmal1*, opposing the effect of *Rev-Erbα* at the RORE. Mice lacking both *Rev-Erbα* and the closely related *Rev-Erbα* have major disruptions of metabolic

and behavioral rhythms [67]. Consequently, definition of the «core» clockwork progressively loses its focus as a network of transcriptional interactions develops [65]. Additional transcription factors, Dec-1 and Dec-2, and an auxiliary loop consisting of *Dbp* and *E4BP4*, further refined the current understanding of the circadian clock architecture, consisting of inter-nested transcriptional loops [68].

Retinal innervation of the SCN, carried via the retino-hypothalamic tract (RHT), is the means by which the transcriptional program of the SCN is synchronized to the environmental light-dark cycle. A subclass of retinal ganglion cells (RGCs) expresses melanopsin that confers upon them intrinsic photoreceptivity [69]. These intrinsically photoreceptive RGCs (iPRGCs) are sufficient for circadian entrainment of the SCN. The principal neurotransmitter of RGCs is glutamate. Entrainment by photic-induction of *Per* expression is equally applicable to both diurnal and nocturnal species because the cycle of *Per* expression in the SCN is the same in both, regardless of the animal's behavioral habits [70]. Across the body, other mechanisms are involved in the coordination of circadian rhythms, notably the feeding schedule, endocrine signals (e.g., corticosteroid hormones from the adrenal glands), and information stemming from the autonomic nervous system (e. g., body temperature) [65].

In addition to the core feedback loops, between 5 and 20% of the local transcriptome has been found to be subject to circadian modulation. In the liver, the circadian modulation is most pronounced for transcripts involved in metabolic and signaling pathways [71] as well as cell cycle regulators. The enzymatic components of the cell rather than structural genes are clock-regulated. Some of the rhythmic targets of the core loop factors, such as PPAR and HNF4a, are themselves transcriptional regulators. Cues that entrain the core clock, such as corticosteroids can also act upon clock-controlled genes directly. The transcriptome can therefore be viewed as a resonant network [65].

Post-translational mechanisms coordinating clock outputs implicate the localization and stability of clock proteins. Phosphorylation and ubiquitinylation are involved in supporting rhythmicity and setting the clock's period [72]. Cellular metabolism is intrinsically rhythmic, as observed in mouse liver *in vivo* [73] and in isolated mammalian cells *in vitro* [74]. This means that the cell's metabolic state can directly regulate transcription factor activity, as seen in vitro in the case of the redox state of nicotinamide adenine dinucleotide (NAD and NADP) cofactors [75]. While circadian cycles of gene expression are generally viewed as driving cellular rhythms of metabolism, there is also evidence for circadian cycles of metabolism to drive rhythms of gene expression [76].

Intercellular signaling is a critical aspect not only in synchronizing the SCN cellular transcriptional clocks but also in maintaining them. Altering electrical communication across the circuit may affect the transcriptional clockwork. The consequently reduced secretion of neuropeptides (AVP, VIP and GRP) across the SCN attenuates intracellular cues [77]. The core loop usually drives the circadian rhythms of action potential firing, cAMP and Ca^{2+} concentrations, neuropeptide synthesis and secretion. Non-transcriptional outputs of the core loop within the SCN neuron are thus also its sustaining inputs, acting both within a neuron and between neurons [65]. Both transcriptional and cytosolic components are mutually dependent and act in concert [65]. The fact that a self-sustained rhythm in glucose uptake by embryonic stem cells prior to and following differentiation implies the existence of intrinsic timekeeping that is not reliant on any known transcriptional clock mechanism [78].

In order to study non-transcriptional mechanisms of circadian rhythms in mammals, human red blood cells were used, which have no nucleus (or DNA) and therefore cannot perform transcription [79]. Results showed that transcription is not required for circadian oscillations, and that non-transcriptional events may be sufficient to sustain cellular circadian rhythms, since peroxiredoxins, highly conserved antioxidant proteins, underwent entrainable and temperature-compensated 24-hour redox cycles, persisting for several days under constant conditions. The concentrations of several cellular metabolites (ATP, NADH, NADPH) also appeared to be rhythmically modulated [79].

Beyond Circadian Rhythms

Unicellular organisms represent an attractive model to study time structure beyond circadian rhythms. Early on [80–87], it was known that unicells (e.g., *Acetabularia, Gonyaulax, Euglena, Paramecium*, and *Escherichia coli*) exhibited overt persisting circadian rhythms in variables such as cell division, luminescence, pattern formation, and enzymatic activity, including NADH and NADPH [86]. In *Euglena*, temporal differentiation was documented [86], a large number of diverse behavioral, physiological, and biochemical activities being partitioned in time, thus providing dimensions for both environmental adaptation and, functional integration in time. In *Acetabularia mediterranea,* circadian rhythms of chloroplast movement [88, 89] and oxygen production [90, 91] persisted even when the nucleus of the cell was removed.

One spectral component that has been particularly investigated in unicells is the about-weekly (circaseptan) variation. Following the study design of Hans-Georg Schweiger, two cultures of *Gonyaulax polyedra* were grown on almost opposite lighting regimens, then mixed and released into continuous light (LL). Not only did the light emitted from the mixed culture differ from the arithmetic mean of the two parent

cultures (also kept in LL), the circadian peaks attributable to the two cultures tending to merge, an indication of circadian cellular communication [92], a circaseptan modulation of the circadian amplitude was also detected in the cultures that had not undergone an LD phase shift and in some of the mixed cultures, but not in the shifted cultures [93].

Oxygen production, basal and apical chloroplast migration and the electrical potential of *Acetabularia acetabulum* measured in Hans-Georg Schweiger's laboratory under LD conditions before being released in LL show not only prominent circadian rhythms but also circaseptans, which are amplified by the change in lighting conditions [94]. The circaseptan variation is even more prominent than the circadian rhythm in the case of electrical potential in LL, Figure 3. Such a circaseptan amplification after exposure to a single stimulus had been documented earlier in relation to testosterone self-administration [95], organ transplantation [96], balneotherapy [97], and human birth [98, 99].

Data on *Acetabularia's* light transmission from Woolum's lab [100] showed that circaseptan as well as circadian rhythms persisted after enucleation. Results on the first 6 cells indicated that, as compared to the controls, the enucleated cells had a numerically longer circadian period (26.8±0.5 vs. 25.2±0.5 hours; P=0.124) but a shorter circaseptan period (6.3±0.3 vs. 7.7±0.6 days; P=0.085). Enucleation was also associated with a decrease in the circaseptan-to-circadian amplitude ratio (20.6±3.4 vs. 41.7±3.0%; P=0.018). These results were confirmed after additional cells were similarly investigated [100]. They suggest that the biologic week and day may be subtractively coupled, at least for this particular variable in this unicellular organism.

The SCN does not appear to be necessary for circaseptans to be discernible in dentin formation of Wistar rats, whether they are monitored in LL without surgery, before or after sham-operation or SCN ablation [57]. In these data, the circaseptan variation was more prominent than the circadian rhythm, the circaseptan-to-circadian amplitude ratio assuming values of 2.11, 1.40, and 2.85, respectively. Circaseptans remained prominent after SCN lesioning, suggesting their genetic basis. Interestingly, circaseptans were more prominently expressed in dentin formation, a variable representing growth, than in locomotor activity monitored concomitantly [57, 101].

A compilation of processes for which about-weekly and half-weekly variations were demonstrated with statistical significance indicates that they primarily relate to growth, regeneration, and repair [102]. They are found in organisms that have been on Earth for a very long time, suggesting that their manifestation early in human life [98, 99], and perhaps also early in evolution [100–102] may illustrate the parallel often made between ontogeny and phylogeny. The partly built-in nature of circaseptans and circasemiseptans related to growth and regeneration is substantiated by their demonstration in isolation from society [103, 104] and by their free-running from an exact socio-ecologic counterpart [95].

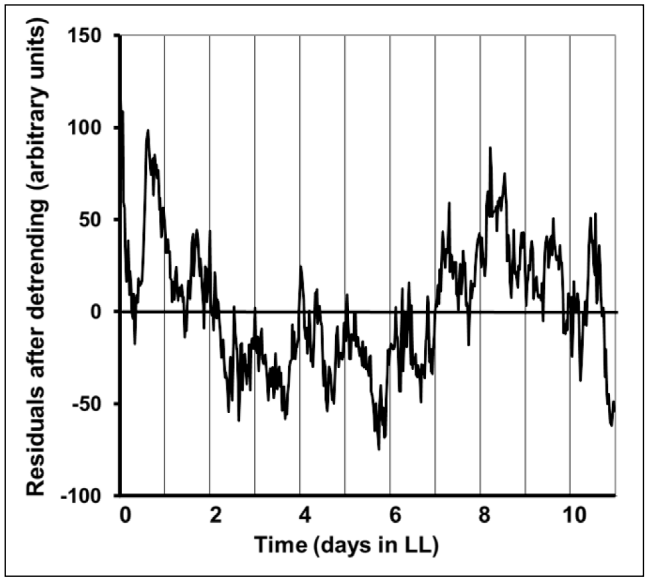

Figure 3. **Electrical potential of Acetabularia Acetabulum. Signal averaged data of cells kept in LD12:12 after release into LL [94]. Both circadian and circaseptan components are documented with statistical significance. © *Halberg Chronobiology Center***

Hours of Changing Resistance

Apart from the spontaneous rhythms, the response to a given stimulus also changes predictably depending on when it is administered. Timing can be as important as dosing since circadian (and other rhythm) stage determines the chances of life versus death in response to the same stimulus, as demonstrated for many drugs under the environmental conditions available in a modern laboratory, with standardized lighting, environmental temperature and humidity, and noise [105]. Circadian rhythms are not only responsible for the organization in time of the various physiological processes, they also play a critical role in health and disease [106]. They are involved in sleep disorders, respiratory diseases, cancer, cardiovascular diseases, neurodegenerative diseases, metabolic disorders, and infectious diseases [107]. In each case, physiological markers underlying each condition are circadian periodic, resulting in a circadian susceptibility-resistance stage-dependence to injury and a circadian stage-dependent response to treatment.

The hours of changing resistance constitute the foundation of chronopharmacology and chronotherapy. The tolerance of seven different anticancer drugs was tested in our laboratory on 5,266 rodents in 35 studies, yielding therapeutic gains that led to the doubling of sur-

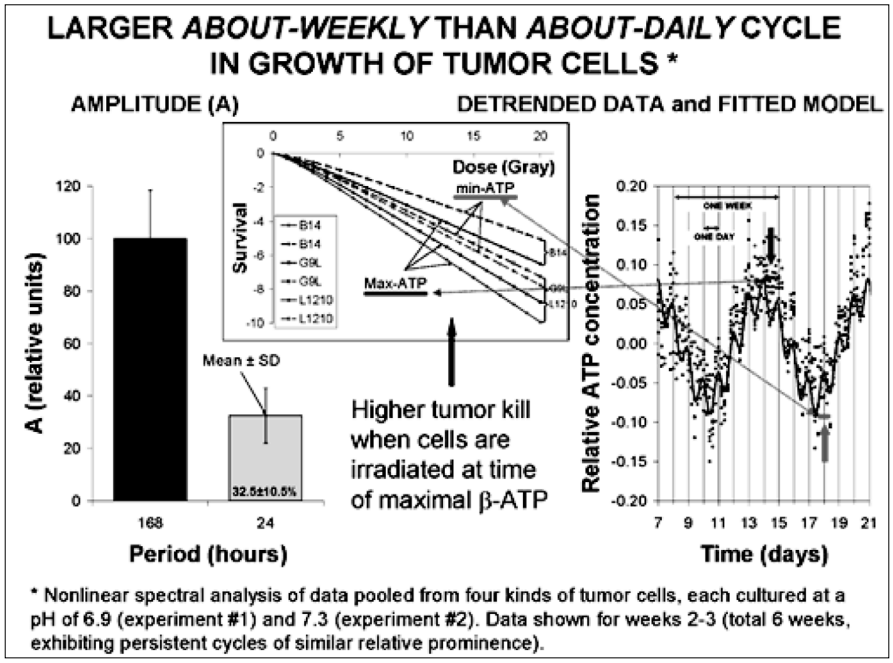

Figure 4. **Larger circaseptan than circadian amplitude of tumor cell growth (left), documented in four kinds of cells (C3H-MA grown from a murine mammary adenocarcinoma, B14 cells from a Chinese hamster, 9L glioma cells of a rat, and a monolayer culture of mouse L1210 leukemia cells). Both components affect outcome: irradiation at combined circadian-circaseptan stage of maximal β-ATP (right) results in higher tumor kill (middle). © *Halberg Chronobiology Center***

vival rate [108]. Doubling of the 2-year disease-free survival was similarly achieved for patients with oral cancer irradiated at the time of their peak tumor temperature [109, 110]. Apart from cancer [111], the merits of chronotherapy have also been documented in relation to respiratory diseases [112–114], cardiovascular diseases [115–119], neurodegenerative diseases [120], metabolic disorders [121, 122], and infectious diseases [123–125].

Studies in the clinic usually compare outcomes from patients treated in the morning or in the evening. A few chronobiological investigations used a 6-time-point approach that enabled the assessment of treatment effects of the circadian amplitude and phase in addition to the mean value. Recent work is considering a complementary approach that attempts to «fix the clock» by realigning a misaligned circadian system. Exposure to bright light in the morning and melatonin administration before bedtime [126, 127] are two main avenues to restore a disrupted circadian rhythm that have met with some success in relation to sleep disorders [128–132], depression [133–135], and neurodegenerative conditions [136–139]. Because sleep has a strong circadian component, many disease conditions are associated with a decrease in sleep quality. Improving sleep by means of bright light and melatonin intervention thus also helps improve patients with different disease conditions. Now that clock genes have been related to human health and disease, researchers are searching for new therapeutic modalities that target clock genes directly [140, 141].

Circaseptan rhythms can also be exploited to optimize treatment by timing. In studies of the immunomodulation of malignant growth in LOU rats bearing an immunocytoma, the effect of a 7-day pre-treatment with lentinan or saline was compared. The growth of the malignant tumor was inhibited and survival time was lengthened when this immunomodulatory was administered during the daily light span in doses varying sinusoidally from day to day with a period of 7 days [142]. Just as the circadian rhythm in RNA, DNA, phospholipids and mitoses prompted the circadian chronotherapy of cancer, merit of a circaseptan chronotherapy is supported by the presence of sharp peaks occurring about every 7 days in DNA labelling and mitotic activity during the regeneration of the kidney after unilateral nephrectomy or contralateral ischemia [143]. A circaseptan response to immunization has also been reported in mice [144]. Moreover, cyclosporine chronotherapy of pancreas-allotransplanted rats suggests that beyond the circadian stage-dependence of equal daily doses further gain in graft function can be obtained from doses varying from day to day according to a weekly periodicity [145]. Perhaps the more striking results relate to the time dependence of the β-ATP peak of different tumor cells in vitro gauging overall metabolism [146]. Gains to be derived from modulating the administration of radiotherapy according to a weekly schedule is suggested by the large circaseptan-over-circadian prominence of the time dependence of the β-ATP peak, as illustrated in Figure 4.

Concluding Remarks

Finding ways to restore a healthy circadian system by manipulating its endogenous component is a step forward. The procedures currently available to follow this approach are not invariably amenable to implementation in clinical studies. Future chronobiologic studies, however, ought to overcome the limitation of being restricted to comparing morning to evening dosing. As our work in the field of blood pressure has extensively demonstrated, blood pressure disorders can greatly differ in their presentation from one patient to

another. Diabetic patients with autonomic nervous dysfunction may be more likely to have a reverse circadian variation of their blood pressure but not of their heart rate [147], whereas patients at a higher risk of cerebral ischemia may have an amplified circadian variation of their blood pressure [148, 149]. Elevated values of blood pressure will thus occur at night or during the daytime, respectively. The optimal time to administer anti-hypertensive medication should thus be adjusted accordingly. In other words, chronotherapy needs to take the chronodiagnosis into consideration [108]. As one clinical study already showed, the optimal time to administer a given anti-hypertensive drug combination differs from one patient to another, thus clearly indicating the merit of personalized chronotherapy [119].

The idea of adjusting the treatment to individual needs emerged as molecular biology tools became available to provide rapid and accurate diagnostic assays [150]. The profile of metabolites present in urine before drugs are administered may also help identify whether a patient is a good candidate for a drug. Pre-dose metabolic profiles can predict how a patient might respond to a particular drug, using pharmacometabonomics [151]. Not only can the use of biomarkers for personalized medicine help reduce drug risks [152], the danger of relying on averages that can hide individual differences in clinical trials has also been pointed out [153]. In the ATLANTIS B trial, the outcome of stroke patients treated 3 to 5 hours after the onset of symptoms showed no difference between t-PA treatment or placebo collectively, but found a benefit for one-third of the patients who had the least risk of hemorrhage [153].

The ubiquitous, broad time structures that have repeatedly been shown to make the difference between life and death in the experimental laboratory [108] or between the success or failure of a given treatment in the clinic [109], however, remains mostly missing from current clinical practice. The recent development of several technologies for (1) monitoring health status, (2) screening for disease conditions as well as for risk elevation, (3) administering treatment scheduled according to bodily rhythms, and (4) continued surveillance of the patient's response to treatment should facilitate the implementation of a chronobiologic approach in everyday healthcare. Data on physiological variables acquired by personal long-term ambulatory monitors, analyzed statistically and interpreted chronobiologically in the light of time-specified reference values derived from clinically healthy peers can guide the programmed scheduling of treatment. Portable devices such as pacemakers, defibrillators, and drug pumps can be further programmed to account for circadian (and other) rhythms. Parameter tests [154] and self-starting cumulative sum (CUSUM) control charts [155] are tools available for personalized chronotheranostics, using N-of-1 designs [156]. All these technologies could lead to marker rhythms-guided chronotherapy adjusted for the chronodiagnosis of each individual patient.

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