Vinod Kumar *Editor*

# Biological Timekeeping: Clocks, Rhythms and Behaviour

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*Dedicated to my mother and father who always encouraged me to be honest, hardworking and truthful.*

# Foreword

Biology is the study of life in its full richness from the evolution of cyanobacteria three billion years ago to the human menagerie of 7.5 billion souls on earth today. This compendium of 31 chapters edited by Vinod Kumar is really a tribute to the biology of life from the perspective of time. Life and evolution are dynamic and cannot be severed from the inevitable march of time. Because life on earth is slave to the energetic cycle of the solar day (both as a resource and as a detriment), essentially all life forms evolved internal timing systems to anticipate and measure the time of the (now) 24-h day. This collection of essays is truly a rich and vibrant set that spans not only the range of organismal diversity (from cyanobacteria to humans) but also the spectrum of time (from ultradian, to tidal, to circadian, to circannual). Part I addresses the "History, Concepts, Evolution, and Basic Features of Biological Clock" and is a refreshing view of the history and development of the field and the evolution of timekeeping, as well as an excellent primer for understanding circadian concepts and analytical methods. Part II on "Animal Clocks: Complexity and Diversity" reviews the physiological organization of insect and vertebrate circadian systems, including *Drosophila*, fish (and zebrafish), amphibians, reptiles, birds and mammals. Part III on "Human Circadian Rhythms: Entrainment and Sleep Regulation" discusses the physiology and behavior of human circadian rhythms, their implications for health, their regulation by environmental factors, as well as their impact on the timing of sleep. Part IV on "Clock Interactions Within and Between Individual and the Natural World" brings into perspective the novel questions that have arisen from the discovery of cell-autonomous oscillators throughout the body and how this new system architecture of the physiological clock is organized and regulated, the implications of circadian photoentrainment arising from the discovery of intrinsically photosensitive melanopsin-containing retinal ganglion cells, the paradoxical role of non-photic entrainment cues in both nocturnal and diurnal animals, and the important role of temperature as an entraining signal. Part V on "Circadian Clocks, Metabolism, and Immune Functions" highlights the recent explosion of work linking the circadian clock at the molecular level to metabolism, immunity, and cancer. This is a huge growth area in

the field that has wide-ranging implications for human health and medicine. Part VI on "Pineal, Melatonin, and Biological Timekeeping" reprises the long history of melatonin as one of the first biomarkers of the clock system in animals, its divergent role in mammals and nonmammalian vertebrates, and its central role in avian circadian organization. Finally, Part VII on "Circannual Rhythms, Photoperiodism, and Seasonal Behavior" brings us full circle and addresses biological rhythms that are synchronized with seasonal cycles driven by the orbit of the earth around the sun. The rich biology of annual cycles in migration, hibernation, and reproduction and behaviors associated with these cycles are highlighted, as well as more recent work on the mechanisms underlying seasonality and circannual rhythms.

In looking back at the modern era of the field of circadian rhythms, first crystallized at the 1960 Cold Spring Harbor meeting, the field has been revolutionized. As described in the opening chapter by Schwartz and Daan, the field, its phenomena, and its formal properties were conceptualized and codified by Colin Pittendrigh and Jurgen Aschoff. Initial forays into the genetics of clocks by Ron Konopka and Seymour Benzer in *Drosophila* and by Jerry Feldman in *Neurospora* heralded the molecular era of clocks that emerged decades later and guides us today. In a parallel universe (at the time in the 1970s), physiological approaches by Michael Menaker, Fred Stephan and Irving Zucker, and Robert Moore and Eichler Victor led to the discovery of the avian pineal gland and the mammalian suprachiasmatic nucleus as critical anatomical foci for the regulation of organismal rhythms. Now many decades later, the field is fully integrated with the knowledge of circadian clock genes, the cell-autonomous mechanisms of clock systems from cyanobacteria to humans, the multiplicity of clocks throughout the body, and the intricate and intimate role that clock genes play in all aspects of biology. Today, clocks equal biology equal life!

In closing, *Biological Timekeeping: Clocks, Rhythms, and Behavior* is an important contribution to the field and will be a valuable resource for students, faculty, and anyone interested in the biological significance of circadian clocks.

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Joseph S. Takahashi

# Preface

Time is neither an entity nor a process and hence, cannot be measured and defined. In physical terms, however, the temporal measurement is observed in the form of the output from a clock – a mechanistic process that progressively counts the passage of time in seconds, minutes and hours. This in turn provides a calendar, which is a mathematical abstraction of 'clock time' over a longer period, e.g. day, month or year. In the biological context, time almost serves as the 'sixth' sense. All living beings have the sense of time and use it to sequence and restrict their most, if not all, biological activities to a specific time during the day and during a season of the year. Central to this is the evolution of an elaborate timekeeping mechanism(s), the 'clock' that keeps on 'ticking' and 'telling' an organism with great precision about the 'correct' time of day and time of year. Based on the temporal information coming out, we designate these clocks as the circadian (*circa* = about; *dian* = day), circannual (*circa* = about; *annum* = year), ultradian (shorter than the day) and tidal/lunar (periods matching the ocean tides and lunar day) clocks. The clock-driven measurable recurring processes are appropriately called as the circadian, circannual, ultradian and tidal/lunar rhythms.

Natural selection has operated in such a way that all rhythmic events occur synchronized (entrained) by external agents in the geophysical environment. In the natural environment, therefore, the rhythms are exhibited as daily, annual and tidal/lunar rhythms. The main agents (factors, cues) from the environment that synchronize (entrain) these endogenous rhythms to local time include the daily cycle of changes in the illumination, temperature, food availability, social factors, etc.

The endogenous timekeeping is achieved through a layered system of the organization and control, which consists of the molecules, cells, tissues, circuits and networks. These layers add to the complexity of orchestration of body rhythms to a concerted action in synchrony with changes in the local environment. Thus, unless carefully operated in sync with the external world a species or individual inhabits, there are heightened chances of the disruptions and hence dysfunctions; anything can go wrong anywhere. The self-sustained and robust timekeeping mechanisms, therefore, exhibit interesting individual and species between the

habitats and latitudes, since each individual and species can define its own relationship with the environment it inhabits.

The time perspective in biology was fully recognized some 50 years ago in a Cold Spring Harbor symposium. Since then, the study of biological timekeeping – called chronobiology – has evolved as a truly interdisciplinary science and attained a global appeal, expanding from the biodiversity, physiology and genetics to health issues, fitness and survival. Spectacular advances have been made in the field of chronobiology during the last years, with contributions by some of the best brains drawn from different disciplines, viz. biology, chemistry, physics and medicine. Much effort has been directed towards deciphering the clock mechanisms, particularly the cellular and network interactions within and between individuals as well as with the environment. In the last decade, there have been significant advances in our appreciation of the role of clocks in metabolism, sleep, cognition, immune functions and diseases. Increasingly evident is the application value of the biological clock in the human society, especially among the people working at 'unnatural' times of day (e.g. shift workers). Intensive research has shown associations of several chronic disorders and lifestyle-related diseases with disruptions of the circadian rhythm or the clock networks. No wonder, with outlets open $24 \times 7$, eating at the wrong time or consuming food more than required by frequent eating can lead to obesity and in turn have consequences on fitness and health. The ever-growing list of diseases linked with clock disruptions includes obesity, diabetes, cardiac diseases, depression and cancer. Also, the timing and duration of therapy, called chronotherapy, have been found critical in determining the efficacy of drugs.

The present book treats the organization and importance of clocks in animals and humans alike and covers significant aspects of the timekeeping mechanisms in both the groups. The book attempts at answering questions related to the ecological and evolutionary implications of the clock. I set out clear goals when I conceived the idea of bringing out this volume. Besides clearly outlining the history, origin and basic features of the clock, the book comprehensively includes the organization of clock system in *Drosophila*, fish, amphibia, reptile, birds and rodents, to present a complex and diverse nature of the timekeeping mechanisms among different animal groups, as this is generally not available in recent volumes on the subject. Secondly, the book includes topics that characterize rhythms in human and deal with the potential applications of circadian rhythms to health issues, including the metabolism and immune functions. Thirdly, a few chapters deal with the mechanism(s) of time generation and synchronization to the environment, both at cellular and molecular levels. A small section deals with the role of melatonin in regulation of daily and seasonal functions in animals. Finally, the book details on seasonal timekeeping mechanisms, with emphasis placed on genetic and epigenetic regulations of physiology and behaviour. Overall, the book tends to cater the needs of advanced undergraduates, researchers and professionals engaged in the field of chronobiology. I have avoided duplications or curtailed texts on topics that have been covered in important books published during the last 10 years, unless a particular topic was important for the present book and required a fresh look at this time.

A well-known expert in the area has authored the chapter. When presenting a state-of-the-art account, the text provides a consistent thematic coverage with adequate illustrations and feeling of the methods of investigations to arrive at the statements. The citation of references within the body of the text adequately reflects the literature as the subject is developed. Full references at the end of each chapter are useful to a reader interested to go deeper into the subject.

I am grateful to all the authors who have very kindly agreed to be part of this book. They have been extremely cooperative all the time. However, I am fully aware that the present volume of *Biological Timekeeping: Clocks, Rhythms and Behaviour* by no means can be considered as a full account of the subject. And I am solely responsible for the sins of omissions and commissions that are inevitable in an endeavour of this nature.

Undertaking such a mammoth task along with regular teaching, research and many other responsibilities requires immense goodwill and huge support. I have no hesitation that a strong team of doctoral and postdoctoral students during more than three decades of my professional career has made it possible by enabling me to learn more and find newer ways to overcome the challenges. I have very sincere words of praise and acknowledgement for all of them. Also, I have excellent friends both in India and abroad, who have full trust in me and always have encouraged my academic pursuits. My whole family supports me in whatever I do in the academics, and I am sure they would derive some satisfaction that their constant support has allowed me to produce something worthwhile. Finally, Springer (India) Pvt. Ltd., in particular Dr. Mamta Kapila and her excellent team, deserves special appreciation for the full support they have extended all along in bringing out the present volume.

Delhi, India Vinod Kumar
Thursday; 28 April 2016

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# About the Editor

**Dr. Vinod Kumar** is currently Professor of Zoology at the University of Delhi, India. He has about four decades of research and three-and-half decades of teaching experience in different Indian Universities. He has spent about 5 years abroad as a researcher working in different institutions of other countries viz. the United Kingdom, Canada, Germany and Japan, and more than a year-and-half as Visiting Professor working in Texas A&M University, USA. He has been awarded several international fellowships under The Indian National Science Academy – The Royal Society exchange program, DST-European Community bilateral cooperation, CIDA-NSERC Canada program, MaxPlanck Society post-doctoral and visting fellowship (over 5 years). He is honored with: 2010 Platinum Jubilee Lecture Award, ISCA, SRM University, Chennai; 2008 Vijay – Usha Sodha Scientific Research Prize, University of Lucknow 2006; P. Govindarajulu Gold medal from the Society of Reproductive Biology and Comparative Endocrinology, India; 2000 Young Investigator Award Gordon Research Conference in Pineal Cell Biology (Oxford, UK). He has received recognition from different international forums in the form Associate membership, regular membership and member of the scientific committees and Editorial Board of prestigious journals. He has published about 140 research publications, in most reputed journals of organismal biology, including the *Proceedings of National Academy of Sciences, USA, Proceedings of Royal Society B, Journal of Pineal Research, BMC Genomics, Journal of Biological Rhythms, Chronobiology International, Molecular and Cellular Endocrinology, General and Comparative Endocrinology, Journal of Neuroendocrinology, Journal of Comparative Neurology, Physiology and Behavior, Journal of Comparative Physiology A&B, Comparative Biochemistry and Physiology A, Behavioral Brain Research, Journal of Chemical Neuroanatomy, Molecular Brain Research, Journal of Ornithology*, etc. He is the editor of the book *Biological Rhythms* and the author of *Animal Behaviour*. He has been the most visible Indian face in the international meetings and conferences in the area of his research. He has

established the state-of-art research facility in his area of research in at least three different institutions in India, and has trained a generation of scientists to take the field forward. He has contributed heavily for the growth of the subject by organizing Schools, conferences and Symposia, and individual trainings. He is the Secretary (and President-Elect) of the Indian Society for Chronobiology.

# Part I
# History, Concepts, Evolution, and Basic Features of Biological Clock

# Chapter 1
# Origins: A Brief Account of the Ancestry of Circadian Biology

**William J. Schwartz and Serge Daan**

**Abstract** Who were the investigators and what was the path that enabled the launch of modern mechanistic research on circadian biology in the 1970s? Here we trace the origins of ideas from antiquity to the experimental study of the daily movements of leaves; on to the twentieth-century realization that circadian rhythms are widespread, endogenous, and innate; and finally to the appreciation that such rhythms could be utilized by organisms for the measurement of time. The conceptualization of the internal "clock" metaphor was key to the wave of mathematical, neurobiological, and molecular genetic advances that has transformed the field over the last 50 years.

## 1.1 Introduction

> We are like dwarfs sitting on the shoulders of giants. We see more, and things that are more distant, than they did, not because our sight is superior or because we are taller than they, but because they raise us up, and by their great stature add to ours. (John of Salisbury (c. 1120–1180) *Metalogicon*, 1159)

The last 50 years have seen a remarkable transformation in our understanding and application of the science of biological timekeeping, especially in the circa 24 h domain. We have moved forward from debating the existence of an endogenous "clock" to identifying a pathological point mutation in the human homolog of a fruit fly gene that regulates behavioral rhythmicity. Of course, such an explosion of knowledge does not take place *de novo* or by chance. Who made the antecedent

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observations, experiments, and insights that (paraphrasing Louis Pasteur) prepared the minds of contemporary researchers for discovery?

Here we provide a short account attempting to highlight the scientific path that preceded and launched modern mechanistic research in circadian biology, which, by our subjective estimation, dates from the 1970s; the astounding progress made since then is clearly described in the chapters of this volume. The literature already includes notable accounts of the history of chronobiology and the memoirs and reflections of those who made it, including Erwin Bünning [1], Jürgen Aschoff [2], and Colin Pittendrigh [3]. A complete review of the historical development of chronobiological concepts [4] has been composed by one of the present authors (SD).

## 1.2 From the Mists of Antiquity

The ancient Greeks distinguished their sense of time with two words. *Chronos* (χρόνος) refers to the passage of chronological time, linearly from the past to the future. *Kairós* (καιρός) represents the suitable (right or opportune) moment in time, especially for taking action; perhaps *timing* is our modern English equivalent. Thus, *kairós* is the meaning of the translated aphorism in the poem *Works and Days* by the Greek rhapsode Hesiod c. 700 BCE, "Observe due measure, for right timing is in all things the most important factor." So, arguably, the differentiation of biological from geophysical time, and the notion of optimal phases under entrained conditions, had already been realized thousands of years ago, presaging modern compilations of the ideal times of day for conducting disparate behavioral activities and physicochemical functions.

Aristotle (384–322 BCE) is credited with the proverb, "It is well to be up before daybreak, for such habits contribute to health, wealth, and wisdom." It is tempting to imagine that this ancient endorsement of the benefits of an early chronotype might have reflected even earlier beliefs that derive from solar worship and the need to greet the rising sun. In any case, this advice has been passed down across the millennia, as recognized in English-speaking countries from the quotations of the American polymath Benjamin Franklin (1706–1790) ("Early to bed and early to rise, makes a man healthy, wealthy, and wise." [5]) and English essayist Samuel Johnson (1709–1784) ("I have, all my life long, been lying till noon; yet I tell all young men, and tell them with great sincerity, that nobody who does not rise early will ever do any good." (1773) [6]). Earlier versions of this admonition have been identified in fifteenth-century English writings [7].

## 1.3 In the Beginning: The Daily Movement of Leaves

Theophrastus of Eresos (371–287 BCE), widely considered the "father" of botany for his work *Historia Plantarum*, cited the observations made by Androsthenes of Thasos, a trierarch (commander of a trireme), on his voyage from the Indus River to the Persian Gulf following Alexander the Great's Indian campaign (326–324 BCE). On Tylos (present-day Bahrain), Androsthenes described the tamarind tree as ". . .another tree with many leaves. . .that closes at night, but opens at sunrise, and by noon is completely unfolded; and at evening – again it closes by degrees and remains shut at night, and the natives say that it goes to sleep." Such daily leaf movements, known by the term *nyctinasty*, were further tabulated by others in additional species over the centuries, but reports of experimentation are not known until the eighteenth century.

Jean-Jacques d'Ortous de Mairan (1678–1771) had already published dissertations on subjects in physics, mechanics, and optics before his now famous experiment was reported to the Royal Academy of Sciences in Paris in 1729. He was very well respected, was elected to various leadership positions in the Academy, and eventually was inducted to membership in learned societies throughout Europe (at the time, he was considered one of the most outstanding scientists of the century, and a crater on the moon, Mairan, was named after him in 1935). A one-page "botanical observation" [8] reported on de Mairan's placement of "sensitive plants" in a dark spot, away from the sun and the open air. The plants seemed to continue opening their leaves in daytime and closing them at night. Thus, while de Mairan deservedly receives credit for moving a plant from sunlight, his chronobiological legacy rests on a paper without any description of how dark the place was or of the timing of the observations. Certainly no claim (or even speculation) was made that the plant opened its leaves in the dark by using an internal clock. On the contrary, de Mairan's conclusion was that the plant "sensed the sun without seeing it," so he presumed an external cause.

De Mairan's paper was not ignored, and within decades the work was pursued by Henri-Louis Duhamel du Monceau (1700–1782) in France, John Hill (1716–1775) in England, and Johann Gottfried Zinn (1727–1759) in Göttingen (then in the Kingdom Hannover, now Germany). De Mairan's basic finding was confirmed, and additional evidence was provided that the perception of daytime did not appear to be due to light leaks or varying temperature [9–11]. A breakthrough came only after the turn of the nineteenth century, by Augustin Pyramus de Candolle (1778–1841), a Swiss botanist. Like de Mairan, he was celebrated in his time, especially for his work on taxonomy [12], a word he coined in 1813; he was the first of four generations of de Candolle botanists, and the journal *Candollea*, published by the Conservatory and Botanical Garden of the City of Geneva, is named after him. De Candolle studied the leaf movements of *Mimosa pudica* in continuous light and found that the periodicity of "sleep and wakefulness" was shorter than 24 h and varied from plant to plant ("in several pots the acceleration amounted to one and a half to two hours per day") [13]. From this deviation from 24 h, de Candolle

concluded (p. 861) "that the sleep-wake movements are connected with a disposition for periodic motion that is inherent in the plant." This was the first time, in 1832, that an endogenous cause of the periodicity was postulated.

De Candolle's view was adopted by prominent botanists, notably Julius Sachs (1832–1897) [14], but the first person to write a book about plant movements, the German plant physiologist Wilhelm Pfeffer (1845–1920) – whose interest extended beyond nyctinasty to thermonasty and photonasty and whose opinions carried great weight – was not at all persuaded. He concluded that the movements were "after-oscillations" of a system returning to a stable position after a disturbance ("The experiments reported here show irrefutably that the daily periodic movements can not be considered historically established properties, since they indeed slowly stop in continuous illumination.") [15]. Notably, Charles Darwin (1809–1882) was convinced of the inherited endogenous nature of daily movements. Just 5 years later, he too wrote a book, together with his botanist son Francis, entitled *The Power of Movement in Plants* [16] and even offered a specific idea for selective benefits. He stated (p. 284):

> The fact that the leaves of many plants place themselves at night in widely different positions from what they hold during the day, but with the one point in common, that their upper surfaces avoid facing the zenith, often with the additional fact that they come in close contact with opposite leaves or leaflets, clearly indicates, as it seems to us, that the object gained is the protection of the upper surfaces from being chilled at night by radiation. There is nothing improbable in the upper surface needing protection more than the lower, as the two differ in function and structure. All gardeners know that plants suffer from radiation.

In Leipzig, Pfeffer had moved his attention to other aspects of plant physiology; he did pioneering research on osmosis and acquired a reputation for exacting standards and innovative instrumentation (including photography). He was only drawn back to daily rhythms by the work of the German biologist Richard Semon (1859–1918), who went on to consider the nature of memories, coining the term engram. In 1905, Semon published data on young bean sprouts (*Phaseolus*) that had never seen any light; were then exposed to light-dark cycles of 12, 24, or 48 h; and afterward returned to constant conditions. He expected to see these periodicities reflected in the periods of the ensuing after-oscillations, but these always showed only a circa 24 h period, even when they had never seen one in their own life [17]. Pfeffer then returned to the problem, now applying continuous automatic recording of movements using the kymograph. In 1907, he finally accepted the existence of "autonomous" movements (p. 465) but with a much shorter period than the daily periodicities and their "aftereffects" (p. 472) [18]. Eventually, he found an ingenious way of preserving the rhythm of movements in constant light by darkening the leaf joints in the plants with black cotton. After conducting an extensive series of experiments in this way, he too was forced to conclude that the daily rhythm was sometimes persistent and not driven by an external 24-h force. He was 70 years old when he published his carefully obtained results in 1915 [19] (Fig. 1.1) in a verbose and unclear writing style and in a little-read journal – *Abhandlungen*

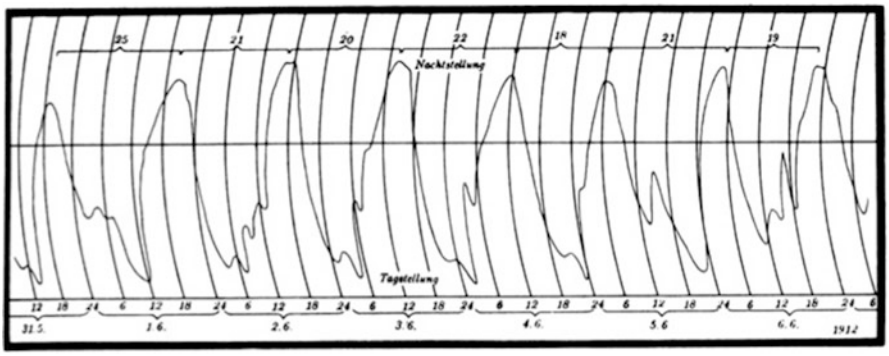


**Fig. 1.1** Recording of bean leaf movements in continuous light by Pfeffer [19] (Reproduced with permission [80])

*der mathematisch-physikalischen Klasse der Königlich sächsischen Gesellschaft der Wissenschaften* – and so its impact at the time was limited.

## 1.4 Circadian Rhythms: Endogenous and Innate

As the twentieth century progressed, philosophical arguments began to yield to empirical evidence, and the case for an endogenous origin of daily rhythmicity, or at least a component of it, continued to build. Looking back, the experiments of the Dutch botanist Anthonia Kleinhoonte (1887–1960) on jack bean plants (*Canavalia ensiformis*) were especially prescient [20]. In the 1920s, she demonstrated that leaf movements exhibited a circa 24 h rhythm in constant darkness even if the seedlings were first maintained in an 8 h:8 h light-dark cycle and that brief light pulses – as short as one minute in duration – could reset rhythm phase depending on the phase of administration of the light. Plants could be induced to express a range of different phases in darkness, arguing against the view that such rhythms represented a direct response to an unknown exogenous factor [20].

Erwin Bünning (1906–1990), although entering the field as a *bona fide* botanist at the University of Jena in Germany, could be considered to be the first real circadian biologist. He helped to legitimize circa 24 h rhythms by referring to them by a name (endodiurnal; only later was the term circadian introduced by Franz Halberg (1919–2013), in 1959 [21]), and he wrote what was arguably the first accessible "textbook" on the subject (*Die Physiologische Uhr*, 1958). But perhaps most importantly, his research was not restricted to a single organism but spanned both plant and animal kingdoms. In the 1930s, he showed that endodiurnal rhythmicity in *Phaseolus* was clearly and significantly longer than 24 h, that raising the plants in anomalous light-dark or constant conditions did not affect the periodicity of succeeding generations, and that studying hybrids of plants with different periods implicated a genetic basis [22–24]. And then he proceeded to become among the

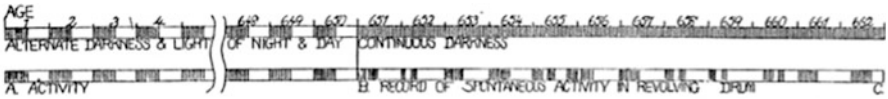


FIG. 19. SCHEMATIC RECORD SHOWING PERSISTENCE OF ACTIVITY RHYTHM AFTER WITHDRAWAL OF ORIGINAL STIMULUS

**Fig. 1.2** Recording of rat locomotor activity in light-dark cycles and continuous darkness by Richter [27] (Figure 19, reproduced with permission [27])

first (see also the work of Hans Kalmus (1906–1988) in Prague [25]) to undertake studies of another 24 h rhythm, that of adult emergence from pupae (eclosion) in populations of fruit flies (*Drosophila*), reporting its persistence in the progeny of flies maintained in dim continuous light for multiple generations [26].

As mid-century approached, it had become clear that 24 h biological rhythms were a general phenomenon, encompassing a wide range of functions in a variety of organisms (although their endogenous generation had not been universally accepted). Vertebrate animals were no exception. By the end of the 1920s, the American psychobiologist Curt Richter (1894–1989) had already accumulated a wealth of locomotor activity data from rats [27] (Fig. 1.2), including those with access to "revolving drums" (running wheels) [28] invented some years earlier by Colin Stewart at Clark University in Worcester, Massachusetts [29]. For larger animals – including humans – body temperature became the assay of choice and a natural one for a new cadre of investigators entering the field after training in medicine and physiology. Sutherland Simpson (1863–1926), a Scottish MD who later moved his physiological laboratory to Cornell University in New York, performed heroic recordings of the axillary temperature rhythms of rhesus monkeys (*Macaca mulatta*) in light-dark, reversed light-dark, and constant dark conditions [30]. American physiologist Nathaniel Kleitman (1895–1999), considered a founder of modern sleep research, was intrigued by the regulation of the timing of sleep and wakefulness. In 1938, he and his assistant Bruce Richardson isolated themselves in Mammoth Cave, Kentucky, for 32 days, attempting to adjust to a 28 h day (19 h awake with lights on and 9 h in bed with lights off) at a subterranean-constant temperature of 54 °F (12.2 °C). Their body temperature recordings published later (Fig. 1.3a) hint that different periods were expressed by the two men [31]. Incidentally, decades later he and his PhD student Eugene Aserinsky (1921–1998) discovered REM sleep [32], effectively overturning the notion of a quiet, resting brain during sleep.

Jürgen Aschoff (1913–1998) was the youngest son of the famous German cardiac pathologist remembered eponymously by generations of medical students as the Aschoff-Tawara node (now known as the atrioventricular node) and Aschoff bodies (myocardial nodules following rheumatic fever). The younger Aschoff also studied medicine and in 1939 began to investigate the physiology of human thermoregulation; through self-experimentation, he discovered a previously unreported daily rhythm in heat loss under constant conditions without food intake, sleep, or locomotion [33]. He became fascinated by the subject, and – in the course of expanding his knowledge to include ethology and applied mathematics and

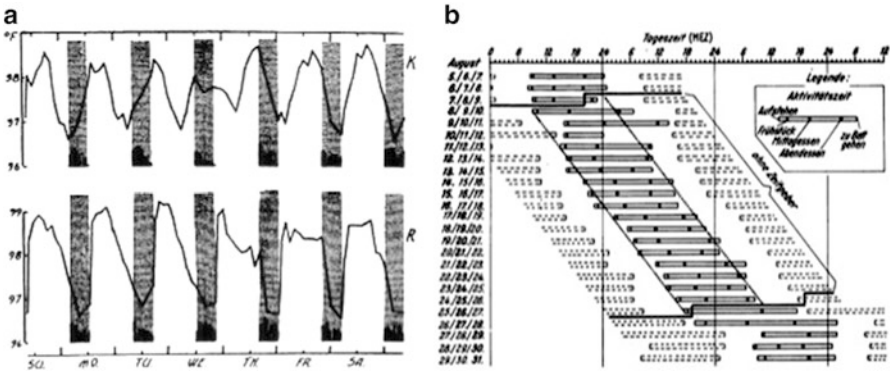


**Fig. 1.3** Recordings of human rhythmicity. (**a**) Recording of Kleitman's (K) and Richardson's (R) body temperature rhythms in Mammoth Cave (1938) (Figure 18.4, reproduced with permission [31]). (**b**) Demonstration of the free-running endogenous circadian oscillator in humans by Aschoff and Wever [34] (Reproduced with permission [34])

working with rodents, birds, and humans – he produced a rigorous oeuvre on the properties and entrainment of physiological rhythms. Later, in 1961, he had the chance to use a civil air-raid shelter deep underground below the university's surgery clinic in the center of Munich and studied nine human subjects who lived there, one after the other, each for 2–4 weeks in isolation without any contact with the outside world and without access to any clocks or timing signals. Aschoff demonstrated in all of the subjects continuing rhythms with a period in excess of 24 h (Fig. 1.3b) [34]. After this first demonstration of human circadian rhythms, Aschoff built an underground bunker near his institute in Erling-Andechs (less than 50 km from Munich), specifically designed as a temporal isolation facility in which two subjects could be studied simultaneously. This led to a series of unprecedented experiments on nearly 450 subjects over 25 years that would set the standard for analyses of human circadian rhythmicity [35]. As an energetic, enthusiastic, and dramatic spokesperson for the field, Aschoff succeeded in bringing its emerging insights to international biomedical attention.

## 1.5 Paradigm Shift: Rhythms as Clocks

A few individuals, well ahead of their times, anticipated the concept that the internal "clocks" that were generating overt 24 h oscillations could be utilized by organisms for actual time measurement. Julien-Joseph Virey (1775–1846) was chief pharmacist at the Val-de-Grâce hospital in Paris until he was awarded an MD degree in 1814. Having observed the periodic nature of clinical phenomena in the hospital, he had the temerity to speculate (in his doctoral thesis [36]) on the existence of a kind of living clock – "une sorte d'horloge vivante" – *entrainée* (entrained), as he called it for the first time, by the movements of the sun and earth.

In 1936, a 30-year-old Bünning hypothesized how a 24 h clock could be part of a mechanism for measuring changing daylength [37], and in 1939 the American zoologist Maynard Johnson published activity records (cage movements) of deer mice (*Peromyscus leucopus*) in continuous light and concluded that the animals have ". . .an exceptionally substantial and durable self-winding and self-regulating physiological clock, the mechanism of which remains to be worked out." [38]. It was Johnson's final contribution to circadian biology (see below for a key earlier contribution); he left the Biological Laboratories of Harvard University for the Lake Mattamuskeet Wildlife Refuge in North Carolina.

It was in the 1950s that the conceptualization of rhythms as clocks truly began to take shape. In order to use an internal clock for the measurement of external time (both the time of day and the passage of time), the clock's oscillation must adopt a stable phase relationship to the environment. American biologists J. Woodland ("Woody") Hastings (1927–2014) and E. Beatrice ("Beazy") M. Sweeney (1914–1989), assaying the circadian bioluminescence rhythm of a photosynthetic dinoflagellate (*Gonyaulax polyedra*, now *Lingulodinium polyedrum*), and Patricia DeCoursey (b. 1932), recording the circadian wheel-running rhythm of the flying squirrel (*Glaucomys volans*), rigorously quantified the resetting responses of these rhythms to light pulses presented across the circadian cycle. The resulting "phase-response" curves [39, 40] revealed how the interaction of an endogenous oscillation with a rhythm of light responsiveness could lead to precise and accurate entrainment to the environmental light-dark cycle.

Colin S. Pittendrigh (1918–1996) earned his initial degree in botany at the University of Durham in England, and during World War II, he was posted to Trinidad to tackle malaria control; it was there that he first noted the daily activity rhythms of mosquitoes. He then studied genetics and evolutionary biology for his PhD (1948) at Columbia University in New York, under the famous geneticist Theodosius Dobzhansky (immortalized by his axiom that "nothing in biology makes sense except in the light of evolution" [41]). Pittendrigh remained in the United States, and his influence on circadian biology, and on the formulation of the "clock" metaphor, was pivotal, continuing even after his formal retirement (1984) to homes in Arizona and Montana. While Kalmus had claimed that the period of the *Drosophila* eclosion rhythm depended on temperature [42], Pittendrigh argued that temperature independence (or temperature compensation) of circadian period must be a fundamental feature of a reliable clock – actually, that it must hold true for the spontaneous frequency of any sensory organ, if such organs are to be employed for purposes other than temperature sensing [43]. In a masterful and lucid set of experiments on the timing of eclosion in *Drosophila pseudoobscura* [44], he rigorously analyzed the rhythm and the effects of light and hypoxia, demonstrated the rhythm's near independence of ambient temperature (Fig. 1.4), and provided an explanation for Kalmus' mistaken conclusion (he had measured only the first cycle after a temperature step). Moreover, Pittendrigh laid a foundation, in this and future work on flies and on nocturnal rodents with colleagues, for crucial conceptual advances that the mechanism of the circadian clock is separate (and separable) from the downstream behaviors it regulates and that the clock is used not only to

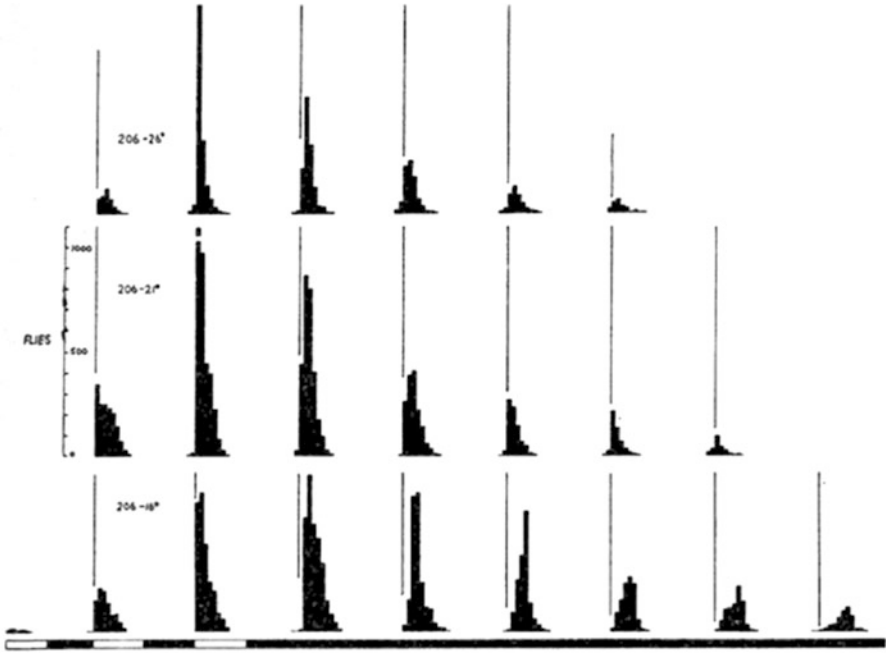


**Fig. 1.4** Temperature compensation of the *Drosophila* eclosion rhythm in constant darkness, at 26°, 21°, and 16°C (1954). *Vertical lines* represent 24 h intervals (Reproduced with permission [55])

align the timing of an organism to the environment but also to temporally organize rhythmic subsystems ("slaves") within an organism. Imperious and insightful, at 45 years of age, he was the first circadian biologist to be elected to the US National Academy of Sciences. Notably, for "Pitt" (as he was called by his colleagues), teaching (including to undergraduates) served a critically important role; he was a passionate and electric speaker, pacing the floor and usually drenched in sweat by the end of his lectures.

Pittendrigh himself acknowledged the great influence of the brilliant German ornithologist and naturalist Gustav Kramer (1910–1959) on his conception of an innate, functional circadian clock. Kramer elucidated how migrating European starlings (*Sturnus vulgaris*) could stay on course by using the sun during their seasonal migration. Through ingenious experiments using circular cages and mirrors that artificially changed the apparent position of the sun in the sky, Kramer tracked the orientation of the birds' migratory restlessness and revealed the existence of a time-compensated sun compass [45], viz., navigation by the direction of the sun, taking into account its movement over the course of the day. In later experiments, a circadian mechanism was implicated by phase shifting the birds' clocks and then exposing them to the natural sun, leading to predictable deviations in their flight direction [46]. Kramer's accidental death while hunting rock pigeons on a Calabrian mountainside – just as he and Aschoff were poised to collaborate – was a tragic loss for the field and for ecology, as memorialized in the obituary [47]

by Konrad Lorenz, 1973 Nobel Laureate. Of note, Karl von Frisch, the other 1973 Laureate (along with Nikolaas Tinbergen), concluded, simultaneously with Kramer, that honeybees also possess a time-compensated sun compass [48] as an orientation mechanism for their remarkable time-of-day-dependent choice of feeding location (referred to as "time memory") [49].

The 1960 edition of the annual symposium of the Cold Spring Harbor Biological Laboratory, on Long Island, New York, attracted 150 scientists; it was entitled *Biological Clocks* [50] and chaired by Pittendrigh. It has since assumed a vaunted place in the history of chronobiology. Here Bünning, Aschoff, and Pittendrigh presented a conceptual and experimental framework for the new interdisciplinary field, using the precise language and quantitative methods of oscillator theory: Bünning on photoperiodic time measurement and the heritability of circadian period; Pittendrigh on temperature compensation, transients and aftereffects, nonparametric entrainment, and the distinction between clock and driven rhythms; and Aschoff on zeitgebers, parametric entrainment, and exogenous and endogenous rhythm components. It was also the setting for a memorable exchange [43] between Pittendrigh and Frank A. Brown, Jr. (1908–1983), the persistent champion of the exogenous origin of overt rhythmicity.

> Brown: "...there is no logically defensible proof that the clocks underlying circadian rhythms possess a timing system, a self-sustaining oscillation...such proof has been precluded by the fact that one can never establish through negative evidence alone that nothing on the outside provides essential timing signals....in insisting upon a self-timed, or fully autonomous autonomous, living clock, there always lurks the possibility that we are pursuing a ghost."
>
> Pittendrigh: "...the question of the ghost is simple – either it is an aspect of living organization, or an unknown geophysical variable. My taste in ghosts suggests the latter but, as scientist, I must agree that Dr. Brown may prove right; and as scientist he will doubtless agree he may prove wrong."

For all practical purposes, the search for the exogenous ghost ended some years later, when circadian rhythmicity was found to be sustained in a laboratory setup a few hundred meters from the South Pole with turntables rotating once per 24 h against – and hence fully canceling – the earth's own rotation (in hamsters [*Mesocricetus auratus*], fruit flies [*Drosophila pseudoobscura*], bean plants [*Phaseolus vulgaris*], and fungi [*Neurospora crassa*]) [51]. Additional proof was obtained in the 1980s in *Neurospora* placed in geocentric orbit aboard Spacelab 1, up to 157 miles above earth and circling the globe with a period of 89.5 min [52].

## 1.6 Ushering in the Modern Era: Mathematics, Maps, and Molecules

As attention turned to the clock itself in the 1960s and 1970s, mechanistic questions recruited a fresh wave of mathematical biologists, neuroscientists, and molecular geneticists newly attracted to the problem: What kind of oscillator is it? Is it

physically located in a discrete part of the body? How does it actually work at a tissue, cellular, or molecular level?

American theoretical biologist Arthur Winfree (1942–2002) was a graduate student of Pittendrigh at Princeton University, where he applied his undergraduate background in engineering physics to model the clock as a stable, attractive limit cycle and predict how perturbations affect its behavior. The topology of such a multidimensional nonlinear system led him to reason how light pulses, depending upon their phase of administration and intensity, could generate "strong" and "weak" resetting or even propel the oscillator to an undefined phase ("singularity") (as though transported to the North Pole, where all lines of longitude converge and there is no time of day); he then proceeded to successfully test his predictions by resetting and desynchronizing the *Drosophila* eclosion rhythm with critical photic stimuli [53]. Winfree also considered the dynamics of populations of weakly coupled limit cycle oscillators, realizing that their behavior could be quite different from that of one oscillator alone, and he surmised, as we now know, that intercellular synchronization might determine as much about clock dynamics as does its autonomous cellular mechanism [54].

As early as 1957, Pittendrigh and Victor Bruce (1920–2009) constructed a block diagram of a canonical timekeeping system including input, pacemaker, and output [55] (Fig. 1.5a), a schematic that evolved over 20 years to become Arnold Eskin's iconic blueprint [56] (Fig. 1.5b). This was an era that exploited new techniques and circumscribed lesions in an effort to map various brain functions to distinct sites (e.g., the lateral hypothalamus as a feeding "center," the ventromedial hypothalamus as a satiety "center," various pleasure "centers," and so on). But how could a circadian

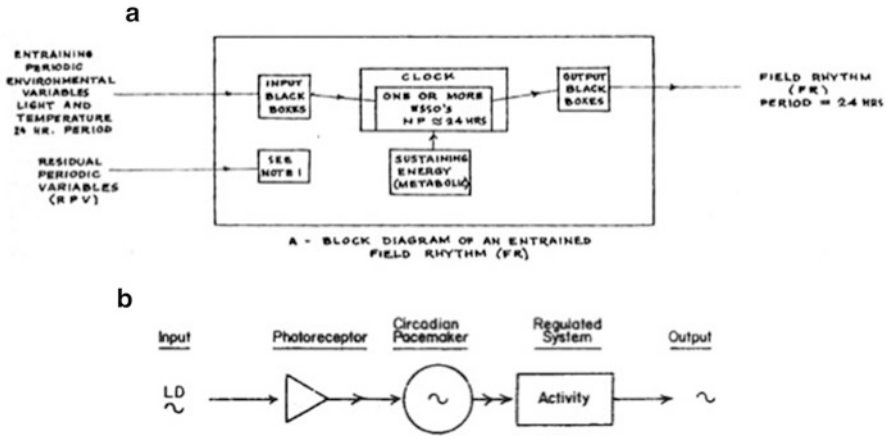


**Fig. 1.5** (**a**) Pittendrigh and Bruce's diagram of an entrained circadian system [55], with a clock composed of one or more endogenous self-sustaining oscillators (ESSOs). "Note 1" posits that all periodically fluctuating variables other than light and temperature are not coupled to the oscillator (Reproduced with permission [55]). (**b**) Eskin's original diagram [56] (Reproduced with permission [56])

"center" be distinguished from its downstream effector ("hand of the clock") if a lesion of either would lead to the same arrhythmic phenotype? An answer to this problem was provided in 1970 by James Truman (b. 1945) and Lynn Riddiford (b. 1936) with a daring transplantation experiment that elucidated the timing of ecdysis (a phase of pupal emergence) in two species of silk moths [57]. In a 17 h:7 h light-dark cycle, *Hyalophora cecropia* and *Antheraea pernyi* were shown to emerge in the morning and evening, respectively, a phase difference that was lost in brainless moths. However, when *cecropia* brains were transplanted to the abdomens of brainless *pernyi* and vice versa, the *timing* of ecdysis was restored – with a phase predicted by the donor brain, not the recipient body – while the species-specific behavioral *pattern* of ecdysis remained characteristic of the host. Thus, the clock was in the brain, communicating with the actual behavioral generator (elsewhere) via a neuroendocrine mechanism. Further transplantation studies followed, and the circadian clock regulating locomotor rhythmicity was localized to the brain in *Drosophila* [58], the optic lobes in cockroach (*Leucophaea maderae*) [59], and the pineal gland in house sparrow (*Passer domesticus*) [60]. In 1972, lesions of the suprachiasmatic nucleus (SCN) in rat (*Rattus norvegicus*) [61, 62] implicated this hypothalamic nucleus as the site of the clock in mammals; the decisive transplantation experiments came much later, interchanging fetal SCN tissues between wild-type and short-period mutant hamsters (*Mesocricetus auratus*) [63].

Despite the rise of the "master clock" or "pacemaker" metaphor, it was already clear in the 1960s that the circadian system includes a number of interacting body clocks. In particular, American physiologist G. Edgar Folk, Jr. (b. 1914) cultured hamster adrenal glands and measured oxygen consumption and steroid secretion over a few days [64]. Evidence was presented for a circadian metabolic rhythm in culture, with its period relatively temperature independent, and its phase dictated by the light-dark cycle before animal sacrifice. In 1965, Folk also reported a circadian rhythm of heart rate in isolated rat hearts, proposing that ". . .the circadian rhythms of resting heart rate are controlled by a "clock" located within the heart cell" [65]. It was another four decades before the further study of such mutually interactive "peripheral" clocks could be undertaken in earnest.

It was in 1959 that Pittendrigh and associates reported a circadian rhythm of asexual spore formation (conidiation) in the filamentous fungus *Neurospora crassa* [66]; this had been the model organism exploited by Norman Horowitz to demonstrate the "one gene-one enzyme" hypothesis of George Beadle and Edward Tatum (1958 Nobel Laureates). Despite Pittendrigh's vehement indifference to searching for "clock genes" – after all, inheritance of clock properties must be polygenic – his graduate student Jerry Feldman (b. 1942), upon completing his PhD in 1967, headed to the California Institute of Technology in Pasadena for a postdoctoral fellowship with Horowitz. The eventual result of his chemical mutagenesis screen with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was the discovery of a set of single gene mutants that all mapped to the same genetic locus, which he named *frequency* (*frq*) [67]. Remarkably, he isolated mutations at this locus that could either shorten or lengthen the free-running period, with a gene-dosage effect on period length. A friend of Feldman's when he was at Cal Tech, Ronald J. Konopka (1947–2015), the first graduate student of Seymour Benzer (1921–2007) there, set up an ethyl methanesulfonate (EMS)

mutagenesis screen in 1968 and generated three *period* (*per*) mutants of *Drosophila melanogaster*, all affecting the same functional gene on the X chromosome, exhibiting either a short (19 h) period, a long (28 h) period, or arrhythmicity, in rhythms of both population eclosion and individual locomotion [68]. Like the skepticism that had previously greeted the evidence for an endogenous clock, there were some who did not readily embrace the fact that single gene alterations could dramatically affect a behavior as complex as the clock's internal mechanism. As recounted by Benzer, Max Delbrück, 1969 Nobel Laureate, was dumbfounded:

> . . .[Konopka] had shown that you could make mutations that changed the biological clock in Drosophila. And he was telling this to Max, and Max said, "No, that's impossible." And then I said, "But Max, he's already done it." And Max said, "No, that's impossible." That was not a completely unusual kind of event. [69]

While 200 years had to pass to realize the significance of de Mairan's botanical observation, it took less than 20 to capitalize – spectacularly – on Feldman's *frq* and Konopka's *per* mutants.

## 1.7 A Paean to Actograms

No historical account of circadian biology should fail to mention the actogram, a graphical display method that is essentially unique to the field. Figure 1.6 illustrates how plotting a time series in such a format serves to accentuate rhythm phase and period. To our knowledge, the first actograms were published – although not named as such – by Curt Richter in 1922 [27] (his Figs. 17 and 18), but it was in 1926 that they assumed their familiar form, as part of the PhD thesis of Maynard Johnson

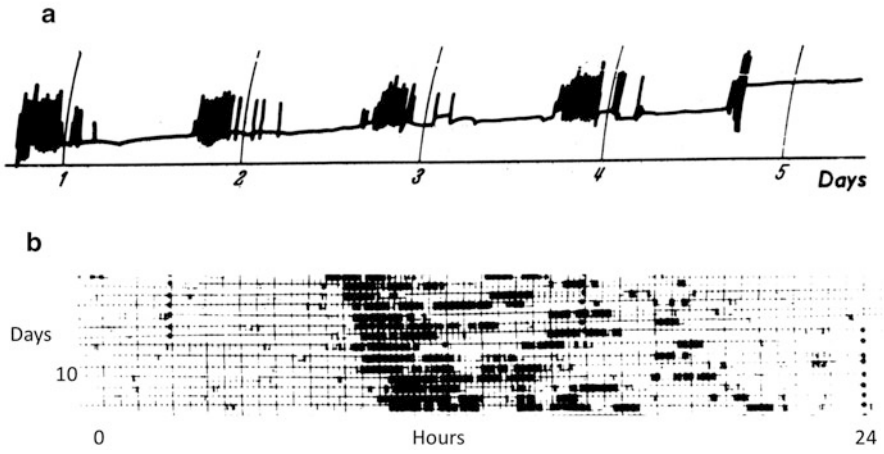


**Fig. 1.6** Recordings of locomotor activity rhythms in two different hamsters in constant light (**a**) or constant darkness (**b**), with the latter graphed in actogram format ((**a**) Reproduced with permission [81]; (**b**) modified from [82])

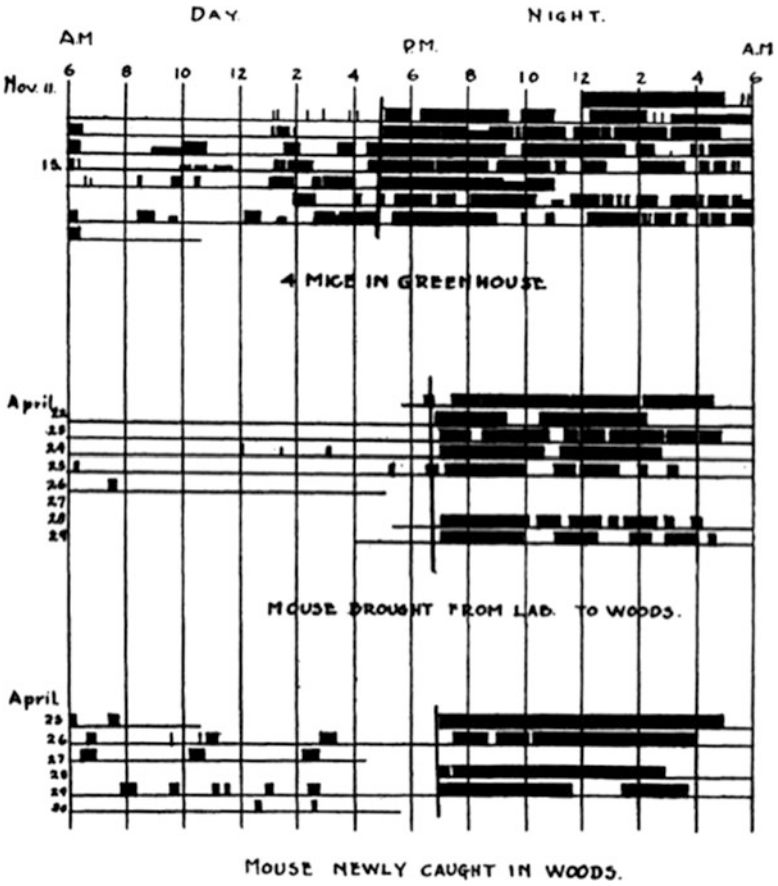


**Fig. 1.7** Recordings of rhythmic mouse cage movements in natural light-dark cycles by Johnson [70] (Reproduced with permission [70])

(referenced earlier), graphing rhythms of cage movement in deer mice (*Peromyscus leucopus* and *P. maniculatus*) (Fig. 1.7) [70]. Although his paper provided extensive detail on his recording apparatus, nothing was mentioned about his choice of stacking each day's activity vertically from top to bottom. The origin of the term for such an activity plot is unclear; perhaps it arose from the Prussian psychologist J.S. Szymanski's *Aktograph* apparatus for recording animal activity, with the inscribed trace on a smoked drum called an *Aktogramm* in a 1918 publication [71] (although multiple days were not arranged vertically). In any case, while many actograms appeared in the 1960 Cold Spring Harbor Symposium *Biological Clocks*, none of them was referred to by that name, and as late as the 1981 Handbook of Behavioral Neurobiology volume on *Biological Rhythms* [72], J. T. Enright wrote "actogram" in quotations, John Brady referred instead to actographs, and others used the rather nonspecific term "raster plots."

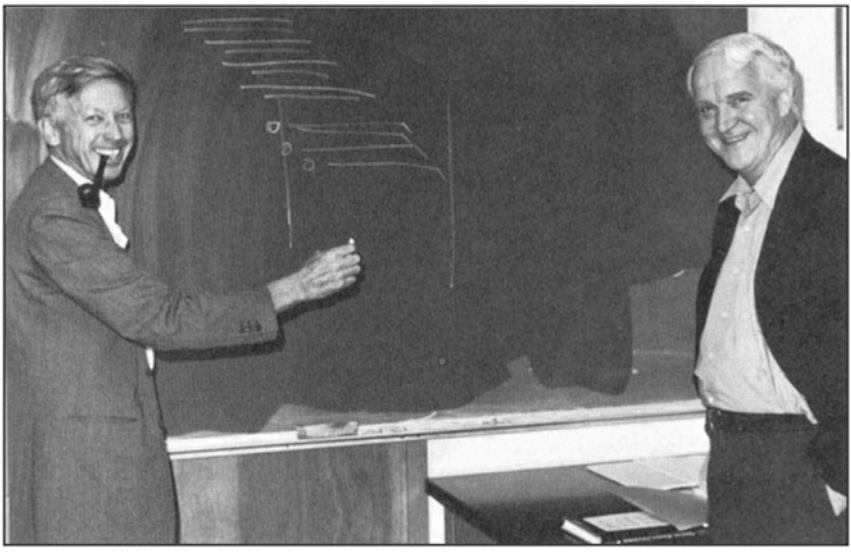

**Fig. 1.8** Jürgen Aschoff (*left*) and Colin Pittendrigh in 1970. Their diagrammed actogram clearly shows phase and period but no amplitude (Reproduced with permission [83])

As a means to analyze the burgeoning studies on rodent rhythmicity [73], actograms meshed well with the field's emphasis on clock phase and period (Fig. 1.8) and with the technical limitations of most of the recording equipment of the time, especially pen and ink chart recorders (e.g., Esterline-Angus) that could not provide reliable measures of rhythm amplitude (Fig. 1.6b) [74]. There were attempts to incorporate amplitude in actographic displays [75] (Fig. 1.9), but these failed to achieve widespread acceptance, and rigorous analyses of rhythm amplitude had to await the computer.

## 1.8 Coda

Our brief essay cannot do justice to all who have contributed to the success of modern circadian biology; the giants whom we have highlighted stood on and by the shoulders of many others. And here we have not reviewed the historical development of other domains of biological timing, including biomedical rhythmicity, non-24 h functions that depend on the clock, and adaptation to the seasons and the tides. Future historians will have access to fantastic resources, including video histories of lectures (e.g., a 1992 lecture by Pittendrigh at the University of Virginia [76]) and investigator interviews [77, 78].

Our review has taken us to the end of the beginning. Near the end of his life, Pittendrigh realized that the *clock* paradigm, which had proven so useful for the field's development, needed to be re-framed as a temporal *program* of events at

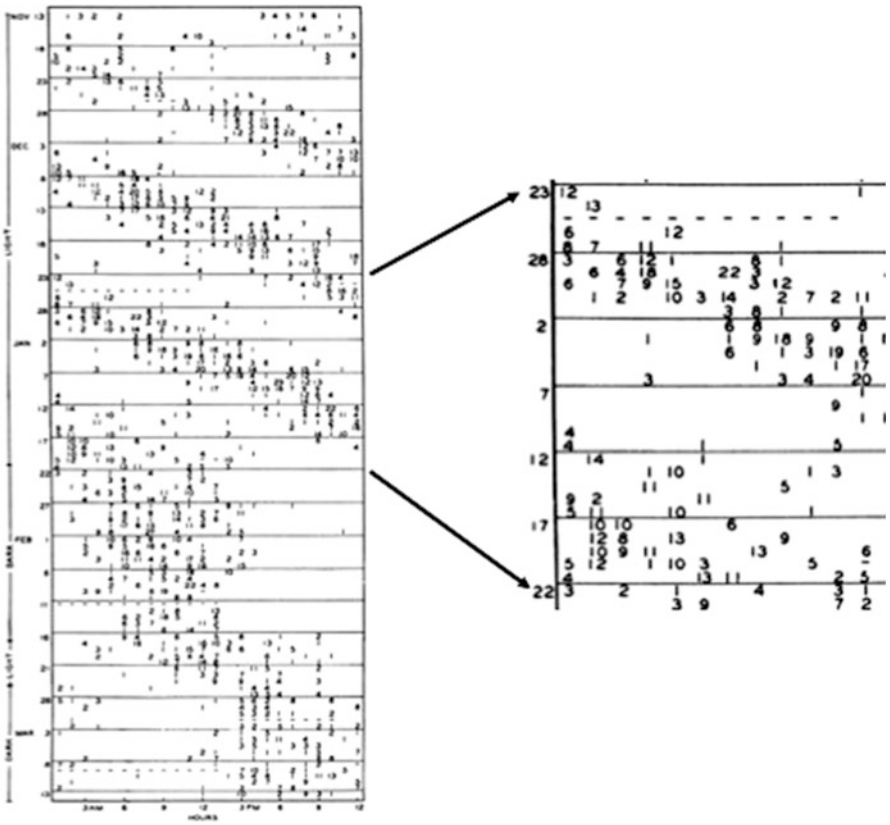

**Fig. 1.9** Plot of rat locomotor activity, with amounts in arbitrary units, in constant dim light (Modified from [75])

different times of day, orchestrated by a multiplicity of circadian oscillators ultimately entrained by light [79]. The interdisciplinary investigation of that concept – from molecules, to cells and tissues, and even to communities – is now well underway. The far-reaching importance of circadian organization as a ubiquitous property of living things that evolved on this rotating planet, as well as a prominent phenomenon in human life, behavior, and health, has come to be widely appreciated by both science and society at large.

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# Chapter 2
# Interpreting Circadian Rhythms

**Dietmar Weinert and James Waterhouse**

**Abstract**

(A) Most daily rhythms are impure reflections of the body clock's output due to exogenous components and effects of the sleep-wake cycle.
(B) The constant routine protocol enables effects due to the body clock (endogenous component) to be separated from those due to the environment and sleep-wake cycle (exogenous component), but it is arduous to perform and inappropriate for body clock assessment on consecutive days and for studies upon animals and young subjects, for example.
(C) The components due to the body clock and the sleep-wake cycle can be separated by the forced desynchronisation protocol, but this also is an impractical protocol.
(D) The endogenous component of a rhythm is accurately described by a sinusoid.
(E) The rhythm of melatonin secretion (measured in dim light) is currently the favoured method for assessing the phase of the body clock, but it is expensive, sampling during the sleep period (when the rhythm is at its peak), requires a venous catheter or continually waking the subject and is inconvenient for field studies. Core temperature is easier to measure, but masking effects need to be removed at source or corrected mathematically ("purification"). Current purification methods are not widely accepted.
(F) For mental performance tasks involving cognition, the endogenous component consists of a homoeostatic effect (due to time awake) and a clock-driven component. These two components can be separated by the forced desynchronisation protocol but not by a constant routine.
(G) For physical performance, constant routine and forced desynchronisation protocols have been used far less frequently, and so the exact origin of measured rhythms is less well understood. Also, the problem of muscle fatigue can limit the frequency of measurement. Insofar as many physical actions also involve

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reflex activity and cognition, it seems likely that effects of muscle fatigue, time awake (mental fatigue) and exogenous factors will all be superimposed upon a clock-driven component.

## 2.1 Background

Homoeostasis – maintaining variables within viable limits in spite of an individual's lifestyle and environment – is fundamental to physiology. Nevertheless, if a variable is measured regularly throughout a period of 24 h in subjects living normally (eating/being active in the daytime, sleeping at night), daily rhythms are found. Even though details of these rhythms might vary day by day, the general profile is consistent both between and within individuals.

Chronobiology considers how an individual's biology depends upon time of day, the seasons or some other influence with a different timescale. In this chapter, daily (circadian) rhythms – changes during the course of the solar day, a 24-h period – will be considered.

Two obvious causes of such rhythms are an individual's environment and lifestyle: the environment shows marked day-night differences – in light intensity and temperature, for example – and an individual's lifestyle normally comprises daytime activities (physical, mental and social and food and fluid intakes) and nocturnal rest and sleep. Do such causes account for the observed rhythms?

## 2.2 Separating the Endogenous and Exogenous Components of a Rhythm: The Constant Routine Protocol

### *2.2.1 Rationale*

Many of the above factors can be standardised when measurements are made under "control conditions", the subject sitting quietly in a standardised environment for a few minutes before any reading is made. This procedure has been extended for studies of daily rhythms – the "constant routine". In this protocol (more details of which are given in [1], for example), the subject is required to remain awake and sedentary (or, preferably, lying down but remaining awake) for at least 24 h in an environment of constant temperature, humidity and lighting; engage in undemanding activities throughout this period (reading or listening to music) and take identical meals at regularly spaced intervals. Lighting levels were initially normal domestic values (ca. 200 lx) when the rhythm of core temperature was of

interest, but have more recently become lower (ca. 15 lx) as the rhythm of melatonin secretion is investigated. Measurements are made over a 24-h period, so assessing a daily rhythm. The protocol is rather more than making a series of measurements under control conditions since variables that take longer than a few minutes to stabilise can be measured. Such variables would include, for example, core temperature (where a heat load takes about 30 min to dissipate) and the renal excretion of water (where the homoeostatic control of plasma osmolality takes at least 30 min to be achieved).

It is common for a constant routine to last about 27 h, results from the first hours – before equilibrium due to the imposition of standardised conditions has been established – being discarded.

### 2.2.2 Results for Core Temperature

Figure 2.1 compares the mean hourly temperatures of a group of subjects who lived normally (daytime activity and food intake; nocturnal inactivity and fasting) and then undertook a constant routine. The rhythm of core temperature remained during the constant routine, even though its amplitude decreased. Three deductions can be made:

(i) The rhythm which remained during the constant routine must have arisen from within the body. It is described as "endogenous" and is attributed to the body clock, and its waveform is close to a sinusoid.
(ii) Effects of the environment and individual's lifestyle existed in the "normal" record, since the two curves differ; this difference indicates the "exogenous" component of the original recording. For temperature, the exogenous component is mainly due to physical activity (raising daytime temperatures) and sleep (lowering nocturnal temperatures). Effects from mental and social activity will normally be present also, as will those from food intake (the "specific dynamic action" of food intake). Since these exogenous influences normally obscure effects due to the endogenous component of the measured rhythm, they are known as "masking effects".
(iii) The endogenous and exogenous components act in synchrony when subjects live normally, both raising temperature diurnally and lowering it nocturnally.

### 2.2.3 Modifications of the Constant Routine

The above protocol can be used to separate the endogenous and exogenous components of other variables affected by activity and sleep. However, some modifications of it are needed to deal with exogenous factors specific to a particular variable. For example:(i) water or sodium excretion by the kidneys; the intakes of these substances need to be controlled more accurately than is likely to be achieved

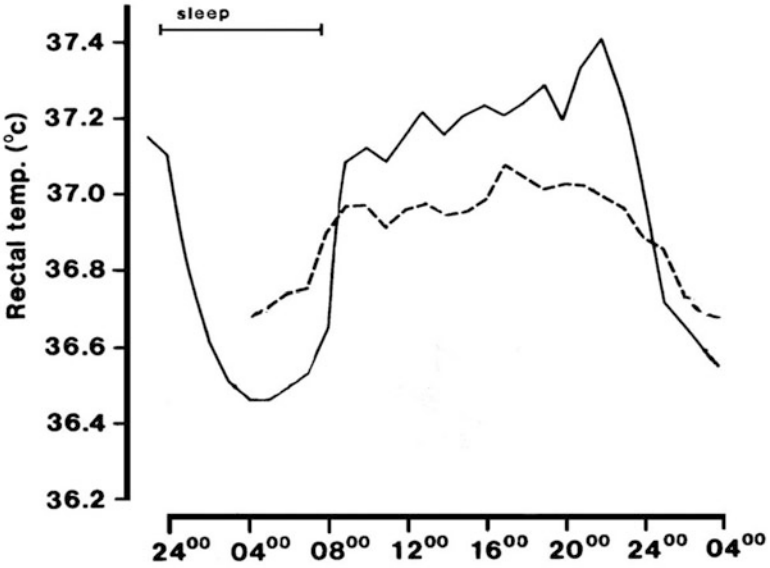


**Fig. 2.1** Constant routine protocol. The mean temperature rhythm is shown for a group of eight subjects living normally (*full line*, time of sleep shown by the *horizontal line*) and then undertaking a constant routine (*dashed line*) (From [1])

merely by "a regular intake of identical snacks". (ii) ADH secretion; water intake and posture need to be controlled; and (iii) hormones associated with carbohydrate metabolism; intravenous infusion of glucose to maintain plasma glucose levels is required. Eating identical meals regularly does not guarantee a constant uptake of glucose into the bloodstream, both digestion and absorption from the gut showing daily variation.

### *2.2.4 What the Constant Routine Demonstrates*

The constant routine protocol is the best way to characterise the exogenous and endogenous components of a daily rhythm. Since the endogenous components of body temperature, heart rate and blood pressure closely resemble a sinusoid [2, 3], the cosinor method may be used to estimate their amplitude and phase. Because rhythmic effects of the exogenous component have either been removed (sleep disallowed) or made uniform throughout the period of measurement (activity), cosinor analysis gives information about the phase and amplitude of the endogenous component, but not about its mesor.

The protocol also indicates that the relative size of these two components is specific to a variable; some – for example, the heart rate, the blood pressure, the excretion of water by the kidneys and the pituitary secretion of growth hormone

(promoted by sleep) – have a much larger exogenous than endogenous component. Accordingly, measurement of these rhythms normally, when masking effects are present, discloses little information about the body clock. Thus, even though the heart rate rhythm adjusts readily to shift work, this result only shows that the exogenous component of the rhythm has changed; the amount of adjustment of the body clock cannot be inferred. By contrast, the excretion of potassium in the urine and, particularly in subjects studied indoors, the secretions of melatonin and cortisol into the plasma have larger endogenous components, and so the rhythms of these substances reflect the output of the body clock more accurately. Core temperature has endogenous and exogenous components of similar magnitude (compare the two curves in Fig. 2.1), some implications of which will be discussed later.

### *2.2.5 Measuring the Phase of the Body Clock*

One implication of this is that the rhythms of cortisol and melatonin secretion are better "markers" of the body clock than is that of heart rate, for example, and the rhythm of core temperature is of intermediate value. In practice, cortisol secretion is rarely used because its secretory profile is complex; in addition to its daily rhythm, it shows episodic secretory bursts, and so its daily component is difficult to quantify. In addition, the profile of a rhythm might change in certain diseases, but it is often not possible to attribute this change to the body clock itself.

#### 2.2.5.1 Melatonin

Melatonin secretion is often used as a marker of the body clock, provided that samples are taken in dim light. Secretion into the blood normally rises a few hours before bedtime, probably contributing to the desire to go to sleep. Secretion continues throughout the night and falls in the morning, around the time of waking, this fall normally promoted by increased light exposure at this time.

The melatonin rhythm can be assessed by hourly or half-hourly sampling of plasma or saliva or by excretion of its metabolite in urine. More frequent sampling enables the rhythm to be described more accurately but is inconvenient (requiring a catheter) and expensive. The phase of the body clock is often inferred from the time at which melatonin secretion starts to rise (DLMO, dim light melatonin onset), this requiring a shorter period of sampling. Total nocturnal secretion can be assessed from an overnight urine sample.

It is unclear if DLMO varies with the intensity of daytime light, before the subject is exposed to evening dim light. It might be argued that daytime suppression of melatonin secretion by bright light would delay DLMO due to some "carry-over" effect, or daytime suppression of melatonin secretion would advance DLMO due to some "rebound" effect. Even though most people are exposed to low daytime levels

of light, often with a red bias (through indoor artificial lighting), light exposure in the hours before sleep poses a problem.

These issues remain to be resolved; nevertheless, the current view is that DLMO provides the best estimate of the phase of the body clock in humans.

#### 2.2.5.2 Core Temperature

Core temperature is also used as a marker of the body clock and has the advantage that it can be measured repeatedly, conveniently and cheaply using a rectal probe or a thermistor that has been swallowed and passes through the gastrointestinal tract. However, since physical activity and sleep affect core temperature, these effects either need to be removed or made constant (constant routine) or corrected for in some other way (Sect. 2.4). Moreover, it has to be considered that thermoregulatory responses to exercise vary with the time of day due to rhythmic changes in the thresholds and sensitivities of cutaneous vasodilatation and sweating, the main causes of the circadian temperature rhythm.

### *2.2.6 Limitations of Constant Routines*

The constant routine is often regarded as providing the "gold standard" for uncovering the endogenous component of a rhythm, but suffers several limitations:

(A) Exogenous effects need to be standardised, and it is assumed that this has been achieved a few hours after the constant routine has begun. Such an assumption might not always be true – for longer-term metabolic changes, for example.
(B) It is too demanding for field conditions and so is "laboratory bound". Even in the laboratory, it might cause stress and thus affect the body clock.
(C) It is unsuitable for experiments with animals and for individuals who cannot undertake the demanding protocol (babies or those who are severely ill or mentally inadequate).
(D) It cannot be used on consecutive days – for assessing the process of adjustment to a time-zone transition or during night work, for example – because sleep is prohibited.
(E) Any variable affected by sleep loss or the amount of time awake cannot be assessed satisfactorily; indeed, time-awake effects become marked towards the end of a constant routine, an issue that will be discussed in Sect. 2.5.

For (A), the constant routine protocol would need to be incorporated into a longer-term metabolic study with appropriate dietary controls. Items (B–D) require a different approach (Sect. 2.4). Item (E) is very important when mood and mental performance are considered; it can be tackled by a different protocol, that of forced desynchronisation.

## 2.3 Separating the Endogenous Component of a Rhythm from Effects Due to the Sleep-Wake Cycle: The Forced Desynchronisation Protocol

### *2.3.1 Rationale*

The "fractional desynchronisation" protocol is based on the observation that the body clock cannot adjust to an imposed lifestyle whose period differs substantially from 24 h. Thus, if a subject lives on regular 27-h "days" (9-h sleep, 18-h activity each "day"), the endogenous component of a rhythm is no longer entrained but rather "free runs" with its autonomous circadian period (about 24.3 h). Also, $8 \times 27$-h days equal $9 \times 24$-h solar days, this length of time being called a "beat cycle". With such a protocol, the endogenous component of the rhythm becomes desynchronised from the component due to the imposed lifestyle, the two components moving out of phase and then back into phase during the course of a beat cycle. This is illustrated in Fig. 2.2 for an individual whose free-running period during days 9–29 averaged 24.3 h and who underwent two beat cycles on this protocol.

Line A represents the minimum of the temperature rhythm; it is initially entrained to mid-sleep (full line) but then free runs with a period averaging 24.3 h (dashed line). During days 9–29, the minimum of the temperature rhythm is coincident successively with mid-activity, soon after rising, the sleep phase, just before retiring and progressively earlier phases of the activity cycle – before becoming coincident once again with the sleep phase (days 24–26) and then the activity phase (days 27–29). Considering all temperatures on the dashed part of line A, they will have been affected by all stages of the sleep-wake cycle. The same argument can be used with regard to the temperature maximum (line B) and, indeed, with any other line parallel to line A or B. In other words, the forced desynchronisation protocol enables an estimation of the endogenous rhythm of temperature to be made with the sleep-wake effects having been averaged and, therefore, no longer exerting a rhythmic effect.

The dotted lines (C, D and E) represent temperatures obtained just before retiring. These temperatures coincide with, successively, midway between the temperature minimum and maximum, the temperature maximum, the falling phase of the temperature rhythm, the temperature minimum, the rising phase of the temperature rhythm (days 18–20) and so on. Considering all temperatures on lines C, D and E together, it can be seen that they will have been affected by all phases of the circadian rhythm. The same argument can be used with regard to other lines that are parallel to C, D or E and placed at a different phase of the sleep-wake cycle. That is, the protocol also enables an estimation of the effects of the sleep-wake upon temperature to be made with the endogenous effects having been averaged and, therefore, no longer exerting a rhythmic effect.

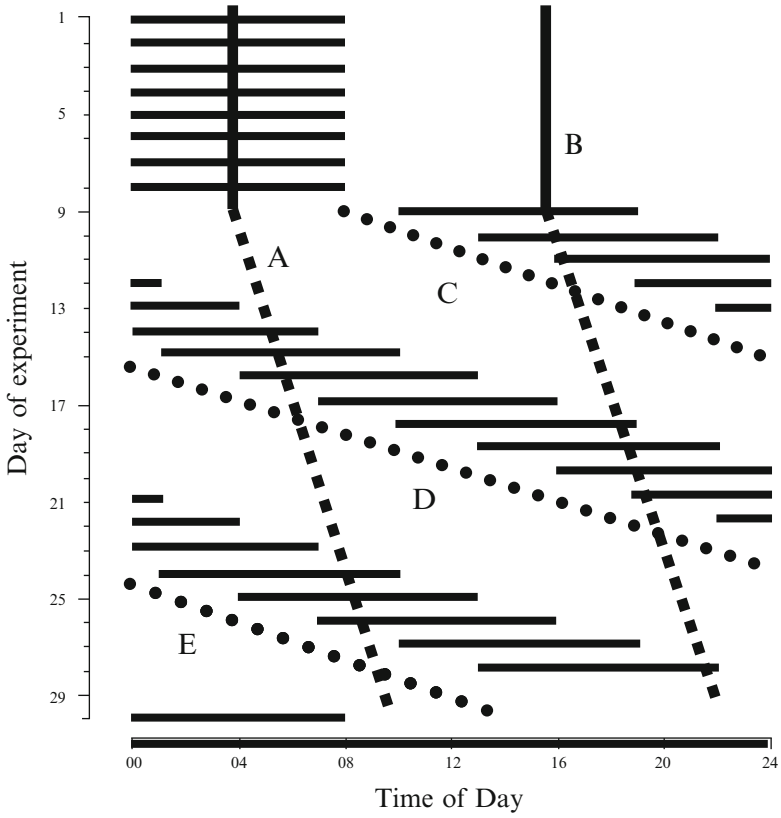


**Fig. 2.2** Forced desynchronisation protocol. The *horizontal black bars* indicate times of sleep. Days 1–8, control days – sleep from midnight to 8:00 and meals at conventional times followed by a constant routine lasting for 26 h. Days 9–29, living on 27-h "days" with 9-h sleep and 18-h awake (From [4])

### *2.3.2 Results for Spontaneous Activity and Core Temperature and Their Interpretation*

Results for core temperature and activity (measured actimetrically) in which the above protocol was undertaken by a group of volunteers are shown in Fig. 2.3a, b.

Figure 2.3a shows that the endogenous components of core temperature and motor activity (spontaneous activity which is independent of the sleep-wake cycle and due to a direct effect of core temperature) manifest synchronised circadian rhythms. Both rhythms are sinusoidal, their profiles closely resembling that of the endogenous component in Fig. 2.1 – that is, the endogenous component extracted from a forced desynchronisation protocol is almost identical to that given by the constant routine (which provides a direct estimate of this component).

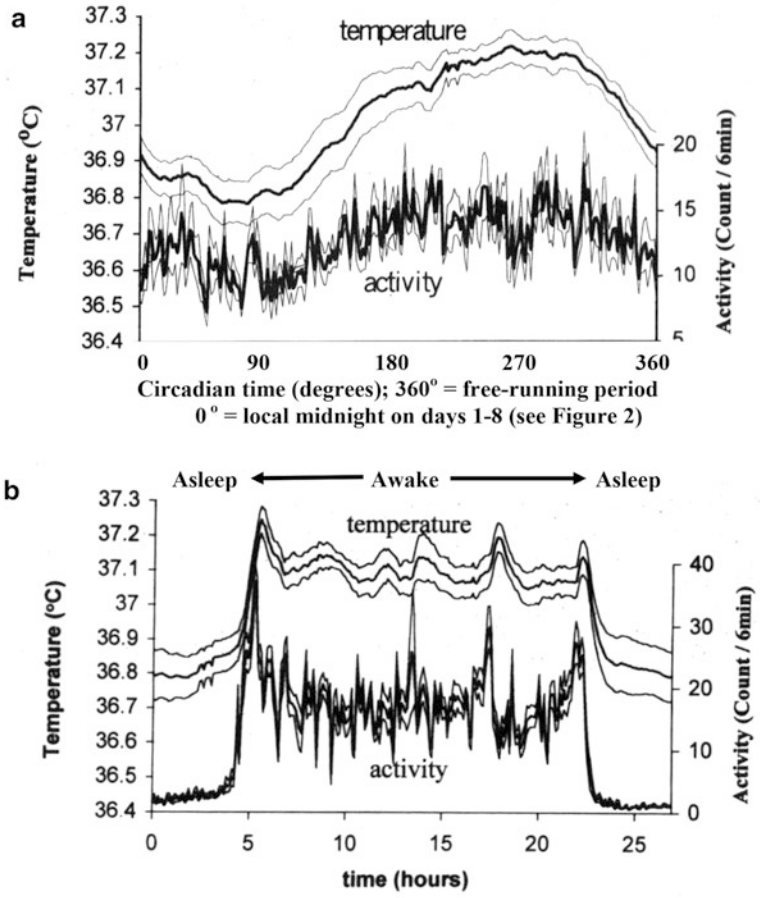


**Fig. 2.3** Results for core temperature and activity (measured actimetrically) from a forced desynchronisation protocol. (**a**) Circadian (endogenous) component, time-awake and sleep effects having been averaged out. (**b**) Effects of the sleep-wake cycle, circadian effects having been averaged out. For more details, see text (From [4])

Figure 2.3b shows the temperature and activity records after correction for circadian effects. There is a clear dichotomy between "nocturnal" inactivity (asleep) and "daytime" activity (awake), and this is of a much greater amplitude than is seen in Fig. 2.3a. This dichotomy is reflected in the temperature record – differences in heat load produced by waking activity vs. sleeping inactivity being largely responsible for it. This is a clear example of the direct masking effect of the sleep-wake cycle upon the temperature rhythm, an effect confirmed (Fig. 2.3b) by the observation that regular bouts of activity (rising, preparing meals and retiring) produce transient rises in core temperature. Such a rhythm is not satisfactorily described by a sinusoid.

The plateau of temperature during the hours of waking (4.5–22.5 h) is almost exactly horizontal. That is, temperature regulation does not change with time awake – the thermoregulatory system does not "fatigue". This is in strong contrast to mental performance (see Sect. 2.5).

### *2.3.3 Summary of the Problem (1)*

Both constant routine and forced desynchronisation protocols indicate that a measured rhythm comprises an endogenous component and masking factors due to the environment and the individual's sleep-wake cycle. However, if the measured rhythm is to reflect accurately the phase of the body clock, then masking factors need to be removed at source (a constant routine) or small (melatonin). The problems associated with constant routines and the measurement of the melatonin rhythm have already been described; the forced desynchronisation protocol, due to its length and complexity, is also a tool that cannot be used in field conditions, upon babies, etc.

Accepting that there are problems caused by the protocols that can be used to isolate the endogenous component of a rhythm, that nocturnal measurements of melatonin are inconvenient, and that effects of the sleep-wake cycle upon core temperature might mask the phase of the body clock (even though the rhythm is comparatively easy to measure under field conditions), is there an alternative way to deal with temperature data?

## 2.4 Further Ways to Estimate the Endogenous Phase of a Measured Rhythm

### *2.4.1 Correcting Masked Temperatures Mathematically*

An alternative approach is to accept that raw temperature data are masked (Fig. 2.4a, b) and then attempt to correct for this mathematically ("purification"), so giving more accurate information about the endogenous component. Two methods have been used.

#### 2.4.1.1 Purification by Categories

This method (Fig. 2.5, top) assumes that the endogenous rhythm is described by a sinusoid (see Figs. 2.1 and 2.3a) and that, when the subject is lying down but awake (as in a constant routine), the associated temperatures are not masked but accurately reflect the endogenous component. It is proposed that the sinusoid is displaced

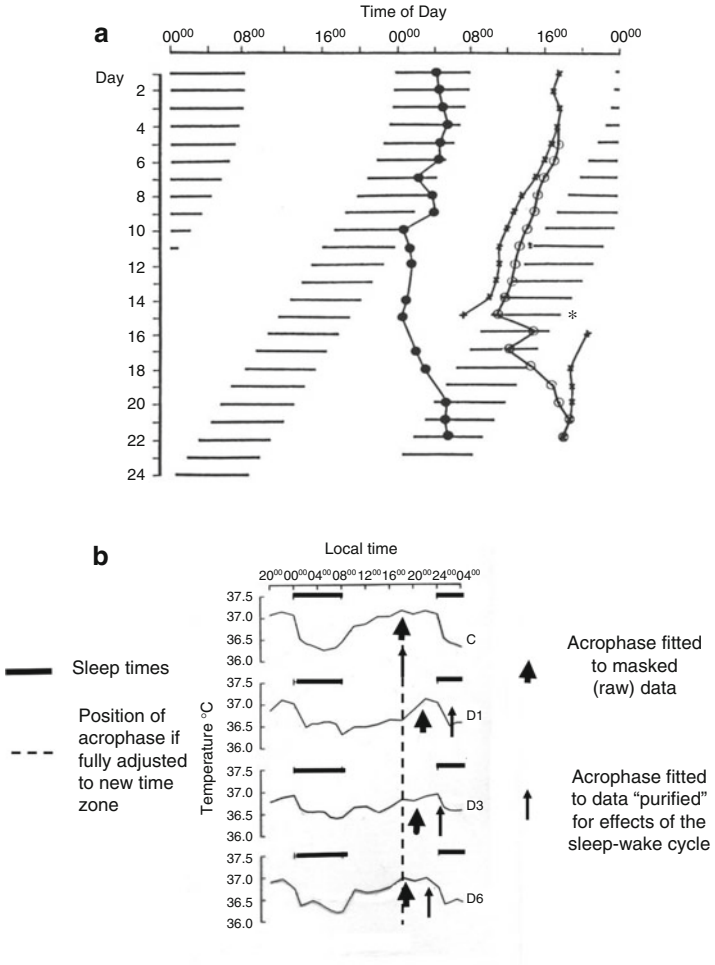


**Fig. 2.4** (**a**) The daily acrophases of melatonin secretion (*filled circles*), raw (masked) core temperature data (*crosses*) and purified temperature data (*open circles*) in an individual living on a normal day (days 1–3) and then 22.8-h "days" (days 4–24). Times of sleep are shown by *horizontal lines* (From [5]). (**b**) Raw core temperature curves (*line*) and acrophases fitted to masked and purified temperature data on a control day (C) and days 1, 3 and 5 (D1, D3 and D5) after a simulated time-zone transition to the east of 8 h (Based on [6]). For more details, see text

downwards by sleep and upwards by different levels activity, as indicated in Fig. 2.5. Due to the thermal capacity of the body, the effect of activity will not be immediate, and so its effect upon body temperature is estimated from activity in the previous 30 min. That is, a series of synchronous sinusoids exists, and the measured temperature switches from one curve to another according to the type of activity. The phase of the set of sinusoids and the vertical differences between the curves are

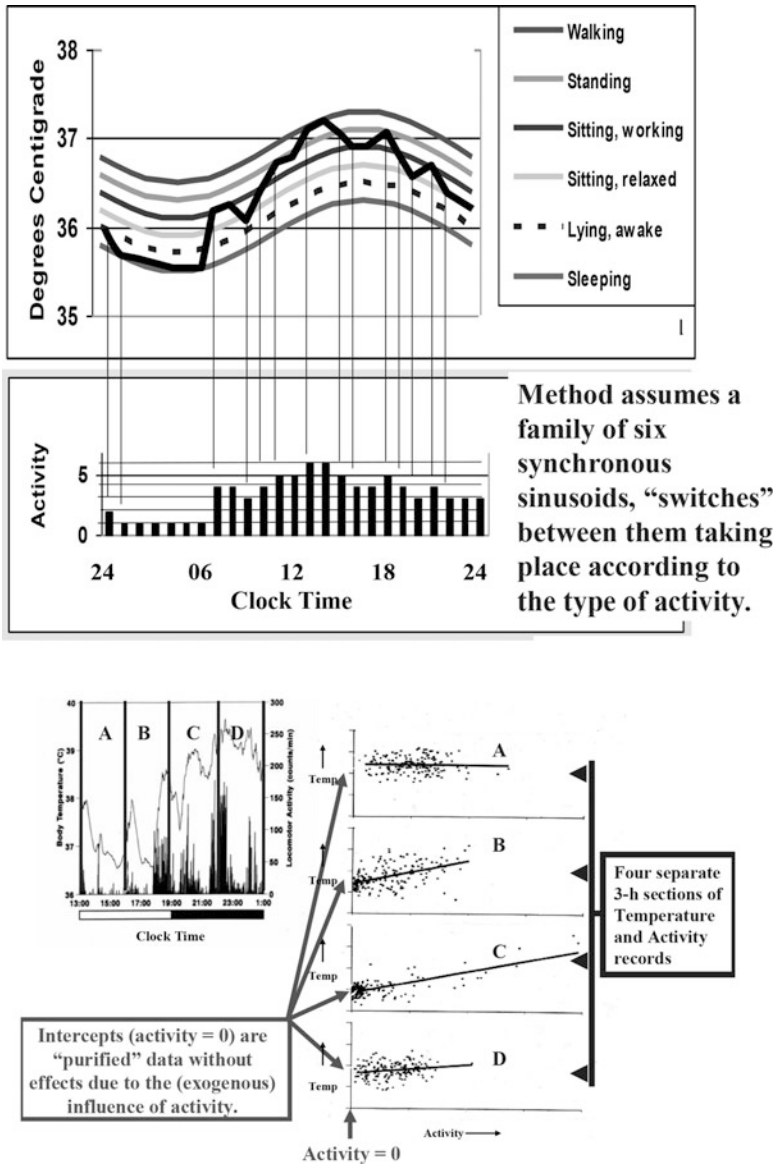


**Fig. 2.5** *Top*: Purification by categories. *Bottom*: Purification by intercepts (From [7]). For more details, see text

estimated iteratively, the aim being to duplicate the profile of the raw data as closely as possible by using the set of sinusoids.

The process is performed separately for each day and for each individual. Results indicate that temperatures need to be corrected in proportion to the amount

of activity; whilst this is not surprising, it is stressed that it is not a requirement of the method, each category of activity being treated independently.

#### 2.4.1.2 Purification by Intercepts

The second method was developed from work on mice and considers a putative phase dependence on exogenous effects [7]. Its basic assumption is that there is a linear effect of activity upon core temperature (at least for lower activity levels). Therefore, a linear regression of measured temperatures upon mean activity in the previous 30 min enables an intercept temperature, $T_{int}$, to be estimated, a hypothetical temperature that would have existed in the absence of any physical activity (Fig. 2.5, bottom). The regression analysis is performed upon successive blocks of data – for example, hourly blocks – and separately for each day and for each animal. Using this method in animals showed that the shape of the purified temperature rhythm approximated closely to a sinusoid. This result supports the view that the purified temperature rhythm is no longer masked (see the endogenous component in Figs. 2.1 and 2.3b), even though it is stressed that the method does not specify the shape of the purified rhythm.

The method was initially less successful in humans, the temperatures during sleep being depressed (see the raw data in Fig. 2.5, top). This further fall of temperatures during sleep in humans is caused not only by decreased activity during sleep (which is taken into account by the analysis) but also by a period of consolidated sleep and change in posture (neither of which occurs in rodents). When temperatures were corrected for this extra fall during sleep, the method was as successful in dealing with data from humans as from mice [8].

### *2.4.2 Comparing Results from the Two Purification Methods and from Constant Routines*

Acrophase estimates using the two purification methods are almost identical [8].

Two examples illustrate the interpretive problems with masked data that can arise:

Example A. Figure 2.4a compares the acrophases (times of peak) of cosinor curves fitted to daily rhythms of melatonin and body temperature in an individual living on 22.8-h "days". The melatonin rhythm adjusts poorly to this sleep-wake cycle, probably "free running" from around day 10 onwards. By contrast, the temperature rhythm follows the sleep-wake cycle more closely until about day 15, when it suddenly jumps to a new phase before continuing to follow the sleep-wake cycle. Does this result indicate that there are two body clocks (one reflected by the melatonin rhythm and the other by the temperature rhythm) or is the difference due to greater masking effects upon the temperature rhythm?

Example B. Figure 2.4b shows the temperature rhythm measured during a control day and on the first, third and fifth days after a simulated eastward flight over eight time zones. Temperature falls due to sleep are clearly evident, even though effects of waking activity are slight due to the subjects' sedentary lifestyle. Acrophases fitted to the temperatures suggest that adjustment to the new local time is complete by day 5. Is this the true rate of adjustment of the body clock, or has the rate been "contaminated" by masking influences from the exogenous component (which will be in phase with the new local time)?

Figure 2.4 also illustrate the effects of purification. Figure 2.4a shows that, when daily acrophases were fitted to the purified temperature data, the daily shifts in temperature mirrored those of melatonin. That is, there was no longer any need to postulate the presence of two body clocks – the original problem had been due to masking effects of the sleep-wake cycle upon temperature data. Figure 2.4b shows that the acrophases fitted to the daily rhythms of purified data adjusted far more slowly than those for masked data, adjustment being only about two thirds complete by day 5.

Accepting that the two purification methods produce very similar results, even though they are based upon different assumptions and mathematical treatments of the data, do they indicate accurately the phase of the body clock (the endogenous component)?

To investigate this, a simulated eastward time-zone transition across eight time zones was performed [9]. Rectal temperatures were measured throughout the study, the phases of the daily rhythms of both the raw and purified data being assessed each day. The two methods of purification gave almost identical results. From these phase estimates, the process of adjustment to the new time zone of raw and purified temperature rhythms could be measured. Two constant routines were performed, the first during the 7-day control phase and the second 4 days after the transition; the difference in phase between these two constant routines was taken to indicate the "correct" phase adjustment of the body clock. This shift was compared with that predicted by extrapolation of results from days 1–3 after the time-zone shift to day 4. As expected, the raw data predicted a significantly greater shift than was measured by the constant routine data; by contrast, there was no significant difference between the shift measured by the constant routines and that predicted from either set of purified data. These results confirm that masked temperature data showed phase shifts that overestimate the process of adjustment of the body clock to a time-zone transition (see also Fig. 2.4b) and also indicate that the purification methods remove this problem and produce results not significantly different from that given by the constant routine.

### 2.4.3 Summary of the Problem (2)

Whereas the constant routine and forced desynchronisation protocols experimentally separate the endogenous component of a rhythm from that due to the environment and the sleep-wake cycle, they are too demanding for field experiments; unsuitable for animals, babies and those who are severely ill, and inappropriate for use on consecutive days. The purification methods offer a different approach; they attempt to correct mathematically data that are masked and, therefore, "impure" reflections of the output of the body clock. So far, these methods have been used upon core temperature data only, but they indicate that the masking effects due to an individual's sleep-wake cycle can be removed from this variable, so producing phase estimates that do not differ from those found using a constant routine.

Nevertheless, the methods have not gained general acceptance, possibly due partly to the large amount of computation involved. The view has also been expressed that, since the amount of masking depends upon the phase of the endogenous rhythm when the masking occurs, masking effects and the endogenous rhythm cannot be separated unambiguously, a criticism that can be levelled particularly at the purification by category method. However, the authors believe that, by making separate estimates for each day and subject and even within a period of 24 h in the case of the purification by intercept method, this problem can be mitigated.

Whatever the true position, a method which corrects masked data, enabling estimates of the phase of the body clock to be made, is required for chronobiological studies in the field and upon animals, etc. – even if such a method has not yet been devised.

## 2.5 Mental Performance Rhythms: Time-Awake Effects

### 2.5.1 Types of Mental Performance Task and Their Rhythms

It has already been mentioned that the endogenous component of any variable affected by sleep loss or the amount of time awake cannot be assessed satisfactorily by a constant routine protocol and that, by contrast, even though the forced desynchronisation protocol does not remove time-awake effects, it does enable them to be assessed since it separates them from the endogenous rhythm.

As indicated by Fig. 2.3b, core temperature does not show any effects of time awake, the plot during the waking phase being horizontal. By contrast, there is clear evidence that some types of mental performance show effects of time awake. "Mental performance" refers to a mixture of tasks which range widely in their nature and complexity; in the present context, a "simple" task is one that requires little cognition (e.g. a simple reaction time and a simple search task) as opposed to a "complex" task, where cognitive processes (interpretation and decision making) are

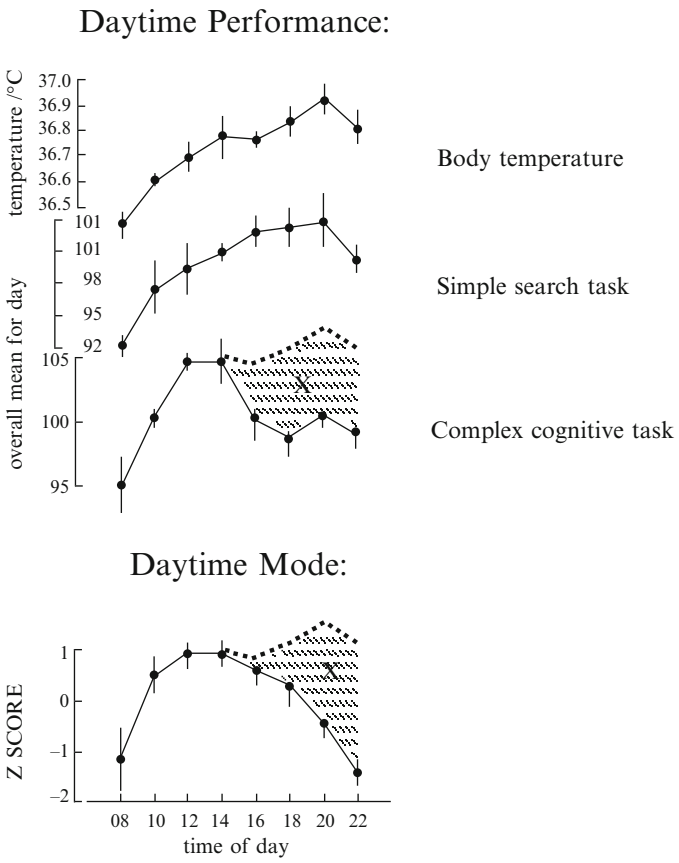


**Fig. 2.6** Dependence on daytime of body (core) temperature, a simple search task, a complex cognitive task and mood. For explanations of the hatched areas ("X"), see text (Based on [10])

important. "Mood" shows properties closer to those of a complex than a simple performance task.

Figure 2.6 shows daytime values for body temperature, performance at two different types of mental task and mood. For all variables, the rise early in the daytime parallels that in body temperature, but differences begin to emerge from the early afternoon onwards. Whereas the diurnal rhythm of a simple task tends to remain parallel to core temperature and peak in the late afternoon, the rhythms for a complex task and mood peak earlier in the daytime, with falls occurring sooner than for core temperature. Accordingly, the acrophase for the simple task is close to that of core temperature, but those of the complex task and mood are earlier. The times of best performance will differ according to the type of task, therefore, but there will be further differences if the phase of the body temperature rhythm is different, for example, due to an individual's age or circadian chronotype [11, 12].

### 2.5.2 Possible Reasons for the Observed Differences

These differences in time course are considered to indicate variations in the rate at which decrements occur due to time elapsed since waking; they illustrate the homoeostatic role of sleep. As the task becomes more complex (its cognitive element increases), this rate of deterioration increases and results in an earlier acrophase. The decline in performance is often referred to as "fatigue" (though this is mental, not muscular, fatigue).

In Fig. 2.6, the profiles of performance at a complex cognitive task and of mood that would be expected in the absence of fatigue (profiles parallel to core temperature) are indicated by the dotted lines, and the differences between these hypothetical curves and the observed result are indicated by hatched areas, labelled "X". That is, it is suggested that observed rhythms in mental performance (when exogenous components have been standardised) are determined by an interaction between a rhythmic component (parallel to core temperature) and a decrement resulting from fatigue due to time awake. Do results from the forced desynchronisation protocol bear out this interpretation?

### 2.5.3 Evidence from the Forced Desynchronisation Protocol

Figure 2.7 shows results from a forced desynchronisation protocol in which subjective alertness, body temperature and performance at a task requiring cognition were measured. There are clear circadian rhythms for body temperature and the two performance tasks, their profiles being closely parallel. Such results are in agreement with those obtained for spontaneous activity (Fig. 2.3a) and indicate that all have a clock-driven component. By contrast, the changes associated with the sleep-wake cycle show very different profiles, from which the following conclusions can be drawn:

(A) Waking temperatures show little effect of time awake (see also Fig. 2.3b). This confirms that the thermoregulatory system does not fatigue.
(B) By contrast, performance declines with time awake, supporting the above explanation of the earlier fall in performance during the daytime (Fig. 2.6).
(C) Performance often shows a transient *improvement* in the first hour or so after waking; this is "sleep inertia", indicating that any benefits of sleep or nap might not be apparent immediately after waking.

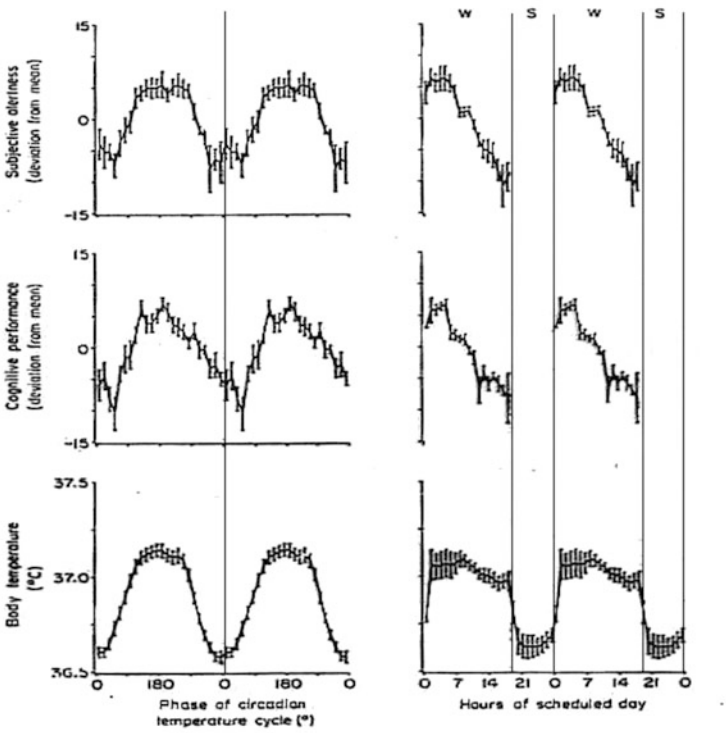


**Fig. 2.7** Results for (*top*) subjective alertness, (*middle*) cognitive performance and (*bottom*) body temperature from a forced desynchronisation protocol (28-h "days"). *Left*: Circadian (endogenous) component; *Right*, time-awake component. *W* wake time, *S* sleep time (For more details, see text (Based on [13]))

### *2.5.4 Summary of Causes of Daily Changes in Mental Performance and the "Ultrashort Sleep-Wake Cycle" Protocol*

Mental performance is affected by circadian and time-awake factors, which can be separated by the forced desynchronisation protocol and can both be considered to be "endogenous". The endogenous component of a mental performance rhythm is more complex than that of core temperature, consisting of a component that shows circadian rhythmicity (parallel to the rhythm of core temperature, possibly with some causal link) and one that causes performance deterioration due to the length of time awake. This interaction between the two endogenous components has been modelled by the "two-process model of alertness" [14], and it is likely that this type of model can also be applied to cognitive performance tasks. The relative

importance of the circadian and time-awake factors depends upon the nature of the performance task, simple tasks being less influenced by time awake than are mood and complex tasks (see Fig. 2.6).

Other factors affect cognitive performance. Not only is it affected by sleep inertia (see above) but also it is adversely affected by sleep loss, whether total or partial, acute or chronic; sleep loss of as little as 2 h can produce measurable decrements [14].

A protocol designed to reduce time-awake effects is the "ultrashort sleep-wake cycle". In this protocol, the sleep-wake cycle lasts less than 24 h; as a result of which, time-awake effects are reduced below the normal value of 16 h. A common example of such a protocol is when subjects undertake 3-h sleep-wake cycles (1 h allocated to sleep and 2 h to waking). Accumulation of fatigue due to time awake is minimal, but the extent to which sleep inertia will affect the results is unclear, and it cannot be assumed that sleep will be obtained equally well in all the 1-h intervals allocated to it. Nevertheless, such a protocol indicates that rhythms of mental performance continue, with minima when the wake time coincides with the night and maxima somewhere in the afternoon, and, importantly, mental performance shows smaller falls due to decreased time awake.

Recent work indicates that individuals differ in their resilience to the effects of time awake and sleep loss. This has been termed "trototype", and there is evidence for a genetic component to this attribute [15].

Figure 2.8 illustrates some of the endogenous factors that affect performance at tasks with different amounts of cognition. Detailed phase positions, but not the internal phase relationships, might vary with chronotype.

## 2.6 Physical Performance Rhythms: Effects of Muscular and Mental Fatigue

### *2.6.1 Types of Physical Performance and the Problems They Present*

As with mental performance, there are many types of physical performance, differing in the intensity and duration of exercise, the number of muscle groups involved, and the relative importance of muscular, neural and cognitive components.

Assessments of physical performance generally involve *maximum* effort (often to exhaustion), which will produce muscle fatigue. Until full recovery has taken place (and full biochemical recovery might take hours), physical performance will be compromised, which has implications for the repetitive measurements required to describe a rhythm. Measuring only one time point per day can overcome this problem, but it greatly increases the total time required to collect the data.

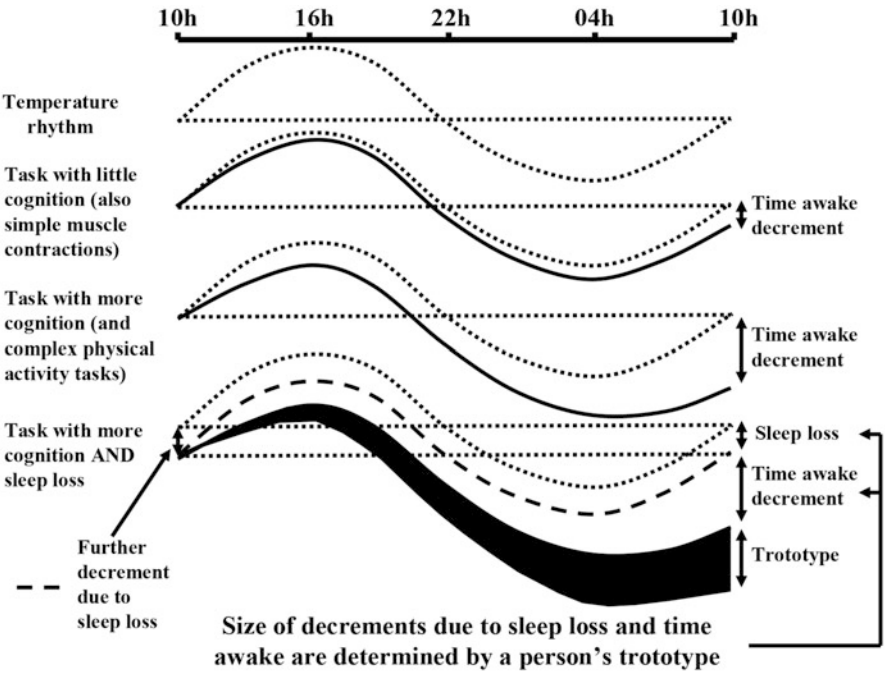


**Fig. 2.8** Diagram to illustrate how the endogenous component of a rhythm in mental or physical performance depends upon the cognitive content of the task, time awake and sleep loss. For more details, see text

Successful performance in many sports is not determined wholly by muscle strength and power; accuracy at aiming at a target, for example, requires manual dexterity and hand-eye coordination and is associated with little muscle fatigue; that is, such tasks show rhythmicity that is due to some (quantitatively unknown) combination of muscle and neural activity. Several complications due to neural involvement exist: (A) Most movements (of a limb, for instance) involve coordination between groups of muscles, requiring reflex control. (B) Movements often affect much of the body's musculature, not just the moving limb, so requiring reflex control of posture and balance. (C) Many physical tasks also involve cognition – when to perform an action and exactly how to do so (e.g. passing a ball in soccer); in such complex situations, a distinction between "central fatigue", including motivation, and "peripheral fatigue" due to the muscles has been made.

Therefore, physical performance will be affected also by those factors which affect mental performance (Sect. 2.5). Further, not all types of physical activity are amenable to a study of rhythmicity; it is difficult to study rhythms at events requiring endurance (e.g. the marathon and triathlon), since the concept of a "time point" – fundamental to assessing a rhythm – becomes meaningless with tasks which take a long while to perform.

### 2.6.2 Observed Daily Rhythms and Their Explanation

Many aspects of physical performance display a daytime rhythm, with higher values in the late afternoon than in the early morning and with differences in timing depending on circadian chronotype. As might be predicted, there is also evidence that movements which require high levels of sensorimotor coordination peak earlier and are more susceptible to effects of sleep loss than are those dominated by muscle strength.

The observation that a variable shows a daily rhythm requires the clock and time-awake contributions to the endogenous component and exogenous effects to be separated. The constant routine protocol has rarely been used in exercise physiology, but protocols that can be considered to be variants of it (see the review [16] for more details) are:

(A) Heart rate and the amount of activity were measured over about 4 days in subjects undergoing a "marathon" football match for charity. Subjects were allowed 5-min "comfort breaks" every hour, when they could drink and eat, but sleep was prohibited. Daily rhythms were present (with peaks around the late afternoon and troughs around the end of the night), superimposed upon downward trends in performance and mood and an upward trend in subjective fatigue. These trends were probably due to both physical fatigue (continuous physical activity), mental fatigue (increasing time awake and sleep loss) and declining motivation. The rhythm that remained after "de-trending" the results can be considered to approximate to effects of the clock-driven component of the tasks.

(B) Subjects were required to cycle on an ergometer at a constant speed for 24 h and then to stay awake and rest for a further 24 h. The maximum torque developed by voluntary isometric knee extension was estimated at 4-h intervals throughout the 48 h. Rhythms peaking at about 19:00 h were observed on both days, and, again, decrements due to physical, as well as mental, fatigue were evident.

(C) Effects of time awake were reduced in a study using the ultrashort sleep-wake cycle protocol. Swimmers were required to be awake for only 2 h and were then allowed to sleep for 1 h, this 3-h sleep-wake schedule being repeated for a total period of 50–55 h. Maximum swimming performance was assessed six times at 9-h intervals. A rhythm was present, peaking around 23:00 h and showing a trough around 05:00 h (coincident with the temperature minimum). However, there was clear evidence for physical fatigue as the assessments progressed.

No studies using the forced desynchronisation protocol have been reported, research groups appearing to be reluctant to face the challenges posed by the rigour of this research design.

### 2.6.3 Summary of Physical Performance Rhythms

Protocols which enable the endogenous and exogenous components of a rhythm to be separated (a constant routine) and the relative contributions of a clock-driven component and the sleep-wake cycle to be ascertained (forced desynchronisation) have not been applied to studies of physical performance. Accordingly, the detailed causes of rhythms in physical performance remain to be ascertained. The position is complicated by the fact that a physical performance task often contains neural as well as muscular components, and so there will also be a mixture of effects due to physical (muscular) and mental fatigue to disentangle. Even so, it seems likely that the factors that affect mental performance will also be relevant to physical performance, as indicated in Fig. 2.8.

## 2.7 General Conclusions

With regard to inferring the phase of the body clock from a measured rhythm, the general conclusions are:

1. The impact of exogenous and time-awake factors on circadian rhythms differs between variables. However, the widespread use of variables that are masked comparatively little (e.g. melatonin) is restricted.
2. Experimental protocols to remove masking effects at source are “laboratory bound” and inconvenient.
3. Some method to estimate the phase of the body clock under normal circumstances is required. Current purification methods have some value but are not widely accepted; an alternative, possibly based upon them, is needed.

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# Chapter 3
# Basic Principles Underlying Biological Oscillations and Their Entrainment

**Theresa Floessner and Roelof A. Hut**

**Abstract** Biological oscillators govern life-supporting processes from unicellular organisms to the most complex life forms. The timescales may vary from tidal rhythms to annual rhythms. Here we will focus on the organisation of daily rhythms and the underlying circadian oscillators. The molecular mechanisms of circadian oscillators are discussed for mammals and insects. Different light resetting mechanisms and input pathways of circadian oscillators are explained and used to evaluate basic principles of light entrainment.

## 3.1 Introduction

Throughout evolution life is maximising reproductive output by optimising behaviour to the environment. Rhythmic fluctuations in the environment have facilitated the evolution of timing mechanisms to generate an internal representation of these fluctuations for the organism [1]. Conceptually, such an internal representation allows the organism to anticipate the reoccurring rhythmic changes in the environment. The most prominent timescales at which rhythmicity in the environment occurs are tidal (0.5173611 day), daily (1 day), lunar (29.530556 days) and annual (365.256363004 days) rhythms. Internal biological oscillators for tidal, daily, and annual rhythms have been described for various organisms, and recently understanding the molecular basis for tidal and annual rhythms has progressed considerably [2–5]. Nonetheless, the molecular oscillatory mechanisms and their entrainment are best understood for daily (circadian) rhythms. Here we will focus on circadian rhythms in insects and mammals to illustrate concepts and definitions of daily rhythms, free-running rhythms, phase-response curves, principles of entrainment, and light resetting mechanisms.

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## 3.2 Synchronisation and Entrainment

Circadian oscillators are internal biological mechanisms located in many tissues and cells throughout the body. They provide timing signals to the organism. Behaviour, physiological processes, and cellular processes are affected by such oscillating mechanisms. In animals, including vertebrates and arthropods, a central neuronal pacemaker optimally synchronises these various tissue and cell autonomous oscillators. In insects and mammals, the central pacemaker consists of clock neurons with strong molecular rhythmicity and which are closely communicating amongst each other to maintain coherence. In mammals, the neurons of the central circadian pacemaker are located in the supra-chiasmatic nuclei (SCN), located dorsally on the optic chiasm in the ventral hypothalamus.

The central oscillator needs to become synchronised to the 24-h rhythm of day and night in the environment in order to provide a reliable representation of external time to the internal body. The synchronisation process of the internal rhythm of the body to the external daily rhythm in the environment is called *entrainment*. Entrainment (in biology) or resonance (in mathematics) refers to the interaction between two oscillators that leads to mode or phase locking, a notion first observed by the Dutch astronomer, mathematician, early science fiction writer, and inventor of the pendulum clock Christiaan Huygens (1629–1695). In 1665 he wrote a letter to his father Constantijn Huygens, a close friend of Descartes and Galileo Galilei, in which he described his first observation of oscillator entrainment as *odd sympathy*:

> *While I was forced to stay in the bed for a few days and made my observations on my two clocks from the new workshop, I noticed a wonderful effect that nobody could have thought of before. The two clocks, while hanging side by side with a distance of one or two feet between, kept in pace relative to each other with a precision so high that the two pendulums always swung together and never varied. While I admired this for some time, I finally found that this happened due to an odd sympathy: when I made the pendulums swing at different paces, I found that half an hour later, they always returned to synchronism and kept it constantly afterwards, as long as I let them go.*

He published this observation in 'Horologium oscillatorium sive de motu pendularium' (1673):

> *It is quite worth noting that when we suspended two clocks so constructed from two hooks imbedded in the same wooden beam, the motions of each pendulum in opposite swings were so much in agreement that they never receded the least bit from each other and the sound of each was always heard simultaneously. Further, if this agreement was disturbed by some interference, it re-established itself in a short time. For a long time I was amazed at this unexpected result, but after a careful examination finally found that the cause of this is due to the motion of the beam, even though this is hardly perceptible.*

Indeed, the beam on which Huygens' clocks were suspended transferred a small force between both pendula, leading to synchronisation of the two oscillators. In analogy, there also must be some kind of biological force, or better signalling, from the daily environment to the internal circadian pacemaker causing synchronisation between the two oscillating processes. Such entraining stimuli are called *zeitgebers* (time givers), and they will effectively generate a precise 24-h rhythm in the

circadian oscillator, even if the intrinsic period of the oscillator deviates from 24 h under constant conditions (free-running conditions). In general, the daily light-dark cycle can be considered as the most prominent external stimulus (zeitgeber) that entrains circadian systems in various organisms.

## 3.3 Daily Rhythms Versus Free-Running Rhythms

In nature, rhythms in activity patterns are most obvious (Fig. 3.1a). Activity rhythms of animals can be either diurnal (e.g. ground squirrels, butterflies, jewel wasp, most birds), nocturnal (e.g. mice, rats, moths, owls), crepuscular (e.g. *Drosophila*, most ungulates), or arrhythmic (e.g. arctic reindeer, mole rats). Although such activity rhythms may change depending on environmental conditions [6], they can be helpful to define markers and timescales for circadian research (Fig. 3.1a). Under entrained conditions the period of the activity rhythm equals the period of the zeitgeber cycle (T). The time axis of one zeitgeber cycle can be divided into 24 zeitgeber hours (ZT0–ZT24), and either light onset is defined as zeitgeber time 0 (ZT0) or light offset is defined as ZT12 (Fig. 3.1a). This, however, poses a problem in studies where different day lengths (duration of the light phase) are used. Daan et al. (2002) therefore defined another environmental time axis, external time, which, similar to our local timescale, uses midnight as the phase

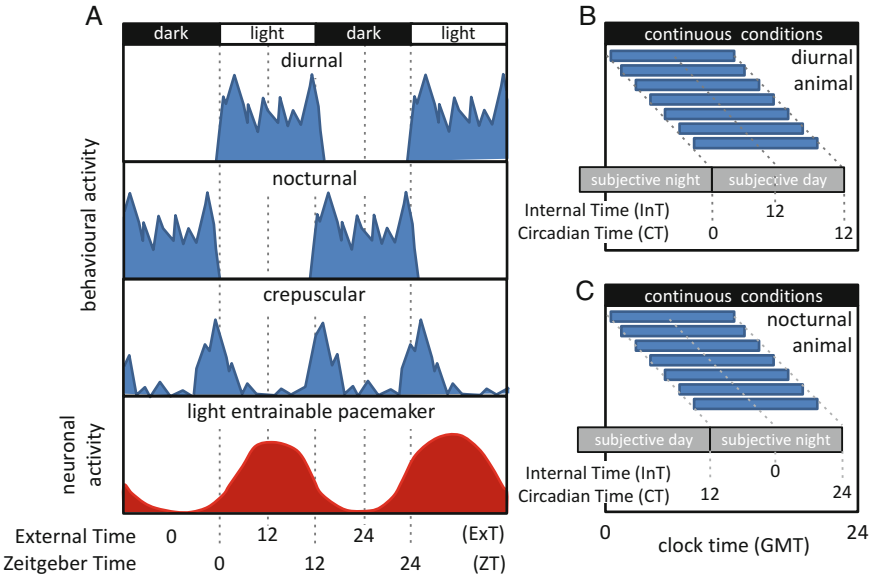


**Fig. 3.1 Daily and circadian activity patterns and timescale definitions in chronobiology** in entrained (**a**) and free-running continuous (**b**, **c**) conditions

reference point (ExT0, Fig. 3.1a). Interestingly, data so far indicate that the light-entrainable pacemaker in the SCN of nocturnal and diurnal mammals roughly maintains the same phase angle relative to the external light-dark cycle (Fig. 3.1a, lower panel), which leads to the conclusion that activity patterns are driven by a slave oscillator or a relay downstream of the SCN [6] and that the SCN under entrained conditions forms a reliable internal representation of the external light-dark cycle independent of the overt activity rhythm.

To establish the endogenous origin of a rhythm, it is essential to show that the rhythm persists under continuous conditions, albeit with a different period (Fig. 3.1b, c). To define phase markers under continuous conditions, we need to know the activity rhythm under entrained conditions. Under continuous conditions, where the activity rhythm of the animal free-runs, we define the subjective day and night as the active and rest phase in a diurnal animal and as the rest and active phase in a nocturnal animal (Fig. 3.1b, c). The circadian timescale (CT) is defined by phase markers drawn from the zeitgeber timescale, where CT0 is defined as activity onset in diurnal animals and CT12 is activity onset in nocturnal animals. Again, this may pose a problem when different durations of activity ($\alpha$) or rest ($\rho$) are compared. As a solution, Daan et al. (2002) also defined an internal time axis (InT) where activity midpoint of a diurnal animal is defined as InT12, while activity midpoint of a nocturnal animal is defined as InT24 (Fig. 3.1b, c) [7]. Both the circadian timescale (CT) and the internal timescale (InT) cover one circadian cycle (with duration $\tau$) and are divided in 24 equal time steps (circadian hours, ch). When $\tau > 24$ h, a circadian hour lasts longer than one clock hour (local timescale), while $\tau < 24$ h results in a circadian hour shorter than one clock hour (thus, ch $= \tau/24$).

## 3.4 Phase-Response Curve and Phase Transition Curve

To understand circadian entrainment, it is useful to understand the perturbing effect of a single stimulus (e.g. light pulse) on a certain circadian rhythm. The effect of such single stimulus depends on the phase ($\varphi$) at which the stimulus is applied. The perturbation (advance or delay) of the observed rhythm can be described in terms of a change in phase or a phase shift ($\Delta\varphi$). The relationship between $\Delta\varphi$ and $\varphi$ is called the phase-response curve (PRC, Fig. 3.2). Numerous experiments have described PRCs to a variety of stimuli in a multitude of organisms [8]. One rule of thumb can be learned from this collection of PRCs: the phase-shifting response to a stimulus is largest at those time points when the organism is usually not exposed to such a stimulus under entrained conditions. Indeed, in many organisms the phase-shifting response to light is maximal during the subjective night (i.e. the dark phase under entrained conditions, Fig. 3.2; [9]).

Two types of PRCs can be distinguished: weak resetting PRCs (type 1; Fig. 3.3a, c green) and strong resetting PRCs (type 0; Fig. 3.3 red). The terms type 0 and type 1 PRC originate from an alternative way to present the phase-shifting effect of a stimulus. Here, the phase of the applied stimulus is expressed relative to the rhythm

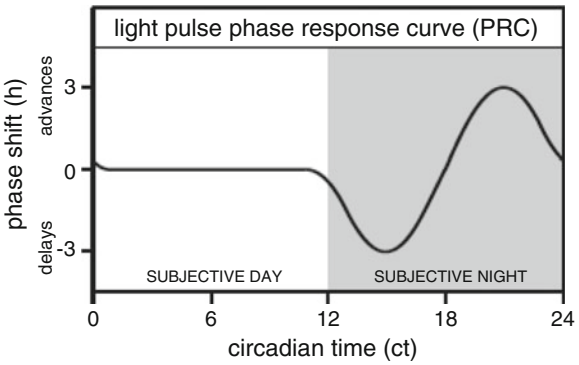


**Fig. 3.2 The circadian phase-response curve** represents the phase-shifting response to a stimulus plotted against the phase of the rhythms at which the stimulus was given. Advances of the rhythm are defined as positive, delays as negative. Under constant conditions, the rhythm of an organism will free-run with a period that can deviate from 24 h. The phase at which the pulse was applied thus has to be expressed on a circadian timescale (CT), where 1 circadian hour (ch) = (period of the free-running rhythm)/24. Subjective day is defined between CT0 and CT12 and subjective night between CT12 and CT24. The dead zone (no response) falls in the middle of the subjective day where normally light would lead to stable entrainment of the rhythm. The largest responses are found in the subjective night where it would be normally dark when the rhythm is entrained by a light-dark cycle. When activity is used as a phase marker, the subjective day is defined as the active phase in diurnal animals, while the subjective night is defined as the active phase in nocturnal animals

before the perturbation ('old phase') and relative to the rhythm after the perturbation ('new phase'). This phase transition can be described in a phase transition curve (PTC; Fig. 3.3b, d) when new phase is plotted against old phase. The average PTC slope of a strong resetting stimulus is 0, while the average slope of a weak resetting stimulus is 1, respectively, type 0 and type 1 resetting.

The PRC and PTC can be considered as a property of the pacemaker and its shape will depend on the response and sensitivity to the stimulus. With increasing stimulus strength (or increasing sensitivity of the pacemaker to the stimulus), a weak resetting PRC (or type 1 PTC) can change into a strong resetting PRC (or type 0 PTC). Theoretical arguments predict a discontinuity during this transition, which becomes clear from the 0 transition during the subjective night (between CT12 and CT24) in a weak resetting PRC, which should jump to a 12-h phase advance (or −12-h phase delay) in a strong resetting PRC when stimulus strength increases.

## 3.5 Entrainment Explained from the PRC: Parametric or Phasic Entrainment

PRCs are useful tools to understand entrainment and, more importantly, phase angle of entrainment. Because free-running rhythms usually do not occur in nature, natural selection could not directly select for a certain intrinsic period of the

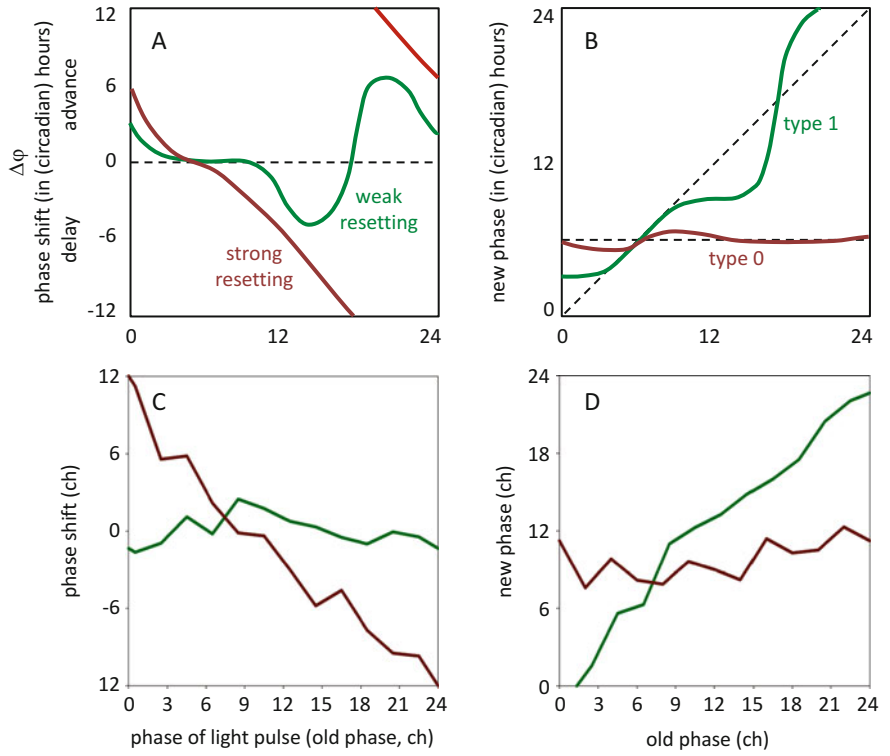


**Fig. 3.3 Circadian phase-response curves (PRCs) and phase transition curves (PTC) for strong (type 0) and weak (type 1) resetting.** The phase-shifting effect of a stimulus can be plotted against the phase of the circadian rhythms when the stimulus was presented to generate a PRC (**a**). The phase resetting response can either be strong (**a**, *red curve*) or weak (**a**, *green curve*). When *plotted* as a PTC (new phase against old phase, **b**), the average slope of the weak resetting stimulus becomes 1 (type 1 PTC; **b**, *green*), while the average slope of the strong resetting stimulus becomes 0 (type 0 PTC; **b**, *red*). The PRC of a jewel wasp (*Nasonia vitripennis*) iso-female line was measured with two different stimulus strengths to observe the transition from weak to strong resetting. Comparing a weak 1-h light stimulus (**c**, *green*) with a strong stimulus 8-h light stimulus (**c**, *red*)) indeed reveals larger phase shifts for the strong stimulus. When plotted as a PTC, the type 0 (**d**, *red*) and type 1 (**d**, *green*) can be distinguished by their average slopes

circadian system. Natural selection could possibly only select for a certain intrinsic circadian period because of its influence on the phase angle of entrainment. Oscillator theory predicts that the ratio between intrinsic period and the period of the zeitgeber ($\tau$/T) determines the phase angle of entrainment ($\psi$). To see this, we can take the weak resetting PRC (Fig. 3.2) and let it entrain to a 24-h light-dark cycle (T = 24 h). Under entrainment, the period of the rhythm = T $\neq \tau$. This means that daily phase shifts are needed to obtain stable entrainment according to the following relationship: T = $\tau - \Delta\varphi$. Thus, if $\tau < 24$ h the circadian rhythm needs

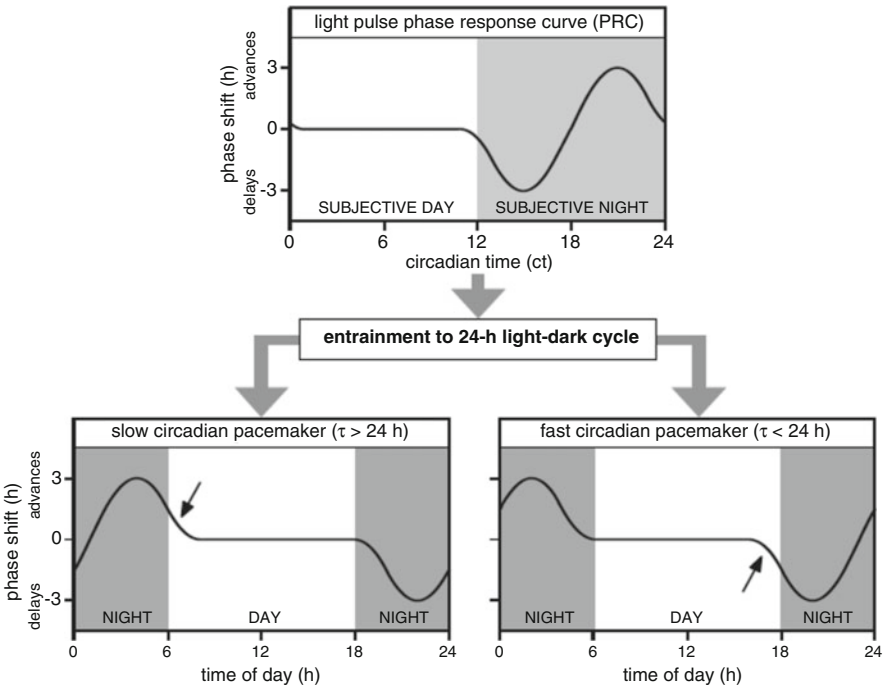


**Fig. 3.4 The PRC as a tool to understand phase angle of entrainment.** If $\tau > 24$, daily advances will lead to entrainment, and if $\tau < 24$, daily delays will cause entrainment. From this it follows that the phase angle of entrainment is determined by the shape of the PRC and the $\tau/T$ ratio

daily delays to entrain to $T = 24$ h, whereas if $\tau > 24$ h daily advances are needed (Fig. 3.4, arrows).

From this it follows that $\tau/T$, together with the shape of the PRC, determines (1) the phase angle ($\psi$) of entrainment and (2) the limits of the range of entrainment to different T cycles (Fig. 3.5a, [9]). This can be simply understood in common language: With a relatively fast clock ($\tau/T < 1$), one tends to be too early, and with a slow clock ($\tau/T > 1$), one tends to be too late (see Fig. 3.4). Because the amplitude of the PRC increases with increasing zeitgeber strength, the range of entrainment will also widen with increasing zeitgeber strength (Fig. 3.5b).

This widening of the range of entrainment is visualised in Fig. 3.5b, where zeitgeber strength is plotted against the $\tau/T$ ratio, thereby creating an area where entrainment will occur: the so-called Arnold tongue, after the Russian mathematician Vladimir Arnold (1937–2010), who also happened to be a great admirer of Christiaan Huygens.

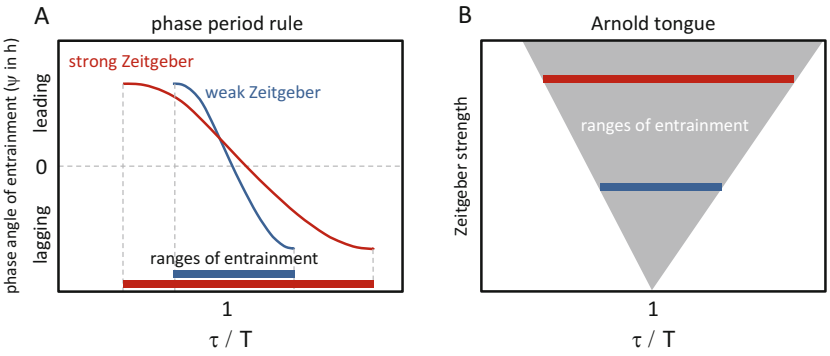


**Fig. 3.5 Phase period rule, range of entrainment, and Arnold tongue.** (**a**) Given a certain shape of the PRC, the ratio between pacemaker and zeitgeber period (τ/T) will affect phase angle of entrainment (see Fig. 3.3). If $\tau/T < 1$, the rhythm will lead relative to the zeitgeber (positive ψ), and if $\tau/T > 1$, the rhythm will lag relative to the zeitgeber (negative ψ). The exact shape of this relationship depends on the shape of the PRC. Because PRC amplitude will increase with increasing stimulus strength, the range of entrainment will increase with increasing zeitgeber strength (**a**, compare *red* and *blue bar*). For a range of zeitgeber strengths, the ranges of entrainment are collectively called an Arnold tongue (**b**)

## 3.6 Parametric Versus Non-parametric Entrainment

The theory of entrainment explained by *phase shifts* (as explained above) is called non-parametric or phasic entrainment and relies on timing of the light-dark transitions [10, 11]. Despite its exceptional explanatory power, non-parametric entrainment theory was challenged by the observation that exclusively diurnal ground squirrels never see dawn or dusk under natural entrainment [12]. Non-parametric entrainment can be contrasted with parametric or tonic entrainment, where the intrinsic *period* of the pacemaker is affected by light in a phase-dependent manner [13–16]. This phase dependency of light affecting the speed of the circadian cycle could work in synergy with PRC-based entrainment in such a way that light in the early subjective night would slow the pacemaker down, whereas light in the late subjective night would speed the pacemaker up. This could improve entrainment stability especially in diurnal burrowing animals (like the ground squirrels), which receive large fluctuations in their light environment during the day. In an attempt to combine both models of entrainment, it could be shown that entrainment stability indeed increases in diurnal animals when non-parametric entrainment works in synergy with parametric entrainment [14, 17].

## 3.7 Light Input to the Transcriptional-Translational Negative Feedback Loop: Two Alternatives

Type 0 phase-response curves for circadian light resetting may theoretically be present in all animals when the light stimulus is strong enough, but so far they were described mostly in insects (i.e. *Drosophila*). Even with very long bright light pulses, mice do not respond with strong resetting to light [18]. The explanation may lie in the neurobiological molecular mechanism of light entrainment in different vertebrate and invertebrate species groups.

In mammals, it has been well established that melanopsin plays an important role as circadian photoreceptor in the ganglion cells in the retina, while classical photoreceptors like rods and cones also provide light input [19–24]. The pacemaker cells in the mammalian SCN themselves are not intrinsically light sensitive. In *Drosophila*, however, circadian pacemaker cells in the brain are intrinsically light sensitive. *Drosophila* Cryptochrome (dCry) is a photosensitive clock molecule and plays an important, albeit not exclusive, role as circadian photoreceptor [25–27]. Mammalian Cryptochrome (mCry) has lost its light sensitivity over the course of evolution. Also in fish and crustacea, Cryptochromes seem to have lost their role as a light receptor and, so far, a role for Cryptochrome as a circadian light receptor has only been discovered in Diptera (flies, *Drosophila melanogaster*) and in Lepidoptera (butterflies, [28]; Table 3.1). Next to the photoreceptive dCry, Lepidoptera also express the non-photoreceptive mCry (Table 3.1; [29] for a complete phylogenetic Cryptochrome review).

**Table 3.1 Phylogenetic tree of Cryptochrome as a circadian photoreceptor.** Light sensitive Drosophila Cryptochrome (dCry or Cry1) seems to be present only in diptera (flies) and lepidoptera (butterflies). Non-light sensitive Cryptochrome (mCry or Cry2) seems to be present in all other classas of animals studies so far

| | Drosophila-Cry (photoreceptive) | mammalian-Cry (not photoreceptive) |
|---|---|---|
| animalia | | |
| arthropoda | | |
| crustacea | - | + |
| hymenoptera | - | + |
| lepidoptera | + | + |
| diptera | + | - |
| fish | -/+ | + |
| mammals | - | + |

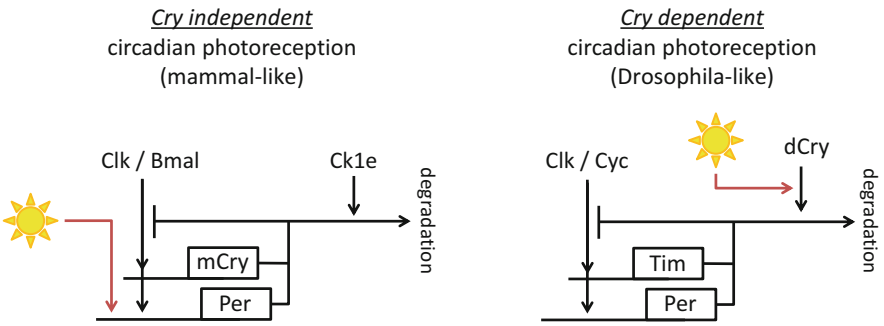


**Fig. 3.6** Cry-dependent and Cry-independent circadian light input to the circadian molecular clock. The transcription-translation feedback loop consists of central transcription factors (Clock and Bmal in mammals **a**; Clock and Cycle in *Drosophila* **b**), which drive transcription of Period (Per) and Cryptochrome (Cry) genes. Light induces Per expression in mammals (**a**), whereas light-activated Cryptochrome causes degradation of Per (when dimerised with Timeless) in *Drosophila*

From Table 3.1 it can be concluded that there are at least two basic circadian light input mechanisms: Cry-dependent and Cry-independent (Fig. 3.6) light input. The striking difference between these mechanisms is that in Cry-independent resetting, the Period gene is induced, leading to an *increase* of the Per/Cry repressor complex over the first 1–2 h of a light pulse (Fig. 3.6a). In Cry-dependent circadian light resetting, however, light-activated Cryptochrome causes *degradation* of the Period/Timeless protein complex, resulting in a reduction of this repressor complex [30–32]. As a result, Per protein expression under normal light entrainment should have an opposite phase in Cry-dependent and Cry-independent circadian light entrainment systems. This seems to be the case indeed. In mammals, Per mRNA in the SCN is peaking around the second half of the light phase [33], whereas Per mRNA levels in Diptera only start to rise after lights off [34].

Another important difference between type 0 and type 1 resetting mechanisms is that the direct light sensitivity of clock neurons in Diptera may lead to strong resetting because of its direct interactions with clock proteins. In mammals, all circadian photoreception is at least one synapse away from the pacemaker neurons. Second messenger pathways within the circadian pacemaker neurons offer more possibilities for the circadian entrainment system to attenuate and regulate circadian phase-shifting properties in response to a light pulse. Such processes may have helped to regulate and stabilise entrainment to light in mammals, which had to rely on a nocturnal and burrow-dwelling lifestyle in their early evolution [35].

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# Chapter 4
# Circadian Waveform and Its Significance for Clock Organization and Plasticity

**Michael R. Gorman, Elizabeth M. Harrison, and Jennifer A. Evans**

**Abstract** The daily rotation of the earth creates a strong selection pressure for the evolution of endogenous circadian clocks that, at least in mammals, are generally phase shifted slowly and incrementally by light. Because the earth's axis of rotation is tilted relative to the revolution around the sun, there is an additional selection pressure for clocks to adjust their waveform (i.e., shape of the daily oscillation) to match seasonal variation in daylength. With a focus on rodents, this chapter reviews protocols demonstrating circadian waveform plasticity and its relationship to the functional organization of the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. Manipulation of waveform uncovers additional novel and unanticipated effects on the lability of circadian timing systems.

## 4.1 Introduction

It is commonly appreciated that circadian clocks evolved to maximize fitness in a world where there is recurrent and predictable change in the environment. In particular, a circadian clock enables organisms to anticipate the changing environmental conditions – from light to dark or from warm to cool, for example – that result from the rotation of the earth around its axis. A key discovery in chronobiology has been the demonstration of endogenous timing mechanisms across biological taxa (e.g., cyanobacteria, plants, invertebrates, mammals, etc.). In the absence of any environmental timing cue, organisms continue to express near 24 h rhythmicity in various outputs. Because the period of these rhythms does not match any known environmental cue or the rhythms of other organisms held under identical conditions, their endogenous origin is indisputable. And indeed, the molecular mechanisms, commonly involving transcription/translation negative feedback loops, have been characterized for a number of model systems. A second

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notable achievement is the increasing understanding of how these not-quite 24 h rhythms can be adapted, or entrained, to 24 h conditions. The most common entrainment signal, or zeitgeber, is light which, when presented acutely, can cause the phase of the rhythm to advance or delay. With a similar pattern observed across many species, the endogenous clock mechanism generates a circadian rhythm in the resetting effects of light. A light pulse presented early or late in the night will differentially reset the phase of the rhythm with the effect of promoting synchronization to the 24 h day. Finally, disruption of clocks, whether by pharmacological, genetic, or environmental means, commonly disadvantages organisms. Indeed, a close match between period of the clock and the environmental cycle improves survival.

Despite an impressive understanding of the mechanisms that generate and entrain circadian rhythms, it remains difficult to practically manipulate mammalian, and particularly human, rhythms in ways that have obvious utility. People asked to work during the night, for instance, are generally unable to use light effectively to realign their circadian clock to promote alertness during their night shift work. And after rapidly crossing multiple time zones, jet travelers require several days to realign the phase of their endogenous clocks to match local time. Thus, there is a remarkable robustness apparently built into the design of the mammalian circadian pacemaker that resists rapid adjustment in the face of abrupt environmental change.

Whereas circadian rhythmicity is arguably necessitated by the earth's rotation, the axis of rotation is tilted relative to the revolution about the sun. The geophysical consequence of this is that, except at the equator, the relative durations of day and night vary systematically throughout the year. To the extent that anticipation of an oscillating light environment is a strong selection pressure, circadian clocks of organisms should be well adapted to such changes. Indeed, plasticity with respect to entrainment to these seasonal photoperiods (i.e., daylengths) should be an expected counterweight to the rigidity considered above.

### *4.1.1 Parameterizing Circadian Rhythmicity*

As time is cyclical, the study of circadian rhythmicity has relied heavily on principles and tools of circular mathematics and statistics. Any recurrent rhythm can be readily characterized in terms of (a) its *period* – the average time interval required to complete one daily cycle, typically measured from one instance of a phase marker to the next; (b) its *phase* – one point in the ~24 h oscillation and *phase angle* – the temporal relation of the oscillator phase to some other marker; and (c) its *amplitude* – a measure of deviation from high and low values of the oscillation. As trigonometric functions have the same specifications, simple sine/cosine curve-fitting techniques are frequently used to estimate and rigorously analyze these variables. While very useful, this approach omits one fundamental dimension of rhythmic organization of interest here, namely, the *waveform* of the oscillation. The waveform is simply the shape of the rhythm over its cycle.

The sine wave is one very specific waveform that is particularly useful mathematically. Most oscillations in nature, however, are not sinusoidal. For example, Fig. 4.1a depicts the daily rhythm in light intensity averaged over one summer month at a temperate latitude. With nearly undetectable levels throughout the night, the light signal would be very poorly fit by a sinusoid. But more significantly, many rhythm waveforms *change* as a function of season of the year. Figure 4.1b depicts the same variables over a 1-month period in winter. Whereas the rhythm *amplitude* is clearly reduced in winter, the *period* of the environmental oscillation does not differ seasonally – it is still 24 h. And considering peak light intensity as a phase marker, neither does the *phase* of the oscillation change with the seasons. But what changes markedly is the relative number of hours of light and dark. Further, relative to the peak phase of the rhythm, the *phase* of these critical environmental transitions (e.g., dawn and dusk) is altered by several hours. Were one to align these two waveforms with respect to the abrupt increase in light intensity (e.g., dawn), they are rendered misaligned with respect to peak intensity and to dusk. Common physiological and behavioral rhythms likewise exhibit deviations from sinusoidal waveforms and may vary seasonally (Fig. 4.1c–f).

Thus, it becomes clear that waveform is a central and critical dimension of circadian organization. Nevertheless, waveform is far less commonly studied than other parameters of rhythmicity. As a crude example, in the PubMed database, a search for the conjunction of "circadian" with "waveform" returns only 2 % of the hits as those with "phase" or "period." By juxtaposing behavioral studies from early dates of chronobiology with those employing modern neurobiological methods, this chapter aims to highlight the importance of circadian waveform for understanding circadian organization and flexibility in mammalian systems.

## 4.2 Multiple Oscillators Comprise Mammalian Circadian Timing Systems

As described in greater detail in subsequent chapters, the circadian timing system of mammals can be considered a hierarchical, multi-oscillator system strongly governed by a dominant pacemaker in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus [1, 2]. As assessed by any number of rhythmic measures (electrophysiology, metabolism, gene expression) monitored *in vitro* or *in vivo*, the SCN is a robustly and indefinitely self-sustaining circadian clock. The SCN receives photic input through the retino-hypothalamic tract, which phase-dependently resets the SCN through induction of immediate early genes that transiently perturb the dynamic interactions of clock genes until they achieve a new steady state with altered phase. Organs throughout the body also demonstrate circadian oscillations in the same set of clock genes as well as in tissue-specific outputs, but unlike the SCN, these tissue-level rhythms are not sustained indefinitely. Instead, they depend on persistent rhythmic inputs from SCN outputs such as

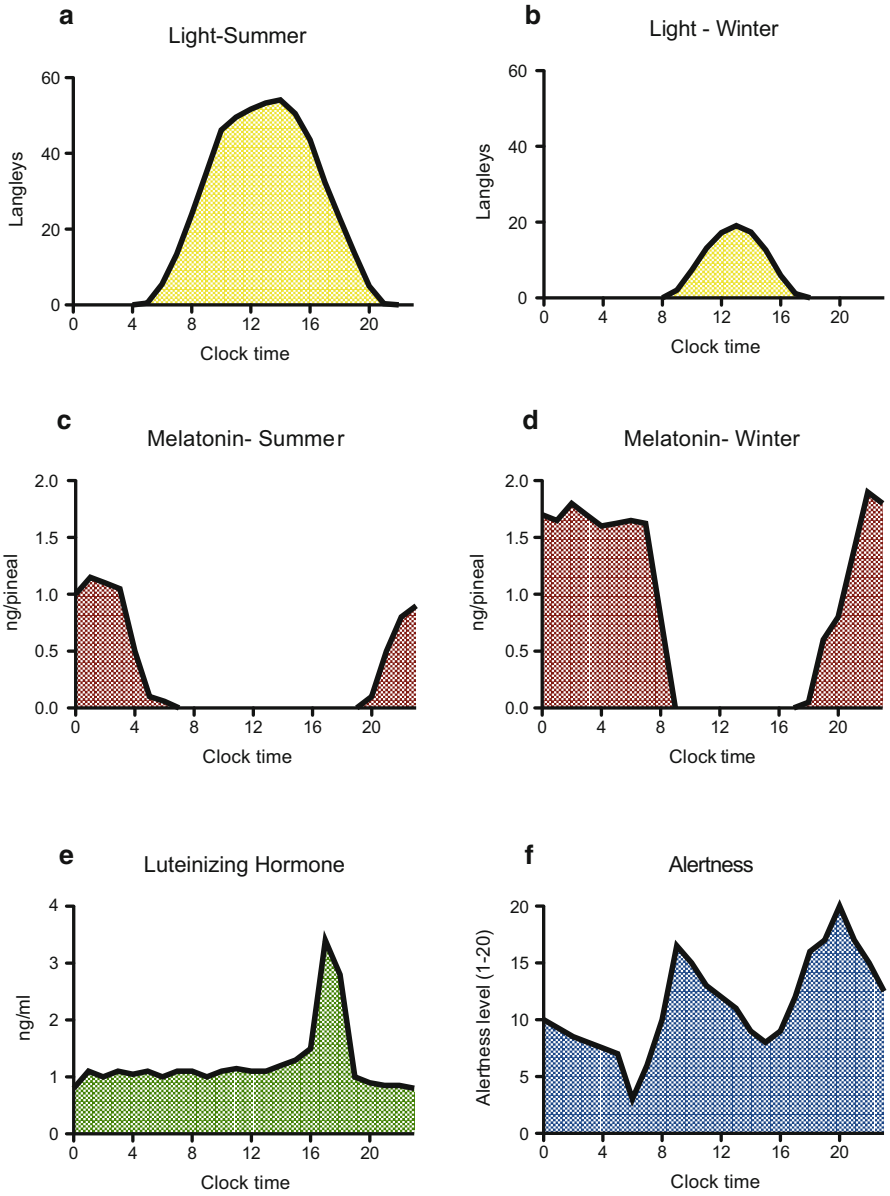


**Fig. 4.1** Examples of waveforms in various environmental and physiological rhythmic oscillations. The daily rhythm in light intensity at a temperate latitude over 1 month differs markedly in summer (**a**) versus winter (**b**; data from https://ndawn.ndsu.nodak.edu). Similarly, seasonal changes in daylength are reflected in waveform differences in the melatonin rhythm in Siberian hamsters in summer (**c**) versus winter conditions (**d**; data adapted from [64]). Concentrations of luteinizing hormone remain at basal levels except for a rapid, burst-like pattern late in the day of proestrus (**e**; data adapted from [65]). Daily variation in alertness in humans is bimodal (**f**; data adapted from http://www.nhtsa.gov/people/injury/drowsy_driving1)

body temperature, locomotor activity, hormone secretion, neural innervation, or other factors to sustain coherent rhythmicity. In the absence of persistent zeitgebers, cells that comprise peripheral organs remain individually rhythmic but become desynchronized as a population.

In the absence of a complete knowledge of clock mechanisms, it is often necessary to use observable measures that may allow inferences about underlying circadian clocks. It is a relatively sure inference that in constant conditions, the period of a measured rhythm must reflect that of its underlying clock. However, with waveform, this need not be the case. Waveform may be the additive product of multiple rhythmic processes that jointly affect the same measure. For example, clocks entrained by light and by feeding, respectively, can jointly shape the daily waveform in locomotor activity [3]: In nocturnal rodents, restriction of feeding to a few hours during the afternoon light phases induces daytime activity just prior to time of food availability. The SCN continues to drive nighttime activity, but a separate food-entrainable oscillator renders the activity rhythm bimodal by virtue of its distinct regulation of activity. On the other hand, any given waveform may be determined by a combination of clock and non-clock processes. A classic example is the two-process model of sleep [4], whereby an endogenous circadian rhythm in alertness interacts with a homeostatic mechanism that accumulates and dissipates sleep debt, to govern the timing and duration (waveform) of sleep and wakefulness. Although undoubtedly pervasive and physiologically important, rhythms from the last two categories will not be emphasized here. Finally, a given waveform may indeed appear because it is programmed specifically by a single clock. Although proof of such control may be elusive, it is these types of waveform rhythms that will be the focus in this chapter because of their direct relevance to circadian clock mechanisms.

## *4.2.1 The Complex Circadian Pacemaker and Waveform Plasticity*

In the early days of formal chronobiology, the study of rhythm waveform was a central concern, and two major phenomena – *photoperiodism* and *splitting* induced by constant light – inspired a highly influential model of the circadian pacemaker.

### 4.2.1.1 Photoperiodic Regulation of Circadian Waveform

As was well appreciated in plants for decades, photoperiod was shown to be a potent modulator of many aspects of vertebrate physiology and behavior including reproduction, metabolism, thermoregulation, social behavior, and migration to name a few [5–7]. Daily rhythms in locomotor activity, likewise, were markedly influenced by daylength: under long summer days, the nighttime wheel-running

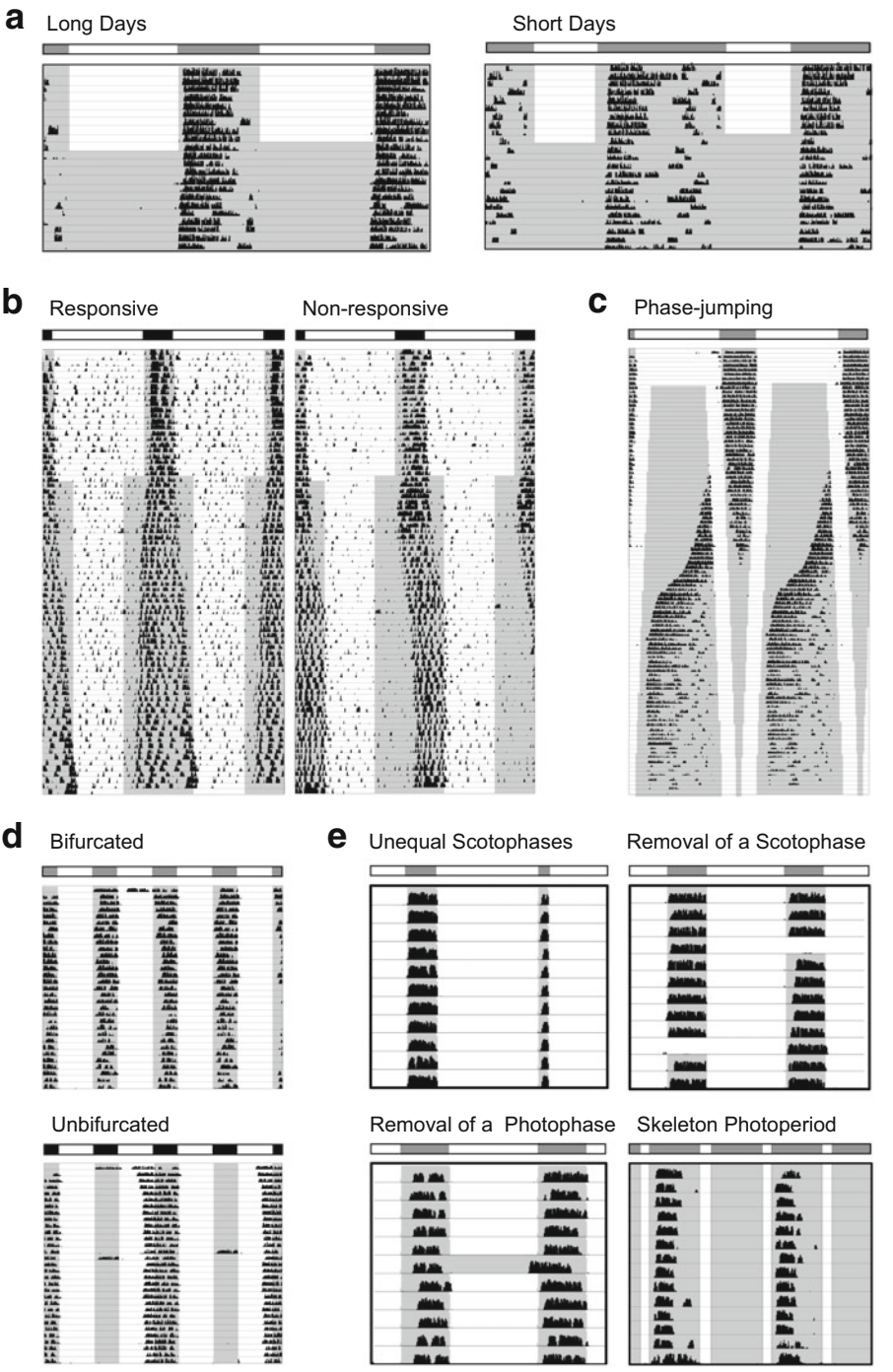


**Fig. 4.2** Behavioral consequences of waveform manipulation in hamsters. Representative double-plotted actograms under long days and short days (**a**). Locomotor activity expands under short

behavior of nocturnal rodents was of short duration, and the interval of non-activity was proportionally long. Under long nights of winter, the relative durations of activity and inactivity were reversed. Moreover, when rodents from these conditions were subsequently exposed to constant darkness, these patterns of rest/activity persisted for weeks, definitively ruling out acute effects of the light-dark cycle as the proximate basis for these different waveforms (Fig. 4.2a). Mirroring the photoperiodically entrained waveforms in activity, the length of the photosensitive phase of the circadian cycle (as determined by light-induced phase shifts) was extended following winter entrainment, and the interval of elevated melatonin secretion was correspondingly longer [8]. Across species it emerged that there was a suite of diurnally and nocturnally phased events the duration of which mirrors the length of the light and dark portion of the LD cycle, respectively, and could be conceptualized as distinct states of "biological day" and "biological night" [9]. Moreover, the correspondence between multiple measures extended even to nonsteady-state conditions such as when activity duration gradually increased after release from long days to DD [10].

The fact that numerous circadian waveforms are modulated in concert suggested that the central pacemaker itself is sensitive to photoperiod. To account for these seemingly adaptive and flexible features of the circadian clock, Pittendrigh and Daan [11]proposed a model of a complex circadian pacemaker (reviewed in [12]). Here, "complex" is used in the sense of made up of more than one unit, rather than the sense of difficult to understand – perhaps an infelicitous word choice from the perspective of encouraging deep engagement with the model. According to the model, changes in circadian waveform were said to derive from adjustments in the phase relationships between two distinct populations of clocks. Based on the differential control of activity onset and offset, respectively, these two clock populations were designated evening (E) and morning (M) oscillators. When E preceded M by 8–12 h, the duration of subjective night (i.e., $\alpha$) was shorter than subjective day as is the case in summer conditions. To account for the increase in activity duration after transfer from long days to short days or to DD, the two oscillators were modeled to have different free-running periods such that $\tau_E < \tau_M$. Under this scenario, the phase difference between E and M ($\varphi_{EM}$) would grow in the absence of entraining light to yield an increasing duration of subjective night. It is further proposed that during entrainment, the E oscillator, with $\tau < 24$ h, is routinely

**Fig. 4.2** (continued) photoperiods and is maintained after release into constant conditions. Individual hamsters respond differently to changes in photoperiod (**b**). Phase jumping occurs under changing skeleton photoperiods, wherein as the original scotophase is shortened, activity "jumps" to the longer scotophase (**c**). Hamsters bifurcate more robustly under dim, not dark, scotophases (**d**). Bifurcation can be maintained under a variety of manipulations of the light cycle, including unequal scotophase duration, transient removal of a scotophase or photophase, and under skeleton LDLD cycles (**e**). Data in E are single plotted, across 24 h (Data are previously unpublished or adapted from Refs. [25, 30, 37, 62])

phase delayed by light at dusk, and the M oscillator, with $\tau > 24$ h, is phase advanced daily by light at dawn.

If two independent oscillators with different periods were jointly programming the activity rhythms in DD, one would expect the activity waveform to show activity bouts that periodically diverged and converged to produce a "beating" pattern when monitored over many cycles. Indeed, this is rarely the case, and typically the duration of subjective night increases and then becomes fixed under DD. In fact, DD-induced increases in $\alpha$ and melatonin secretion are typically proportional to the length of the scotophase under the previous LD cycle [10], with little to no further expansion occurring after release from very short days. Thus, if two oscillators (a pacemaker complex) underlie the rhythm waveform, then these two oscillators must be coupled to one another. In other words, they interact so that they do not merely free-run with respect to one another.

But simple models that do not posit the existence of multiple oscillators may just as easily account for the waveform phenomena considered above. Consider, for example, a simple sinusoidal clock mechanism paired with a threshold that initiates and terminates biological night. If the threshold is gradually lowered, night duration will increase gradually and $\tau_E$ will appear shorter than $\tau_M$. More subtle differences in phase markers can easily be modeled if the underlying oscillation is postulated to deviate from a pure sinusoid. Such a model, moreover, does not require coupling mechanisms.

With the discovery of the role of the SCN as a dominant circadian pacemaker, it became possible to test whether photoperiodic regulation of waveform inhered in the SCN itself (i.e., did the SCN have the properties of a complex oscillator) or whether it derived from the interaction of a waveform-invariant SCN with other extra-SCN rhythmic or homeostatic processes. Supporting the former conclusion, direct measures of SCN function indicate that it does indeed encode photoperiod. SCNs of animals entrained to long or short photoperiods show different waveforms of rhythms in electrical firing, in endogenous c-fos expression, and in rhythms of clock genes and their protein products [13, 14]. Moreover, these differences persist for multiple cycles in LD, and there is a gradual transition upon transfer from long to short daylengths [15].

#### 4.2.1.2 Changes in Circadian Waveform Under Constant Light

Simultaneously informing this early model of a complex oscillator were findings that radical changes in waveform could be induced by exposure to constant light. In hamsters, for instance, such exposure induced gradual reductions in the duration of wheel-running behavior similar to that seen in very long photoperiods, except also free-running. In a substantial fraction of animals, however, this single short activity bout devolved into two components, each of which might free-run with its own period until the two bouts reached antiphase and from there adopted a common free-running period. Although study of melatonin was precluded by the required exposure to constant light, as with photoperiodism, the two split bouts of behavior

were shown to be accompanied by other clock outputs (e.g., the LH surge) suggesting an origin in a central clock mechanism. Unlike the findings related to photoperiodism, however, a simple threshold mechanism did not easily explain this phenomenon, termed "splitting." Rather, the simultaneous appearance of two oscillations with different periods strongly suggested the existence of at least two distinct clock mechanisms. As in the case of photoperiodism, the fact that they eventually (and universally) adopted a common phase relationship again suggested that they were not independent but were instead coupled.

As with photoperiodic variations in waveform, electrophysiological and lesion studies strongly suggested that splitting reflected altered circadian organization intrinsic to the SCN. More recently, splitting has been convincingly related to antiphase oscillations of the left and right SCN [16], which are connected by contralateral projections. Finer-grained analysis in hamsters additionally shows antiphase oscillations between cellular compartments *within* each of the two SCNs [17]. As lateral asymmetries in SCN cycling are uncommon under any other conditions, it can be concluded that LL alters the coupling, or interactions, between the left and right SCNs, by mechanisms that remain to be understood.

#### 4.2.1.3 Photoperiodic Nonresponsiveness and Arrhythmicity in Siberian Hamsters

In many rodent species, some individuals fail to adopt a complete short-day phenotype in response to winter photoperiods. In Siberian hamsters, *Phodopus sungorus*, this short photoperiod nonresponsiveness has a basis in circadian entrainment. Nonresponsive Siberian hamsters permanently express both a short interval of locomotor activity and a short melatonin signal despite prolonged exposure to long nights, with each rhythm phase locked to dawn in the large majority of animals (Fig. 4.2b) [18]. The incidence of nonresponsiveness is highly sensitive to artificial selection establishing a strong genetic basis [19]. However, its expression is additionally photoperiod history dependent: If never exposed to long daylengths, artificially selected strains will show typical winter responses. Conversely, unselected strains can be induced to become nonresponsive if exposed to very long daylengths [20]. Thus there appears to be genetic polymorphism in the environmental conditions for inducing the nonresponsive phenotype. This model system represents a unique example in which there are genetic differences in the regulation of circadian waveform by ambient light. Unfortunately, the genetic basis of this trait remains unanalyzed, and comparable circadian patterns of nonresponsiveness have not been reported in other species. A second waveform phenomenon apparently unique to *Phodopus* is the induction of permanent behavioral arrhythmicity following a single-phase advance and a subsequent phase delay. Rhythmicity is not restored even under regular light-dark cycles [21].

Both behavioral phenotypes are associated with alterations in SCN function. In the former case, *in vivo* light sensitivity of the SCN and *in vitro* electrical activity rhythm of hypothalamic slices are both markedly delayed in the nonresponsive

phenotype [22]. In the latter case, rhythms of clock gene expression in SCN are eliminated, and expression values are markedly suppressed [23].

#### 4.2.1.4 Transient Changes in Circadian Waveform

As described briefly above, photic entrainment is facilitated by the fact that light falling in early subjective night produces phase delays where light late in the subjective night produces phase advances. But acute light pulses may also cause transient perturbations in circadian waveform: Following late-night light pulses, for example, the offsets of activity and melatonin secretion are readily advanced, but onsets are not shifted commensurately for several cycles [24]. The different resetting kinetics of distinct phase markers thus produces "transient" cycles where subjective night is compressed and which are resolved as activity onset shifts gradually over subsequent days. In extreme cases, transient cycles may be characterized by the complete loss of nocturnal events, such as melatonin secretion [24]. In contrast, α compression is less pronounced during light-induced phase delays because both phase markers reset with similar kinetics following a light pulse applied during early night. Direction-dependent transients in circadian waveform also emerge following shifts of the LD cycle that simulate travel across time zones, although shifts in activity onset and offset can be masked by light under these conditions. Transients observed in melatonin regulation and behavior, and believed to reflect oscillator interactions, are closely mirrored by rhythms of light sensitivity in the SCN [13, 24].

A "skeleton photoperiod" is produced when a full, uninterrupted photophase is replaced with only two short light pulses simulating light transitions at dusk and dawn. The remaining portion of the day is left unilluminated. Across a range of ecologically relevant conditions, entrainment under skeleton photoperiods generally resembles that elicited by full photoperiods. However, if the skeleton photoperiod simulates very long daylengths, a "phase jump" may result: Often occurring suddenly, activity traverses one of the entraining light pulses; the pacemaker re-entrains with activity phased to the longer of the two available scotophases, and a longer α is adopted (Fig. 4.2c) [25, 26]. As large phase jumps are not seen under comparable full photoperiods, manipulation of the zeitgeber waveform alone, without a change in period or phase, is sufficient to strongly modulate the stability of the pacemaker.

#### 4.2.1.5 Waveform Bifurcation

Over the past 15 years, a new class of entrained variations in circadian waveform has been characterized in hamsters and mice. Coining the phrase "behavioral decoupling," Mrosovsky and Janik [27] demonstrated that the pattern of nighttime locomotor activity could be altered by repeatedly transferring hamsters to novel wheel-running (NWR) cages during the middle of subjective day. The lights were

turned off for the 3 h interval of NWR opportunity. With repeated exposure, the onset of the home cage running at night progressively delayed, and running duration in the home cage was curtailed. Extending the study of this waveform change to steady-state conditions, Gorman and colleagues proceeded with daily NWR until the nighttime was approximately half of its former duration. Hamsters were then left in the home cage but continued to receive a 3 h exposure to darkness during the subjective day, effectively exposing them to a 24 h LDLD cycle. Under such conditions, the hamsters exhibited stably bifurcated activity rhythms characterized by robust activity in the latter half of the long original night and nearly equal duration activity in the second afternoon scotophase [28].

Although the role of novelty-induced wheel running appeared critical to hamsters under this protocol, it shortly became apparent that manipulations of the light environment alone were sufficient to induce a comparable entrainment state. Specifically, exposure to 24 h LDLD cycles could reliably induce "bifurcation" of activity rhythms in mice, Siberian hamsters, and Syrian hamsters, provided that two conditions were met. First, the duration of the individual nights had to be short ($<$ ~6 h) to induce bifurcation. Second, the twice-daily nights could not be completely dark but needed to be very dimly illuminated (at an intensity comparable to that from the stars or a dim moon). Under such conditions, a majority of animals in each species can rapidly and reliably adopt the bifurcated entrainment pattern (Fig. 4.2d) [29]. Moreover, within these constraints, there is tremendous latitude in the duration and relative phasing of the LDLD components. For examples, the two scotophases may or may not be in antiphase (e.g., 12 h apart or 9 h apart), and they may or may not be the same duration (e.g., both 5 h or one 5 h and one 3 h) (Fig. 4.2e). Deviation of the LDLD pattern for one or more 24 cycles induced acute changes in behavior, but the stable bifurcation was immediately recovered upon restoration of the LDLD (Fig. 4.2e) [30].

A critical question is to what degree masking by light contributes to the bifurcated entrainment state. Perhaps the animals are adopting a short photoperiod waveform that is merely interrupted by light falling in the middle of the long subjective night. To discount this possibility, hamsters were exposed to skeleton photoperiods of the LDLD cycle. For example, each original 7 h L phase was replaced by two one-hour light pulses with 5 h of darkness between, yielding a LD1:5 cycle repeating four times per 24 h. Under such conditions, bifurcated entrainment of hamsters is maintained with activity confined to alternate 5 h dark periods, discounting the role of negative masking (Fig. 4.2e) [29].

As would be expected on the basis of behavioral results, bifurcation also appears to represent alterations in SCN function. Thus in NWR-induced bifurcation of hamsters, each activity bout was associated with melatonin secretion and light sensitivity of the SCN as measured by c-fos expression and inactive periods with heightened per gene expression [31, 32]. After bifurcation induced in hamsters without timed NWR, we observed that rhythms of Per1 protein cycled in antiphase in shell versus core regions of the SCN [33]. A similar temporal reorganization of *Per1* and *Bmal1* mRNA of SCN compartments is seen in mice [34] and contrasted with global unimodal expression patterns of these transcripts in LD12:12 mice.

Unfortunately, neither the hamster nor mouse study was able to make comparisons with non-bifurcated rodents under the same LDLD cycle. Thus, it is still unclear to what extent these altered clock gene product rhythms relate to the entrainment status versus the LDLD exposure. Lateral asymmetries were absent in all studies, however, definitively distinguishing this entrainment phenomenon from LL-induced splitting.

#### 4.2.1.6 Role of Dim Light in Waveform Modulation

The critical role of dim nighttime illumination in bifurcation was surprising as the irradiance fell far below putative thresholds of the circadian system sensitivity and had been incorporated into activity recording chambers only to facilitate nighttime experimental manipulations. Its biological significance was only suspected after a cohort of hamsters failed to bifurcate, and it was determined that the dim lights had become unpowered. Controlled experiments confirmed its critical influence on the entrained waveforms of activity to LDLD cycles of Syrian hamsters [35], and subsequent experiments assessed its significance in additional plastic waveform paradigms. In Siberian hamsters, its facilitated role in bifurcation was replicated, but, additionally, dim light accelerated elongation of subjective night after transfer to short photoperiods, prevented circadian nonresponsiveness induced by long-day exposure, and promoted arrhythmicity in constant conditions (dim versus dark) [36, 37]. These results suggested a critical role of dim light on the coupling of multiple oscillators hypothesized to underlie waveform regulation. Indeed, in the outbred Siberian hamster, which is genetically suitable for studies of individual differences, there was significant correlation between the effects of dim light in multiple waveform paradigms suggesting a convergent effect on oscillator coupling [36].

Nighttime light exposure has recently attracted great attention as a result of numerous documented adverse outcomes in humans and animal models [38–43]. What other authors call “dim” (e.g., 5 lx) are orders of magnitude brighter than the “dim” light employed in our studies (0.01–0.1 lx). And whereas other authors aim to simulate light pollution or rodent equivalents to artificial light exposure of humans, our nighttime illumination does not exceed levels occurring under natural conditions of rodents.

## 4.3 Waveform Variations in the SCN

The SCN functions as a network of multiple, coupled oscillators [2]. Like cells throughout the body, individual SCN neurons are self-sufficient cellular clocks that continue to express circadian rhythms in clock gene expression and electrical activity even when synaptic communication is prevented by pharmacological blockade and/or physical dispersal [44, 45]. Alternatively, when oscillating

independently in dispersed cell cultures, individual SCN neurons have period lengths that may differ from one another by several hours. Because cells in an organotypic hypothalamic slice preparation adopt a common period, the cells must be functionally coupled to adjust their periods. The ability to maintain period synchrony at the tissue level under constant conditions appears to be a property that is not shared by other tissue clocks [46].

Although coupled SCN neurons generally adopt a common period, they do not all adopt a common phase. Indeed, as reported by bioluminescent reporting of *Per1* mRNA or PER2 protein rhythms, the explanted SCN shows regional variation of 2–4 h in peak phase of each reporter [47, 48]. The critical role of synaptic communication in organizing these phase differences can be demonstrated by blocking $Na^+$-dependent action potentials with tetrodotoxin. Under such conditions the individual cells are free-running with a range of periods, and thus their phases are progressively scrambled. Conversely, the phases of cells can be tightly synchronized by first inhibiting protein synthesis with cycloheximide and then restarting cellular rhythms at a common phase upon washout of the drug. Regardless of how the original phase relationships are desynchronized or synchronized, when allowed to resume coupled interactions, the SCN cells return to their prior phase relationships [48]. Thus, phase organization across the SCN is a highly regulated feature of the network.

How does this relate to waveform plasticity? Considerable work has characterized the spatiotemporal dynamics of the SCN network following entrainment to different photoperiods. In one approach, electrical activity of single units and of neuronal ensembles was monitored in SCN explants. Firing rhythms in slices from short versus long photoperiods were distinguished by the duration of elevated activity, in proportion to the length of the light phase. Across photoperiods, individual units were characterized by relatively short (~4 to 5 h) periods of increased electrical activity, suggesting that the population waveforms were an emergent property of the phasing of the cell population [49]. In short photoperiods, units are highly synchronized, whereas the phases of their firing intervals become dispersed following entrainment to long photoperiods. The patterns of synchronization of electrical activity, moreover, vary regionally. In mice from LD12:12, SCN electrical activity is less phase synchronized dorsally than it is ventrally. However, these regions encode photoperiod differentially: cells of the ventral SCN modulate their phase synchrony whereas cells of the dorsal SCN additionally modulate their individual waveforms of electrical activity [50].

As electrical activity is a clock-controlled output, it need not faithfully report the phase of the underlying cellular clock. Therefore, it is important to assess whether photoperiod manipulations alter the phase distributions of clock genes. Indeed, as monitored by *Per1* mRNA luciferase reporter, cells in the posterior SCN tracked lights on (e.g., morning) over a wide range of photoperiods. In contrast, two populations of cells were distinguished in the anterior SCN: one that tracked lights off and another that became increasingly phase segregated as photoperiod lengthened [51]. Using a PER2 reporter, Evans et al. likewise described marked variations in the phase maps of SCN cells as a function of photoperiods lengthened above

LD12:12 [52]. Over time in culture, these cell populations modulated their phase relationships systematically, rendering the SCN explant a powerful model in which to assess coupling interactions in real time.

Beyond encoding photoperiod, regionally and temporally distinguished cell populations model the resetting kinetics of behavior following exposure to advancing and delaying light pulses. The evening and morning peaks of hamster SCN electrical activity revealed in a horizontal slice preparation are differentially shifted by application of glutamate [53]. Similarly, following a 6 h phase advance in the photoperiod, differential resetting kinetics of ventral versus dorsal cell populations are apparent in the distribution of peak phases of PER2 luciferase reporting [54].

Thus, phase dispersion of neural clocks appears to be an organizing principle of altering the SCN's waveform. This conclusion, however, does not exclude the possibility that individual neurons are themselves altering their individual waveforms as was the case in the electrical activity rhythms of cells in the dorsal SCN of mice [50]. Indeed, with a green fluorescent protein reporter of Per1 activity, the waveform of SCN neurons was persistently modulated by the developmental photoperiod [55]. This apparent imprinting was not as large as the overall effect on SCN waveform suggesting that both individual neuron and network interactions contribute to this modulation of SCN waveform.

Although convergent evidence suggests a strong link between waveforms in behavior and in SCN network dynamics, much work remains to understand their relationship and specific mechanisms. Whereas a role of synaptic communication from network organization is clearly established, multiple neurotransmitters may regulate the network in a complex manner. Vasoactive intestinal polypeptide (VIP) signaling contributes to neural synchrony but also has the potential to desynchronize the network as a function of timing and dose [56]. $GABA_A$(-γ-aminobutyric acid) signaling has been demonstrated to desynchronize SCN neurons, at least when VIP signaling is attenuated[52, 57]. The effects of $GABA_A$, moreover, are sensitive to the prior photoperiodically entrained state of the network [52].

## 4.4 Consequences of Waveform Manipulation

Besides providing a mechanistic basis for an internal calendar through which to regulate seasonal physiology, photoperiodic entrainment appears to modulate core features of the pacemaker itself. As mentioned above, the fraction of the endogenous cycle in which light induces phase resetting is expanded in winter versus summer conditions [13]. But more surprisingly, experiments examining the effects of photoperiod on phase shifting by brief light pulses indicate that animals under short, winter-like days have a higher amplitude PRC and thus respond to brief light pulses with phase shifts of greater magnitude than long-day counterparts [8, 58].

Whereas the PRCs to bright light pulses establish photoperiodic differences in the response to light, there appears additionally to be variations in light sensitivity.

Photic sensitivity can be operationalized either in terms of the threshold of light capable of inducing a phase shift, the irradiance sufficient to generate the maximum phase response, or most commonly, the irradiance that produces half of the maximal phase shift response. Each measure is separated from the question of the size or direction of phase shifts. In Syrian hamsters tested late in subjective night, approximately 40× more light (~1.5 log units) was necessary to produce half of the maximum phase shift response following entrainment to summer conditions than after winter conditions [59]. The disparate irradiances demonstrated to generate comparable phase advances under short versus long days likewise yielded comparable phase delays. Moreover, equal photon doses produced significantly larger delays in the short photoperiod condition, as well as markedly greater pERK, Per1, and c-fos immunoreactivity in the suprachiasmatic nuclei. Patterns of immunoreactivity in all three proteins were related to the size of the phase shift rather than the intensity of the photic stimulus, suggesting that photoperiod modulation of light sensitivity lies upstream of these events within the signal transduction cascade. An analogous effect of resetting sensitivity was generalized to Siberian hamsters [60]. In that species, however, the regulation of melatonin was not comparably modulated.

Until recently, photoperiodic modulation of pacemaker properties has tended to focus on acute (i.e., nonparametric) effects of light on the circadian clock despite ample evidence that, even in rodents, daytime light exerts additional, parametric effects on clock function [61, 62]. As noted above, acute light pulses can induce transient changes in circadian waveform, typically reducing α and perhaps reducing PRC amplitude as a result. Therefore, it is unclear whether winter entrainment should be expected to produce any meaningfully enhanced resetting to changes in full photoperiods. To test this prospect, a novel assay was designed in which winter- and summer-entrained hamsters were transferred to six identical summer photoperiods phased at successive 4 h intervals. Because changes in waveform introduce different size phase shifts with respect to different phase markers, there can be no unambiguous identification of the zeitgeber shift. Nevertheless, phase-resetting kinetics across all the groups can be plotted on circular coordinates to provide an objective measure of phase lability. Syrian hamsters under short photoperiods were, overall, able to reset to new time zones twice as quickly on average as long-day counterparts (Fig. 4.3).

The same protocol was used to evaluate the speed of resetting to traditional, long photoperiods after bifurcation. Here again, where the total light exposure was the same as in long photoperiods, the circadian system adapted more rapidly to phase shifts of varying magnitude and direction (Fig. 4.3). Moreover, previously bifurcated hamsters were shifted to antiphase time zones (12 h apart) and released into DD [63]. As assessed by the free-running locomotor activity rhythm, the master pacemaker was fully shifted into antiphase time zones in only a matter of days, confirming that the rapid behavioral resetting seen after bifurcation is not a behavioral artifact of masking. While the mechanisms for enhanced rates of resetting after waveform manipulation are unknown, we hypothesize that they will be relatable to the phase and waveform dynamics of oscillators within the SCN.

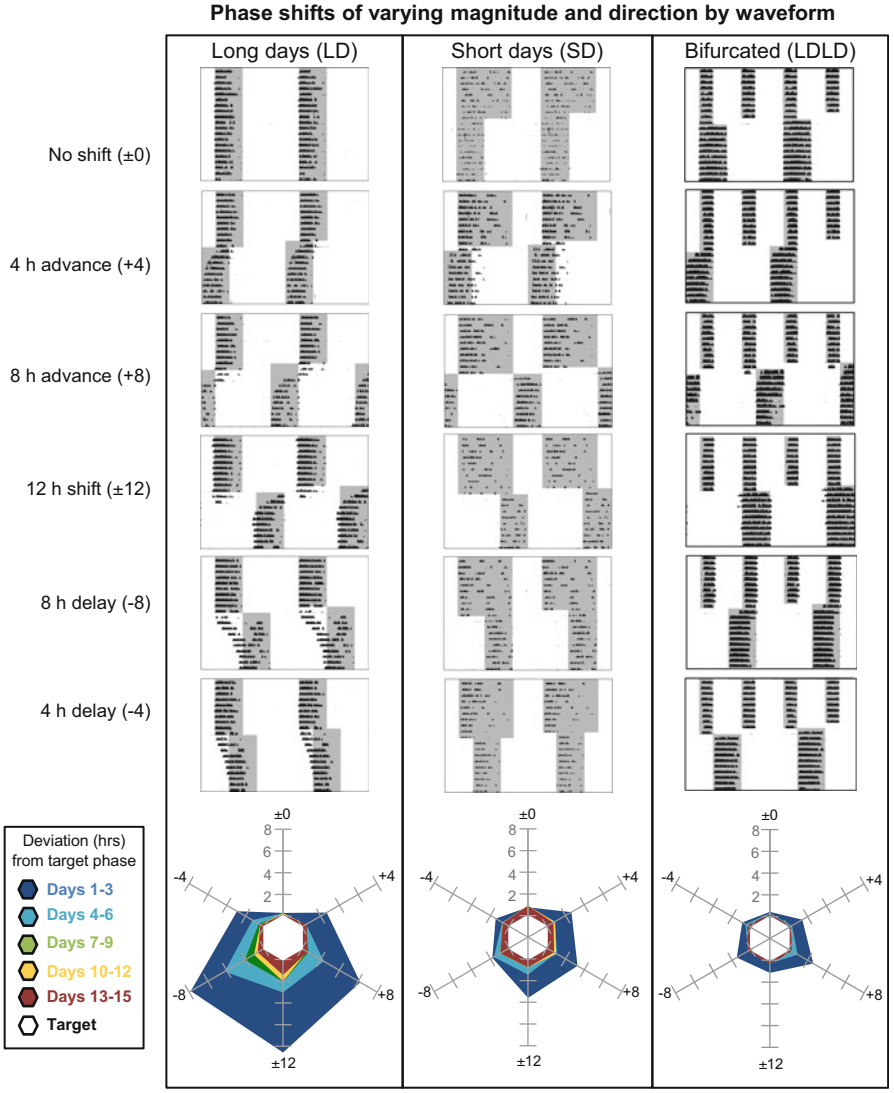


**Fig. 4.3** Manipulations of waveform prior to a phase shift accelerate re-entrainment. Shown here are representative double-plotted actograms of wheel-running activity in Syrian hamsters first exposed to one of three different waveforms (LD, SD, and LDLD) and then exposed to each of six phase shifts to a new LD 16:8 schedule in a global assay of phase resetting. Actograms are organized in columns by waveform group (LD, SD, and LDLD). For each actogram, 10 days of the baseline waveform and 10 days following the shift are shown. The size of the phase shift is denoted on the left of each row and reflects the change in lights off (+4 h represents a 4 h advance of lights off in all groups). For LDLD animals, the reference point for lights off was derived from the pre-bifurcation scotophase. Radar plots below each column reflect the phase mismatch at progressive 3-day intervals post-shift for that waveform manipulation. Each axis of the radar plot represents a shift of varying magnitude and direction. The white hexagon in the center of each plot represents the "target" phase in the new schedule (for activity onsets, this was the new lights off). Each colored polygon represents the absolute value of the average 3-day deviation of the mean onset from the new lights off in hours, with colors progressing from coolest (days 1–3 post-shift) to warmest (days 13–15). Similar results were found when midpoints and offsets were examined (Data adapted from [63])

## 4.5 Summary

- Waveforms of behavioral and physiological rhythms of rodents can exhibit tremendous variation, both transiently and under steady-state conditions. While waveform plasticity has obvious ecological utility in the context of photoperiodism, its various manifestations in non-ecological context likely reflect nonadaptive by-products of its mechanistic organization. Although heritability of waveform plasticity has been demonstrated in mammals [19], there has been little attention paid to the genetic basis of this fundamental dimension of circadian organization.
- The SCN, as a result of its multi-oscillator network organization, may encode and modulate pacemaker waveform through changes in phase of SCN neurons, changes in individual SCN neuron function, and perhaps via other yet-to-be-discovered mechanisms. Recent investigations of SCN neural circuitry establish the roles of multiple, interacting neurotransmitter and signaling mechanisms in the regulation of multicellular pacemaker network.
- Earlier reports that winter photoperiods increase the amplitude of the phase response curve [8] and have now been shown to generalize to shifts in full photoperiods that are of greater relevance to time-zone travelers and to shift workers. Moreover, novel, but unecological, entrainment conditions such as bifurcation itself – a novel entrainment condition worthy of consideration for shift workers [30] – likewise demonstrate a linkage between waveform and circadian resetting that may afford new approaches to circadian adaptation [63].

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## Key Questions of Interest and Suggested Readings

- How do taxonomically diverse organisms adapt to photoperiodic change? [6]
- How might circadian waveform manipulations be relevant to human shift workers? [30]
- What are the emergent properties of SCN networks? [12, 14]

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# Chapter 5
# On the Origin and Implications of Circadian Timekeeping: An Evolutionary Perspective

**K.L. Nikhil and Vijay Kumar Sharma**

**Abstract** The ubiquity of circadian rhythms (driven by underlying circadian clocks) in various behavioural and physiological processes across a variety of life forms supports the hypothesis that such rhythms are probably adaptive. This is further substantiated by studies demonstrating that dysfunctional circadian clocks are associated with multiple aberrant physiological processes. However, owing to the complex interplay of life-history traits that collectively mediate realised fitness of organisms, rigorously testing whether circadian clocks are indeed adaptations turns out to be quite challenging. Here, we review our current state of knowledge on the adaptive benefits of circadian clocks, and discuss the pros and cons of various studies, followed by a brief discussion on our recommendations for how improved experimental designs can be employed in future.

## 5.1 Introduction

A majority of life forms exhibit circadian (Latin *circa* = about/approximately; *diēs* = day) rhythmicity in behaviour and physiological processes driven by the underlying circadian clocks (see glossary) and have been the topic of study for several decades. Even though records (by Androsthenes) suggest that the earliest mention of daily rhythms dates back to fourth century BC, such rhythms appear to have been extensively noticed and their importance realised much before Androsthenes (Box 5.1). Today, it is commonplace to encounter statements such as 'circadian clocks evolved in response to rhythmic selection pressures imposed by environmental variables as a consequence of earth's rotation about its axis' implying that it is a well-established knowledge. Nevertheless, if one were to critically assess all chronobiology studies, it would become evident that studies on this topic are relatively few and that the majority of the existing body of evidence demonstrate that circadian clocks provide certain advantages to its bearers under specific contexts. However, from the perspective of an evolutionary biologist, being

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advantageous may not necessarily mean being adaptive as will be discussed in the next section.

**Box 5.1: Pre-Androsthenes Mention of Circadian Rhythms**
The majority of literature discussing the history of circadian rhythms invariably refers to Androsthenes, a Greek philosopher and admiral of Alexander III of Macedon who during his march to India in fourth century BC observed the rhythmic movement of tamarind tree leaves. This is followed by another mention of sleep/wake rhythm in bees by Aristotle in his book *History of Animals* (Greek, Τῶν περὶ τὰ ζῷα ἱστοριῶν 'Inquiries on Animals'; Latin, *Historia Animālium* 'History of Animals' as translated from Greek by D'Arcy Wentworth Thompson) [cf 1] around the same time (350 BC). However, it appears that human knowledge of daily rhythms can be traced further back to the Vedic period predating Androsthenes and Aristotle by almost a thousand years. In fact, unlike mere observations of rhythmic behaviours by Androsthenes and Aristotle, the relevance of rhythmicity in physiological variables appears to have been extensively studied as can be inferred from the fact that it even formed the basis of the traditional medicinal system 'Ayurveda' whose origin is attributed to the 'Atharvaveda' composed around 1500–1000 BC [2]. The principles of Ayurvedic medicinal treatments reside on three fundamental physiological measures (called 'doshas') – Vata, Pitta and Kapha believed to exhibit 24-h rhythmicity with each of them peaking at specific times of the day [3, 4 and citations therein]. It was observed that sleep/wake cycles when not synchronised with the external day/night cycles would lead to an imbalance of the three doshas resulting in physiological disruption, a concept now acknowledged as circadian misalignment. Thus, even though rigorous scientific analyses of circadian rhythms were initiated relatively recently, the importance of temporal order within the human body seems to have been acknowledged for over 3000 years now. However, extensive and, more importantly, exact translations of Vedic scriptures and related literature will help further uncover the rich history of circadian rhythms.

To begin with, we will briefly revisit some of the key concepts of adaptation which we feel are essential for rigorous evaluation of methodologies and interpretations of studies aimed at unravelling adaptive significance of circadian clocks. Later, we will discuss the evolutionary significance of circadian clocks by reviewing literature on theories and evidence about their origin and adaptive value, followed by the discussion on the pitfalls of some of the experimental methodologies used, and suggest improvements that we believe can aid relatively better experimental designs for future studies.

## 5.2 Adaptation

Even though adaptation as a concept has enjoyed unanimous acceptance, its precise definition has remained controversial for over a century [5]. It is conventional to use the term adaptation in two contexts. One may refer to it as either a continuous process by which organisms adapt to a given environment, or as a character/trait that confers higher fitness to organisms in a given scenario [6]. However, we will be using adaptation in the latter context throughout this review (see glossary).

The earliest definitions regarded adaptations as traits 'optimally' designed in an organism for a specific function relevant to its ecology. This idea seemed to be so convincingly adopted that it even inspired William Paley to propose in his book *Natural Theology* [7] that such complex designs could have been shaped only by an intelligent designer and use this argument to support the existence of an 'intelligent deity' or 'god' who functioned as the creator of life. Now pinned under the umbrella term 'the argument from design', such arguments were widely used to explain several phenomena by geologists Sedgwick, Buckland and Murchison and naturalists like Agassiz which underscores the influence of theological temperament in scientific inquiry [8]. Ironically, even Darwin (as a student) upon reading Paley's work was convinced of the idea of an intelligent 'deity' driving adaptations [9]. However, philosophers like Hume and Kant in their works *Dialogues Concerning Natural Religion* [10] and *Critique of Judgment* [11], respectively, have been credited to have demolished this argument [5, 8].

While these ideas were eventually dismissed by Darwin himself in his theory of natural selection [12], the argument over the exact definition of adaptation continued for over a century. Following debates by GC Williams [13] and Gould and Lewontin [14], the definition of 'adaptation' underwent subsequent refinement with a large number of proposed alternatives which are broadly categorised as 'historical' and 'ahistorical'. Some authors have also discussed another category called 'teleological definitions' [15] which will not be considered here. A detailed discussion on this topic can be found in Lauder et al. [16 and citations therein].

Ahistorical definitions focus on the current utility of the trait considered to be an adaptation without reference to the historical reasons that might have led to its origin. For instance, Bock [17] stated that 'An adaptation is, thus, a feature of the organisms that interacts operationally with some factor of the environment so that the individuals survive and reproduce'. A more recent definition proposed in this regard is, 'An adaptation is a phenotypic variant that results in the highest fitness among a specific set of variants in a given environment' [15]. Historical definitions, on the other hand, highlight the importance of viewing an adaptation ($A$) in the context of ancestral populations which experienced a given selective regime that eventually drove the evolution of $A$, thus implying adaptation as a relative (to its ancestral state) concept. For instance, Gould and Vrba [18] proposed that a trait is an adaptation only if the historical selection pressures that led to its evolution for a given utility to the organism are the same selection pressures that currently maintain $A$. Sober [19] proposed that '$A$ is an adaptation for task $T$ in populations $P$ if and

only if *A* became prevalent in *P* because there was selection for *A*, where the selective advantage of *A* was due to the fact that *A* helped perform task *T*' as also retained by Coddington [20], and similar versions of it are now widely accepted.

The above-discussed categories, even though primarily differ in considering the relevance of the origins of adaptations, it is clear that both define adaptations as traits that confer certain benefits over others in a given environment. The traits conferring such benefits would be naturally selected for if they enhanced the ability of organisms/species to survive and reproduce in that environment (referred to as the reproductive fitness), and thus individuals that maximally contribute to the gene pool of the succeeding generation are considered fitter than others [21]. Therefore, the ideal way to assess whether a trait is indeed an adaptation is to study the influence of that trait on fitness of the Organism.

*But how does one measure fitness*? While the measures of fitness are highly context dependent (as will be discussed later), for practical purposes, fitness is generally partitioned into multiple components. For instance, in a population of sexually reproducing organisms, the ability to reach sexual maturity is dependent on several factors including survivorship of zygotes until birth following which the ability to find mates and successfully reproduce becomes relevant. All these factors collectively influence the reproductive fitness of organisms, and, therefore, various components such as survivorship till sexual maturity, mate procurement, fecundity, fertility and competitive ability (the ability to outcompete others for resources) are used as general fitness measures. With this background we will now discuss, in detail, the rationale behind the proposition that circadian clocks are adaptive, their origins and evolutionary implications.

## 5.3 Circadian Clocks as Adaptations

Several features of circadian rhythms are suggestive of the adaptive nature of the underlying clocks. Firstly, the proximity of the period ($\tau$) (see glossary) of circadian rhythms to that of 24-h day/night cycles is unlikely to be a mere coincidence. Further, had circadian clocks originated just by chance and not due to selection, it is unlikely that they could have attained such a widespread distribution across life forms. There have also been several examples suggesting the convergent evolution of circadian clocks which imply that they might have independently evolved multiple times (discussed later) which once again is an unlikely scenario had clocks not been adaptations. All these evidences are suggestive of the idea that circadian clocks might have been shaped up by natural selection. Therefore, it is logical to hypothesise that circadian clocks probably confer adaptive advantage to organisms.

### 5.3.1 How Can Circadian Clocks Be Advantageous?

Circadian clocks are hypothesised to confer advantages to organisms in two ways: one by establishing an internal temporal order among various physiological processes within the organism, referred to as 'intrinsic advantage', and the other by facilitating entrainment (see glossary) of circadian rhythms to the external cycles, known as the 'extrinsic advantage'.

#### 5.3.1.1 Intrinsic Advantage

Intrinsic advantage hypothesis proposes that circadian clocks evolved to ensure temporal segregation of cellular and physiological processes within organisms. Since the efficient operation of multiple processes at the same time would require large energy expenditure, timing them with appropriate temporal lag may help efficiently partition resource/energy. Also, biological processes may differ in their requirement for optimal cellular/tissue conditions such as ionic strength, *pH* and so on, and, therefore, temporal segregation may help the same tissue regulate multiple processes. Additionally, temporal segregation can prevent undesired physiological consequences arising from incompatibilities between components or products of some of the processes (for instance, photosynthesis and nitrogen fixation in *cyanobacteria*). In this regard, Oatley and Goodwin [22] proposed that systems with oscillatory components tend to be more stable as compared to their non-oscillatory counterparts, and thus mutual synchronisation would enable better homeostatic regulation, but remains to be systematically tested.

A classic example cited to highlight the importance of temporal segregation of physiological process is that in *cyanobacteria* which exhibit rhythmic but antiphasic $N_2$ fixation (during the night) and photosynthesis (during the day) [discussed in 23]. It is postulated that since the products of the two processes are toxic to each other, they are segregated so as to avoid interference [24]. While this seems to be a convincing explanation, it is not the complete story. Based on the above argument, it can be expected that growth of *cyanobacteria* will be retarded under constant light (LL) due to the unavailability of dark phase for $N_2$ fixation, the main source of their metabolic nitrogen. However, contrary to this, *cyanobacteria* exhibit normal and sometimes even faster growth in LL [discussed in 25]. Additionally, not all *cyanobacteria* fix nitrogen, and in some cases individuals of the same genus use both spatial and temporal segregation of these processes, while others balance the rates of the two processes such that the net oxygen produced is zero [26]. Therefore, the current evidence in hand does not convincingly support the temporal segregation hypothesis; nevertheless, several other evidence do favour it.

An interesting study by Harker [27] highlighted the relevance of circadian timekeeping where she experimentally manipulated cockroaches to disrupt their internal temporal order. Harker transplanted the subesophageal ganglion (SG) known to drive circadian activity/rest rhythm in cockroaches such that hosts which received a transplant from a donor entrained to LD cycles either in-phase or anti-phasic with that of the host. Hosts which received SG transplants from donors entrained to anti-phasic LD cycles developed tumours as compared to those that received transplants from in-phase donors suggesting that internal desynchrony induced in the former case might have led to physiological disturbances. Physiological variables including core body temperature, hepatic degradation, growth hormone, plasma cortisol and potassium levels in humans [28], plasma corticosterone, N-acetyltransferase activity and serotonin in rats [29] and temperature and urinary potassium concentration in squirrel monkeys [30 and citations therein] exhibit daily variation. Fuller et al. [31, 32] reported that when monkeys are devoid of rhythmicity in the external environment, reduction in environmental temperature by 8 °C leads to a 2 °C reduction in body temperature due to the failure of thermoregulatory homeostasis, whereas when entrained to LD cycles, reduction in ambient temperature had no measurable effect on body temperature. Studies on several circadian clock gene mutants have also highlighted the importance of temporal order in physiology. Kondratov et al. [33] reported that *bmal* double knockout mice exhibit reduced lifespan and display symptoms of premature ageing including sarcopenia, cataract, reduced subcutaneous fat and organ shrinkage. Similarly, *clock* mutant mice have been observed to exhibit metabolic dysfunctions including hyperleptinaemia, hyperlipidaemia, hepatic steatosis, hyperglycaemia and hypoinsulinaemia [34] and also develop neurological problems [35]. Deficiency of another mammalian clock gene *per2* has also been observed to result in increased bone mass and higher propensity for cancer [36–38], while recent studies on *rev-erb-α* mutant mice reported observing obesity, aberrant lipid metabolism and thermogenesis [39–41].

The advent of genomics has facilitated exploration of circadian clocks' influence on the genome of organisms. Studies have revealed that over a third of the *Arabidopsis* genome is under the influence of circadian clock [42], 10–15 % of all cellular transcripts exhibit circadian oscillation in mammals [43, 44] and over 43 % of all protein-coding RNAs cycle in a circadian manner in at least one tissue [45]. Regulatory pathways involved in glucose homeostasis and lipid metabolism have also been reported to be under direct circadian control [43, 44, 46, 47]. More importantly, such rhythms also maintain stable phase relationship with each other thus highlighting the role of circadian clocks in establishing temporal order at the genome level. Collectively these results extend support to the idea that circadian clocks might play a crucial role in maintaining internal temporal order thus extending support to the intrinsic advantage hypothesis.

#### 5.3.1.2 Extrinsic Advantage

The 24-h periodic rotation of the earth about its axis drives cyclic variation of several abiotic (and consequently biotic) factors the most prominent of which include light, temperature, humidity and to some extent barometric pressure as well. While some of the environmental factors may be beneficial to organisms, others may be detrimental. For instance, light during the day may facilitate navigation, search for food and photosynthesis, whereas high temperature and low humidity may increase the risk of desiccation (especially in smaller life forms), and furthermore, harmful cosmic rays during the day might increase the probability of errors in DNA replication and cell division [48]. Thus, it is logical to presume that internal timekeeping mechanisms that can anticipate such rhythmic changes in abiotic factors, and accordingly time behaviour and physiological processes would be advantageous to organisms. Based on this reasoning, it is hypothesised that circadian clocks may confer adaptive advantages by facilitating entrainment and appropriately timing behaviours so as to avoid harsh environmental conditions, enhance food and mate procurement and facilitate predator evasion thereby establishing a temporal niche to reduce interspecific competition [49]- referred to as extrinsic advantage.

Support for the idea of extrinsic advantage of circadian clocks comes from multiple studies. Rhythmic vertical migration is ubiquitously observed in several planktonic species where individuals migrate upward during dawn and dusk, and sink down at other times of the day [50–53]. Most of these species occupy the first 1000 m from the top which is the approximate depth to which light can penetrate [54], and such diurnal migration has been asserted to daily changes in the light which consequently affects temperature, salinity and aeration which contribute to photosynthesis. Additionally, Hardy [55] discussed that water masses move more rapidly at the surface than at lower levels which facilitates rapid exchange of nutrients and other factors in the upper surface of the water body. Therefore, vertical migration is presumed to facilitate nutrient replenishment essential for the physiological functioning of the organism. Several woodlice and millipede species exhibit diurnal rhythm in activity with preference to wander out mostly at night so as to avoid harsh desiccative/low humidity conditions during the day. This is supported by experiments which report that when exposed to light, woodlice wander aimlessly till they find a dark and damp zone [56–58]. While this can also be a passive response to light, choice chamber experiments demonstrated that the tendency to seek damp regions is lower during the night as compared to the day [59, 60], thereby suggesting that such photic avoidance behaviours are at least partly driven by an endogenous timekeeping mechanism.

While the above-mentioned studies, in addition to several others [reviewed in 49], are suggestive of the idea that circadian clocks time behaviours so as to enhance survivability, studies discussing other aspects of extrinsic advantages

such as facilitating predator evasion have been controversial. Various studies have proposed that temporal partitioning between competing taxa in predator-prey systems is essential for promoting coexistence in ecological communities [reviewed in 61]. However, such partitioning is considered to be driven primarily by biotic factors, while the role of circadian clocks in driving such partitioning has remained largely speculative due to the lack of substantial empirical evidence. Schoener [62] reported that temporal partitioning is significantly less common in comparison to that of habitat or food-type partitioning and used theoretical models to predict that temporal resource partitioning on the circadian scale should be relatively rare, requiring severe depletion of resources before such behaviours can be observed. This is further supported by the relatively limited observations of shift from nocturnality to diurnality in nature. Unstriped Nile rats (*Arvicanthis niloticus*) are generally diurnal but become nocturnal when provided with a running wheel [63]. A similar shift from nocturnal to diurnal behaviours is also observed in rats *Sigmodon hispidus* [64], *Octodon degus* [65] and mice [66]. More importantly, careful analysis of the observed activity patterns suggested they were masked behaviours and not entirely clock-driven. However, Daan [67] proposed that since the evolution of nocturnality and diurnality requires different physiological mechanisms (for instance, nocturnal and diurnal animals differ in their retinal photoreceptors and adaptations to vision in a given light intensity range which reduces the efficiency at other intensities) [68, 69], closely related species, competitors or individuals of a predator-prey system generally remain active at a specific phase (day/night/twilight) of the circadian cycle depending on the physiological adaptation to nocturnality or diurnality. In other words, he proposed that rather than driving shifts in behaviours from nocturnality to diurnality (or vice versa), circadian clocks promote partitioning within the activity phase. Subsequently, DeCoursey and colleagues examined if dysfunctional circadian clocks would perturb the ability of animals to time behaviours at ecologically relevant phases and whether such mistimed behaviours have any negative implications. Daily behaviours of SCN (see glossary)-lesioned free-living diurnal antelope ground squirrels [70] and chipmunks [71, 72] were studied in seminatural enclosures. SCN-lesioned individuals in DeCoursey et al. [70, 72] and DeCoursey and Krulas [71] studies exhibited higher night-time restlessness resulting in animals wandering during the night and consequently incurring higher night-time predation as compared to their SCN-intact counterparts (Fig. 5.1). However, similarly treated golden-mantled squirrels *Spermophilus lateralis* and Siberian chipmunks *Eutamias sibiricus* [73, 74] lived for as long as 2.5 years in laboratory conditions. These results suggest that the observed deaths in SCN-lesioned animals were indeed due to mistimed behaviours and not a physiological consequence of SCN ablation. This is among the most convincing field studies to date that support the idea that circadian clocks ensure optimal timing of behaviours in nature to evade risks from predators.

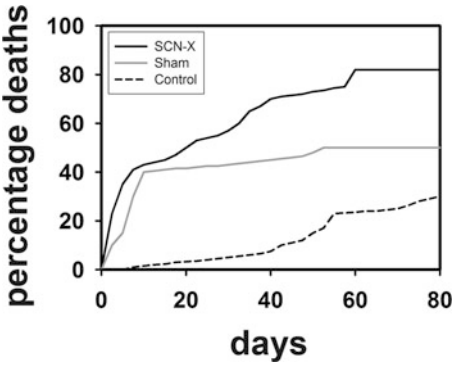



**Fig. 5.1** Figure depicting the proportion of SCN ablated (*SCN-X*), sham and control individuals lost due to deaths over 80 days following radio collaring and repatriation. SCN-X individuals were observed to incur a higher proportion of deaths as compared to controls indicating that absence of internal timekeeping mechanisms may prove detremental for the survival of organisms in nature. SCN ablation was performed with a stereotaxic lesioning device by drilling a 2-mm (diameter) hole in the skull and passing an electric current using an electrode to lesion the site of interest. 'Sham' controls were also subjected to the same procedure but without the use of any electric current, while 'controls' were not subjected to any such procedures (Figure modified after Ref. [72])

## 5.4 Evolutionary Origins of Circadian Clocks

### *5.4.1 When Did Circadian Clocks Originate?*

The period of earth's rotation is estimated to have been ~8 h some 3.4 bya (billion years ago) [75] and was the same until 3 bya as estimated from the daily growth rings resulting from swimming and settling of algal colonies forming stromatolites [76]. Wells [77] used annual growth series added to fossilised corals and estimated that the annual duration on earth was ~400 days in the Devonian period (~375 mya). Since the time taken for the earth to revolve around the sun has remained constant over the past 4 by [78], day length during this period was estimated to be ~22 h. Based on these estimations, it has been speculated that the period of earth's rotation was only ~4 h when *cyanobacteria* originated [25, 79, 80], and, therefore, the earliest clocks in *cyanobacteria* probably had a period of ~4 h which gradually extended to 24 h over time as the earth's angular velocity reduced [81]. *Cyanobacteria* are currently the oldest known life forms with functional circadian clocks and, therefore, cater as a useful tool to test if its clocks comprising the *kaiABC* [82] gene cluster were indeed the ancestral clocks. While the *cyanobacteria Synechococcus elongatus* has only one copy of the *kaiABC* cluster, multiple copies have been observed due to gene duplication in other related genus such as *Nostoc linckia* and *Synechocystis* [83]. However, not all the three genes appear to have evolved simultaneously. The *kaiC* gene is distributed in almost all major taxa of Archaea except *Methanopyri* and *Thermoplasmata* [83–85] but is not

well represented in eubacteria barring a few major taxa [83]. *KaiB*, on the other hand, is distributed in a few *Proteobacteria* including *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides* along with *kaiC* [83, 86] but is observed only in one archaeon, *Methanobacterium thermoauotrophicum* [83]. The presence of *kaiB* in other prokaryotic domains is speculated to be due to lateral gene transfer from *cyanobacteria*, whereas no reverse transfer from prokaryotes to *cyanobacteria* has been reported. However, *kaiA* seems to be restricted only to *cyanobacteria* thus suggesting that this might be the most recent addition to the cyanobacterial clock network. Thus, the representation of *kaiC* in archaebacteria, eubacteria and *cyanobacteria* is suggestive of it being the oldest (over 3.5 by) of the three *kai* genes, with the subsequent addition of *kaiB* between 3.5-2.3 bya to form the *kaiBC* cluster. Upon formation of the cluster, both *kaiB* and *kaiC* genes evolved as a unit before the final addition of *kaiA* to the cluster (Fig. 5.2). Studies on the *cyanobacteria Synechococcus* spp. indicate that expression of all three genes is essential for circadian clocks' function and that a lack of even one of the three renders the clock dysfunctional [82]; however, *kaiA* is not observed in most of the primitive *cyanobacteria* and other photosynthetic proteobacteria [83]. *Does this mean that even though kaiBC genes were present, these organisms did not have a functional clock until the addition of kaiA*? It is not entirely possible to arrive at such a conclusion since other genes apart from *kaiA* or *kaiB* might have partnered with *kaiC* to form a functional clock.

Other than *cyanobacteria,* circadian expression of certain gene transcripts (*Lhcb2*) has also been reported in primitive forms of plants such as the moss

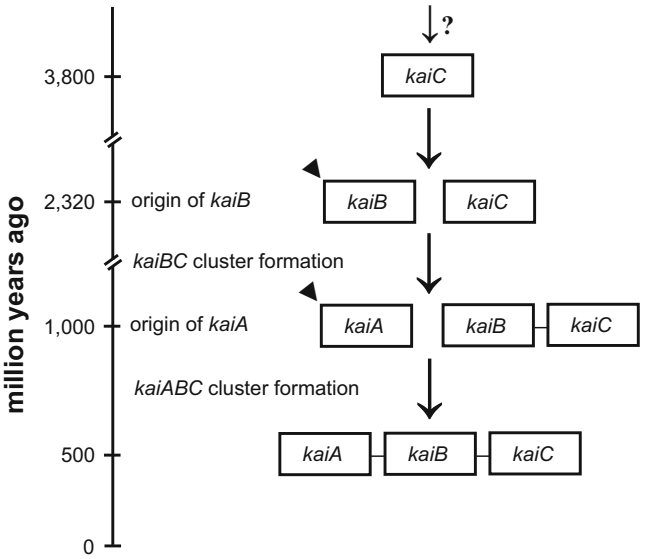



**Fig. 5.2** Pictorial timeline representation of the evolution of *kaiABC* cluster which forms the functional unit of circadian clocks in present-day cyanobacteria (Figure modified after Ref. [83])

*Physcomitrella* [87] and in gymnosperm *Ginkgo* [88]. Preliminary data suggests that the *Physcomitrella* genome shares a higher degree of similarity with *Arabidopsis* than *cyanobacteria* [87– 89] suggesting that circadian clocks in higher plants are probably derived from the moss genome and not from their earlier ancestors. This is further supported by the observation that the genomes of most modern-day plants including rice, tomato and *Arabidopsis* do not share any sequence homology with the cyanobacterial clock genes (discussed later). Nevertheless, *Chlamydomonas* genome indicates the presence of homologues of several kinases and phosphatases such as *casein kinase* and *protein phosphatase* [90] which are also ascribed several functions in the circadian clock machinery of *Neurospora*, *Drosophila* and mammals as well as plants [91–93]. However, since proteins encoded by these genes also perform several non-circadian functions, it could be precarious to ascertain whether the observed conservation across species is due to their role in circadian clocks or other general cellular functions [25].

In summary, some of the modern-day components of circadian clocks in *cyanobacteria* have existed for over 3.5 by with subsequent addition of other clock genes. If the primitive cyanobacterial clock period was indeed ~4 h as discussed earlier, a clockwork comprising a single gene *kaiC* might have been sufficient to form a feedback loop with a delay of ~4 h which eventually might have evolved to ~8-h clocks by the addition of *kaiB* as the day length extended to ~8 h around 3.5 bya, which overlaps with the predicted time of *kaiB* addition (3.5–2.32 bya), and similarly the addition of *kaiA* and other associated factors might have led to the evolution of the present-day ~24-h clocks.

However, the mere presence of only one of the three *kai* genes even though suggestive is not a convincing evidence to claim that functional clocks originated over 3.5 bya since other genes might have preceded *kaiB* and *kaiA* or even *kaiC*.

### *5.4.2 Why Did Circadian Clocks Originate?*

Identifying the selection pressures that might have driven the evolutionary origins of circadian clocks can hold the key to understand their possible adaptive values, and in this regard we will discuss two proposed hypotheses concerning the factors that might have influenced the origin of circadian clocks.

#### 5.4.2.1 Escape from Light Theory

The great oxygenation event associated with the ozone layer formation that shields the earth from the majority of UV radiation occurred around 2.3 bya (after the origin of *kai C*, and probably *kai B* as well). Organisms inhabiting the earth prior to this are likely to have been subjected to extreme temperatures and harmful solar

radiations which might have been the potential factors in driving the evolution of timekeeping mechanisms that help organisms avoid such extreme environments (extrinsic advantage). Furthermore, day/night cycles are associated with exposure to harmful solar radiation during the day which is known to increase errors in DNA replication and other photochemical reactions involving RNA and proteins as well [reviewed in 94], thereby rendering organisms vulnerable at specific times of the day. Furthermore, even though some of the cellular factors do not function as photoreceptors, they might nevertheless be sensitive to light due to obligatorily associated cofactors such as cytochromes [discussed in 25]. Based on a similar premise, Pittendrigh [95] proposed that organisms might have evolved timekeeping mechanisms to anticipate and escape from such harmful effects of light, referred to as 'escape from light' hypothesis. If this is true, given the evolutionarily conserved nature of circadian clocks, it can be expected that some of the present-day life forms may still retain phase-specific susceptibility to solar radiation, and indeed *Chlamydomonas* have been observed to exhibit rhythmic sensitivity to UV light with enhanced sensitivity around sunset and early night [96]. More importantly, rhythmic UV light sensitivity also persists in constant conditions suggesting a possible involvement of circadian clocks in driving such responses. Several other observations further support the escape from light hypothesis. For instance, numerous microorganisms exhibit circadian rhythmicity in DNA replication and cell division, with these processes occurring mostly during the dark phase [97]. Based on his hypothesis, Pittendrigh [95] had also anticipated a DNA photolyase type of enzyme to be closely associated with circadian clocks which eventually turned out to be true with studies reporting that molecular circadian clock components involve *cryptochrome* (*cry*) which shares sequence homology with DNA photolyases, a blue light-sensitive UV-damaged DNA repair enzyme [reviewed in 98]. Homologues of *cry* have also been identified in cyanobacterium *Synechocystis* spp. [99] and *Rhodobacter sphaeroides* [100]; however, their role in circadian function (if any) remains to be elucidated. These results suggest that the ancestral photolyases were probably substrates for natural selection to act upon, eventually driving the evolution of *cry* as a core circadian clock component [25, 96, 101]. Interestingly, the role of *cry* is not entirely conserved across species; while it is known to function as circadian photoreceptors in some insects (CRY1), it acts as a core clock component (CRY2) in mammals [102]. This distinction cannot be merely explained on the basis of differences between invertebrate and mammalian clocks as phylogenetic and functional classifications revealed that non-drosophilids such as silk moths, mosquitoes and butterflies express both *cry1* and *cry2*, whereas honeybee (*Apis mellifera*) [103] and red flour beetle (*Tribolium castaneum*) possess only *cry2* [104]. Thus, it is not clear as to whether *cry* in circadian clocks originated from its function as a photoreceptor or as a core circadian clock component.

#### 5.4.2.2 Endosymbiotic Coordination Theory

First proposed by Levandowsky [105] and later elaborated by Kippert [106], the endosymbiotic coordination theory suggests that since evolution of eukaryotes was facilitated by endosymbiosis of prokaryotes that formed the precursors for currently observed organelles in eukaryotes, such spatial compartmentation of autonomously functioning organelles might have required a coordination mechanism to temporally regulate processes among themselves which otherwise would lead to chaotic cellular systems. Thus, the necessity for the establishment of internal temporal order within the cell has been hypothesised to have led to the origin of circadian clocks. If this is true, then the circadian clock genes (*KaiABC* cluster) in *cyanobacteria* which are presumed to be the ancestors of chloroplasts in modern-day plants must have been transferred to higher plants during endosymbiosis. Unfortunately, no putative homologue of *KaiABC* genes has been observed in either the chloroplasts or the nuclear genomes of *Arabidopsis*, rice, tobacco and *Medicago* [25 and citations therein]. Eukaryotic alga *Chlamydomonas* which is considered to be a closer ancestral relative of plants also do not harbour any of the cyanobacterial *KaiABC* genes. Moreover, comparison of *Chlamydomonas* and *Arabidopsis* genomes revealed that the former does not have any putative homologue of *Arabidopsis* circadian clock genes including *elf3, gpif3* and *tej*, while other genes such as *cca1, lhy, col, ztl, ado1, ado2, ado3, prr1, prr3, prr5, prr7* and *prr9* share minimal homology [25, 90]. Therefore, current evidence barely supports the hypothesis that circadian clocks have evolved to coordinate endosymbiotic processes within the cell.

In summary, results from all the studies discussed in this section clearly indicate that we are far from precisely understanding the evolutionary origins of circadian timing systems. Alternatively, the lack of clarity in shared sequence homology between genomes of modern and primitive photosynthetic life forms suggests that clocks might have evolved independently in multiple life forms possibly by convergent evolution.

## 5.5 Adaptive Significance of Circadian Clocks

It can be noted that majority of the previously discussed studies reported severe consequences of lack of temporal coordination on various aspects of the physiology of organisms including humans (Box 5.2) which highlights that circadian clocks are advantageous. However, barring a few, results from the majority of the studies cannot be used as a conclusive demonstration of the adaptive nature of circadian clocks, primarily because a trait can be termed adaptive only upon fulfilment of certain criteria that stem from the definition of adaptation as discussed in Sect. 5.2. In the subsequent sections, we will discuss studies that adopted various experimental strategies to provide relatively direct line of evidence in support of the adaptive significance of circadian clocks.

**Box 5.2: Chronotypes and Circadian Desynchrony in Humans**
Humans are known to exhibit variation in preferred timing of sleep/wake behaviour with individuals who prefer to wake up early and sleep early termed as 'early' chronotypes, while those who wake up late and sleep later are termed 'late' chronotypes [107]. Furthermore, chronotypes have been observed to be associated with circadian clock period [107]. Owing to the societal schedule that generally favours early chronotypes, late chronotypes often struggle to remain in sync with the societal schedule resulting in them experiencing 'social jet lag' (desynchrony between the internal clock and external cycle). Consequently, late chronotypes have been reported to experience several psychological, physiological and metabolic disturbances [107]. Several studies have also directly examined the impact of circadian desynchrony on human physiology. Such studies adopted protocols that involved placing subjects under dim LD (*T*20 or *T*28) cycles with scheduled sleep bouts and mealtimes. Since the human circadian clock cannot entrain to such extreme *T*-cycles, it tends to free-run, thus desynchronising behavioural cycles (eating and sleeping) with internal clocks and the external environment referred to as forced internal desynchrony. Forced internal desynchrony has been reported to be associated with sleep disturbances, decreased vigilance and cognitive performance [108, 109]. Such desynchrony also results in elevated blood glucose, reduced insulin sensitivity and circulating leptin, altered post-prandial insulin release and advent of hypertension [110–114]. Furthermore, analysis of blood transcriptome in desynchronised subjects revealed a strongly attenuated rhythmic gene expression (going from 6.4 % of the transcriptome at baseline to only 1.0 %) during circadian misalignment [114]. These results are strongly suggestive of the idea that ensuring synchrony with external environment is critical for optimal functioning of organisms, a concept acknowledged even in the ancient Indian medicinal practice-'ayurveda' [Box 5.1].

### *5.5.1 Effects of Dysfunctional Circadian Clocks*

One of the straightforward ways to test the functional significance of rhythms governed by circadian clocks is to assess the effect of arrhythmic behaviours on fitness. Kumar et al. [115] assayed the lifespan of spontaneously arrhythmic individuals from inbred and outbred strains of *D. melanogaster* and found that the arrhythmic individuals exhibited ~37 % reduction in lifespan in the outbred populations, whereas the inbred line exhibited a more severe reduction (~60 %) in lifespan as compared to their rhythmic counterparts. Other studies have adopted approaches such as the use of mutant strains with non-functional circadian clocks, surgical ablation of the master clock or use of constant light (LL), both of which are known to render most circadian behaviours arrhythmic and studied its effect on fitness measures.

#### 5.5.1.1 Effect of Circadian Dysfunction Realised by Genetic Manipulations

Molecular analysis of circadian clocks has revealed that the clock architecture is highly conserved across organisms and that the disruption of core clock genes renders clocks dysfunctional leading to arrhythmic behaviours [reviewed in 116]. Multiple studies have taken advantage of the ability to genetically abort molecular clockwork functioning and investigated its effect on organisms' fitness.

Loss-of-function mutations of core clock genes in *Drosophila*, namely, *period* (*per*), *timeless* (*tim*), *clk* (*clock*) and *cycle* (*cyc*) have deleterious effects on the fecundity of *D. melanogaster* with such mutants showing ~40 % reduction in egg output [117]. Further analysis revealed that the reduction in fecundity was associated with reduced fertility (as assessed by sperm count) of the mutant males [117]. Additionally, clock mutations have also been reported to influence oogenesis in *D. melanogaster* females; however, such effects do not seem to involve circadian clocks but arise from pleiotropic effects of clock genes. Hendricks et al. [118] reported that *cyc* null mutants (*cyc*°) of *Drosophila* exhibit reduced lifespan in both LD and DD conditions as compared to their rhythmic wild-type controls. Interestingly, such a reduction in lifespan was observed only in males, while the lifespan of *cyc*° females did not differ from controls. Thus, the observed effects of clock gene mutations on life-history traits may not necessarily be mediated through circadian clocks per se but by the pleiotropic effects of clock genes. Additional evidence for the effect of clock gene disruption on fitness measures has come from studies on plants as well. Constitutive expression of the *cca1* (*circadian clock associated 1*) gene in *Arabidopsis* (*CCA1-ox*) results in loss of circadian rhythms under both LL and DD [119]. Similar arrhythmic behaviour is also observed upon overexpression of another clock gene *elf3-1* (*early flowering 3-1*), but only in LL and not in DD [120, 121]. Green et al. [122] studied the effect of loss of circadian rhythmicity on the fitness of *Arabidopsis* and observed that *CCA1-ox* and *elf-3* mutant plants were less viable as compared to their wild-type counterparts as also demonstrated by Schaffer et al. [123]. However, the authors quite rightly pointed out that even though their results are suggestive of circadian clocks' role in determining the fitness of *Arabidopsis*, such effects can also arise from the pleiotropic influence of the genes under study and may be independent of the circadian defects caused by the mutations.

#### 5.5.1.2 Effect of Circadian Dysfunction Realised by Environmental Manipulations

Several studies have also adopted LL-induced arrhythmicity to assess fitness costs of harbouring dysfunctional circadian clocks as such an approach would help surpass the pleiotropic effects of gene mutations observed in studies using genetic manipulations discussed in the previous section.

Allemand et al. [124] assayed the lifespan of *D. melanogaster* maintained under LL, LD and DD and reported that the lifespan of individuals under LL was significantly shorter than that under LD followed by DD which had a significantly higher lifespan (~20 % longer for males and ~43 % for females) thus demonstrating negative implications of loss of circadian rhythmicity on the organisms' lifespan. While these studies reported fitness costs incurred due to the loss of circadian rhythmicity, the observed phenotypes might have also stemmed from deleterious effects of LL and not necessarily due to clock-driven arrhythmicity, and, therefore, such effects may not necessarily be a consequence of circadian dysfunction. Several other studies have also assessed the effect of LL on circadian behaviours, but since these studies also used other light regime combinations, they will be discussed in the succeeding section.

### 5.5.2 *Circadian Resonance and Consequences of Circadian Mismatch*

If synchrony with the external environment does confer any fitness advantages, then it can be hypothesised that circadian clocks might have evolved to facilitate the synchrony between organisms and the external environment. This forms the basis of the 'circadian resonance' hypothesis according to which organisms are expected to perform their best (enhanced physiological efficiency) when their clock period matches with that of the environmental cycles [125]. The importance of circadian resonance with external cycles has been studied in a variety of species spanning unicellular *cyanobacteria*, insects, mammals and plants. Among such studies, few assessed the consequence of entraining genetically manipulated or mutant strains with non-24-h clock periods to 24-h LD cycles, while other studies entrained individuals with ~24-h period to various light regimes of non-24-h periods ($T$). The rationale being individuals whose endogenous period does not match with that of the external cycles would exhibit lower fitness as compared to those whose period matches with external cycles.

#### 5.5.2.1 Effect of Circadian Resonance on Development

Pre-adult development time in insects is one of the commonly studied fitness traits, and multiple studies have tested the proposition that development time is influenced by the period of the environmental cycles as would be expected by the circadian resonance hypothesis. For instance, when flesh fly *Sarcophaga argyrostoma* were reared under different $T$-cycles (see glossary), their pre-adult development time was longer under $T$-cycles closer to 24 h which was approximately the $\tau$ of flesh flies [126]. Similarly, another study by Lone et al. [127] reported that pre-adult development was significantly faster when *Camponotus* ants were reared under $T24$ as

compared to *T*20 and *T*28. When growth rate of tomato plants was assessed under different *T*-cycles, the ones growing under *T*24 outperformed those growing under *T*12, or *T*48, or even LL thus providing evidence in favour of the circadian resonance hypothesis [128–131]. Furthermore, growth rates under high and low temperatures exhibited a $Q_{10}$ (see glossary) value of about 1.2 suggesting that it was temperature compensated which being a key property of the circadian clock further highlights the clocks' role in regulating growth rates [132, 133]. However, it appears that the results from tomato plants cannot be generalised. For instance, the growth rate of *Arabidopsis* under LL was found to be significantly higher as compared to that in LD12:12 [reviewed in 25]. Another study on clock-disrupted *Arabidopsis* mutants reported that under LD4:20, such strains produce fewer viable seeds as compared to wild-type strains [122].

#### 5.5.2.2 Effect of Circadian Resonance on Lifespan

Pittendrigh and Minis [134], while testing the circadian resonance hypothesis, observed that wild-type strains of *D. pseudoobscura* maintained under different *T*-cycles (*T*21 or *T*27) or in LL lived considerably shorter than those under *T*24, but these results could not be reproduced in a different laboratory [25]. A similar trend was also observed in blowflies (*Phormia terraenovae*) which exhibited ~22 % lifespan reduction in individuals subjected to repeated changes in *T*-cycle duration as opposed to their controls experiencing *T*24 [135]. Another study on *D. melanogaster* used wild-type ($per^{+}$; $\tau \sim 24$ h), short-period ($per^{T}$; $\tau \sim 16$ h) and long-period ($per^{L}$; $\tau \sim 28$ h) strains to examine the cost of circadian mismatch [136]. The authors assayed the lifespan of all three strains under symmetric *T*16 and *T*24 cycles and found that males of $per^{T}$ and $per^{L}$ strains lived significantly shorter as compared to wild-type males under *T*24. Moreover, $per^{T}$ whose $\tau$ deviated by 8 h from the 24-h LD cycle exhibited a larger reduction in lifespan as compared to $per^{L}$ that deviated by only 4 h. Furthermore, lifespan difference between $per^{T}$ and wild-type flies was further reduced under *T*16 (close to the period of $per^{T}$) as compared to the difference between them under *T*24. While these results are partly in accordance with the expectations from the circadian resonance hypothesis, the fact that $per^{T}$ did not exhibit higher lifespan in *T*16 is intriguing.

#### 5.5.2.3 Effect of Circadian Resonance on Competitive Ability

Even though some of the above-discussed studies reported evidence favouring the circadian resonance hypothesis, it is important to note that most of these studies involved assessment of fitness measures under simple non-competitive conditions. In a more realistic scenario, an organism can be considered to be fit in an environment only when it can outcompete its competitors inhabiting the same environment. Therefore, demonstrating the competitive abilities of different individuals/strains is a more rigorous strategy to assess the fitness of an organism. Here we discuss two

such highly interesting experiments that have provided some of the strongest arguments to date supporting the circadian resonance hypothesis and thus the adaptive significance of circadian clocks.

In a first study of its kind, wild-type strains of *cyanobacteria S. elongatus* (AMC149 and AMC343; $\tau = 25$ h) were competed with two period mutant strains, SP22 and P28 of $\tau$ 23 h and 30 h, respectively [137]. The experimental design comprised pairwise competition experiments under multiple *T*-cycles whose period matched with the $\tau$ of at least one of the strains subjected to competition. The authors observed that, when equal proportions of two strains were mixed and allowed to compete, the wild-type strain outgrew all others under *T*24, while the SP22 strain outcompeted others under *T*22. Furthermore, the P28 strain which performed poorly under *T*22 and *T*24 was observed to outgrow other strains when competed under *T*30 (Fig. 5.3). To further test if the observed performance differences between strains were indeed due to period match with the environmental cycles, and not a consequence of the mutations harboured, the authors also performed similar competition experiments for period mutants harbouring point mutations in other core clock genes in *S. elongatus* and reported similar results [138]. Similarly, Dodd et al. [139] used wild-type *col-o* ($\tau = 24$ h), long-period *ztl-l* ($\tau = 27.1$–$32.5$ h) and short-period *toc1-1* ($\tau = 20.7$ h) strains, reared them under LD cycles with varying periods (*T*20, *T*24 and *T*28) and assessed their photosynthetic activity and growth rates. The chlorophyll content was found to be higher in plants reared under *T*-cycles of periods matching their $\tau$ with *col-o* performing better than other two strains in *T*24 than in *T*20 and *T*28. Similarly, compared to *T*24, *ztl-l* and *toc1-1* strains performed better under LD cycles matching their endogenous periods. Consequently, all strains exhibited higher $CO_2$ fixation and greater biomass when reared under *T*-cycles with periods matching their $\tau$. In the competition between *ztl-l* and *toc1-1* strains which were grown as mixed populations under *T*20 and *T*28, each strain exhibited higher chlorophyll content, leaf number and aerial biomass as compared to its competitor when grown under *T*-cycles with periods matching $\tau$. In accordance with the circadian resonance hypothesis, these results demonstrated that period match between endogenous clocks and external cycles enhances the fitness of individuals.

#### 5.5.2.4 Effect of Circadian Resonance on Other Traits

A naturally identified autosomal gene mutation (*tau*) in hamsters causes a copy number-dependent change in the circadian period with the homozygotes exhibiting 20 h (and fails to entrain to LD14:10 cycles) while the heterozygotes exhibit 22-h rhythms [140]. Heterozygous animals were observed to exhibit reduced lifespan under LD14:10, while the homozygous mutants did not show any lifespan deficit [141]. Moreover, when housed under *T*24, heterozygous individuals showed multiple physiological defects such as cardiomyopathy with fibrosis and impaired contractility and renal dysfunction. Interestingly, when housed under *T*22 which is proximal to their clock period, no such physiological defects were observed in

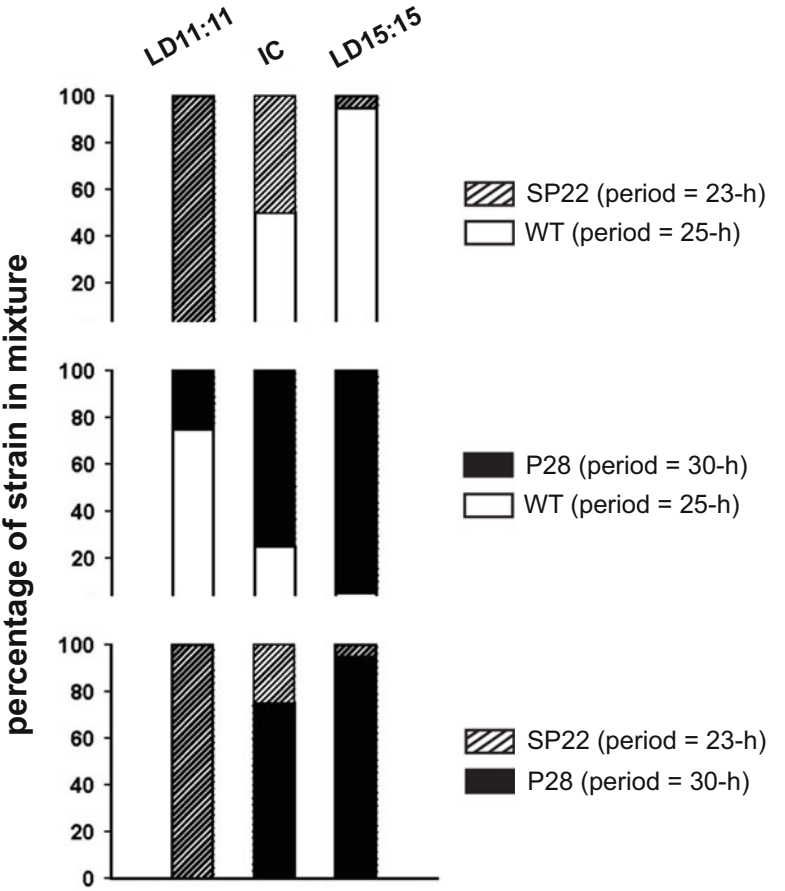



**Fig. 5.3** The figure depicts percentage of short period (SP22), long period (P28) and wild type (*WT*) strains in a mixed culture maintained under different light regimes after 27 days of competition. Different proportions of strains were mixed as pairs at the beginning of the experiment (*IC* initial condition) and were let to compete with each other under LD11:11 and LD15:15 for 27 days. Strains whose clock period closely matched with the period of LD cycle they were maintained in, outcompeted other strains thereby providing evidence for the circadian resonance hypothesis (Figure modified from Ref. [137])

heterozygous individuals thereby encouraging the authors to conclude that the observed physiological disturbances stem from circadian mismatch.

Another interesting study assessed the importance of circadian mismatch in natural populations of pitcher plant mosquito *Wyeomyia smithii*. Individuals of this species are known to enter larval diapause under short photoperiod, and this response is terminated under long photoperiods [142]. Emerson et al. [143] subjected populations of *W. smithii* to LD10:14, LD10:36 and LD10:25, all of which are known to induce diapause in this species. The authors further measured various fitness parameters such as egg-to-puparium survivorship, fecundity,

embryonic viability and adult lifespan and observed that individuals that entered diapause under LD cycles with periods 24 h (LD10:14) or multiples of 24 h (LD10:36) exhibited higher fitness as opposed to those under non-24-h cycles (LD10:25).

In another study on plants, Yerushalmi et al. [144] made use of crosses between long-$\tau$ (*prr7prr9*, $\tau = 36.55$ h) and short-$\tau$ (*prr5prr7*, $\tau = 22$ h) mutant strains of *Arabidopsis* to generate greater variability in $\tau$ (20.79 h and 45 h) among the F2 progeny. These strains were then reared under *T*20 (LD13:7; *T*20) and *T*28 (LD19:9; *T*28). As a measure of the reproductive success of the short- and long-$\tau$ variants, the $\tau$ and frequencies of *prr5prr7* and *prr7prr9* alleles were assessed in the F3 populations. Interestingly, $\tau$ of the F3 plants was positively correlated with the period of *T*-cycles (*T*20 or *T*28) in which their respective parents were reared. Also, the F3 individuals from parents reared in *T*28 inherited long-$\tau$ *prr7prr9* alleles in higher frequencies. However, surprisingly the frequency of short-$\tau$ allele *prr5prr7* did not differ significantly between the F3 populations coming from F2 parents reared under *T*20 or *T*28. Nevertheless, these results suggested that long-$\tau$ phenotypes are favoured under *T*28 thus providing further evidence in support of circadian resonance hypothesis.

## *5.5.3 Studies on Natural Populations*

### 5.5.3.1 Geographical Clines

Among the most prominent variations in environmental conditions are changes in day length (photoperiod), light intensity and temperature observed across latitudes with such variation reaching extremes towards the poles [145]. Such gradation is likely to drive latitude-dependent selection pressures on rhythmic behaviours, and, therefore, studying how circadian rhythms vary in populations inhabiting different latitudes (clines; see glossary) will be highly insightful.

The earliest comprehensive analysis of clines in circadian behaviours was undertaken by Lankinen who studied emergence rhythm and seasonal diapause in European *D. littoralis* populations collected from 30 to 70°N. Lankinen assessed phase and $\tau$ of emergence rhythm in 12 southern (28°N) and northern (56–63°N) strains of *D. subobscura* and found that phase of emergence was advanced, clock period shortened and amplitude lowered in the northern strains as compared to the southern strains [146]. Pittendrigh and Takamura [147] also studied four Japanese strains of *D. auraria* collected from latitudes between 34 and 43°N and observed that the phase of emergence rhythm varied by 2 h and period by 0.5 h across latitudes. Allemand and David [148] reported observing a latitudinal cline in circadian oviposition rhythm in *D. melanogaster* collected from equatorial Africa (0°N) to Scandinavia (62°N). However, it remains to be tested if the observed clines were indeed driven by differences in local environmental conditions and, if so, their role in local adaptations.

The *Drosophila per* gene is known to encode variable-length Thr-Gly residue repeats with $(Thr\text{-}Gly)_{17}$ and $(Thr\text{-}Gly)_{20}$ being the two major variants accounting for 90 % of the repeat length variation in the European populations [149, 150]. A study on natural populations of *D. melanogaster* collected from different latitudes reported that the two repeat length variants were statistically correlated with latitudes with $(Thr\text{-}Gly)_{17}$ occurring primarily in the southern latitudes, while $(Thr\text{-}Gly)_{20}$ was predominant in the northern latitudes (Fig. 5.3) [149]. The authors speculated latitudinal variation in temperature as being the primary driving force and thus assessed the role of Thr-Gly repeat lengths in temperature compensation (see glossary). It was observed that flies with $(Thr\text{-}Gly)_{20}$ variant exhibited better temperature compensation ability as compared to the $(Thr\text{-}Gly)_{17}$ variant, and therefore the authors concluded that the observed cline in the frequency of Thr-Gly repeat length was shaped by natural selection acting on the ability of circadian clocks to remain stable even at lower temperatures experienced at higher latitudes [151]. A similar latitudinal cline for $(Thr\text{-}Gly)_{20}$ repeat length variant was observed in *D. melanogaster* populations sampled from Australia where the $(Thr\text{-}Gly)_{20}$ variant was positively correlated with latitude [152]. However, this was disputed by another study which failed to reproduce the same [153]. Nevertheless, observing similar trends in evolutionary footprints across multiple studies on independent populations further strengthens the idea that circadian clocks facilitate adaptation to local environmental conditions.

Natural polymorphism in *tim* (*ls-tim* and *s-tim*) results in two versions of the translated proteins (TIM) with *ls-tim* coding for both long and short variants of TIM, while *s-tim* codes for only the short variant [154]. Tauber et al. [155] reported a latitudinal cline in the frequencies of *ls-tim* which was higher in southern latitudes (Italy) and gradually decreased towards northern latitudes (Sweden; Fig. 5.4). To further investigate what aspects of the phenotype did *ls-tim* influence, the authors tested its role in temperature compensation as in Sawyer et al. [151]. However, temperature compensation was found to be similar in individuals carrying *s-tim* and *ls-tim*. Northern Europe is characterised by lower temperatures and shorter day lengths, which is known to induce diapause in *Drosophila* [157, 158]. Therefore, the authors further tested if the two variants affected the ability of flies to enter diapause and found that the *ls-tim* females exhibited a higher incidence of diapause as compared to the *s-tim* females. Similarly higher incidence of diapause in northern latitudes is observed in *D. melanogaster* populations sampled from the Eastern USA [157]. Furthermore, females with a higher incidence of diapause have been reported to show lower stress resistance and enhanced fitness under undesirable conditions suggesting that enhanced diapause confers greater adaptive advantages [157, 158]. Observing a similar trend in the European populations suggests that the observed latitudinal cline was probably due to adaptation to local environmental conditions. However, contrary to this, *ls-tim* which favoured diapause in females was observed to be lower in the northern latitudes as compared to the southern areas. Therefore, the source of clinal divergence in the *tim* allele frequencies reported by Tauber et al. [155] remains unclear. Clock genes have been implicated in diapause

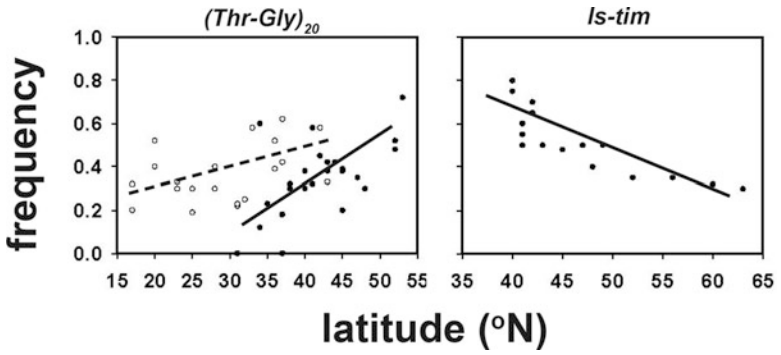



**Fig. 5.4** (**a**) Frequencies of *per (Thy-Gly)*$_{20}$ across latitudes in populations of *D. melanogaster* sampled in Australia (*open circles*) and Europe (*closed circles*). The *dashed* and *solid* regression lines indicate the latitudinal cline of frequencies in Australian and European populations, respectively. Both regression *lines* reveal that the frequencies of *per (Thy-Gly)*$_{20}$ alleles increase towards northward regions. (**b**) Frequencies of *ls-tim* across latitudes in populations of *D. melanogaster* sampled in Europe (south-eastern Italy to Sweden) (Both the figures are modified after Ref. [156])

induction in multiple insect species including the pitcher plant mosquito *W. smithii* [159], *Chymomyza costata* [160], *Riptortus pedestris* [161], *Drosophila triauraria* [162] and *Sarcophaga bullata* [163, 164]. While this is suggestive of a causal link between circadian clocks and diapause mechanisms, clock-independent pleiotropic effects of clock genes cannot be entirely ruled out. Therefore, the observed clines in *ls-tim* frequencies even though might have been associated with diapause response, do not necessarily imply the role of circadian clocks.

Latitudinal clines for the effects of natural zeitgebers have been studied in birds and mammals but not an evolutionary adaptation of their circadian systems to latitude [145]. Similar studies on humans have also been reported but appear to be controversial. For instance, Ciarleglio et al. [165] proposed that variation in clock genes among populations may primarily be due to genetic drift and not due to directional selection (see glossary), whereas Cruciani et al. [166] reported that genetic variation in the *hper2* gene is under directional selection. Studies reporting latitudinal clines in clock gene polymorphisms in vertebrates have been inconclusive. Fidler and Gwinner [167] examined sequences of three core clock genes, *bmal1*, *clk* and *per2*, in owls and found the *clk* polyglutamine (*clock*-polyQ) region to be polymorphic within and among species. In support to this, the non-migratory blue tit *Cyanistes caeruleus* and the migratory bluethroat *Luscinia svecica* populations showed considerable polymorphism in *clock*-polyQ (*clock*-polyQ)$_9$ and (*clock*-polyQ)$_7$ and displayed a positive association between allele length and breeding latitude in the blue tit, whereas bluethroat populations failed to show such correlations [168]. Several other studies on the swallow genus *Tachycineta* sampled across North, South and Central America [169], barn swallow *Hirundo rustica* [170] and Pacific salmon species (genus *Oncorhynchus*)

failed to show consistent patterns of latitudinal clines in *clock*-polyQ frequencies [171, 172].

Among the studies in plants, Mayer [173] observed that $\tau$ of circadian leaf movement rhythm of various plant species was generally shorter northwards which is also observed to be true for within genus comparisons in two dandelion (*Taraxacum*) species. When circadian leaf movements were monitored in a collection of 150 natural accessions of *A. thaliana*, their clock periods were found to correlate positively with day length at the latitude of their origin [174]. In many plants, circadian clocks have been attributed to synchronise flowering with favourable conditions of the day depending on the latitude. For instance, the circadian expression of GmCRY1a which functions in the input pathway of circadian timing system has been observed to be correlated with latitude and also with flowering time in *Glycine max* (soybean) [175]. Therefore, latitudinal clines in clock gene polymorphisms are yet to be well established in plants and vertebrates, and further studies may help shed more light on these aspects.

In addition to latitudinal clines, studies aimed at exploring the functional relevance of circadian rhythms in organisms that live in similar day lengths but different ambient temperatures (altitudes) show considerable potential to help explore interesting aspects of circadian clocks. Sorensen and Loeschcke [176] examined heat tolerance in populations of *Drosophila buzzatii* originating from different altitudes in north-western Argentina. Interestingly, the authors found that heat tolerance varied across the day in an altitude-dependent manner with heat tolerance in the afternoon and early evening being significantly lower in the higher-altitude populations as compared to their low-altitude counterparts. Although the authors did not examine differences in circadian rhythmicity across altitudes, the timing of heat tolerance was controlled by light phase thus suggesting a possible involvement of circadian clocks. As a further support to this, other studies have also reported circadian regulation of temperature stress resistance in plants [177, 178], and additionally, majority of circadian clock-regulated genes in the *Arabidopsis* genome are known to be involved in stress resistance pathways [42]. In addition to studies on lifespan and developmental rates, these results suggest that circadian regulation of stress responses may be another substrate for natural selection to act upon, thus underscoring the adaptive role for circadian clocks.

#### 5.5.3.2 Populations in Constant Conditions

A corollary to the hypothesis that circadian clocks evolved in response to rhythmic selection pressures imposed by cycling environmental conditions is that, if organisms are devoid of cyclic conditions for several generations, their circadian clocks would regress since there would be no obvious advantage of timing behaviours in an otherwise aperiodic environment.

Several species inhabiting constant conditions show considerable changes in morphological features due to regression of traits that are of little utility in their respective habitats. One prominent example is reduced visual sensory system in deep-sea life forms. Based on a quantitative assessment of reduction in visual systems, such species have been sorted into three categories: (1) *macrophthalmic* species in which the visual system is not substantially reduced in comparison with that in allied surface-dwelling species, (2) *microphthalmic* species characterised by a reduction of the eye ranging from mild to almost complete absence and (3) *anophthalmic* species which are the extreme types defined by a complete lack of any visual system [179]. However, it is also essential to identify species in which visual sensory systems never evolved versus those in which such systems evolved and eventually regressed.

Studying circadian rhythms in natural populations that have evolved in constant environments for several thousands of generations is thus an ideal strategy to test the adaptive roles of the circadian clocks. In this regard, deep underwater caves and similar deep-sea habitats have served as excellent laboratory setups [180] for studying the evolution of circadian clocks. Even though several studies have assessed circadian rhythms in all three macro-, micro- and anophthalmic species, we will restrict our discussion to the studies on the microphthalmic and anophthalmic species which show relatively extreme features of visual system regression. A detailed review of other categories can be found elsewhere [179].

*Fish* Several studies have assessed circadian rhythm properties in natural populations of troglofauna and stygofauna (land- and water-dwelling troglobites, respectively). One of the earliest studies in this line tested for temporal activity patterns in the cave crayfish *Cambarus pellucidus* and reported the absence of any rhythmic activity behaviour in constant laboratory conditions [181]. However, 20 years later, Brown [182] analysed the same data with improved statistical tools and surprisingly detected significant circadian rhythmicity in the activity of this species. In continuation, another study reported circadian rhythmicity in oxygen consumption in *O. pellucidus* [183]. However, this might not be surprising since *O. pellucidus* harbours vestiges of the eyes [184] and exhibits photonegative responses [181] suggesting that they probably have functional light input pathways that might underlie the observed weak circadian behaviour. Among the vertebrate microphthalmic troglobionts whose circadian behaviours have been studied is the catfish *Rhamdia enfurnada*. Morphological assessments indicate that the visual system of *R. enfurnada* is highly reduced but is never completely missing; however, this species is characterised by extensive population genetic variation of troglomorphic traits. Consistent with this, individuals, when tested for rhythmic behaviours in DD, exhibited large variation in periodicities ranging from circadian to ultradian values [185], a possible indication of circadian clock regression in progress.

Studies on anophthalmic species have reported several interesting features of circadian clock evolution under constant environments. The anophthalmic cyprinid Somalian cavefish *P. andruzzii* is endemic to a completely enclosed cave habitat. It is estimated to have diverged from its common ancestor and

subsequently evolved in constant cave environment for 1.4–2.6 my [186]. The effect of such long-term evolution under constant condition is evident from the loss of scales, eyes and pigmentation that are presumed to have regressed in due course. However, *P. andruzzii* exhibit negative phototactic behaviour [187, 188] at four different wavelengths (480, 539, 615 and 692 nm) which is most pronounced at blue light which coincidentally (or not) is also the wavelength that circadian clocks are most sensitive to. Interestingly, the genome of *P. andruzzii* possesses homologues of major core clock genes (*clk1a*, *clk2*, *per1* and *per1b*) [189], suggesting that their clocks might have been functional earlier but regressed over time. Laboratory studies revealed that neither the central nor the peripheral clocks of *P. andruzzii* are light-entrainable. However, non-visual zeitgebers such as periodic feeding have been reported to entrain circadian activity rhythm as well as peripheral clocks in this species [189]. Interestingly, the circadian clock of this species does not seem to be temperature compensated. Partial exhibition of circadian rhythms and loss of temperature compensation suggests that *P. andruzzii* is probably in the course of evolutionary regression of circadian clocks. However, this can also be reasoned alternatively. Temperature compensation has been hypothesised to be a key adaptive mechanism to ensure that changes in environmental temperatures do not alter $\tau$. Cave environments, unlike terrestrial habitats, do not experience any temperature changes across the year, in which case a temperature-compensated clock may not necessarily be adaptive, and, therefore, it is plausible that only mechanisms underlying temperature compensation might have regressed in these populations while functional circadian clocks might still persist. Another cavefish, the Indian loach *Nemacheilus evezardi*, which exhibits extreme variation across populations including both micro- and anophthalmic forms, has also served as a model for various circadian rhythm studies [190]. The microphthalmic forms have been documented to exhibit time-dependent phototactic behaviour [191] and also exhibit circadian rhythmicity in burrowing and air-gulping [190, 192–194]. Furthermore, scheduled feeding has been speculated to entrain rhythmic activity in *N. evezardi* similar to that observed in the Somalian cavefish [195, 196]. Nevertheless, there are also examples of the virtual absence of rhythmic behaviours in some of the anophthalmic fish species such as the characiform *Stygichthys typhlops* which do not exhibit any rhythmic activity behaviour either in LD or DD [197]. Studies on catfishes from Brazil reported that a majority of individuals exhibit ultradian and infradian patterns in activity, while no significant circadian component was observed [198 and citations therein].

*Arthropods* The cave-dwelling arthropod amphipod *Niphargus puteanus* exhibits a reduction of both peripheral and central visual systems but appears to be nocturnal under LD conditions [199, 200]. While this may also be due to photic avoidance behaviour and not necessarily endogenous, an exhibition of weak free-running rhythm following LD entrainment suggests the presence of degenerating circadian clocks. Studies that tested for persistence of circadian rhythmicity include those on the ground beetle species *Laemostenus navaricus* and *Typhlochoromus stolzi* which

have reported that majority of individuals of *L. navaricus* exhibit arrhythmic activity behaviour in DD [201] similar to those in the *T. stolzi* species [201, 202]. A relatively recent study by Koilraj et al. [203] which investigated locomotor activity of millipede *Glyphiulus cavernicolus sulu* under LD, DD and LL conditions found that majority of the individuals were rhythmic in LD but close to half of them exhibited circadian rhythmicity in DD and a negligible proportion were rhythmic even in LL. The most convincing evidence thus far supporting the idea of regression of circadian timing systems in organisms adapted to constant conditions comes from the eyeless species of ground beetles and small carrion beetle [204, 205]. None of the individuals in these species were observed to exhibit rhythmic activity patterns either in LL, LD or DD. More importantly, these data seem to be consistent across multiple independent studies on Nearctic and Palearctic species indicating that loss of behavioural rhythmicity due to convergent evolution is plausible and further substantiates the hypothesis discussed earlier.

*Chordata Proteus anguinus anguinus* a subspecies of Cave salamander *Proteus anguinus* has degenerated visual system unlike its epigean counterpart *Proteus anguinus parkelj* [206]. Studies aimed at assessing circadian rhythms in *P. anguinus anguinus* have reported mixed results. While one study demonstrated the presence of circadian and ultradian rhythms in activity [207], another study recorded activity for over 350 days and could only gather tentative evidence supporting the presence of circadian rhythms with mild incidences of ultradian and infradian rhythms [208]. In addition, studies in DD did not observe any significant behavioural rhythm [209]. However, it is believed that the reported discrepancies might be due to low activity levels in *P. anguinus anguinus* because of their inherently lower metabolic rates [discussed in 179] which generally complicates time series analysis to identify rhythmic trends in data.

Even though we discussed selected examples of troglobionts and other organisms known to have evolved in constant environments for several generations, Friedrich [179] extensively compared studies across 40 vertebrates and arthropod species revealing a compelling trend of the regression of visual system being correlated with clock-regulated rhythmic behaviours (Fig. 5.5). It can be observed that among the nine microphthalmic species studied, not surprisingly, a large portion (88 %) exhibit rhythmic behaviours which include circadian, ultradian and infradian periodicities, whereas one species studied thus far was arrhythmic (Fig. 5.5). Nevertheless, with over 60 % of the species studied exhibiting arrhythmic behaviours, anophthalmic species which represent more extreme phenotypes in terms of adaptation to constant conditions provide strong support (Fig. 5.5). The higher incidence of rhythmic behaviours in microphthalmic species might not be surprising as light input through the incompletely degenerated visual systems might drive rhythmic behaviours. It is also possible that the visual systems might not be used for navigation, food and mate procurement and other behaviours; however, its regression might have been constrained by the necessity of photic input pathways for circadian clocks' functioning. Either ways, these evidence further highlights the adaptive value conferred by circadian clocks.

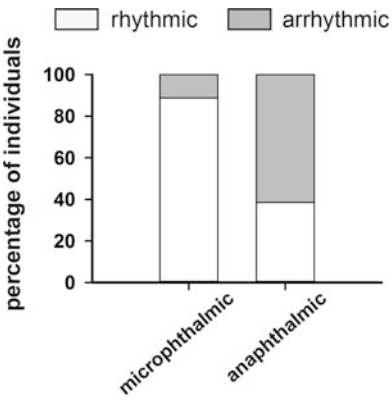



**Fig. 5.5** The graph depicts percentage of microphthalmic and anophthalmic species (among the ones tested) observed to exhibit rhythmic (including circadian, ultradian and infradian) and arrhythmic behaviours. Anaphthalmic species which are characterized by extreme levels of visual system degradation were also observed to exhibit higher proportion of behavioral arrhythmicity (Figure modified after Ref. [179])

In addition to organisms inhabiting in constant conditions, there have been a few efforts to test whether evolving in pseudo-aperiodic conditions leads to the evolution of the degenerate circadian system. Naked mole rats (*Spalax ehrenbergi*) have degenerated eyes which have been accredited to their subterranean habitat where exposure to light occurs infrequently. However, studies thus far have not found convincing evidence for lack or regressive circadian clocks in naked mole rats as several studies have reported circadian oscillation in clock genes *per1* and *per2* [210] and also *cry* [211] as well as rhythmic locomotor activity behaviour [212]. Reindeer *Rangifer tarandus platyrhynchus* and *Rangifer tarandus tarandus* inhabit regions around 78°N and 70°N respectively. These regions are characterised by low amplitude day/night cycles of extremely long photoperiods almost mimicking LL where exhibition of circadian rhythmicity does not appear to have any obvious advantage. Several studies have tried to assess circadian timing systems in these species, and their activity/rest behaviour was not found to have a circadian component, but several ultradian components were identified [213, 214]. Moreover, in accordance with their local habitats, species inhabiting the further northern latitudes exhibited weaker rhythms than those inhabiting relatively southern latitudes. Furthermore, in accordance with the locomotor activity behaviour, melatonin secretion in these species was not observed to be under circadian control but appeared to be masked by photoperiod [215]. Similarly, the bird Svalbard ptarmigan (*Laops mutus hyperboreus*) that inhabits 76–78°N was observed to not exhibit any rhythm in melatonin in summers, while their melatonin levels were below detectable range in winter [216]. These results were explained based on Gwinner et al. [217] who had proposed that reduced amplitude of melatonin increases the sensitivity of the circadian timing system to light and

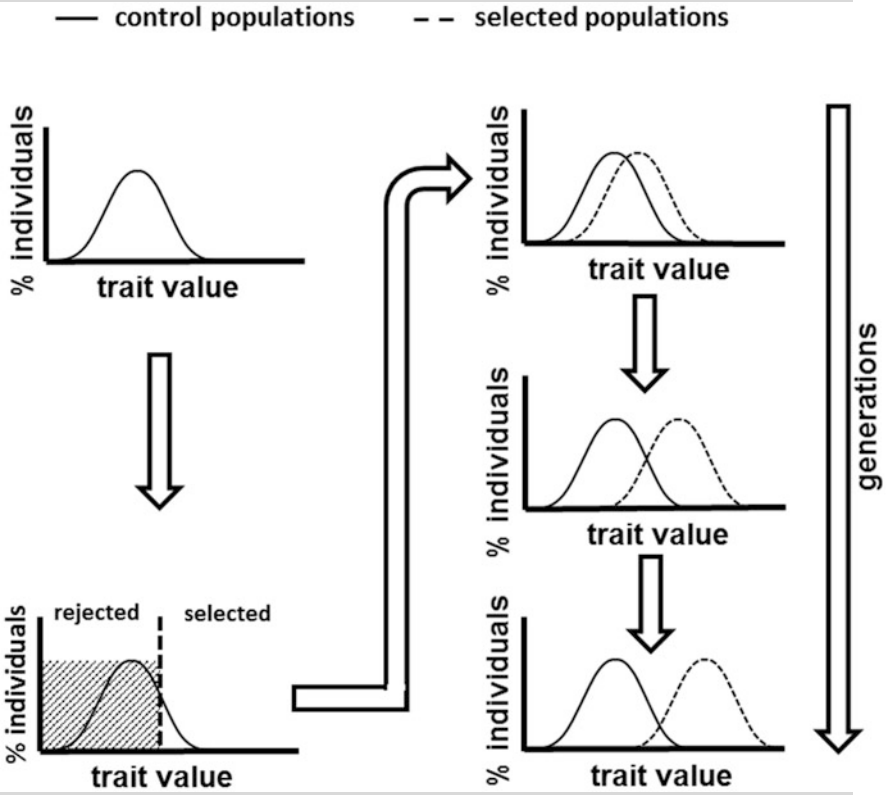
thus its ability to adjust to photoperiodic changes. Similar explanations were also provided by van Oort et al. [213, 214] to explain the low amplitude rhythms observed in reindeer species at higher latitudes. Thus, these results further extend support to the idea that circadian clocks confer adaptive advantages under rhythmic environments in the absence of which the clock organisation tends to exhibit signatures of regressiveness.

In addition to studies on natural populations, a couple of studies on populations living under constant laboratory conditions have also provided interesting insights into the evolution of circadian clocks under constant conditions. In a study, the authors' laboratory studied *D. melanogaster* populations that were maintained under LL for over 600 generations in constant temperature and humidity. Interestingly, even after 600 generations, these populations were observed to exhibit circadian rhythms in emergence, activity/rest and oviposition behaviours [218–220]. Similarly, another recent study on *D. melanogaster Oregon-R* strains reared under DD for over 1340 generations also reported persistence of rhythmic activity/rest behaviour [221]. From calculations based on the mutation and fixation rates in *Drosophila*, and considering Kimura's neutral theory of evolution, the authors roughly estimated that based on the effective population size ($N_e$) in their populations (see glossary), it would require close to 3000 generations for the fixation of an arrhythmic mutant allele in their populations [221]. In light of these results, it is not surprising that the LL flies discussed previously continued to exhibit rhythmic circadian behaviours. However, the two studies differ in several critical aspects. The population size in the Imafuku and Haramura [221] study largely fluctuated between 50 and 200 individuals, while that in Sheeba et al. [218–220] was ~1200 flies. Additionally, no details of population replicates are provided in the former study, while Sheeba et al. [218–220] used four replicate populations. Also, Imafuku and Haramura [221] used *Oregon-R* strain which to begin with is an inbred line, and therefore, a combination of small and fluctuating population sizes and isogenic background of the founding populations would increase chances of random fixation of alleles which might underlie the observed phenotypes. Alternatively, in addition to the rationale presented by Imafuku and Haramura based on the neutral theory of evolution suggesting that the evolutionary time scales are considerably smaller for observing any loss of circadian rhythmicity, lack of regression of circadian rhythms in both the above-mentioned studies as well as those from natural populations can also be used as a support for the intrinsic advantage hypothesis. While circadian clocks in organisms under constant environments might not require synchronisation of behavioural rhythms with the environment, the same cannot be said for maintenance of internal temporal order within the organisms. Therefore, it is not surprising that circadian clocks may continue to persist even under arrhythmic environmental conditions by virtue of the intrinsic advantages conferred by them.

### 5.5.4 Evidence from Laboratory Selection Experiments

Laboratory selection is ideal to test if evolved traits are indeed adaptations to the imposed selection regime (Box 5.3) but is among the relatively less adopted strategies to study the evolution of circadian clocks.

**Box 5.3: Laboratory Selection**




*Pictorial representation of a general laboratory selection protocol*: In a typical laboratory selection protocol, the experimenter sets a cut-off trait value such that all individuals beyond the cut-off trait value are artificially selected from a control population. The individual thus selected (selected population) form the breeding pool of the successive generation, and the same selection protocol is employed for succeeding generations. As this is continued over several generations, the mean of the trait value distribution from the control populations will shift towards the cut-off value imposed by the

(continued)

**Box 5.3** (continued)
experimenter. An additional set of control populations (from which selected populations are derived) are also maintained in parallel without imposition of any selection, and function as the ancestors for the selected population. Since trait value distributions can also change due to population bottlenecks, inbreeding and random genetic drift, all laboratory selection experiments are performed on large outbreeding replicate populations to account for such possibilities. Thus, laboratory selection method provides a useful platform to test the adaptive value of traits especially in the realms for 'historical definition' of adaptation.

Among the earliest studies of this kind was by Clayton and Paietta [222] who imposed artificial selection for morning and evening emergence in *Oregon-R* strain and a wild-caught strain (W2) of *D. melanogaster*. After over 16 generations of selection, the populations responded by evolving higher emergence in the morning and evening hours, respectively. However, the study did not test for the co-evolution of circadian clock properties, and, therefore, it is difficult to assert the evolved differences in phase of emergence to the underlying circadian clock. Pittendrigh [223] and Pittendrigh and Minis [224] selected for 'early' and 'late' emergence under LD12:12 in populations of *D. pseudoobscura* and *Pectinophora gossypiella*. Subsequently, Pittendrigh and Takamura [225] also imposed a similar selection in *Drosophila auraria* under a short photoperiod of LD01:23. In all cases, the populations responded to selection pressure with the early- and late-emerging populations exhibiting differences in phase of emergence. The *D. pseudoobscura* populations diverged by 4 h, *P. gossypiella* by 5 h and *D. auraria* by 6 h. Additionally, correlated changes in $\tau$ were observed in all the three cases, albeit in different directions. The early flies of *D. pseudoobscura* and *P. gossypiella* evolved longer $\tau$, while the late flies evolved shorter $\tau$, whereas the early and late flies of *D. auraria* evolved shorter and longer $\tau$, respectively. Although Pittendrigh explained this paradox in the realms of master-slave oscillator model and to differential coupling between pacemaker and zeitgeber, we believe that such a discrepancy could have arisen from other factors which pose to be considerable limitations of the experimental design. For instance, the selection protocols used in these studies did not appear to have (at least not mentioned in their respective publications): (a) population replicates within a selection regime, (b) any details of population ancestry and (c) details of population size and employed selection protocol. Even moderately small differences in the above-mentioned factors have been acknowledged to considerably influence the response to selection since the evolutionary trajectories of populations greatly differ depending on microenvironmental conditions and the genetic variation harboured by the baseline populations [reviewed in 226]. Therefore, even though these studies demonstrate the co-evolution of circadian clocks in response to selection, whether such changes were indeed responses to selection cannot be clarified. Furthermore, since these

studies did not assess the effect of selection on fitness measures, whether the timing of emergence does confer adaptive advantages remains unaddressed.

With such shortcomings in consideration, a long-term laboratory selection study was initiated in the authors' laboratory to examine the evolutionary trajectories of circadian clocks in response to the selection for different phase of emergence (emergence chronotypes) and subsequently circadian clocks' role in mediating chronotype differences. In contrast to studies on inbred, so-called wild-type strains, Kumar et al. [227] utilised four independent replicate outbreeding populations of *D. melanogaster* and reported that after 55 generations of selection, the early populations exhibit an increased emergence of ∼60 % during the morning as compared to ∼45 % emergence in the control populations, whereas the late populations exhibit an enhanced emergence of ∼25 % in the evening as opposed to ∼15 % observed in the control populations. Consequently, the two populations evolved divergent emergence waveforms with the mean phase of emergence differing by 4.5 h [227]. Furthermore, contrary to previous studies by Pittendrigh [223] and Pittendrigh and Minis [224], the early and late populations in Kumar et al. [227] evolved shorter and longer $\tau$ differing by ∼50 min, divergent photic phase response curves (PRCs; see glossary) for emergence rhythm [227] and activity/rest rhythms [228], and the two populations differentially utilised light in the morning and evening hours to exhibit their respective emergence chronotypes [229]. This is in accordance with the earlier studies that have reported correlations between $\tau$ and chronotypes in humans [230, 231]. Also, the morning and evening chronotypes in humans are observed to be correlated with the nature and duration of light exposure [231–234] which can partly explain the observations by Vaze et al. [229]. The contribution of circadian clocks to early and late emergence is further substantiated by Nikhil et al. [235] who reported that the molecular clocks of these populations also exhibited phase relationships in accordance with their behavioural phenotypes. The evolution of similar circadian phenotypes in independently evolving populations strongly suggests that the early and late emergence chronotypes can be attributed to differences in the underlying circadian clocks. The late populations also evolved high amplitude circadian clocks as compared to the early populations [236], which is in accordance with other latitudinal cline studies [147]. These results collectively suggest that laboratory selection can help reproduce several aspects of natural populations, and with the ability to precisely control the environmental conditions, the selection pressures driving the evolution of various clock properties can be accurately studied thereby highlighting experimental evolution as a strong experimental platform for 'evolutionary chronobiology'. Furthermore, in a recently concluded set of studies, various life-history measures including egg-to-puparium and egg-to-adult duration, egg-to-puparium and egg-to-adult survivorship, dry-weight at pupation and emergence, fecundity and lifespan were assayed in the early and late populations. The *late* populations were observed to exhibit significantly longer egg-to-puparium and egg-to-adult durations, higher fecundity and reduced female lifespan as compared to the *early* populations [237]. Thus, the evolution of various life-history traits in response to selection further underscores

the possible genetic correlations between the mechanisms governing the time of emergence (probably circadian clocks) and various life-history traits.

Precision (see glossary) of circadian clocks is considered to be critical for efficient timekeeping in organisms [238]. To study the evolution of precise circadian clocks and associated clock properties, another laboratory selection study was initiated in the authors' laboratory that involved imposing selection on *D. melanogaster* populations for a higher precision of emergence within a very narrow window of 1 h every day. It was observed that the selected CP (chrono-precise) populations showed a gradual increase in emergence during the 1-h selection window as compared to the control populations [239]. The CP populations also evolved higher accuracy for both emergence and activity/rest rhythms which persisted under multiple environmental conditions suggesting that the evolved mechanisms driving higher accuracy (see glossary) constitute a common unit (circadian clock) governing both the rhythms [239, 240]. This was substantiated by the correlated evolution of $\tau$, with the CP populations evolving a shorter $\tau$ as compared to the controls [240]. Furthermore, it was observed that CP populations exhibit reduced variance in development times, and the females of these populations exhibited higher midlife fecundity and reduced lifespan as compared to the controls [241]. Collectively, these results strongly suggest that natural selection acting on timing of rhythmic behaviours can be associated with correlated changes in life-history traits that fitness is partitioned into, and thus highlights the possible role of circadian clocks in mediating fitness of organisms in specific environments.

Other selection studies have also implicated the circadian clock's role in one of the life-history measures – the development time. When two lines of melon fly *B. cucurbitae* were subjected to selection for faster and slower pre-adult development under LD14:10, the lines responded by evolving faster and slower development times, respectively. The faster developing lines after 21 generations of selection exhibited about 3 days reduction in development time as compared to the controls, while the slower developing lines took about 5 days longer after 16 generations of selection [242]. Interestingly, the selection response was also associated with correlated changes in the $\tau$ of activity/rest rhythm with the mean $\tau$ of faster developing lines having reduced by ~2 h as compared to the controls (24.7 h), while that for the slower developing lines was lengthened by ~3.5 h [242]. Similarly, in two recent studies, the genetic correlation between development time and $\tau$ was reported in *D. melanogaster* populations [243, 244]. Such correlation between pre-adult development time and $\tau$ of activity/rest rhythm was also reported in the *per* mutants of *D. melanogaster* with homozygous individuals of the short-$\tau$ allele of *per* ($per^s$) exhibiting shorter development time as compared to the wild-type flies, and individuals homozygous for the long-$\tau$ allele $per^l$ had longer development time [245]. However, whether the genetic basis for the observed difference in $\tau$ in the selected lines from Shimizu et al. [242] can be attributed to *per* remains unanswered since the authors did not undertake further genetic analyses. The results by Shimizu et al. [242] may seem quite intuitive since biological timing mechanisms which time several processes can alter the rate of such processes just by a

change in the $\tau$. In other words, slower-running clocks could drive developmental processes at slower rates and, consequently, delayed development time, and the same would be true for faster-running clocks as well. Another study on melon flies reported that circadian clocks may, in fact, be associated with the timing of several other life-history events as well. Miyatake [246] observed that *B. cucurbitae* when selected for different ages of reproduction co-evolve divergent mating phases and $\tau$. The set of strains selected for early age (10–15 days) of reproduction (Y-lines) for 65 generations and later age (55–60 days) of reproduction (O-lines) for 24 generations showed a correlated response to selection such that the Y-lines were observed to mate earlier in the day as compared to the O-lines that mated later. Also, $\tau$ of Y-lines was significantly shorter (males, 22.8 h; females, 23.05 h), while that for the O-lines was longer (males, 26.5 h; females, 27.6 h). Even though the observed co-evolution of $\tau$ strongly suggests their role in timing life-history events, there is one critical aspect of the study that needs to be considered before arriving at any conclusion. Due to the nature of selection protocol employed, the O-lines selected for reproduction at 55–60 days old would have incurred greater mortality, therefore reducing the effective population size ($N_e$) in these populations as compared to the 10–15 days at reproduction in the Y-lines. Over several generations, this might have led to a higher degree of inbreeding (see glossary) depression in the O-lines, and, therefore, the change in $\tau$ cannot be entirely attributed to selection but can also be an artefact of inbreeding. This is further substantiated by the large variation in phenotypes observed among the three replicate populations used in the study [246].

The broader picture that arises from all of the above-discussed studies can be summarised as follows. Selection on the specific timing of an overt behaviour leads to the co-evolution of various clock properties such as $\tau$, precision, zeitgeber sensitivity and amplitude of underlying circadian clocks thus emphasising the role of such properties in timing rhythmic behaviours. More importantly, such evolutionary responses are also associated with changes in multiple life-history traits spanning the pre-adult and adult stages which demonstrate the underlying genetic correlations between circadian timing mechanism and life-history traits. Therefore, it is reasonable to infer that circadian clocks by virtue of genetic correlations with life-history traits confer adaptive advantages to the organisms by appropriately timing rhythmic behaviours so as to enhance the organism's fitness in a given environment.

## 5.6 A Critique on the Currently Used Methodologies and the Way Forward

Earlier, we briefly discussed the concept of adaptation and criteria that are considered essential to test the adaptive nature of a trait. If one were to critically compare these criteria in the context of studies discussed thus far, several obvious experimental design and methodological shortcomings become evident. While we

appreciate the enormous efforts invested by a large number of researchers, we also take the privilege of critically assessing the methodologies and shortcomings (even though some of them may be unavoidable) of some of the above-discussed studies and suggest pointers for improving the previously adopted experimental designs. In this regard, we may at certain places choose examples from actual studies, but such examples will be chosen purely to elaborate on our arguments and do not reflect our intentions to specifically target the study or personnel involved.

(a) *Assessment of fitness*: Majority of the studies that assess fitness under various experimental setups implicitly assume that the measures used truly reflect the overall fitness of organisms or sometimes are even synonymous with the term fitness. Several studies reported that clock manipulation either by genetic or environmental means severely reduces lifespan thus concluding that circadian clocks are adaptive in nature. However, life-history theory highlights that the overall fitness of an individual can be partitioned across multiple life stages and among different fitness measures. For instance, all else being equal, if an individual-*K* lives longer but eventually produces smaller number of viable offspring as compared to another individual-*L* that lives considerably shorter than *K* but has a higher offspring output, it is obvious that *L* would be fitter than *K*. However, if lifespan alone is used as a fitness measure, then one is bound to disastrously conclude *K* to have higher fitness than *L*. Even though estimating the overall fitness is practically impossible due to several constraints, we believe that it is essential for experimenters to assess a large number of fitness measures before arriving at conclusions regarding the fitness benefits of harbouring circadian clocks.

(b) *Choosing appropriate fitness measures*: In addition to assessing multiple fitness measures, it is equally critical to ensure that the fitness measures chosen are ecologically relevant to the organism. For instance, *D. melanogaster* populations used in Kumar et al. [227] and Kannan et al. [239, 240] were maintained on a 21-day discrete generation cycle which means that eggs for succeeding generation were collected on day-21 post egg collection from the previous generation. In this regard, if parents in a given population were to ensure the representation of their progeny to the next generation, survival till day 21 and fecundity on day 21 are the only critical fitness components as eggs laid post or pre day 21 will not contribute to the succeeding generation. Therefore, how long the parental populations survive post day 21 is irrelevant as their realised fitness beyond day 21 is essentially zero. In this regard, if one were to estimate fitness of these populations, only traits that would influence the populations' survival till day 21 such as pre-adult development time, egg-adult survivorship, starvation and desiccation resistance, and fecundity until day 21 are among the adequate fitness measures whereas other measures such as lifetime fecundity and longevity even though are relevant to address other unrelated questions might not be essential in this scenario. Therefore, it is important to bear in mind that the measures of fitness are relevant to the ecology of the organism under study.

(c) *Use of genetic manipulations*: Several studies reported fitness consequences of circadian clock disruption in conventional laboratory strains such as *Oregon-R* (as in *Drosophila*) or other mutants of core clock genes. It is well known that life-history traits have complex genetic architecture involving a large number of genes and multiple allelic combinations [247]. However, conventional laboratory wild-type and mutant strains generally are severely inbred and might have accumulated multiple random mutations within their genomes. Inbreeding is known to have several dire consequences on the genome such as increased homozygosity, reduction in effective population size and recombination rates, random fixation of alleles and also increased linkage disequilibrium (see glossary) in comparison with those of the outcrossing natural populations. Such factors might reduce the organism's fitness [248] independent of the trait in question, and, therefore, employing such strains even though is indispensable in certain experimental designs, their use should be avoided to the maximum possible extent especially for the assessment of fitness.

(d) *Studies on natural populations*: Most studies testing the evolutionary benefits of circadian clocks in natural populations either use latitudinal or altitudinal clines, or organisms that have evolved in constant conditions such as deep sea habitats. Inferential arguments drawn from such studies are often rendered weak primarily due to the lack of knowledge about the environmental history of the geographical area in which the organisms evolved. Considering the complex interplay of several biotic and abiotic factors in an ecosystem, a plethora of factors might have led to the evolution of observed traits in a given environment. For instance, floods and earthquakes or epidemics might have led to severe population bottlenecks in the past consequently changing the evolutionary trajectories of the traits being studied. In this regard, we propose that rather than relying only on the evidence from restricted sampling of members of a given clade, a comprehensive comparison across members of the clade or closely related clades occupying both similar and dissimilar geographical areas might provide a more reasonable estimate of how and why the trait in question might have arisen. This information along with the knowledge of environmental conditions around that time would assist identification of possible selection pressures that might have driven the evolution of the trait in question thus reducing the chances of misinterpreting results from such studies.

(e) *Studies relying on environmental manipulations*: We also discussed several studies that assessed the consequences of dysfunctional circadian clocks or circadian desynchrony induced by light regime manipulations. While such studies have provided interesting insights into the role of circadian clocks, not all results can be generalised, primarily because such studies fail to distinguish whether the observed phenotypes are actually clock mediated or are direct consequences of manipulated environmental regime. An example for this is the decreased growth rates observed in tomato plants under LL as compared to LD12:12. This, even though suggestive of the detrimental effects of clock

disruption (due to LL) on growth rate, *Arabidopsis*, on the other hand is observed to grow considerably faster in LL as compared to LD12:12 which questions the plausibility of the interpretations from tomato studies [discussed in 25]. Therefore, it is essential that studies aimed at assessing the effects of circadian clock manipulation on organisms' fitness should adopt multiple approaches to demonstrate the adaptive significance of circadian clocks.

(f) *From lab to nature and back*: It is notable that majority of the studies discussed so far have been performed either entirely under controlled laboratory conditions or on natural populations. Stochasticity in multiple unavoidable factors in nature makes it difficult to attribute the observed phenotype to a given environmental variable. Contrary to this, laboratory studies are performed under relatively simplistic environmental conditions where organisms are generally maintained at optimal population size, constant light or temperature regimes with ad libitum food and water. Thus, it is not possible to directly extrapolate the phenotypes observed in laboratory set up to that in nature. Here, we take the liberty to discuss some of our previous work highlighting the importance of such studies that go from laboratory to nature and back. In one of the studies discussed in the previous section, early and late population that were derived by Kumar et al. [227] had evolved under LD cycles with constant temperature. Interestingly, the divergence in their eclosion profiles was considerably enhanced under seminatural conditions [249], and it was later dissected out that the primary environmental variable driving such a change was, in fact, temperature [250]. Therefore, even though the phenotypes observed in laboratory conditions may sometimes be quiescent and can be attributed to few of the environmental factors, they may not necessarily reflect the dynamics in nature. Also, one possible reason for observing clear differences in competitive ability between strains in Ouyang et al. [137] may be due to the seemingly simplistic environmental conditions used in the experiment. These results, even though are suggestive of the adaptive nature of circadian clocks, whether the same is observed in a more naturalistic environment remains to be addressed. Therefore, studies aiming to assess the adaptive nature of circadian clocks in controlled laboratory setups should try and reproduce the same under more realistic natural setups which would provide a stronger support to the hypothesis being tested. Such studies may additionally allow appropriate dissection of the contribution of the environment to various life-history traits.

(g) *Adopting genomic technologies:* The bulk of studies discussed in this chapter have relied primarily on behaviour to explore the adaptive benefits of circadian clocks. Another potential tool that can further assist this is the genome sequencing technology. With the considerable reduction in sequencing costs, sequencing whole genomes of wild-caught populations which was once a far-fetched dream is now a possibility. Whole genome sequencing helps identify several genomic signatures that can reveal whether certain regions have experienced natural selection, as highlighted by a recent study that identified loss of multiple circadian genes in fishes evolving in constant

darkness for several millions of years [251]. Thus, combining behavioural studies with genome sequencing approach can serve as a potential tool for studying the evolution of circadian clocks.

While we have tried to review majority of the studies that have made phenomenal contributions to our understanding of these marvellous circadian timekeeping mechanisms, we sincerely apologise for failure in citing any studies and acknowledge that such oversights were purely unintentional. This being said, a comprehensive analysis of the current state of knowledge pertaining to the evolutionary origins and implications of circadian clocks leaves an impression that even though we have substantial evidence that strongly underscores the relevance of circadian clocks across all life forms, we still have a long road to take before arriving at undisputable conclusions regarding the origin of circadian clocks and their adaptive significance. Nevertheless, advances in technologies combined with continued collaborative efforts from researchers across disciplines to address such questions in future are bound to be an exciting and intellectually enlightening journey for the field of chronobiology.

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## Glossary

**Adaptation** The process by which organisms evolve traits that confer higher fitness in the organisms' habitat. Alternatively, the trait that confers higher fitness to organisms in a given environment is termed an adaptation.

**Circadian clocks** Biological timekeeping mechanisms that drive circadian rhythms.

**Circadian rhythms (Latin *circa* = about/approximately; *diēs* = day)** Biological rhythms in behaviour and physiology expressed with a period of ~24 h under constant conditions (absence of external time cues/zeitgebers).

**Clines** Gradual phenotypic variation across a geographical area as a consequence of variation in geophysical features such as latitudes (latitudinal clines) or altitudes (altitudinal clines).

**Directional selection** Selection for a phenotype that constitutes the extremes of the phenotype distribution in the population.

**Effective population size** The size of an ideal population that would undergo an equal amount of genetic drift as that of a nonideal population of size $N$ is defined as the effective population size ($N_e$).

**Entrainment** Entrainment refers to the process of synchronisation of circadian rhythms to external time cues (zeitgeber) such that (a) the period of the entrained rhythm match that of the zeitgeber, (b) the rhythms attain a stable and reproducible phase relationship with the zeitgeber (also known as phase of entrainment) and (c) upon removal of the zeitgeber, the free-running rhythm initiates from the phase of entrainment established with the prior zeitgeber.

**Fitness** Fitness is the measure of an organism's or a population's ability to survive and reproduce in a given environment.

**Free-running period ($\tau$)** The period of the circadian rhythms exhibited under constant conditions.

**Inbreeding** Mating among individuals with high genetic relatedness leading to increased homozygosity, isogeny and random fixation of deleterious alleles over generations.

**Linkage disequilibrium** Also known as gametic phase disequilibrium, this is the occurrence in members of a population a particular combination of linked alleles in non-random proportions.

**Phase response curve (PRC)** A PRC maps the magnitude of response (measured as phase shifts) to zeitgebers at different phases of the circadian cycle and, therefore, is a measure of the circadian clocks' zeitgeber sensitivity.

**T-cycle** Zeitgeber cycles of periodicity *T*. For instance, *T*24 indicates a 24-h zeitgeber cycle with the durations of light/dark or thermophase/cryophase summing up to 24-h, *T*30 a 30-h zeitgeber cycle and so on.

**Temperature compensation** Temperature compensation refers to the ability of circadian clocks to maintain a stable and constant $\tau$ across different temperatures by compensating for temperature-induced changes in the rate of biochemical reactions.

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# Part II
# Animal Clocks: Complexity and Diversity

# Chapter 6
# The *Drosophila* Clock System

**Charlotte Helfrich-Förster**

**Abstract** The present chapter describes the circadian clock system in fruit flies starting with the molecular mechanisms that generate circadian rhythms and then moving to the organismic level, whereby most importance is given to the clock network in the brain. It is shown that the molecular clock controls neuronal excitability and synaptic plasticity of the clock neurons in the brain, that these clock neurons communicate with each other via neuropeptides, and that they have different roles in controlling activity rhythms. The mechanisms by which the clock in the brain is entrained to light and temperature cycles are explained as well as the known output pathways by which it controls behavior. The chapter ends by discussing the significance of the clock as well as its immediate and evolutionary adaptations to different environmental conditions. Throughout the chapter, comparisons are made to the circadian system of other species.

## 6.1 The Molecular Mechanisms of *Drosophila's* Clock

Until the 1970s of the last century, the molecular mechanisms of circadian clocks remained enigmatic. By selecting two bean plant strains that possessed fast and slow clocks, respectively, and genetically crossing them, Erwin Bünning (1932) was the first to show that the circadian clock has a genetic basis [1]. However, the genes that control circadian rhythms remained unknown until the late Ronald Konopka (1947–2015) and Seymour Benzer (1921–2007) performed their legendary screen for clock mutants in *Drosophila melanogaster* [2]. The clock causes adult flies to emerge from their pupal case (eclose) around dawn. This eclosion rhythm persists in constant darkness, with ~24-h periodicity. The screen therefore searched for mutant flies that eclosed in aberrant fashion and was remarkably successful. They found a short-period mutant (about 18 h), a long-period mutant

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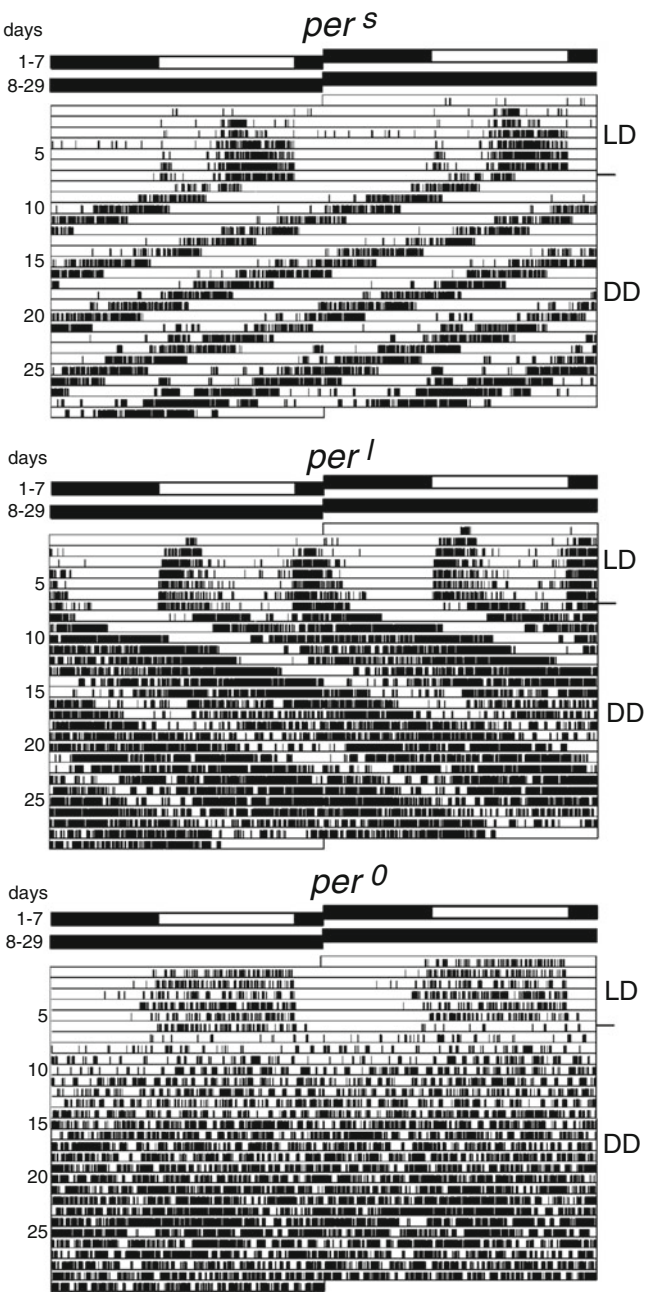


**Fig. 6.1** The *period* mutants of *Drosophila melanogaster*. Typical actograms are shown for $period^{Short}$ ($per^{s}$), $period^{Long}$ ($per^{l}$), and $period^{zero}$ ($per^{0}$) mutants. Locomotor activity of the flies was first recorded for 7 days in 12-h light-dark (LD) cycles and subsequently for 3 weeks in constant darkness (DD). The bars on top of the actograms indicate the light program. Under LD conditions, the $per^{s}$ and $per^{l}$ mutants show a bimodal activity pattern, whereas that of the $per^{0}$ mutants is uniformly distributed during daylight hours. Under DD conditions, the $per^{s}$ mutant free-

(about 28 h), and an arrhythmic mutant. Konopka and Benzer assumed that the mutants were central to circadian rhythms for a number of reasons. Firstly, the three mutants affected not only the eclosion rhythm but also an independent circadian rhythm, locomotor activity, in exactly the same way (Fig. 6.1). Secondly, genetic analysis indicated that all three mutations were alleles of a single gene, which they named *period* (*per*). Thirdly, the results indicated that this single gene was of key importance for circadian timing, as it was necessary for rhythmicity and determined the speed of the clock. The molecular characterization of the *per* gene and of its protein took another 15 years. These essential studies were predominantly carried out by three laboratories: the ones of Jeffrey Hall and of Michael Rosbash at Brandeis University and of Michael Young at Rockefeller University. This early work is covered by several reviews of which the ones of Jeffrey Hall are the most elaborate (e.g., [3]). Furthermore, I warmly encourage anyone interested in the beginnings of behavioral genetics to read the book of the popular science writer [4].

Most importantly, the Period protein (PER) and its function appears conserved throughout the animal kingdom. Thus, one cannot overstate the importance of the study of Konopka and Benzer [2] for the entire circadian field and its subsequent development.

### *6.1.1 Transcriptional Feedback Loops*

As accurately phrased in the review of Hardin [5], the PER protein sequence didn't offer many clues about its role in the clock because its only distinguishing features were a stretch of threonine-glycine repeats, later shown to be involved in adapting to different thermal environments and a region that mediates protein-protein interactions, called PAS domain. However, the discovery that *per* mRNA and protein cycle in a circadian manner [6, 7] and that the PER protein is required for cycling of *per* mRNA [6] suggested that *per* contributes to circadian timekeeping via a feedback loop in which the rhythms of *per* mRNA expression are controlled by the PER protein [6]. Studies demonstrating transcriptional control of *per* mRNA cycling and PER-dependent inhibition of *per* mRNA expression further refined the role of PER in this feedback loop as a transcriptional repressor [8, 9]. Subsequent studies not only supported the view that this transcriptional feedback loop keeps circadian time in *Drosophila* but also showed that similar feedback loops keep circadian time in diverse species including *Cyanobacteria*, plants, fungi, and animals (recent reviews: [10, 11]).

**Fig. 6.1** (continued) runs with a period of ~19 h and the $per^{l}$ mutant shows a period of ~29 h. The $per^{0}$ mutant shows an arrhythmic activity pattern. To better visualize the short and long free-running rhythms of $per^{s}$ and $per^{l}$ mutants the actograms are shown as double plots, meaning that the activity of two consecutive days is plotted in one row, additionally to plotting the activity of each consecutive day (day 1–29) vertically one below the other. For comparison with the activity pattern of a wild-type fly, see Fig. 6.6 (After Ref. [176])

Certainly, *per* is not the only gene involved in this feedback loop. In the following years, more core clock genes were discovered. The first one was *timeless* (*tim*) in 1994 [12], succeeded by *Clock* (*Clk*) [13] and *cycle* (*cyc*) [14] in 1998 and *vrille* (*vri*) [15] in 1999. The PAR domain protein 1ε encoding gene *pdp1ε* followed in 2001 [16] and the basic helix-loop-helix orange-domain putative transcription factor *clockworkorange* (cwo) in 2007 [17–19]. Last but not least, the nuclear receptor genes *unfulfilled* (*unf*) and *ecdysone-induced protein 75* (E75) are required for a normally functioning clock [20–22]. These clock genes and their encoded proteins interact in three transcriptional feedback loops among which the *per*/*tim* loop is the most important one (Fig. 6.2).

Furthermore, kinases such as the Casein kinase 1δ/ε alias double time (DBT), the Casein kinase 2 (CK2), GSK-3ß alias shaggy (SGG), ribosomal s6 kinase (S6K2=RSK), phosphatases (as the protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1)), the O-GlcNAc transferase (OGT), and the E3-ubiquitin ligases SLIMB and CTRIP turned out to be important for clock function (reviewed by [5, 11, 23–25]). These posttranscriptional modifications are especially important for setting the speed and amplitude of the clock, since they determine the stability of the clock proteins and/or their ability to form complexes as well as their ability to move into the nucleus [11, 26]. To some degree, the posttranscriptional modifications can drive molecular rhythms even in the absence of rhythmic gene transcription showing that these are very important [24].

Besides rhythmical modification of the clock proteins, almost all events from splicing, polyadenylation, and mRNA stability to translation are rhythmically controlled (reviewed by [11, 27]). Even the noncoding functions of microRNAs are involved in clock gene regulation. This complex control most probably enhances robustness of the clock as well as the ability of the flies to adapt to different environmental conditions. In the following section, the most important posttranscriptional modifications will be explained in more detail.

### *6.1.2 Posttranscriptional Modifications Determining Clock Phase and Speed*

Once again, the importance of posttranscriptional modifications of the clock proteins can be best illustrated taking PER as an example. The first described kinase phosphorylating PER is DBT [28, 29]. DBT associates with PER soon after its translation in the second half of the night and subsequently phosphorylates it at many sites. This phosphorylation finally leads to binding of the E3-ubiquitin ligase SLIMB and degradation of PER in the proteasome [30, 31]. For a long time, it was thought that this SLIM-mediated degradation occurs in the cytoplasm directly after PER translation and continuously hinders PER's accumulation until PER is eventually stabilized by forming dimers with TIM. Consequently, PER's nuclear entry

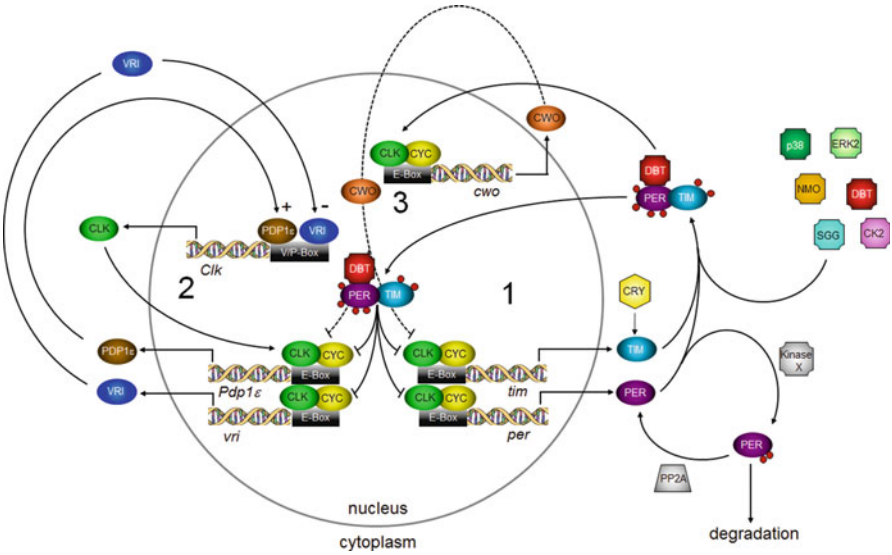


**Fig. 6.2** Three transcriptional feedback loops lead to a cycling in clock gene expression. In the main feedback loop (1), the transcription of *per* and *tim* is activated in the late morning by the CLK-CYC dimer binding to the E-boxes in the 5′ upstream region of the two genes. *per* and *tim* mRNAs move into the cytoplasm (not shown), and translation into PER and TIM proteins starts (probably after a time delay as mentioned in the text). PER gets then phosphorylated (phosphate groups are depicted as red dots) which results in its destabilization and degradation. PER's phosphorylation is counterbalanced by the phosphatase PP2A. Overall, PER's degradation slows down its accumulation in the cytoplasm. The accumulation of TIM is also slow during the day, because TIM is permanently degraded by light via interaction with CRY. However, with the coming night, TIM accumulates and dimerizes with PER. This dimerization stabilizes PER and both proteins accumulate in the cytoplasm. PER and TIM then undergo a cascade of phosphorylations. Both are phosphorylated by SGG, and PER additionally by proline-directed kinases (NMO, p38, ERK2, see Fig. 6.3), CK2, and DBT. DBT forms a complex with PER and TIM. The phosphorylations allow the PER/TIM/DBT complex to translocate into the nucleus in the middle of the night. In the nucleus, the complex binds to CLK-CYC and inhibits *per*/*tim* transcription plus the transcription of other genes with E-boxes. At dawn, CRY is again activated and leads to TIM's degradation (not shown). PER still remains and represses transcription. However, it is further phosphorylated by DBT, which leads to its degradation by SLIMB a few hours after lights on (see Fig. 6.3). Subsequently, CLK and CYC can reactivate *per* and *tim* transcription and the entire cycle starts again. In the second feedback loop (2), CLK-CYC bind E-boxes to activate *vri* and *Pdp1ε* transcription. VRI accumulates immediately, moves into the nucleus, and binds to VRI-/PDP1-boxes (V-/P-boxes) in the *Clk* promoter, thereby repressing *Clk* transcription. PDP1ε accumulates later, moves about 4 h after VRI into the nucleus, and activates *Clk* transcription. Overall this leads to a cycling of CLK, which reinforces the main feedback loop (1). In the third feedback loop (3), CLK-CYC bind to the E-box in the gene *cwo* that codes for the bHLH-orange transcription inhibitor CLOCKWORK ORANGE (CWO). After translation CWO moves into the nucleus and inhibits CLK-CYC-activated transcription through competitively binding E-boxes. This feedback independently reinforces transcriptional inhibition by the PER/TIM/DBT complex. The third feedback loop is depicted by broken lines because it is mainly based on in vitro studies

and the subsequent block of its own transcription are delayed. This delay is obligatory for the functioning of the main negative feedback loop, since an immediate block of *per* transcription would not lead to an oscillation in *per* mRNA, but instead stop the whole cycle. Indeed, there is a 6-h time lag between *per* mRNA and protein accumulation, and PER is virtually absent in $tim^0$ mutants that lack the stabilization of PER by TIM [32]. However, more recent data indicate that PER is probably not degraded via SLIMB, because the key phosphorylation by DBT at serine 47, which is essential for SLIMB binding, does not occur before dawn, when PER is entirely nuclear [33]. Thus, PER must be degraded by other still unknown mechanisms at this early stage in the cycle. Recent data suggest that there are even more putative delaying steps in the cycle. At least in certain clock neurons in the brain (the $LN_v$, see later), several factors are crucial to promote *per* translation: TWENTY-FOUR (TYF), ATAXIN 2 (ATX) RNA-binding protein, and the POLY-A-binding protein (PABP) [34–36]. Without these factors, PER translation is slow, and consequently PER levels are extremely low and flies show weak rhythms with long period.

Although PER phosphorylation in the middle of the night appears not leading to PER degradation, it is essential for the nuclear translocation of PER. Kinases promoting PER nuclear entry are CK2, SGG, and a still unknown proline-directed kinase, which has a priming role [33]. Proline-directed kinases phosphorylate serines (S) or threonines (T) followed by a proline; S661 is such a proline-directed kinase target (Fig. 6.3). Its phosphorylation primes the phosphorylation of S657 by SGG and perhaps also the phosphorylations of S149, S151, and S153 by CK2. Mutations at serine 661 provoke a delayed nuclear entry of PER indicating that S661 phosphorylation is a key phospho-signal regulating the timing of PER's nuclear entry [37]. Preliminary evidence from in vitro studies suggests that the mitogen-activated protein kinases ERK2 and p38 are genuine candidates for the unknown proline-directed kinase [37, 38]. Most significantly, mutating or downregulating *ck2*, *sgg*, or *p38* delays PER's nuclear entry and slows down the speed of the rhythm, indicating that the three kinases normally co-act to accelerate both. Furthermore, P38 points to an interesting link between stressful external stimuli and the circadian clock [38]. The acceleration of PER's nuclear entry is counterbalanced by the rhythmic glycosylation of PER at S and T residues with O-linked N-acetylglucosamine (O-GlcAC) which is maximal at around midnight. PER glycosylation enhances PER stability and delays nuclear entry [39]. Another limiting factor for PER nuclear entry is TIM. Dimerization of PER with TIM is a prerequisite for nuclear localization, although the two proteins appear to enter the nucleus separately [40–42]. The $per^l$ mutation is located in the PER/TIM interaction domain and leads to a slower dimerization and thus to a delayed nuclear entry of PER resulting in a long period [43].

Once in the nucleus, PER (complexed with DBT and TIM) binds to CLK, and this interaction leads to hyperphosphorylation of CLK and to the dissociation of the CLK-CYC dimers from the E-boxes upstream of *per*, *tim*, *vri*, and *pdp1ε* ([44, 45]). Consequently, transcription of these genes stops (Fig. 6.2). Around dawn, the

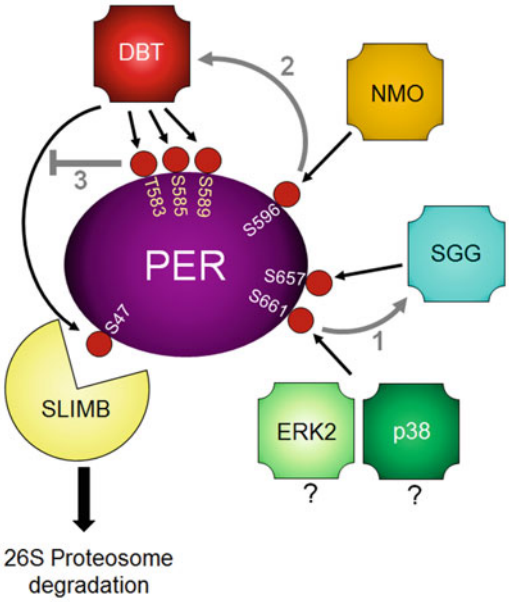


**Fig. 6.3** Few key phosphorylation sites on PER that affect PER function. Phosphorylation at serine 661 (S661) by a proline-directed kinase (perhaps ERK2 and p38, hence the question marks) primes (1) the phosphorylation of serine 657 (S657) by SGG, promoting nuclear translocation. Phosphorylation at serine 596 (S596) by NEMO promotes phosphorylation by DBT (2) of the $PER^S$ phosphocluster (T583, S585, and S589). In turn, this delays (3) phosphorylation of S47 also by DBT (3). The latter is the key phosphorylation event leading to SLIMB-binding and proteasome-mediated degradation of PER (Modified after Ref. [24])

degradation of the PER complex permits the activators CLK-CYC to bind the E-boxes and initiate the next cycle of transcription. The degradation time of the PER complexes is another major speed-determining event in the cycle. It begins with the degradation of TIM by light [46–48]. PER is more stable as it remains in the nucleus for up to 8 h longer than TIM does [40]. As mentioned in the beginning, degradation of PER in the proteasome depends on phosphorylation of S47 by DBT, since phosphorylated S47 is a binding site for SLIMB. However, this final PER phosphorylation is preceded by a hierarchical phosphorylation cascade, in which the phosphorylation by the priming proline-directed kinase, called NEMO/NLK, delays phosphorylation of S47 by DBT [33, 49]. Thus, the action of NEMO/NLK stabilizes PER and prolongs the period of the cycle. On the other hand, mutating or downregulating *nemo* shortens the period as does a mutation of PER in NEMO's phosphorylation site at residue S596 [49, 50]. Most interestingly, NEMO/NLK gates phosphorylation of two serines, S589 (the original $per^s$ mutant site) and S585, and one threonine, T583, by DBT [49, 51]. These phosphorylations also prolong PER stability and their mutations lead to short periods. A model proposed by Edery and colleagues postulates that phosphorylation of PER by NEMO/NLK and DBT within the "short-period domain" (S585-Y601) alters the conformation of

PER, thereby inhibiting the phosphorylation of sites required for SLIMB binding and delaying PER degradation [49]. There are also PER sites, such as T610 and S613, that decrease PER stability when phosphorylated by DBT [51]. These, consequently, enhance PER degradation and shorten the period. Furthermore, the phosphatases PP2A-1 and to a minor degree PP1 can counteract PER phosphorylation at several sites and, thus, contribute to the control of period [51].

As well as PER, the other clock proteins are modified posttranscriptionally too. Here, the reader is referred to the reviews of Hardin [5], Hardin and Panda [11], and Özkaya and Rosato [24] as well as to recent original papers [51–53]. In summary, one can say that phase, speed, and amplitude of the molecular oscillations are controlled on multiple levels. Not all are completely understood and many still need to be discovered.

## 6.2 The Clock Ticks in Many Cells and Tissues

The just above-described molecular clock ticks in many cells and tissues: in the so-called clock neurons and many glial cells in the brain, in the retinal photoreceptor cells, in the antennae and other sensory organs on the surface of the fly, in the gut, in the heart, in the fly kidney (= Malpighian tubules) and the fly liver (= fat body), as well as in the gonads and the cuticle [54–56]. The clocks distributed in many tissues outside of the brain are called peripheral oscillators.

However, how does this molecular clock drive overt rhythms that are different in every tissue? For example, clock neurons show rhythms in electrical activity (which is determined by the ion channels involved), the sensory organs may show rhythms in receptor density, the gut in concentration of digestive enzymes, the fat body in factors controlling metabolism, etc.

Clock regulation of different tissue-specific physiological and metabolic rhythms implies the CLK-CYC transcriptional activators do not only bind to clock genes but also to target genes coding for ion channels, receptor proteins, and diverse enzymes, and they do so differently in each tissue [57, 58]. The studies of Abbruzzi et al. [58] and Meireles-Filho et al. [59] provide a putative mechanism: CLK-CYC bind consensus CAGGTG E-box sequences to drive rhythmic transcription. There are several thousands of such sequences in the *Drosophila* genome, but chromatin immunoprecipitation analysis revealed only ~1500 CLK-CYC binding sites in *Drosophila* heads, indicating that additional sequences contribute to this binding [58]. Indeed, CLK-CYC collaborates with tissue-specific transcription factors that bind to cis-regulatory sequences nearby the CAGGTG E-box sequence [59]. CLK-CYC and the tissue-specific transcription factors synergistically activate different sets of target genes in different tissues (Fig. 6.4). According to these findings, a differential rhythmic control of genes can occur in the diverse tissues. The clock genes themselves contain dual E-box elements that drive higher levels of transactivation than single E-boxes suggesting that they are high-affinity target sites [60].

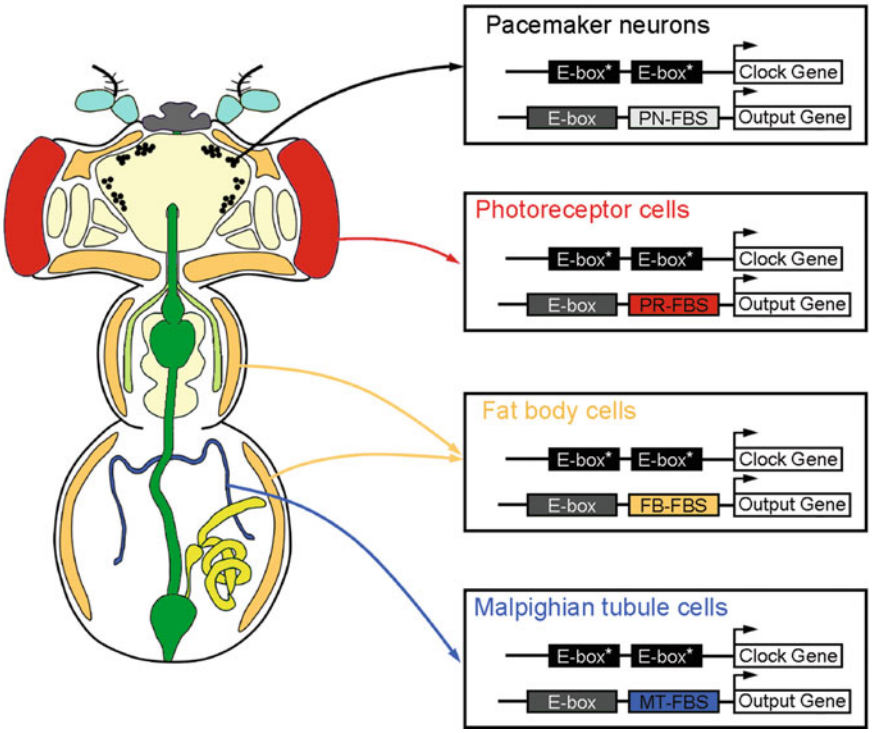


**Fig. 6.4** Model for widespread and tissue-specific expression of CLK-CYC target genes. Clock gene-expressing tissues in an adult *Drosophila* (*left*). *Cyan*, antennae; *cream*, nervous system; *tan*, fat body; *gray*, proboscis; *black*, pacemaker neurons; *red*, photoreceptors; *green*, digestive tract; *light green*, salivary glands; *blue*, Malpighian tubules; *yellow*, male reproductive tract. *Arrows* denote regulation of clock gene and output gene expression in brain pacemaker neurons, photoreceptor cells, fat body cells, and Malpighian tubules (*right*). A pair of closely spaced E-boxes that bind CLK-CYC with high affinity (*) is thought to promote transcription activation of clock genes in all tissues, whereas CLK-CYC bound to an E-box and a tissue-specific factor bound to a tissue-specific binding site cooperatively activate transcription of clock output genes in different tissues. Pacemaker neuron-specific factor binding site, PN-FBS; photoreceptor-specific factor binding site, PR-FBS; fat body-specific factor binding site, FB-FBS; Malpighian tubule-specific factor binding site, MT-FBS (Modified after Ref. [57])

Most interestingly, not every peptide or protein showing a rhythm in abundance is rhythmically transcribed. Microarray studies carried out in different labs yielded only a relatively small number of rhythmically transcribed genes [61–63]. In sum, only ~10 % of the *Drosophila* genes appeared rhythmically transcribed, indicating that rhythmic control occurs at later stages, e.g., during translation, processing, maturation, transport, or degradation. Thus, the rhythmic transcription of a set of key proteins may lead to posttranscriptionally controlled oscillations of many other proteins. Huang et al. [64] revealed the underlying mechanism by showing that the rhythmic transcription of a few factors involved in ribosomal association leads to

the rhythmic translation of hundreds of proteins. In the brain, redox factors, ion channels, or channel regulatory proteins (see also below) as well as enzymes synthesizing neurotransmitters are among the rhythmically translated proteins [64]. Most significantly, protein synthesis was maximal during the resting times of the flies in the night and in the midday (see Fig. 6.6 for a wild-type activity pattern).

In addition to the rhythmic transcription of few factors involved in translation, it is also possible that the mRNAs of other genes cycle in smaller subsets of the clock cells and are therefore not detectable by microarray analysis of entire heads or bodies. Cell-type-specific gene-expression profiling is a new promising approach to find rhythmically controlled genes in selected cells that Nagoshi and coworkers have adopted for the circadian clock network in the brain [65]. Indeed, they identified several interesting oscillating candidates. The clock network in the brain is especially interesting because it controls rhythmic behavior, and, in the following, I will devote the largest part of my chapter to it.

## 6.3 The *Drosophila* Circadian Network in the Brain

Already, the early studies of Konopka show that behavioral rhythms, such as rhythmic eclosion and locomotor activity, are dependent on *per* expression in the brain [66]. PER and the other clock proteins are present in thousands of glial cells and about 150 neurons that are called "clock neurons." In all these cells, PER is oscillating in abundance with a maximum before dawn and a minimum before dusk. Although there is increasing evidence that the PER-positive glial cells are important players in the neuronal clock network (reviewed in [67]), most attention has so far been attributed to the clock neurons (Fig. 6.5a). According to their location in the brain, the clock neurons are traditionally divided into seven groups – three dorsal ones (DN1–3) in the dorsal brain, three lateral ones ($LN_d$, l-$LN_v$, and s-$LN_v$) in the anterior lateral brain, and one additional lateral group in the posterior brain that is called LPN ([68–71]; see below for a more detailed classification). The entity of these clock neurons is also called the "circadian master clock" or the "circadian pacemaker center," because it generates rhythmic output signals that time essential brain functions as, for example, sleep, hormonal and physiological processes in the body, as well as rhythmic behavior. In mammals, the circadian pacemaker center in the brain even coordinates the phase and speed of the peripheral oscillators, whereas the latter have more autonomy in *Drosophila*. In the following, I will concentrate on the clock neurons in the brain that constitute the circadian pacemaker center of the fruit fly.

### 6.3.1 Daily Rhythms in Membrane Excitability of the Pacemaker Center

Neurons have the unique property to change their resting membrane potential and to fire action potentials (= to spike). This works via the opening of membrane-spanning ion channels that change the voltage of the neuron. At a certain voltage threshold, voltage-sensitive $Na^+$ channels open and the neuron rapidly depolarizes and spikes. In the presynaptic terminal, this depolarization leads to a transient flooding of the cell with $Ca^{2+}$ due to the opening of voltage-sensitive $Ca^{2+}$ channels. Influx of $Ca^{2+}$ causes neurotransmitters or neuropeptides to be released from the presynaptic terminal of the neuron.

Circadian pacemaker neurons of mammals spontaneously generate such spikes, and the frequency at which these spikes are generated varies with the time of day (reviewed in [73]). Firing rate is high during the day and low at night for both nocturnal and diurnal species. This rhythm persists even in vitro in cultured neurons that are isolated from each other, demonstrating that the electrical rhythms are cell autonomous [74, 75]. Furthermore, the periods of the oscillations are long in mutants in which the molecular feedback loop is slowed down [75, 76] and short in mutants in which the molecular feedback loop is accelerated [77]. However, the mechanistic links between the molecular clock and the machinery controlling cellular excitability are not well understood.

*Drosophila melanogaster* is not an ideal model for performing electrophysiology due to its small size. Nevertheless, patch-clamp recordings from two groups of clock neurons, the l-$LN_v$ and the $DN_1$, have been successfully done in isolated brains [78–84]. These studies revealed that the two groups of neurons spike spontaneously as do neurons of the mammalian pacemaker. Furthermore, as in mammals, the firing rate of *Drosophila* circadian pacemakers is modulated in a diurnal manner with the highest firing rate in the morning and the lowest firing rate in the first half of the night. In the morning, the neurons are rather depolarized, whereas they are hyperpolarized in the first half of night. The mechanisms by which daily depolarization and hyperpolarization occur appear again conserved between mammals and flies [83], and regarding hyperpolarization they are even the same in mollusks [85]. Daily hyperpolarization is accompanied by an increase in potassium ion conductance (i.e., more potassium channels are open during the night) [86, 87]. An increase in potassium conductance during the night drives the resting membrane potential closer to the equilibrium potential for potassium ions. The latter is more negative than the resting membrane potential and pulls the cell's voltage further away from spike threshold. Thus, increased potassium conductance is expected to decrease spike rate. A recent study on the $DN_1$ neurons of *Drosophila* and on mammalian pacemaker neurons indicates that sodium ion conductance additionally varies during the day [83]. It appears to cycle in antiphase to the potassium conductance and is highest in the morning, provoking a depolarization of the neurons. Most interestingly, experiments in *Drosophila* revealed for the first time a putative mechanism by which the molecular clock controls membrane

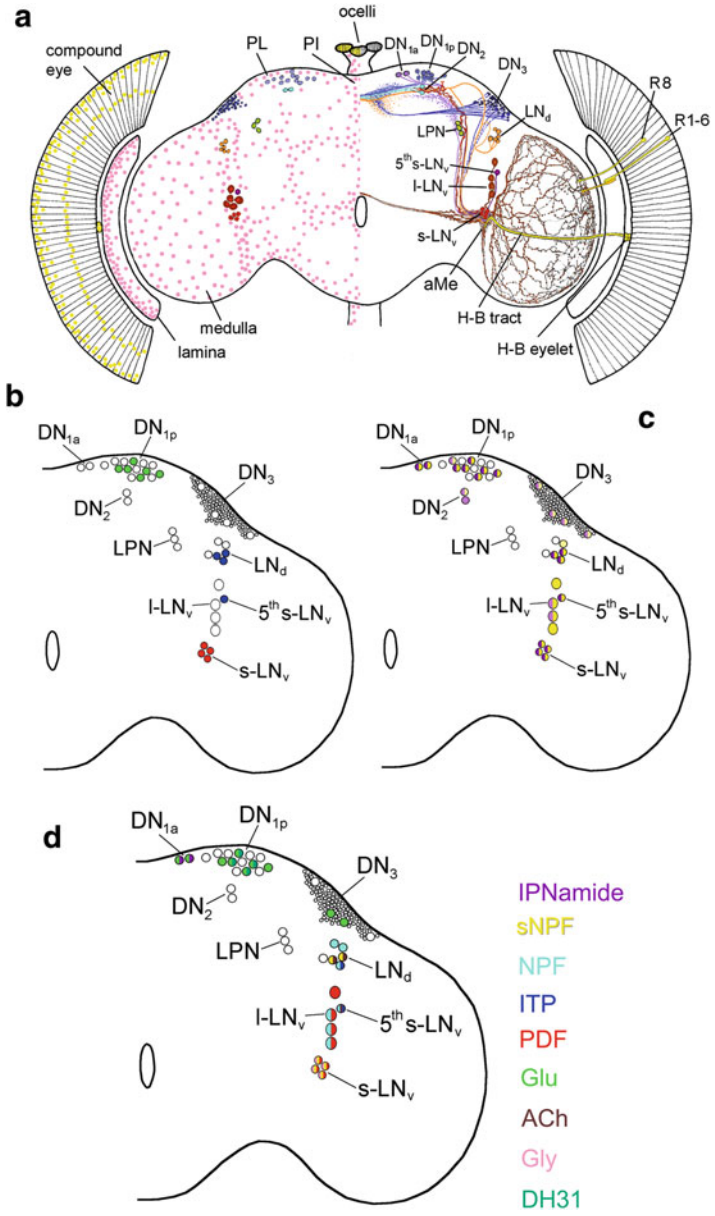


**Fig. 6.5** Clock network organization. (**a**) Clock gene-expressing cells in the head. The cell bodies are shown in the left brain hemispheres, the arborizations of the clock neurons in the right hemisphere. The clock ticks in the photoreceptor cells of the compound eyes and ocelli (*yellow*), in hundreds of glial cells (*pink*), and in the clock neurons that are also called pacemaker neurons. The clock neurons consist of about 150 lateral neurons (LN) and dorsal neurons (DN). Most of the LN are located anterior and are subdivided into the ventral LN ($LN_v$), consisting of five small $LN_v$ (s-$LN_v$) and four large $LN_v$ (l-$LN_v$) and six dorsal LN ($LN_d$). Only the LPNs (lateral posterior neurons) are located in the lateral posterior brain and consist only of three neurons. The DN consist

conductance: the respective sodium ion channel, the $Na^+$ leak channel (NALCN), called "narrow abdomen" (NA) in *Drosophila*, appears to be rhythmically processed and delivered to the axon membrane. The ER protein, NLF-1, that is responsible for this delivery shows robust rhythms in mRNA abundance, and chromatin immunoprecipitation demonstrated rhythmic CLK binding at the *Nlf-1* locus [58, 83]. Thus, CLK-driven transcriptional oscillations appear to be directly linked to sodium conductance of the membrane. Whether similar mechanisms also control potassium conductance still has to be investigated.

A new imaging technique promises in vivo membrane potential monitoring even in those clock neurons that lie deeper in the brain than the $DN_1$ and l-$LN_v$ and are therefore harder to access by whole-cell patch-clamp physiology [88]. This technique relies on the genetically encoded fluorescent voltage indicator "ArcLight" that changes its fluorescence when neurons depolarize [88]. The big advantage of this technique is the possibility to simultaneously record the membrane potential in intact neural circuits and to report membrane potential in somata, neurites, presynaptic terminals, and postsynaptic terminals. Thus, it appears possible to fully characterize the electrophysiological properties of the entire neuronal clock network using solely optical methods. This is very important, as we know that the clock neurons of mammals and flies are quite heterogeneous in their morphology and neurochemistry. Thus, they may also have different electrophysiological properties.

In spite of these promising new imaging techniques, some words of caution should be added. So far, all measurements need to be done in brains which have been isolated from their sensory inputs and neuronal outputs. A recent patch-clamp study showed that the spontaneous firing of the l-$LN_v$ is different when the compound eyes are still attached to brain [84]. This indicates that sensory inputs may change the properties of the entire clock network.

**Fig. 6.5** (continued) of two subgroups of $DN_1$, the two anterior $DN_{1a}$ and ~15 posterior $DN_{1p}$, two $DN_2$, and ~50 $DN_3$. The clock neurons form a fiber network in the accessory medulla (aMe) and the dorsal brain (PI, pars intercerebralis; PL, pars lateralis). Only the l-$LN_v$ invade the optic lobes and form a network in the distal layer of the medulla. Photoreceptor cells R7 and R8 as well as lamina monopolar cells enter the distal medulla in close vicinity to the l-$LN_v$, but appear to have no direct contact. The aMe gets direct input from the Hofbauer-Buchner (H-B) eyelets via the H-B tract. Modified after [71, 72]. (**b**) The lateral neurons can be divided in morning (M, red) and evening (E, blue) neurons, whereas six $DN_{1p}$ (*green*) appear to control the siesta. Only the right hemisphere is shown. (**c**) A subset of the clock neurons express cryptochrome (CRY, *yellow*) and the PDF receptor (PDFR, *violet*). Cells that express only low amounts of CRY or PDFR are marked in pastel colors. (**d**) The clock neurons utilize different neuropeptides and neurotransmitters: pigment-dispersing factor (PDF, *red*), ion transport peptide (ITP, *blue*), neuropeptide F (NPF, *cyan*), short neuropeptide F (sNPF, *yellow*) and IPNamide (IPNa, *magenta*), glutamate (Glu, *light green*), acetylcholine (ACh, *brown*), and glycine (Gly) (*pink*). Neurons that are marked by several colors express several signaling molecules

### 6.3.2 Daily Rhythms in Synaptic Plasticity

Besides showing rhythms in intrinsic excitability, the structure of the circadian clock neurons is remodeled in a daily manner with the number of presynaptic active zones increasing and decreasing rhythmically [89–91]. This is shown for the axonal terminals of the s-$LN_v$ that are regarded as the dominant circadian pacemaker neurons (see below), but it may be true also for other clock neurons. The axonal terminals of the s-$LN_v$ are maximally spread in the morning and show the highest number of presynaptic zone at this time, which coincides nicely with their maximal electrical excitability (see above). In the evening, the axonal material decreases and the volume of the axonal terminals shrinks [92]. Similar changes in volume and number of synapses appear present in the axonal terminals of the l-$LN_v$ that project toward the optic lobe [93]. Recently, the group of Justin Blau identified the molecular mechanism that link the molecular clock to these rhythmic synaptic changes [92]. They found that the GTPase Rho1 is rhythmically active and retracts s-$LN_v$ axonal termini by increasing myosin phosphorylation. The rhythm in Rho1 activity is in turn controlled by clock-regulated transcription of a Rho1 GEF (guanine nucleotide exchange factor that increases the activity of Rho1). They called this GEF "Purathrophin-1-like" (due Pura) due to homologies with a human GEF.

After having discussed the possible ways by which the molecular clock can control the electrical and synaptic properties of the clock neurons in the brain, it is high time to regard the general organization of the circadian clock network in the brain.

### 6.3.3 General Organization of the Circadian Clock Network in the Brain

As mentioned above, there are seven major groups of clock neurons (Fig. 6.5a). However, this classical division of the clock neurons is insufficient, because the majority of these groups are heterogeneous in neuropeptide expression (see below). A further subdivision is particularly appropriate for the s-$LN_v$ and the $DN_1$. Of the s-$LN_v$, four cells express the neuropeptide pigment-dispersing factor (PDF), and one expresses the ion transport peptide (ITP) and is called the fifth s-$LN_v$. Both types of neurons project into the dorsal brain, and the abovementioned rhythm in synaptic plasticity has been shown for the terminals of the PDF-expressing s-$LN_v$. Of the $DN_1$, two neurons are located anterior to the others, express the neuropeptide IPNamide, and are called $DN_{1a}$ [70]. The remaining ~15 $DN_1$ are consequently named $DN_{1p}$. All these clock neurons appear to be mutually connected. During the last decades, the network of the circadian clock in the brain has been revealed in detail and will be briefly described in the following. More detailed descriptions can be found in [71, 94, 95].

The clock neurons have two main projection targets: (1) the accessory medulla (aMe), a small neuropil situated between the central brain and the optic lobes and that had been identified as pacemaker center in many insect species and (2) the dorsal brain that houses the hormonal center (pars intercerebralis and lateralis) of insects and also has connections to most brain areas. The clock neurons form a well-defined fiber network in these two brain areas, putatively allowing considerable crosstalk between them (Fig. 6.5). The aMe is not only invaded by the clock neurons but also by aminergic, glutaminergic, acetylcholinergic, and GABA-ergic inputs from non-clock neurons [96–100]. This emphasizes the role of the aMe in intercellular communication – both among clock neurons and between extrinsic cells and clock neurons. Furthermore, the aMe appears to get light information from the eyes and from the H-B eyelets (Fig. 6.5a) that entrain the clock to the periodic environmental cycles. The large ventral LN (l-$LN_v$), which massively innervate the aMe and the surface of the medulla (Fig. 6.5a), may especially play an essential role in receiving and transferring light signals to the clock network (see below). In the dorsal brain, the clock neurons' fibers terminate close to regions that have been shown to be involved in the control of locomotion, sleep, and metabolism, such as the pars intercerebralis (PI), the mushroom bodies, and the central complex (see below under "Output Pathways from the Clock in the Brain to Rhythmic Behavior"). Clock neurons themselves show rhythms in plasticity, a property which they seem to transfer to other brain regions as is nicely shown for the optic lobe [101–104]. I warmly recommend reading these and the therein-cited papers to get a deeper insight. For the dorsal brain, direct evidence for plasticity rhythms controlled by the circadian clock is so far absent, probably because the dorsal brain lacks the regular columnar structures of the optic lobes and is therefore harder to analyze. Nevertheless, it has been shown that sleep alters synaptic homeostasis in central brain structures [105, 106] and at least the timing of sleep is controlled by the circadian clock.

### *6.3.4 Communication Among Clock Neurons via Neuropeptides*

As already mentioned above, the clock neurons are remarkably heterogeneous in their neurochemistry, most of them being peptidergic (Fig. 6.5b). The neuropeptide pigment-dispersing factor (PDF) was the first to be identified in the clock network. PDF is expressed in 8 clock neurons per brain hemisphere (4 s-$LN_v$ and the 4 l-$LN_v$; Fig. 6.1b) [27], and it is essential for normal locomotor rhythms [107–111]. PDF is released from the s-$LN_v$ in a rhythmic fashion into the dorsal brain where it may transfer rhythmic signals to neurons within and outside the clock network [112]. A similar role was recently also suggested for the ion transport peptide (ITP) expressed in only two clock neurons per brain hemisphere (Fig. 6.5b) [113, 114]. As PDF, ITP appears to be released rhythmically into the dorsal brain

where it seems to act as further clock output factor complementing the actions of PDF [113]. Whereas PDF is necessary for the flies' normal morning activity, ITP enhances their evening activity and inhibits nocturnal activity. In addition to their role in the central brain, PDF and ITP are involved in the regulation of the abovementioned circadian rhythms in the visual system [101, 115].

PDF is not only a putative output factor of the clock but also the most important communication factor within the clock [109, 111, 116, 117]. The PDF receptor is expressed on many clock neurons, including the PDF-positive s-$LN_v$ (Fig. 6.5c) [118–120]. PDF is able to couple the molecular oscillations of individual clock neurons by speeding them up or slowing them down [116, 117]. This differential action appears possible due to individual clock neurons utilizing different signalosome components and allowing distinct PDF-mediated cellular responses [121, 122]. Thus, PDF is the most powerful neuropeptide in the clock network, leading to the hypothesis that the PDF neurons are dominant circadian pacemakers governing the other clock neurons by setting phase and period of their molecular clocks, especially under constant darkness [123, 124].

Other neuropeptides in the clock network are IPNamide (IPNa), expressed in the two $DN_{1a}$ [70]; the diuretic hormone 31 (DH31) in 4 $DN_{1p}$ [125]; the neuropeptide F (NPF), expressed in several LN [126, 127]; and short neuropeptide F that is co-localized with PDF in four s-$LN_v$ and additionally present in two $LN_d$ [114] (Fig. 6.5b). Only the roles of NPF, sNPF, and DH31 in the clock have so far been partly characterized. NPF is implicated in the control of timed courtship behavior, evening activity, and sleep, but most results concerning this neuropeptide are based on cell ablation experiments, and the phenotypes stemming from an actual downregulation of the peptide are only subtle [126–129]. sNPF, on the other hand, appears to promote sleep by an inhibitory action of the s-$LN_v$ on the l-$LN_v$ neurons [130]. There is some evidence that the NPF (NPFR1) and sNPF (sNPFR1) receptors are expressed on clock neurons [130, 131], and thus NPF and sNPF may work as communication factors within the clock network as does PDF, though with clearly less impact. DH31 does not serve as a communicator between the clock neurons at all, but as an output factor controlling sleep ([125]; see below).

Apparently, some clock neurons additionally utilize classical neurotransmitters [100]. The expression of the choline acetyltransferase (Cha) in the fifth s-$LN_v$ and the two sNPF-expressing $LN_d$ (Fig. 6.5b) indicates that these cells might contain acetylcholine [114] and the $DN_1$ and some $DN_3$ were shown to be glutamatergic and to signal to the $LN_v$ via metabotropic inhibitory receptors [97, 132, 133]. Furthermore, the s-$LN_v$ express the inhibitory neurotransmitter glycine and signal to the more dorsally located clock neurons (Frenkel and Ceriani, personal communication).

At the first glance, the mixture of signaling molecules in the clock neurons appears very confusing, but this has nice analogies in the mammalian circadian pacemaker center, the suprachiasmatic nucleus (SCN). Firstly, the SCN is rich of neuropeptides among which the vasoactive intestinal polypeptide (VIP) is the functional analog of PDF and vasopressin (AVP) the functional analog of ITP. Secondly, VIP is expressed in ventral parts of the mammalian circadian pacemaker center as is PDF in *Drosophila's* ventrolateral clock neurons. Both neuropeptides

are main communicators in the circadian pacemaker centers signaling predominantly from ventral to dorsal. Thirdly, AVP and ITP are expressed in the dorsal parts of the mammalian SCN and the fly's dorsolateral clock neurons, respectively. Both of them might be mainly output signals from the circadian pacemaker center to downstream neurons, and both have antidiuretic functions in the body. Last but not least, GABA is the major inhibitory neurotransmitter in the SCN signaling mainly from ventral to dorsal parts of the SCN. Its functional analog glycine has the same function in the fruit fly circadian network (Frenkel and Ceriani, personal communication).

### *6.3.5 The Dual-Oscillator System*

The detailed characterization of the clock neurons, which revealed that they have a different neurochemistry, raises the fascinating question of whether they have different functions in the circadian clock of the fly. Indeed, this seems to be the case. As mentioned above, PDF appears important for normal morning activity, whereas ITP (and NPF) appears vital for normal evening activity [113]. Many animals show two activity bouts in their daily activity rhythm, and, under constant conditions, these activity bouts become sometimes asynchronous (reviewed in [109, 133]). Already in 1976, Pittendrigh and Daan [134] proposed a model in which the two activity bouts of nocturnal rodents are controlled by two circadian oscillators with different properties. This model was verified in hamsters by electrophysiological recordings of horizontally cut SCN sections [135] and also gets support from behavioral observations with *D. melanogaster*. As seen in Fig. 6.6, wild-type fruit flies are active in the morning and evening, with a siesta in the middle of the day and almost complete inactivity during the night. When transferred into constant darkness, the morning activity usually diminishes and sometimes fuses completely with the evening activity. In other cases, the morning activity bout remains separated from the evening activity bout and free-runs in parallel to it (Fig. 6.6b). The interesting cases are the ones in which morning and evening activity bouts do not run in parallel (Fig. 6.6c). Although these cases are rare, they clearly indicate that independent morning (M) and evening (E) oscillators are present in the fly brain and that they are usually coupled with each other, but also that they can desynchronize under certain circumstances.

The anatomical basis of these M and E oscillators remained elusive until Grima et al. [137] and Stoleru et al. [138] genetically manipulated the clock neurons. Grima et al. [137] expressed the *period* gene in different subgroups of clock neurons in a $per^{0}$ mutant background, whereas Stoleru et al. [138] ablated subgroups of the clock neurons by expressing a cell death gene selectively in them. Both laboratories found that – dependent on the manipulation – either the M or the E activity bout disappeared. Consequently, they concluded that the PDF-positive s-$LN_v$ must control the M activity bout and the $LN_d$ control the E activity bout (Fig. 6.5e). In accord with their idea, $Pdf^{01}$ mutants that lack neurotransmission in the M oscillators showed virtually no M activity [110]. Thus, the M and E dual-

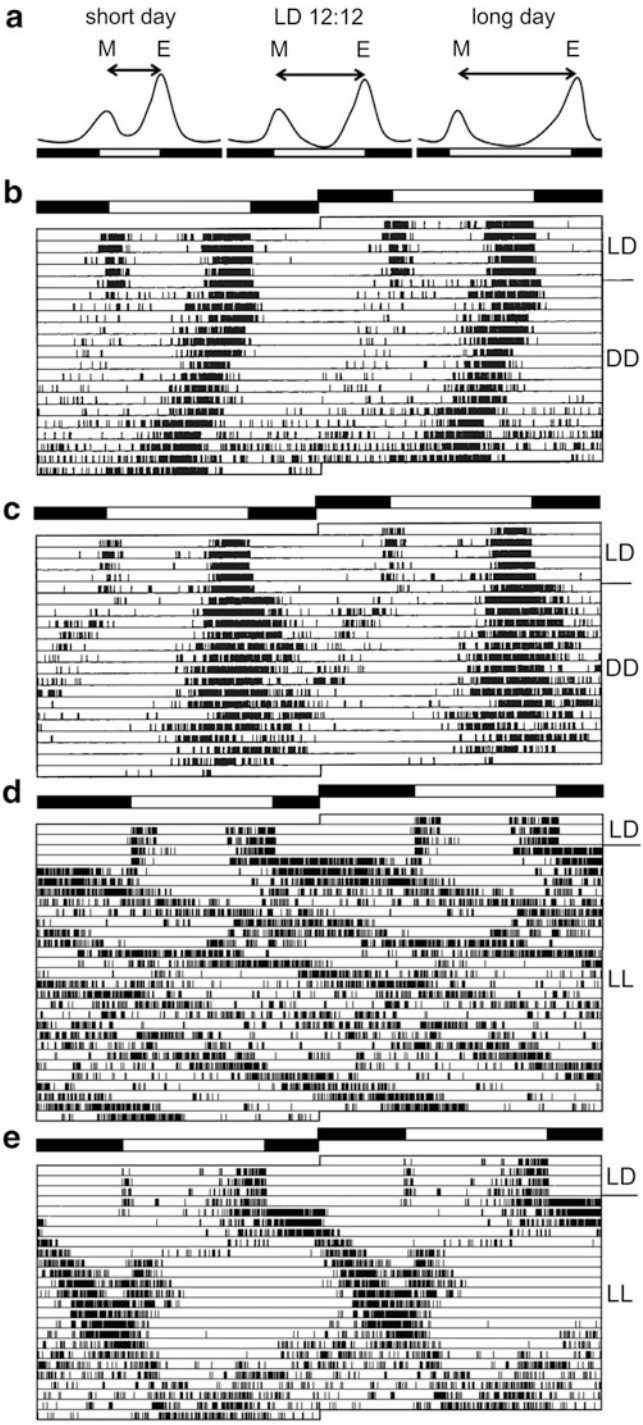


**Fig. 6.6** (**a**) The dual-oscillator model predicts that an animal's morning (M) and evening (E) activity locks to dawn and dusk, respectively. Consequently, in a diurnal animal, the two are close together in short winter days and separated by a prominent siesta under long summer days.

oscillator model was consistent with data in fruit flies. Certainly, this initial M and E oscillator model was much too simple, but it set the stage for further investigations. For example, Rieger et al. [136] found that only three of the $LN_d$ neurons are bona fide E oscillators but that the fifth s-$LN_v$ belongs to the E oscillators. Furthermore, the $DN_{1p}$ seem to be a mixture of M and E oscillators, whereby the ones that express the PDF receptor appear to behave as M oscillators [136, 139]. The reader is referred to Helfrich-Förster [109, 140] and Yoshii et al. [141] for a more detailed view on the M and E oscillator model and its limitations. Most interestingly, one of its main limitations has been solved recently by the group of Paul Taghert. So far, it was puzzling that the molecular oscillations in M and E neurons occurred in complete synchrony with each other and not ~9 h out of phase as M and E activity did. By measuring daily $Ca^{2+}$ oscillations in the M and E neurons of living flies, Liang et al. [142] could now show that these were 9 h out of phase, whereby the $Ca^{2+}$ level in the M neurons reached its maximum about 4 h before maximal M activity occurred and the $Ca^{2+}$ level in the E neurons reached its maxima 4 h before maximal E activity. This demonstrates elegantly that M and E activity are controlled by a differential neuronal activity of M and E neurons.

Several papers indicate that the classification in M and E oscillators is too strict. The clock network may rather consist of a flexible network of interacting clock neurons without the necessity of designated M and E cells [143]. More current models regard the clock network as a composite of multiple independent parts, in which the PDF-positive cells have a large influence on the network and signal via PDF to most of the other cells [144, 147]. A recent paper shows that the glutamatergic $DN_{1p}$ are responsible for controlling the midday siesta by inhibiting M and E oscillators in the lateral neurons [147]. Thus, according to these findings, the glutamatergic $DN_{1p}$ do not belong to the M cells. In the following, I will preserve the classification of M and E neurons but limit it to the lateral neurons as defined in Fig. 6.5e.

The particular power of the dual-oscillator model is that it explains observed adaptations to seasonal changes in day length. The model predicts that the M oscillator will shorten and the E oscillator will lengthen its period when extensively exposed to light [134]. As a consequence, the M activity occurs earlier and the E activity later in long summer days (Fig. 6.6a). Exactly as proposed by the dual-

**Fig. 6.6** (continued) (**b**) Typical activity pattern of a wild-type fruit fly recorded for 5 days under LD12:12 and for 16 days in constant darkness (DD). Under both conditions, the animal exhibited M and E activity bouts. After transfer to DD, M activity is reduced but runs in parallel to E activity (after [133]). (**c**) Activity pattern of a wild-type fruit fly, in which M activity did not run in parallel to E activity under DD conditions, indicating that the two are driven by different oscillators (after [133]). (**d**) Activity pattern of a wild-type fruit fly that was transferred into constant dim light (LL, 0.25 μW/cm$^2$) after 3 days in LD. Under LL is shown an internal desynchronization into two activity components, one originating from M activity that free-runs with a very short period and one originating from E activity that free-runs with a very long period (after [136]). (**e**) Typical activity pattern of a mutant without functional CRY (*cry$^b$*) under LD and bright LL (500 μW/cm$^2$) conditions. Under LL, always two free-running components are visible (After Ref. [136])

oscillator model, the molecular oscillations within *Drosophila's* M cells were shown to be accelerated, while the E cells are decelerated by light, allowing the two activity bouts to follow dusk and dawn in the course of changing day lengths [136, 148].

## 6.4 Light and Temperature Input Pathways to the Clock in the Brain

Circadian clocks have to be synchronized to the 24-h day of the earth by zeitgebers. The most reliable zeitgebers are the daily light-dark (LD) and temperature cycles. Indeed, for most organisms including flies, light is the most important zeitgeber followed by temperature. Therefore, I will start with the effects of light on *Drosophila's* clock and then describe those of temperature and finally the interactions of both.

### *6.4.1 Multiple Light Inputs to the Clock Neurons*

*Drosophila* uses multiple photopigments located in different organs/cells for entraining its circadian activity rhythms: rhodopsins situated in the compound eyes, in the ocelli, and in the Hofbauer-Buchner eyelets and cryptochrome (CRY) placed in the compound eyes and in the clock neurons themselves [149]. Only when all photoreceptors are eliminated, fruit flies are unable to entrain their activity and sleep rhythm to LD cycles, suggesting that the multiple photoreceptors fulfill partially redundant roles and each single one is capable of entraining the molecular feedback loop [150].

CRY is generally regarded as the main photoreceptor of the fruit fly's clock, because it interferes directly with the molecular clock in the clock neurons and can potently reset them to a new phase [151–153]. Upon light activation, CRY interacts with the clock protein TIM and provokes its ubiquitination by the Jetlag (JET) protein and its subsequent degradation via the proteasomal pathway [154–156]. CRY itself is then ubiquitylated via the Ramshackle (Brwd3) protein by an E3 ligase complex and also degraded [157]. The light-induced degradation of TIM in the morning destabilizes CLK-bound PER in the nucleus allowing a new round of p*er*/*tim* transcription (Fig. 6.2). Light-activated CRY in the afternoon prevents freshly translated TIM from accumulating in the cytoplasm, and consequently PER cannot accumulate because it needs the stabilization by TIM. Thus, TIM/PER accumulation is delayed until the onset of darkness.

The described interactions between the PER, TIM, and CRY proteins provide a simple and clear-cut explanation of how the *Drosophila* clock is entrained to LD cycles and phase shifted by light pulses. Without CRY, fruit flies have an attenuated light phase response curve and only slowly re-entrain to phase shifts of the LD cycle

(Fig. 6.7; [151–153, 158]). The action of CRY also explains why fruit flies become arrhythmic under constant light conditions (LL; [159]): LL prevents TIM from accumulating at any time and therefore stops the entire molecular oscillations in the circadian pacemaker neurons.

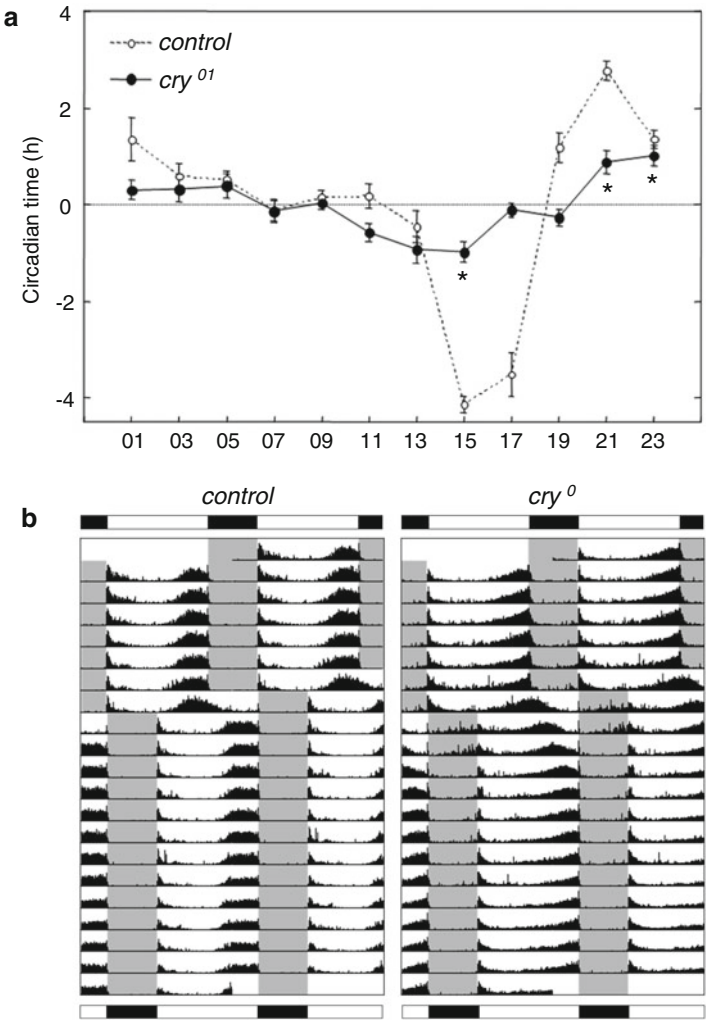


**Fig. 6.7** (**a**) Phase response curves for control flies and $cry^0$ mutants. Flies were pulsed for 1 h with white light (1000 lx) during the first subjective day of DD at the times indicated on the abscissa. Phase changes were calculated by comparing behavioral offsets of light-pulsed flies to the behavior of flies that did not receive a pulse. Asterisks indicate the phase advances/delays in $cry^0$ mutants that were significantly different from un-pulsed flies (after [158]). (**b**) Activity rhythms under 16-/8-h LD before and after an 8-h LD phase shift. The *gray* area in the actograms indicates the dark phase. Bars above and below the actograms indicate light conditions before and after the LD phase shift, respectively. Actograms show mean activity rhythms calculated from the number of flies indicated next to the strain names (After Ref. [149])

However, not all clock neurons express CRY, and levels of CRY expression differ among clock neurons [160, 161]. Furthermore, not all clock neurons show similar responses to light pulses given during the night. For example, Tang et al. [162] demonstrated that a 10-min bright light pulse early in the night (in the phase delay region) induces degradation of TIM in E neurons, but not in M neurons, suggesting that the phase resetting of the clock by a light pulse depends on the type of clock neuron and the time of day. Bright light pulses in the late night (in the phase advance region) always induce TIM degradation in all clock neurons [149, 163]. However, there are still differences in the light responsiveness of the different clock neurons, especially when longer light pulses (up to 120 min) of extremely low light intensity are given [164]. Under such circumstances, the 5$^{th}$ s-LN$_v$ that belongs to the E neurons shows the strongest light response with respect to TIM degradation.

In addition to the above-discussed cell-autonomous CRY-dependent light responses, there is significant cross talk between the clock neurons causing TIM degradation even in those clock neurons that express no CRY [160]. For example, CRY-dependent light input to M neurons can cause CRY-independent TIM degradation in E neurons [124, 165]. Thus, M neurons can direct the light response of E neurons through neuronal communication. Nevertheless, not all of these findings give a coherent picture. The results seem to depend on the experimental conditions suggesting that several mechanism are at play (see contribution of the photoreceptor cells below).

The presence of CRY in M and E neurons has also a different importance in re-entraining the flies' activity to phase-shifted LD cycles. Wild-type flies re-entrain to an 8-h phase delay of the LD cycle nearly within 1 day [149], whereas *cry*$^0$ mutants require nearly 1 week to completely synchronize to the phase-delayed LD cycle (Fig. 6.7b). When CRY is present only in the E neurons, the flies re-entrain as if they were wild type [149]. Thus, the E neurons are most important for re-entrainment to phase delays and CRY plays a crucial role. The M neurons appear to play a larger role for phase advances [166], but the role of CRY in them still has to be determined.

Besides its role in resetting the molecular clock in the clock neurons, CRY has other roles, of which not all are completely clarified. In the peripheral oscillators, including the compound eyes, CRY works as a transcriptional repressor and is an integral component of the molecular clock [167–169]. Furthermore, in the compound eyes and the l-LN$_v$, CRY is additionally associated with the cytoplasm membrane and appears to interfere with the phototransduction cascade [170] and with light-induced membrane depolarization [79, 80]. The latter properties of CRY could contribute to light sensing for entraining the circadian clock. In addition, CRY works also as magnetoreceptor for the clock [171, 172]. Unfortunately, this chapter does not provide the space to elaborate the latter roles of CRY in detail.

Although CRY is *Drosophila's* most potent circadian photoreceptor, entrainment and re-entrainment do also work without CRY (Fig. 6.7). The study of Yoshii et al. [149] revealed a very interesting detail when he looked at the molecular oscillations in the clock neurons of *cry*$^0$ flies after he had phase-delayed the LD

cycle by 8 h. The E neurons of $cry^0$ mutants showed a rapid re-entrainment of the molecular clock [149]. All other clock neurons re-entrained slowly, just in parallel to the behavioral rhythm. This shows unequivocally that light from the photoreceptor organs has phase-delayed the E neurons although this was not visible in the behavioral shift and raises an interesting question: does CRY work as a signaling molecule necessary to transmit phase information to the other neurons? This puzzling detail should be clarified in the future.

Even when we leave this interesting detail aside, the phase-shifting dynamics of locomotor activity in response to LD phase shifts of CRY-less flies reminds on those of mammalian species that need several days for re-entrainment and use solely their eyes for entraining their circadian pacemaker in the SCN. Indeed, in *Drosophila*, the compound eyes and the H-B eyelets appear to play a similar role in entrainment as do the eyes in mammalian species. Besides contributing to entrainment and re-entrainment, the photoreceptor organs are important for adjusting the activity patterns (1) to different day lengths [173], (2) to simulated dawn and dusk [174, 175], (3) to dim light in the night (moonlight; [176, 177]), and (4) to dim constant light (LL) [136, 148]. The LL effects of the compound eyes on the fly's activity pattern can be seen in the $cry^0$ mutant shown in Fig. 6.6e. In LL, the rhythm of flies without functional CRY internally desynchronizes into a component free-running with short period and one free-running with long period. This does not occur in flies that lack CRY plus the compound eyes, meaning that light from the compound eyes accelerates the speed of some clock neurons (the M oscillators) and decelerates that of others (the E oscillators) exactly as predicted by the dual-oscillator model (see above). Obviously, the compound eyes play a major role in mediating these light responses, although CRY in the E neurons appears involved in phase-delaying evening activity after phase delays of the LD cycle.

There are apparent direct connections between the H-B eyelets and the PDF-positive s-$LN_v$ and l-$LN_v$ [178, 179, 191] as there are analogous connections between the retinohypothalamic tract and the ventral part of the SCN. Furthermore, there are indirect connections between the compound eyes and most probably the l-$LN_v$ [84], but the compound eyes could also target other clock neurons indirectly. In any case, the l-$LN_v$ cells are major targets of the H-B eyelets and compound eyes. Consistent with that, several studies show that the l-$LN_v$ and their neuropeptide PDF are important components in the light-input pathway (reviewed in [109]). PDF acts as a light-arousal signal when released from the l-$LN_v$ [180, 181] and seems to play a crucial role in transferring the light information from the compound eyes to the network of the clock neurons [145]. Elevating PDF signals from the l-$LN_v$ in the central brain provokes internal desynchronization of the free-running activity rhythm into two components, as does LL [182]. Furthermore, the M and E clock neurons free-run with a short and long period, respectively, as observed under LL [117]. This suggests that PDF is the factor that accelerates the speed of the M cells and decelerates the speed of the E cells.

The neurotransmitters that transmit the light information from the compound eyes and the H-B eyelets to the l-$LN_v$ and to the other clock neurons are only partly

known. Both photoreceptor organs use the inhibiting neurotransmitter histamine. In addition, acetylcholine (ACh) is involved in transmitting light information, firstly as the second neurotransmitter of the H-B eyelets, and as neurotransmitter of the L2 monopolar cells of the lamina, to which the photoreceptors of the compound eyes are connected [183]. Thus, histamine and ACh may signal on the clock neurons. Indeed, ACh application on isolated brains increases intracellular $Ca^{2+}$ and cAMP in the l-$LN_v$ and s-$LN_v$ [98] and depolarizes the l-$LN_v$ [84, 184]. Furthermore, neuronal activation of the L2 monopolar cells promotes firing of the l-$LN_v$, strongly suggesting that the cholinergic input to the l-$LN_v$ stems from the L2 monopolars [84]. In contrast, the cholinergic input to the s-$LN_v$ stems from the H-B eyelets, because neuronal activation from the latter increased $Ca^{2+}$ and cAMP only in the s-$LN_v$ but not in the l-$LN_v$ [191]. Thus, the l-$LN_v$ and s-$LN_v$ are both excited by ACh, but ACh stems from different sources: excitation of the l-$LN_v$ occurs via the compound eyes and excitation of the s-$LN_v$ via the H-B eyelets. The role of histamine in the light-input pathway is less clear. The l-$LN_v$ decrease their firing upon bath application of histamine, and part of this histamine seems to stem from the H-B eyelets [191]. How the inhibitory action of histamine interacts with the excitatory action of ACh still has to be determined.

The next open question is how light stimuli from the compound eyes and the H-B eyelets are transferred to the molecular clock. Most likely, TIM degradation is not involved. Otherwise, flies that lack functional CRY but retain the compound eyes and H-B eyelets should become arrhythmic under LL as observed in wild-type flies. However, this is not observed, not even at high light intensities [139, 148, 150, 151, 159]. Moreover, the PER/TIM feedback loop runs with high amplitude in these flies under LL conditions without any sign for permanent TIM reduction [139]. In addition, the period shortening of the M neurons in *cry* mutants under LL is hard to explain by TIM degradation. Permanent TIM degradation has the capacity to slow down the clock and eventually stop it but cannot accelerate it. Thus, the light input via the photoreceptor cells must work via a different mechanism. Although not yet proven, it is imaginable that a similar transduction cascade is working in the M neurons as in the mammalian system. There, light input via the retinohypothalamic tract results in an increase of $Ca^{2+}$ and cAMP in certain clock neurons, finally leading to the activation of cAMP-responsive binding element (CREB). CREB binds to CREs in the promoters of *per1* and *per2* genes and activates their transcription. It is not yet established whether there are functional CRE sequences in the *Drosophila per* upstream region, but it was shown that mutations in the *Drosophila* CREB gene affect *per* expression [185]. *per* expression may also be affected indirectly, for example, by the CREB-binding protein (CBP) that influences transcriptional activity of the CLK-CYC heterodimer [186, 187]. Indeed, Mizrak et al. [188] showed that hyperexcitation of *Drosophila's* M cells induced *Clk* gene expression, among the expression of other circadian genes and that CREB transcription factors mediate this effect. In any case, the direct or indirect activation of *per* transcription by light in the morning may advance the M neurons and permanently shorten their period under LL conditions.

This infers that the compound-eye-mediated period lengthening mechanism in the E neurons must be different. Certainly, it could work via TIM degradation as suggested by the group of Michael Rosbash [124, 189]. However, the same group proposed another interesting mechanism that relies on PDF signaling from the l-$LN_v$ [189]: they showed that the activation of cAMP-dependent pathways, including the protein kinase A (PKA), stabilizes PER in the morning. This worked in S2 cells, as well as after in vitro addition of PDF or cAMP to isolated fly brains. Even 4 h after lights on, PER levels were rather high, whereas PER was almost completely absent in control flies. The longer persistence of PER at the Clk/cyc dimer delays the beginning of a new round of *per*/*tim* transcription, slows down the entire molecular cycle, and delays the flies' E activity that usually starts when PER has disappeared [190]. Although the authors did not look at PER stabilization in the E cells, it is possible that this mechanism works in the PDF receptor-expressing E cells. That PDF signaling from the l-$LN_v$ to the E cells does indeed phase delay E activity was recently shown under long days [191].

Leaving all speculations aside, light signaling from the compound eyes and the H-B eyelets does likely operate via mechanisms that are different from TIM degradation.

### 6.4.2 *Clock-Mediated Light Effects Versus Direct Light Effects of the Eyes on Activity Patterns*

In addition to affecting the circadian clock, the compound eyes also mediate effects on the activity pattern that are clock independent. These effects are known as "direct light effects" or as "masking" effect, because they often conceal the clock effects [192]. For example, fruit flies respond with a "startle" activity peak when lights are switched on in the morning [173]. This startle response or "lights-on effect" is similarly present in clock-less flies, indicating that it is independent of a functioning clock [193]. In addition, the increase of nocturnal activity by moonlight can be partly explained by these masking effects [194]. The same is true for the sharp M and E activity peaks induced by simulation of twilight as shown by a recent study [195]. The same study even shows that it is possible to achieve almost wild-type activity patterns in $per^0$ mutants by imitating the natural morning increase and evening decrease of light intensity.

The compound eyes mediate all of these responses, and it is often difficult to distinguish "masking" effects from effects that work via the clock. It is even possible that some of the masking effects (namely the lights-on effect) are mediated by PDF from the l-$LN_v$. As stated above, the l-$LN_v$ are arousal neurons, because they fire in response to light [81]. Since $Pdf^0$ mutants lack the lights-on effect in addition to M activity [110], it is possible that parts of the direct light effects are indeed mediated via PDF. In any case, both light effects, the direct one and the one

via the circadian clock, are of biological importance, because they contribute to adapting the flies' activity to the cyclic environment.

In summary, the fly has at least two principle light-input pathways to the clock – one working via CRY on TIM degradation directly in the clock neurons (though not all details are crystal clear) and the other working via the photoreceptor organs and most likely via neuronal communication, in which the neuropeptide PDF from the l-$LN_v$ plays a prominent role. The CRY pathway enables the fly clock to respond quickly and strongly to light. The photoreceptor input pathway seems to be more subtle but necessary for the adaption of fly activity to different environmental conditions.

### *6.4.3 Temperature Input Pathways*

Daily temperature cycles are the second strongest zeitgeber for the fly clock. The animals can be entrained by temperature cycles under constant darkness and constant light conditions [196–200] and phase shifted by temperature pulses [201–203]. As do light cycles, temperature cycles have different effects on the clock neurons, and the different clock neurons are of different importance for entraining the activity rhythm to temperature cycles. The CRY-negative clock neurons appear to be most vital for temperature entrainment, since flies that lack CRY-positive clock neurons still synchronize to temperature cycles whereas flies with a functional clock only in the CRY-positive clock neurons are unable to do so in a normal way [198, 205]. Among the CRY-negative clock neurons, the posteriorly located LPNs seem to be the most important [197, 199]: the PER/TIM oscillations in the LPNs only synchronize to T cycles but not to LD cycles [205]. The $DN_2$ cells also seem to be more sensitive to T cycles than to LD cycles [205–207]. In contrast, the PDF-positive l-$LN_v$s do only entrain to LD cycles, but not at all to temperature cycles [205]. This result strongly underlines the abovementioned role of the latter cells (and PDF) in the light-input pathway. As does light, temperature has also immediate effects on the activity, especially when temperature increases quickly in the morning. The flies respond to this quick increase with a kind of "temperature-on" (startle) response. This can be completely avoided by increasing temperature in a natural way with the temperature minimum in the morning followed by a gradual increase until ~2 h after noon, when temperature reaches its maximum [205, 208].

The group of Ralf Stanewsky has addressed the question on how temperature cycles are perceived by flies and how they are transferred to the different clock neurons. The starting assumption was that temperature-sensitive neurons in the brain sense temperature via transient receptor potential (TRP) channels and transfer temperature information to the clock neurons. Indeed, such neurons exist in the anterior brain and are called AC neurons [209], but it has become apparent that they are not sufficient for the entrainment of clock neurons to temperature cycles. The Stanewsky group monitored bioluminescence rhythms in isolated body parts and

brains of period-luciferase expressing transgenic flies in LL and found that these were nicely entrained to temperature cycles [210]. However, the bioluminescence rhythms of isolated brains were very different from the rhythms in the peripheral organs. Instead of continuously increasing during the night (cold phase) exhibiting the maximum at the end of the night, as is usual for PER cycling under LD conditions, bioluminescence rose immediately after the increase of temperature in the morning. This speaks rather for an immediate response to the temperature rise and not for proper entrainment. In contrast, in the peripheral organs, *per* luminescence cycled in the expected manner with the maximum at the end of the night. The authors concluded that entrainment to temperature cycles is tissue autonomous in the peripheral organs, but that it depends on signals from the periphery in the clock neurons [211]. They then searched for mutants that are not able to entrain their activity rhythms normally to temperature cycles and found *nocte*, a gene that codes for a large glutamine-rich protein. *nocte* mutants are not able to entrain normally to temperature cycles, and the knockdown of the *nocte* gene in the periphery is enough to perturb temperature synchronization. Most interestingly, *nocte* is important for a normal morphology of the chordotonal organs in the body. Chordotonal organs are largely known for their function as mechanoreceptors (stretch receptors) but have also been linked to temperature sensation in *Drosophila* [212]. *nocte* mutants have structural defects in the dendritic cap of the scolopale cell, which connects the cilia of the chordotonal neuron with the cap cell. Thus, the temperature sensors for synchronizing the circadian clock neurons appear to be not located in the head, but rather in the chordotonal organs of the body [211]. The TRP channel pyrexia (PYX) is one of the temperature sensors in the chordotonal organs [213]. It is expressed in the cap cells that connect the chordotonal organ to the body wall, and it is necessary for entraining the activity rhythm to low-temperature cycles (16–20 °C) [213]. Nevertheless, the pathways from the chordotonal organs in the body to the clock neurons are still completely unknown. There is a pathway from the pyrexia-expressing neurons in the chordotonal organ of the second antennal segment to the AC neurons in the anterior brain [214], but so far there is no evidence that the antenna is involved in temperature entrainment of the clock [210].

The existence of temperature sensors in the periphery does not completely rule out the additional presence of temperature sensors for the circadian clock in the brain. As already mentioned, the warm-activated AC neurons that are localized in the anterior brain close to the antennal lobes are bona fide temperature sensors that are important for the flies' temperature preferences [209]. Activation of the AC neurons depends on TRPA1 channels. Most interestingly, temperature preferences of flies vary over the course of the day: low temperatures are preferred in the morning, whereas higher are preferred in the evening, indicating that there is a connection between the circadian clock and the flies' temperature preference [215]. The AC neurons project into the antennal lobe, to the subesophageal ganglion, as well as to the dorsal protocerebrum, where the dorsal clock neurons are located [209]. Indeed, Kaneko et al. [215] could show that the $DN_2$ clock neurons are important for the circadian control of the temperature preference rhythm. Recently, Head et al. [216] found that *Drosophila's* temperature preference is

influenced by light: flies prefer a ~1 °C higher temperature when they are exposed to acute light. This preference does not depend on the time of day, but, most interestingly, the $DN_{1p}$ clock neurons that express the PDF receptor and receive signals from the eyes via PDF are needed for this light-dependent temperature preference. This suggests that the connection between $DN_{1p}$ and $DN_2$ neurons is vital for integrating light and temperature information (see also below).

Last but not least, two studies show with *trpA1-gal4* reporter lines that TRPA1 channels are expressed in subsets of the clock neurons [150, 217]. The group of Craig Montell revealed TRPA1 channels in the larger subset of clock neurons, namely, the CRY-negative LPN, two neurons of the CRY-negative $LN_d$, and at least one neuron of the $LN_v$, $DN_1$, and $DN_2$, respectively [217]. This suggests that TRPA1 channels can directly work in these clock neurons. Indeed, the entrainment to temperature cycles was impaired after eliminating the *cry*-positive clock neurons in the TRPA1 mutant background, but only subtle phenotypes were observed in the TRPA1 mutant background alone [217]. The latter result weakens the importance of TRPA1 channels for temperature entrainment in the clock neurons themselves. Nevertheless, TRPA1 channels in CRY-negative clock neurons plus neurons outside the circadian system are important for the so-called afternoon activity bout that occurs on warm days under seminatural conditions ([218, 219]; see also under "Adaptive Significance of the *Drosophila* Clock" and the Chap. 19 of Das and Sheeba, this book).

In summary, there are striking parallels between light and temperature entrainment of the clock. In both cases, receptors intrinsic to the clock neurons are involved (CRY for light and TRPA1 channels for temperature) as well as external receptors (the compound eyes/ H-B eyelets for light and the chordotonal organs for temperature). Furthermore, there are direct effects of light and temperature on activity as well as entraining effects on the clock. The next question is how light and temperature cycles are integrated in entrainment.

### 6.4.4 Interaction of Light and Temperature

When natural-like LD and temperature cycles are applied in phase with each other, they work synergistically on entrainment: flies show narrow M and E activity bouts and the molecular clock cycles with high amplitude in all clock neurons [205]. Interesting effects are observed when LD and temperature cycles are applied out of phase with each other or when the clock is restricted to certain clock neurons [140, 197, 204]. Under conflicting LD and temperature cycles, wild-type flies entrain to the LD cycles, but CRY-less flies to the temperature cycles [201]. This shows that (1) light is a stronger zeitgeber than temperature – a fact that was reported already long ago [220] – and (2) it underlines the abovementioned importance of the CRY-positive neurons for entrainment to light.

But what happens when the PDF-positive l-$LN_v$s that seem to be most relevant for light input via the photoreceptor organs are ablated? Busza et al. [197] performed this experiment and found that such flies entrain faster to T cycles

than wild-type flies. Busza et al. concluded that the PDF cells may even inhibit the temperature entrainment of CRY-negative neurons and that this inhibition is important for entrainment in nature because it guarantees that the clock does not overreact to erratic temperature changes caused by weather changes. Gentile et al. [221] came to a very similar conclusion concerning CRY. They measured *per*-luciferase rhythms in the dorsal clock neurons under temperature cycles in the presence and absence of CRY and found that CRY dampens the oscillations. However, very similar to the results of Busza et al. [197] regarding the l-$LN_v$, Gentile et al. [221] found that, in the absence of CRY, rhythmic per expression in a few dorsal neurons is sufficient to entrain the activity rhythm to temperature cycles. Together, the two studies strongly indicate that the light-input pathways via the compound eyes and CRY weaken temperature input to the clock and thereby strengthen the light input.

## 6.5 Output Pathways from the Clock in the Brain to Rhythmic Behavior

After mostly focusing on the input pathways to *Drosophila*'*s* clock, some words should be added about the output pathways from the clock to rhythmic behavior, though I have to admit that the output pathways are less well investigated. As stated previously, in the dorsal brain, the clock neurons' fibers terminate close to regions that have been shown to be involved in the control of locomotion, metabolism, sleep, and learning and memory. These are the pars intercerebralis (PI), the central complex, and the mushroom bodies. Putatively, the circadian clock neurons could modulate the activity of neurons in all these brain regions in a circadian manner.

Nevertheless, so far only few specific output pathways have been identified: Pirez et al. [222] showed that PDF signals on neurons in the ellipsoid body of the central complex and elevates rhythmically their cAMP level. Kunst et al. [125] showed that the four DH31-expressing $DN_{1p}$s are also innervated by the PDF neurons and consecutively release DH31, which wakes up the flies in the morning. DH31 is the fly homologue of the mammalian calcitonin gene-related peptide. Cavanough et al. [223] screened various GAL4 lines with expression in the PI for circadian-relevant neurons and identified few DH44-expressing neurons as components of the circadian output pathway controlling rest/activity rhythms. DH44 is the homologue of the mammalian corticotropin-releasing factor (CRF) that is rhythmically released from the hypothalamus to prepare the organism for activity and stress. Similarly, DH44 may prepare the animal for daily activity. Indeed, ablation of these neurons caused behavioral arrhythmicity. The DH44 neurons are connected to the $DN_{1p}$ (putatively also to the DH31-positive $DN_{1p}$ neurons) that in turn are closely linked to the PDF-positive s-$LN_v$. In 2015, the same authors show a pathway from the dorsal clock neurons to the sleep-promoting centers in the dorsal fan-shaped body, another part of the central complex [224].

A second output pathway from the PDF-positive s-$LN_v$ goes to a pair of leukokinin-expressing neurons in the lateral brain that inhibit leukokinin receptor-positive neurons in the fan-shaped body and ellipsoid body of the central complex [225]. Neurons in the latter structures have been previously shown to promote activity [226]. The model presented by Cavey et al. (2016) predicts that the s-LNv inhibit the leukokinin neurons at dawn to allow the leukokinin receptor neurons to signal and promote locomotor activity.

Another pathway from the clock neurons to the mushroom bodies enables the flies to remember the time of day at which different odors have been presented [227]. Thus, the flies are capable of time memory as are honey bees. However, here the exact connections are still unknown. Altogether, there appear to exist multiple downstream circuits to orchestrate behavioral rhythms.

## 6.6 Adaptive Significance of the *Drosophila* Clock

Possessing a circadian clock is not crucial for fly survival, since also arrhythmic flies are viable and show no evident health problems, at least in the lab. Only three studies indicate that *Drosophila* flies with a functional clock have fitness advantages to clock-less flies. The first study in this direction showed that life span of male clock mutants raised in the lab is marginally shorter than that of their normal siblings [228], the second found that arrhythmic clock mutants have a reduced sperm production and fewer offspring [229]. The third study demonstrated that arrhythmic flies are more sensitive to oxidative stress [230] probably because they lack the rhythm in the response to oxidative stress [231].

A few studies under seminatural conditions even indicate that the activity pattern of clock-less flies resembles that of wild-type flies questioning whether possessing a circadian clock may have any adaptive significance [232–234]. Nevertheless, at closer inspection, there are subtle differences between clock-less and wild-type flies that become more evident when lab conditions simulate some of the natural changes in a controlled manner [195, 234]. Clock-less flies appear to keep a less pronounced siesta and to respond stronger to high temperatures at midday. Furthermore, they also respond with high activity to simulated twilight during midnight, whereas the response of wild-type flies is largely attenuated at this time. The authors concluded that one important function of the clock is to suppress futile activity during midday and midnight and hence to serve energy by doing so.

The daily timing of mating is another important function of the circadian clock in female flies [235]. Fruitful mating is not only important for reproductive success but also for speciation. The timing of mating is different between *D. melanogaster* that mate mainly in the morning and the sympatric species *D. simulans*, which mate predominantly in the evening. This difference could initiate the sexual isolation between them, which may then be enhanced by pheromonal differences playing another key role in mating behavior [236]. The mating of *D. pseudoobscura* occurs also later than that of *D. melanogaster* [237]. Most interestingly, transgenic

*D. melanogaster* carrying the *per* gene of *D. pseudoobscura* show the late mating peak of *D. pseudoobscura* flies [237] indicating that a single clock gene has the potential to provide the permissive conditions for speciation. This remarkable result was among the first to show that the natural variation in a single gene determines complex species-specific adaptive phenotypes (reviewed by [238]). In the following, I will summarize immediate adaptations of *Drosophila's* clock to changes in the environment followed by a summary of long-term evolutionary adaptations. At this point in our discussion, it is important to note that *D. melanogaster* flies that originated from the equatorial regions of Africa invaded large parts of the temperate zones in the north, exactly as humans did. To do so successfully, significant adaptations to daily and seasonal fluctuations in temperature and day length have been necessary.

### 6.6.1 Immediate Adaptations of the Clock to Changes in the Environment

Individual flies can adapt their activity pattern to changes in the environment by splicing their clock genes differently. This was shown for *per* and *tim* splicing. The mRNAs of both genes are differentially spliced in their 3′ untranslated regions, and in both cases the degree of splicing is dependent on the environmental temperature. *Per* shows an enhanced splicing at low and an attenuated splicing at higher environmental temperatures and long days [239, 240]. For *tim* it is the opposite: it is spliced to a larger degree at high temperatures than at low ones [241]. Enhanced *per* splicing at low temperatures leads to a quicker accumulation of per mRNA and PER protein, which accelerates the molecular cycle and advances the evening activity of the flies, whereas reduced *per* splicing at high temperatures slows down the molecular cycle and delays the evening activity. This explains activity of the flies under cold and short autumn days and long and hot summer days [239, 240]: under autumn condition, the flies start evening activity early in the day and lack the midday siesta almost completely, whereas under summer conditions, they have a pronounced siesta and start evening activity late. Most interestingly, differential *tim* splicing enhances these seasonal adaptations. The unspliced TIM isoforms at low temperatures have a higher affinity for CRY what leads to an earlier degradation of TIM and an advance of evening activity under cold days [241]. Under warm summer days, the spliced TIM isoforms have less affinity to CRY, TIM is degraded later, and evening activity is also later. Thus, *per* and *tim* splicing work in the same direction – advancing evening activity under short winter days and delaying it under long summer days.

Most interestingly, there is no thermal regulation of *per* splicing in *D. yakuba* and *D. santomea*, which have a more ancestral distribution in equatorial regions of Africa, where day length and temperature exhibit little fluctuation throughout the year [242]. Thus, thermal regulation of *per* splicing can be regarded as a mechanism

that facilitated the acclimation of the widely colonized *D. melanogaster* (and possibly *D. simulans*) to temperate climates. Consistent with this hypothesis, Low et al. [242, 243] discovered several single nucleotide polymorphisms (SNPs) in *per's* 3′ untranslated region of *D. melanogaster* that modulate its splicing efficiency. Most significantly, there was a latitudinal cline in these SNPs in wild-caught populations of flies originating along the east coast of the United States with the least efficiently spliced versions associated with a longer midday siesta in regions where temperatures can reach high levels. This demonstrates that natural selection can work at the level of splicing signals and that differential splicing plays an important role in the thermal adaptation of life forms.

### 6.6.2 Evolutionary Adaptations of the Clock

Besides *per* splicing, there are other polymorphisms that adapted the circadian clock of *D. melanogaster* populations to their northern habitats. As mentioned in the beginning, the uninterrupted stretch of alternating threonine-glycine (Thr-Gly) repeats in PER is a suspicious feature of this clock protein [244]. This unusual sequence has a natural molecular variation (polymorphism), being shorter in flies stemming from southern *D. melanogaster* populations and longer in flies stemming from northern populations (reviewed by [245, 246]). This latitudinal cline in the Thr-Gly length turned out to be important for temperature compensation of the speed of the clock. The northern populations with long Thr-Gly stretch had the most thermally stable periods that were close to 24 h at all temperatures tested. In contrast, southern populations, with short Thr-Gly stretch, had free-running periods of 24 h at 29 °C, but the periods shortened significantly at cooler temperatures. Thus, the "temperature compensated" PER protein with long Thr-Gly stretch appears better adapted to the colder and thermally variable higher latitudes, whereas the PER protein with short Thr-Gly stretch is more suitable for to the warmer Mediterranean region. Other *Drosophila* species show similar PER polymorphisms [247].

A latitudinal cline in polymorphism was also found for the *tim* gene (reviewed in [238, 247]). In contrast to the *per* polymorphism, the *tim* polymorphism has no role in temperature adaptation but affects the clock's light sensitivity, which is consistent with TIM's role in light resetting of the clock. The original *tim* gene gives rise to a short form of TIM (= S-TIM) that strongly interacts with CRY and is therefore sensitive to degradation by light. However, ~10,000 years ago, after *D. melanogaster* colonized Europe, a mutation occurred in the southeastern Italian populations, in which a single guanosine was inserted in the 5′ coding region of *tim* that resulted in the production of a long TIM isoform in addition to a short one (= LS-TIM). The *ls-tim* mutants are less light sensitive due to a reduced ability of LS-TIM to interact with CRY, and the *ls-tim* mutants gradually invaded Northern Europe [248, 249]. A reduction in circadian photosensitivity of northern fly populations has been documented in Japanese *D. auraria* flies and is interpreted

as being an adaptation of the circadian system to the long summer day lengths [250]. The long exposure to light can make the flies arrhythmic due to enhanced TIM degradation, and a less light-sensitive TIM prevents this. On the other hand, the *ls-tim* mutation causes females to enter diapause (reproductive arrest) earlier in autumn than the *s-tim* allele. Together with the reduced light sensitivity of *ls-tim* flies, the earlier diapause induction is advantageous for a life in the north.

The polymorphisms in PER and TIM offer a fine-tuning of daily rhythms to the environment and can be regarded as evolutionary adaptations of the clock to a life in the warm south or the cold north. When comparing different *Drosophila* species living in the warm south or the cold north, not only differences in clock gene variants become evident but also changes in the clock network. For example, northern *Drosophila* species lack PDF in the s-$LN_v$ and CRY in the l-$LN_v$ [251, 252]. Furthermore, their l-$LN_v$ invade the central brain in addition to the optic lobes. Consistent with the lack of PDF in the s-$LN_v$ and high PDF from the l-$LN_v$ in the central brain, these flies show little to no morning activity and a broad late evening activity [252]. In addition, they do not become completely arrhythmic under constant light, what might be caused by the absence of CRY in the l-$LN_v$. Future studies have to reveal whether these differences in behavior are causally related to the revealed differences in the clock network. In any case, the lack of morning activity at cold times of the day in the north as well as the broad activity in the afternoon and evening when temperatures are milder can be regarded as adaptations to the life in the north. Maintaining a rhythm under very long summer days may also be advantageous for the northern flies.

## 6.7 Conclusive Remarks and Open Questions

Since the discovery of the *per* gene in 1971, the fruit fly developed into a well-accepted model for circadian clocks. This is not only true for the principle molecular mechanisms of the clock that proved to be valid in all animals but also for the neuronal organization of the clock network in the brain and, finally, for the evolutionary adaptations of the circadian clock to the environment, the mechanisms of which are largely conserved among species. An incredible amount of knowledge has accumulated since that day in 1971 impossible for this book chapter to fully cover its breadth. Nevertheless, we are still left with many open questions. I will only mention a few: what is the degrading mechanism for PER shortly after its translation, if it does not go via SLIMB? How are PER and TIM transported into the nucleus? Why is the clock network in the brain so complex? What is the function of the DN3? How does the clock integrate light inputs from CRY and the eyes? Does CRY only work as a photoreceptive molecule necessary for initiating TIM degradation in response to light, or does it play an additional role as signaling molecule? Why, for example, are CRY and the PDF receptor present in the same clock neurons? What is the role of histamine from the photoreceptor organs in the light responses? How does the molecular clock achieve temperature compensation?

What is the activity rhythm of freely moving flies in nature? Do they still show two activity bouts? I have no doubt that the answers to these questions will even rise more questions and that research on the circadian system of the fruit fly remains exciting.

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# Chapter 7
# The Fish Circadian Timing System: The Illuminating Case of Light-Responsive Peripheral Clocks

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**Abstract** This chapter is dedicated to the circadian timing system of fish. In particular, we focus on one unique aspect of fish clocks that is helping us to build a more general understanding of the mechanisms and evolution of the circadian timing system in vertebrates. While in mammals peripheral clocks rely on systemic signals for their entrainment, in fish these clocks are directly light entrainable. Furthermore, in fish the transcription of a set of genes, including key clock genes, is induced upon the direct exposure of cells and tissues to light. We show that studying light-inducible gene expression in fish has revealed how fundamental changes in signal transduction systems have occurred during the evolution of mammals and fish. Furthermore, we explain how blind cavefish can serve as powerful models to further advance our understanding of the complexity of fish photoreceptor systems.

## 7.1 Introduction

One of the profound discoveries that resulted from the cloning of the first vertebrate circadian clock genes in the late 1990s was the finding that circadian clocks existed in most tissues and cell types. As a result, our view of how specialized pacemakers, such as the suprachiasmatic nucleus (SCN) of the hypothalamus, generate circadian rhythms was transformed. We now had to consider the existence of a myriad of independent tissue clocks, so-called peripheral clocks, with the central clock in the SCN playing a coordinating role, much like the conductor of an orchestra. New questions were immediately raised as a result of these discoveries. One, in

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particular, was how environmental zeitgebers such as light entrain this complex network of circadian timers? Providing answers to this question was not only of academic interest. This also had great relevance for understanding the basis of disorders which involve desynchronization of the circadian timing system such as jet lag or during regular shift work. Thus, a considerable amount of attention has been focused on photic entrainment of the circadian timing system in mammals, most notably in rodents such as the mouse. In these animals, light is detected exclusively by the retina, in large part by intrinsically photosensitive retinal ganglion cells (ipRGCs) which express the non-visual opsin, melanopsin. Neural signals from these cells are conveyed to the SCN via the retino-hypothalamic tract (RHT). Thereby, the phase of the SCN clock is indirectly reset in response to light, and in turn, timing information is relayed to the network of peripheral clocks via a complex combination of blood-borne signals, feeding-fasting rhythms and core body temperature changes [1]. Thus the mammalian circadian timing system is highly centralized with regard to light entrainment, relying upon the retina and its regulation of the master clock in the SCN.

This feature of mammalian circadian timing does not appear to be highly conserved in other vertebrate groups. In birds, reptiles, amphibia and fish, photoreception is not exclusive to the retina. Specialized photoreceptor cells are encountered in many other sites including the pineal complex, the parietal eye, regions of the hypothalamus and even dermal melanophores. In addition, a striking observation revealed that all peripheral clocks in the adult tissues of teleosts are directly light entrainable [2]. This means that tissues can be explanted from adult fish into an in vitro culture system and then simply by exposure of the tissues to light; the phase of their peripheral clocks can be reset. Thus, in contrast to the situation in mammals, the peripheral clocks in fish do not need to be linked to dedicated photoreceptive structures or central clocks in order to be entrained by light. These striking results also predict that photoreceptors and associated intracellular phototransduction signalling pathways are widely expressed in teleosts. Interestingly, a similar situation exists in *Drosophila*. Here, tissue-specific clocks, for example, in the epidermis, antennae and excretory tubules have been shown to be directly light entrainable, as a result of cryptochrome blue light photoreceptor function [3–5]. These observations have led to the general conclusion that the ancestral state of the circadian timing system was decentralized with regard to photoreception.

Many questions still remain concerning the directly light-sensing peripheral clocks of fish. What are the photoreceptors? How do they signal intracellularly? Does light influence other aspects of cell biology independently of the circadian clock? In order to provide answers to these basic questions, many studies have exploited the fish genetic model species, the zebrafish using a combination of genetic and in vitro tools. More recently, other models such as blind cavefish have also been studied to explore the evolution and underlying mechanisms of peripheral clock photoreception. In this review chapter, we will present our current understanding of how light affects peripheral clock function, the contribution of specific photoreceptors, how light directly regulates gene expression and finally

how the environment has influenced the evolution of this fascinating aspect of circadian timing.

## 7.2 Light-Sensing Fish Clocks.... in a Dish

The first cloning of vertebrate clock genes, including notably the *period* genes in 1997 and 1998, revealed that clock genes were expressed with characteristic circadian rhythms not only in the SCN and other brain structures but also in most other tissues outside of the central nervous system [6, 7]. This observation represented a key step towards our current appreciation of the crucial role played by peripheral clocks in the mammalian circadian timing system. The cloning of *clock* and *bmal* gene paralogs in zebrafish, and the detailed analysis of their spatial and temporal patterns of expression, not surprisingly, confirmed the existence of peripheral clocks in the largest vertebrate group, the teleosts [8, 9]. Furthermore, the discovery of clock gene expression in cell lines derived from early developing zebrafish embryos [2] mirrored previous findings by the Schibler group showing a widespread expression of clock genes in cell lines and also the ability of serum shock and other transient pharmacological treatments to synchronize rhythmic clock gene expression in cell cultures [10, 11]. However, in the case of the zebrafish tissues and cell lines, rhythmic clock gene expression could be entrained non-invasively, simply by direct exposure to light-dark cycles [2, 12]. The finding of directly light-regulated circadian clocks in the zebrafish heart and kidney and these fibroblast-derived fish cell lines served to reinforce the notion that light-regulated circadian clocks represent a general cellular function in fish. Furthermore, the fish cell lines were also readily transfectable and, so combined with the establishment of clock- and light-regulated luciferase reporters, served as a useful in vitro model to explore the mechanisms underlying light sensing away from the complexity of the whole organism (see Fig. 7.1) [13]. By analysing the effects of light using single-cell imaging, it became apparent that during long periods in constant darkness, individual fish cell clocks continue to tick, but with widely distributed phases as well as marked stochastic fluctuations in free-running periods [14]. The effect of exposure to light pulse is to synchronize all the individual cell clocks as well as to stabilize the subsequent free-running period [14].

### *7.2.1 Light-Inducible Gene Transcription*

The initial challenge was to identify how at the level of clock gene expression, light could impact on the function of the peripheral clocks. The logic was that if the expression of a subset of core clock genes was directly regulated by light, much in the way that light pulses induce period gene expression in the SCN [6, 15], then the phase of the clock could directly respond to changes in environmental lighting

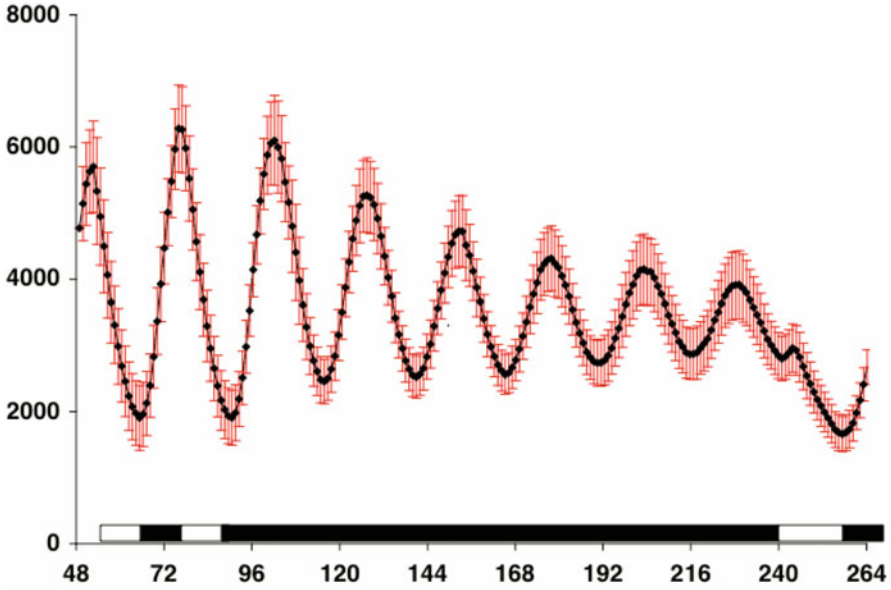


**Fig. 7.1** Light entrainable, rhythmic clock gene transcription in zebrafish cell lines. Plot of bioluminescence (counts per second, y-axis) produced from a zebrafish cell line stably transfected with a luciferase reporter gene under transcriptional control of the *per4* promoter. After addition of luciferin to the culture medium, measurements were performed using an automated scintillation counter. *Black* and *white bars* represent the periods of dark and light exposure, respectively. *Red vertical bars* indicate error bars calculated using data obtained from 12 individual wells. The *black* trace shows mean bioluminescence values. Numbers below the x-axis are hours after the onset of the experiment. Rhythmic gene expression is established under light-dark cycles and then persists with dampening following transfer to constant dark conditions

conditions. Exploring the regulation of clock gene expression in zebrafish was complicated by the existence of extra clock genes in fish. Thus, three *clock*, three *bmal*, six *cryptochrome* (*cry*) and four *period* (*per*) genes have been described [16–20]. Extra copies compared with mammals are not unique to clock genes but represent a property of many genes in fish. Gene duplication is predicted to have resulted from whole genome duplication events that occurred in the early ancestors of teleosts [21]. Comparing the expression of each clock gene under conditions of light-dark cycles and after transfer to constant darkness revealed that two clock genes in particular were directly light inducible, namely, *per2* and *cry1a* [22, 23]. Light-induced expression of both genes which serve as key elements of the negative limb of the core clock was observed in vivo as well as in vitro in zebrafish cell lines. In particular, CRY1a was shown to directly interact with the CLOCK-BMAL complex and thereby to act as a potent repressor of E-box-driven transcriptional activation [23]. By this mechanism, light-induced CRY1a expression serves to entrain the clock as well as to establish and maintain a high-amplitude rhythm [23]. PER2 appears to play a more complex, tissue-specific role in the clock. PER2 has been reported to maintain the zCLOCK:zBMAL heterodimer in the

cytoplasm resulting in transactivation repression [24]. Furthermore, a more recent study of PER2 loss-of-function fish generated by the TALEN method revealed PER2 may serve both as a transcriptional coactivator and a corepressor for Ror/Rev-erb response element enhancers and E-box enhancers, respectively [25].

The identification of these two light-inducible clock genes fuelled two avenues of investigation. The first tested whether these were the only two genes in the fish genome exhibiting light-inducible transcription. This seemed an unlikely scenario given previous studies that had demonstrated the expression of *6-4 DNA photolyase* (*Cry5*) gene were also induced upon exposure of early zebrafish embryos and fish cells to light [26]. This gene encodes a key enzyme of the DNA repair pathway in fish which is interestingly also a close relative of the Cryptochromes [20]. Specifically, DNA photolyase harnesses blue light by the use of flavin adeno-nucleotide (FAD), to serve as an energy source for catalysing the repair of UV light-induced 6-4 pyrimidine-pyrimidone photoproducts in DNA [27, 28]. Transcriptional induction of this gene in response to sunlight has also been observed in other fish species as well as in *Xenopus* and appears to serve as a strategy to enhance the cellular capacity to repair sunlight-induced DNA damage at times of day when UV light exposure is at its maximum [29, 30]. In order to extend the search for light-inducible genes, two studies employed a DNA microarray approach to screen for light-induced transcripts in the zebrafish transcriptome in 9 h post fertilization embryos, larvae, cell lines as well as heart organ cultures [31, 32]. These studies revealed a surprising diversity of light-induced genes, with a notable enrichment for genes involved in circadian clock regulation, DNA repair and stress response. This pattern revealed the importance of light-induced gene expression for coordinating many aspects of how fish cells respond to environmental challenges.

The second avenue of study that was also aided by the identification of many light-induced genes was to identify which promoter elements were responsible for directing light-driven transcription. This also represented an important step towards identifying upstream signalling pathways and ultimately the photoreceptors themselves. Thus, two studies focused on the *per2* and *cry1a* gene promoters using zebrafish cell lines as well as transgenic models to specifically examine pineal gland-specific *per2* gene expression [33]. These studies lead to the identification of light-responsive promoter regions [33, 34]. Further dissection of these regions revealed that both *cry1a* and *per2* shared D-box enhancer elements. Site-directed mutagenesis targeting these enhancers resulted in loss of light-induced gene expression. Furthermore, these enhancers alone, in the context of an artificial promoter, were sufficient to direct light-induced gene expression (See Fig. 7.2) [33, 34]. Interestingly, in the case of *per2*, D-box function was also dependent on the presence of a proximal E-box enhancer element which is the binding site for the core clock factors, CLOCK and BMAL [33]. Benefiting from the knowledge of a wider set of light-induced genes, bioinformatic analysis was performed to identify shared promoter elements. Interestingly, the promoters of many light-induced genes were also enriched for D-box enhancers [31, 32]. Together, these observations firmly established the D-box as a primary nuclear target for photoreception in fish cells and a key link in the clock photoentrainment pathway.

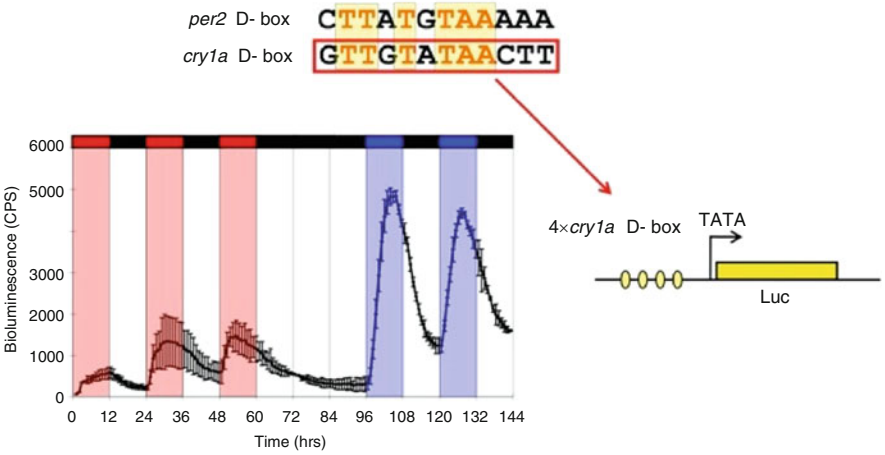


**Fig. 7.2** The D-box enhancers from the *cry1a* and *per2* gene promoters mediate light-driven gene transcription in fish cells. Alignment of the D-box enhancer sequences from both promoters are shown (*upper panel*) with identical sequences highlighted in *red*. Multimerized copies of the *cry1a* D-box were cloned upstream of an artificial basal promoter (TATA) driving expression of a luciferase reporter (Luc) (*right panel*). Plot of bioluminescence (counts per second, y-axis) produced from a zebrafish cell line stably transfected with the D-box luciferase reporter gene. *Black*, *red* and *blue bars* represent the periods of *dark*, *red* and *blue* light exposure, respectively. *Black vertical bars* indicate error bars calculated using data obtained from 12 individual wells. The *black* trace shows mean bioluminescence values. Numbers below the x-axis are hours (time) after the onset of the experiment. *Blue light* preferentially induces D-box-driven transcription

Interestingly, these results were not the first time that D-boxes had been linked with the circadian clock. In mammals, many studies had demonstrated that D-box enhancers play an important role in clock output pathways [35]. The D-box binding transcriptional activators, D-box binding protein (DBP), thyrotroph embryonic factor (TEF) and hepatic leukaemia factor (HLF) belong to the subset of bZIP transcription factors termed the proline- and alanine-rich (PAR) family. Furthermore, the PAR-related bZIP factor E4BP4/NFIL3 also binds to D-box sequences [36]. The expression of D-box binding factors had been shown to be clock regulated and therefore D-box enhancers also directed circadian rhythms of transcription. Thus, in mammals, D-box enhancers serve to convey circadian clock timing information to downstream gene regulatory networks [35]. This striking difference implies that during evolution of the vertebrate lineage, while the D-box enhancer itself has remained highly conserved, its function relative to the core clock mechanism has seen considerable adjustment, from being a target of the clock light input pathway in fish to being a clock output regulator in mammals.

Given the importance of D-boxes for light-regulated gene expression, attention is now focusing on the function of D-box binding transcription factors. Just as in the case of the core clock genes, there are many more D-box binding proteins in fish than in mammals. Thus, there are two genes encoding each of the DBP, TEF and HLF PAR factors as well as six homologues of the transcriptional repressor, E4BP4

[37]. Interestingly, these factors show a range of tissue-specific expression patterns during development; many are clock regulated and E4BP4-6 is an exclusively light-inducible gene [37]. This complex pattern is most likely consistent with these transcription factors fulfilling multiple functions in addition to their light transduction role.

### *7.2.2 Peripheral Photoreception*

Since the first observation of directly light-regulated peripheral clocks in zebrafish, a burning issue has been to identify the photoreceptors. Given the broad distribution of light entrainable peripheral clocks in fish, the simplest prediction was that these photoreceptors should be very widely expressed, even in cell types that are not specialized for photoreception. While the precise nature of these widely expressed photoreceptors remains unclear, several lines of evidence point to the presence of multiple photoreceptors, rather than one single photoreceptor.

The hot favourites for playing this role are opsins. Opsins belong to a large group of G-protein-linked membrane receptors which are normally around 350 amino acids (AA) long and form a palisade of seven α-helical transmembrane regions enclosing a ligand-binding pocket. In this pocket, the chromophore retinal is bound via a Schiff base linkage to a highly conserved lysine residue in the seventh helix. Absorption of a photon by the chromophore and the subsequent photoisomerization of this molecule from an 11-cis to an all-trans state is the first key step in the opsin response to light with its spectral sensitivity being determined primarily by the surrounding structure of the opsin protein [38]. Teleosts possess an unusually large repertoire of opsins (43 to date) [39], with the majority being expressed outside of dedicated photoreceptive tissues such as the retina and pineal gland. These include teleost-specific opsins such as multiple tissue (tmt) opsin as well as homologues of melanopsin, the principle non-visual circadian photoreceptor in mammals [39]. The striking number and expression pattern of opsins in fish tissues would be consistent with them serving as peripheral photoreceptors; however, it might also point to a non-photoreceptor function for these membrane proteins. For example, a recent study has implicated opsins in the thermotaxis of mammalian sperm [40]. Unlike the visual opsins which are expressed in the rod and cone photoreceptor cells, the signal transduction pathways that are triggered upon light activation of the non-visual opsins do not appear to be based upon cyclic nucleotides [41]. In the case of mammalian melanopsin, evidence points to the involvement of $G_{q/11}$-type G proteins and the phosphoinositide signalling pathway [42, 43]. Consistent with a role for melanopsin in zebrafish peripheral clock responses to light, a systematic pharmacological study aiming to dissect the signalling pathways underlying the response of a zebrafish cell line (ZEM-2S) to light has implicated a blue light-dependent mechanism involving phosphoinositide pathway signalling also involving cross talk with nitric oxide (NO) as well as MAPK [44].

A second class of candidates for the widely expressed fish peripheral photoreceptors are flavin-containing oxidases and amine oxidases. These serve many metabolic roles and upon exposure to blue light generate hydrogen peroxide ($H_2O_2$), a reactive oxygen species (ROS) [45, 46]. It has been demonstrated that visible light and UV light exposure all increase cellular $H_2O_2$ levels by activation of these enzymes in different model systems, including zebrafish [47, 48]. Flavin-containing oxidases are versatile flavoenzymes that catalyse molecular oxidation in numerous metabolic pathways, generating ROS as a by-product. However, the main physiological sources of extracellular $H_2O_2$ are the NADPH oxidases (NOXes), which transport electrons from cytoplasmic NADPH to generate $O_{2-}$ and then $H_2O_2$ or directly $H_2O_2$ in different cellular compartments [49]. Hydrogen peroxide has been frequently considered as a toxic cellular metabolite because it can react with various cellular targets such as DNA, proteins and lipids, causing cellular damage. However, it is now clear that it can also function as a signalling molecule that mediates responses to various stimuli in both plant and animal cells. The generation of $H_2O_2$ leads to the activation of protein kinases (mainly by oxidation of cysteine residues) followed by stimulation of downstream signalling systems including MAP kinase and PLCγ [50]. This thereby regulates various physiological systems in response to a variety of extracellular stimuli. In zebrafish it has been hypothesized that $H_2O_2$ transduces photic signals to ultimately activate the transcription of light-responsive genes, including clock genes as well as DNA repair genes involved in photoreactivation [47]. Furthermore, the expression of *cry1a* and *per2* has been reported to be upregulated by treatment of cells with $H_2O_2$ [51]. In turn, clock-driven changes in the endogenous levels of the antioxidant enzyme, catalase, may modulate the amplitude of light-induced clock gene expression via its effect on the levels of accumulating $H_2O_2$ [51]. Another flavin-containing protein that has been proposed as a candidate peripheral photoreceptor in fish belongs to the cryptochrome family. One of the cryptochrome homologues in teleosts, namely, CRY4, bears closer homology to *Drosophila* cryptochrome than to the mammalian cryptochromes [19, 20]. Given the demonstrated photoreceptive function of CRY in *Drosophila*, this has led to some speculation that CRY4 might also serve as a photoreceptor in fish [52, 53]. However, to date, there is little functional data pointing to the involvement of this cryptochrome in the light response of fish cells.

### *7.2.3 Cavefish and Blind Peripheral Clocks*

A large part of what we currently understand about peripheral clock light-sensing mechanisms has been obtained from studying zebrafish and zebrafish-derived cell lines. Access to omics resources as well as genetic tools has enabled the dissection of the molecular genetic basis of light-sensing peripheral clocks in fish. However, more recently other species, notably blind cavefish, have provided unique new insight into the fish circadian timing system and its response to light during evolution.

Blind cavefish represent a group of 100–200 unrelated species distributed worldwide which inhabit subterranean bodies of water and share a set of striking physiological and anatomical adaptations for life under complete darkness, so-called troglomorphisms. These are subdivided into regressive and constructive traits [54, 55]. Constructive traits are frequently linked with enhancing non-visual senses which allow fish to navigate without light as well as metabolic adaptations for surviving with limited sources of food. Instead, regressive traits are associated with loss of light-dependent functions that are predicted to serve no purpose in complete darkness. Regressive traits include most notably eye loss. It is thought that major colonization of cave environments came under the selective pressure of climate change that left surface habitats uninhabitable. For example, major colonization events have been linked with the Messinian salinity crisis during the Miocene (7 Ma) and Pliocene (5 Ma) eras and interglacial aridity in the Pleistocene era (2.5 until 0.5 Ma). Aquatic cave habitats not only lack light but also frequently feature high salinity, high temperatures, hypoxia and high concentrations of natural toxins. Therefore, cavefish also tend to show a significantly enhanced tolerance of harsh environments [56, 57]. In this regard, it is misleading to consider all cave habitats equivalent. The geology of each cave is unique. This defines the degree and duration of isolation from surface water, the properties of the water as well as the food availability. Thus, each cavefish population faces a very different set of challenges to its survival.

One fundamental consequence of life in constant darkness is that it involves isolation from the day-night cycle. Do cavefish that live in completely dark, relatively constant environments still have clocks? If so, do they still fulfil some important function? Do cavefish clocks still respond to light or other environmental signals? Fundamental to addressing these questions is an understanding of to what extent individual cave habitats are isolated from the day-night cycle. For example, although no regular sunlight may reach the cave water systems, it is still possible that food might be carried into the cave periodically by roosting bats and thereby impose a 24-h cycle on the cave environment [58].

To date, the circadian timing system has been studied intensively in only two species of cavefish, namely, *Phreatichthys andruzzii* and *Astyanax mexicanus* [58, 59]. *P. andruzzii* is a Somalian cavefish, exhibiting a particularly extreme troglomorphic phenotype [59, 60]. This fish inhabits layers of water contained within limestone rock formations deep beneath the Somalian desert. The only contact between the water layers where *P. andruzzii* lives and the surface is small, deep natural wells in the desert. Based on the geology of the region, it is predicted that these animals have been isolated from the surface for over 2 Ma as a result of desertification in that region of Africa [60]. Consistent with the relatively long period of isolation in their cave environment, these fish show extreme troglomorphisms such as a complete loss of eyes and body pigmentation as well as an ability to survive long periods of starvation. Upon artificial exposure to light-dark cycles, *P. andruzzii* fail to display activity rhythms that are typical of other surface fish such as the zebrafish (see Fig. 7.3) [59]. Importantly, arrhythmicity is also observed at the level of clock-regulated gene expression in many tissues. Does

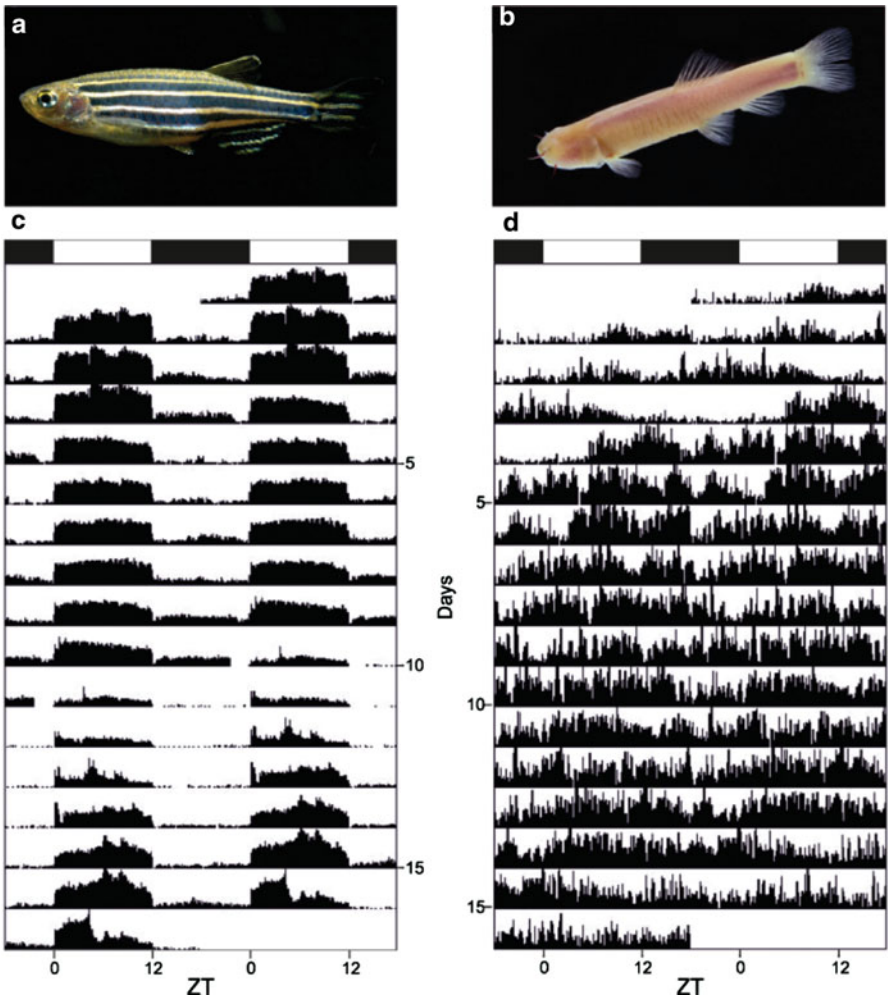


**Fig. 7.3** General locomotor activity of adult zebrafish (panels **a** and **c**) and *Phreatichthys andruzzii* (panels **b** and **d**) exposed to a light (*white bars*) and dark (*black bars*) cycle. Tanks of fish (ten animals per tank) were monitored using an infrared beam/detector system and then double plotted as an actogram

this mean that *P. andruzzii* lack a functional clock, or alternatively, do they have a blind clock? This question has been addressed by establishing cell cultures from *P. andruzzii* fin clips and then artificially inducing clock function in these cells by transient treatment with serum [59]. These in vitro experiments revealed that *P. andruzzii* cells do indeed contain circadian clocks and confirmed that these clocks are effectively blind. Cloning and sequencing candidate photoreceptors revealed that *P. andruzzii* carries mutations in the two non-visual opsins: melanopsin (Opn4m2) and TMT opsin which, as discussed earlier, are both widely

expressed in fish tissues (see Fig. 7.4). By rescue experiments where wild-type zebrafish opsin sequences were ectopically expressed in the cavefish cells, it was confirmed that the loss of function of these two opsins at least in part accounts for the blind peripheral clock phenotype of this cavefish [59]. Thus, this cavefish data further reinforces the notion that opsins serve as central photoreceptors for fish peripheral clocks.

*P. andruzzii* cell culture experiments also revealed another strange property of these cavefish clocks: namely, they tick with an abnormally long, infradian rhythm of around 40 h instead of the approximately 24-h rhythms typically observed for circadian clocks [59]. It is tempting to interpret this striking result as evidence that this species is in the process of losing its normal circadian clock, thus representing a regressive trait. However, regular feeding (one meal provided at the same time each day) is sufficient to establish robust 24-h activity rhythms in the adult cavefish. More specifically, each day the fish become significantly more active just before food is available. Furthermore, in these regularly fed fish, robust 24-h rhythms of clock gene expression are observed in most tissues [59]. This result conflicts with the cell culture data that show abnormal infradian rhythms and indicates that *P. andruzzii* does possess a strongly food-regulated circadian clock. It is tempting to speculate that there may be a strong selection to maintain a food-responsive, 24-h clock in the subterranean environment inhabited by *P. andruzzii*, and thus this could represent an adaptive trait. Specifically, food might be available at a regular time each day and so it is vital that the fish are prepared to consume it.

The second cavefish species where the circadian clock has been studied is the Mexican tetra: *Astyanax mexicanus*. The cave environments of this species differ fundamentally from those of *P. andruzzii* in that they are connected with surface water and rivers. Thus *A. mexicanus* is significantly less isolated than *P. andruzzii*. Combined with a shorter period of isolation in the cave habitat (predicted to be in the order of 100,000 years), this has resulted in less extreme troglomorphic phenotypes than observed in *P. andruzzii*. Furthermore, *A. mexicanus* has successfully colonized both surface and cave environments. The surface forms of this species have normal body pigmentation and eyes and superficially look like different species when compared with their eyeless, pink/white cave relatives. The possibility to cross these surface and the cave forms and to generate fertile hybrid offspring has led to this species being used as a powerful model to study the genetic basis of troglomorphic phenotype [61, 62]. Another important difference between the cave habitats in Somalia and Mexico is that the Mexican caves are also the roosting site for bats. These fly in and out of the cave systems each dawn and dusk in order to feed, and their return to the cave and the associated production of guano represents a regular supply of food for the fish from the outside world [58]. Thus in many ways, *A. mexicanus* has a closer link with the day-night cycle than *P. andruzzii*.

Possibly reflecting these environmental differences, the cave forms of *A. mexicanus* still possess light entrainable circadian clocks showing relatively subtle differences compared with normal surface fish [58]. More specifically, under artificial laboratory light-dark cycle conditions, both surface and cave *A. mexicanus* exhibit cycles of clock gene expression which are more dampened

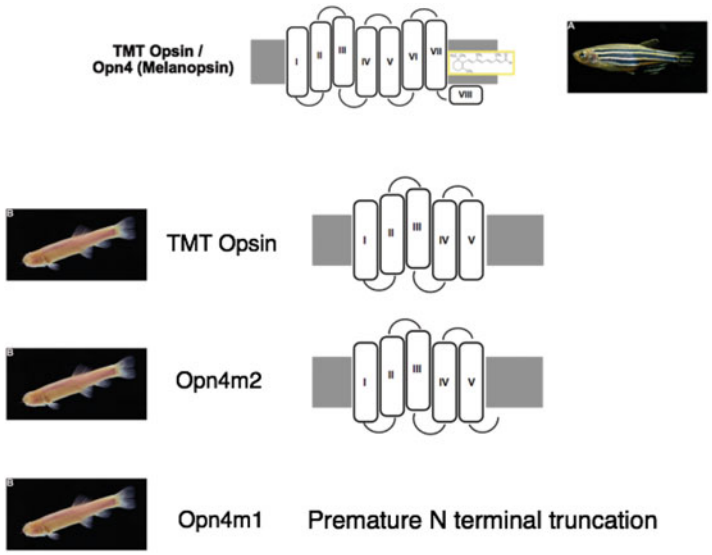


**Fig. 7.4** Schematic representation of the wild-type TMT and Opn4 (melanopsin) photoreceptors in zebrafish (*upper panel*) and the truncated versions in *P. andruzzii* (*lower panels*). The normal location of the retinal chromophore covalently linked to the seventh membrane-spanning segment is shown in the zebrafish cartoon. In the case of *P. andruzzii* Opn4m1, a premature stop codon at the start of the open reading frame leads to no protein being produced

in the cave forms. Curiously, based on the particular expression profiles of a range of clock genes, it has been concluded that these dampened rhythms are characteristic of exposure to constant light [58]. In the actual cave environments, certain *A. mexicanus* populations show no rhythmic clock gene expression but again evidence for sustained upregulation of clock genes normally driven by light. The significance of these molecular changes remains unclear, but these findings reinforce the notion that cavefish adaptations related to circadian rhythms and photoreception are very much environment specific.

### *7.2.4 Future Perspectives*

The organization and many aspects of the function of the circadian timing system have been impressively conserved during evolution, even between vertebrates and *Drosophila*. So the lessons we can learn by studying how fish peripheral clocks respond to light will have direct relevance for pinpointing the most conserved and, thus, the most critical elements linking light with clocks even in mammals. However, we now face some major challenges in order to further advance our understanding of light entrainable peripheral clocks in fish. For a start, we have to address the apparent huge complexity in the repertoire of fish photoreceptors. In order to

test the functional contribution of individual photoreceptor genes, one obvious next step would be to take advantage of genome engineering techniques such as CRISPR-Cas9 in zebrafish and medaka to generate loss-of-function mutations in each of the 43 known opsins as well as other candidate photoreceptors. Subsequent crosses between different mutants would then also allow us to test the effect of combinations of mutations on peripheral photoreception. The inevitable large scale of this analysis would certainly make this a challenging task. For this reason it would also be beneficial to complement this systematic reverse genetic analysis by a comparative study involving the blind cavefish model *P. andruzzii* which during evolution has completely lost the direct light-sensing property of peripheral clocks. Studying which photoreceptor genes are mutated in this model and then testing their functionality by in vitro rescue experiments have proved to be an effective strategy which already highlighted the importance of TMT and Opn4m2 opsin function and could be extended to assess the role of all the other candidate photoreceptors.

Another challenge involves exploring the functionality of the multiple D-box binding transcription factors in fish. Like other bZIP transcription factors, D-box binding factors are able to bind their DNA target as heterodimers as well as homodimers. Thus, the repertoire of D-box enhancer regulatory factors is potentially great. Given the involvement of D-boxes in regulating many physiological systems, it will clearly be imperative to pinpoint which dimeric factor combinations are involved in directing light-induced gene expression. In turn, studying precisely how post-translational protein modifications regulate the function of these transcription factors in response to light combined with knowledge of the upstream photoreceptors will provide us with important clues as to which intracellular signalling pathways link light with the D-box enhancer and changes in clock gene expression. Clearly, exciting times lie ahead!

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# Chapter 8
# Molecular Genetic and Genomic Analyses of Zebrafish Circadian Rhythmicity

**Zhaomin Zhong, Mingyong Wang, Guodong Huang, Shuqing Zhang, and Han Wang**

**Abstract** Evolving from long-term adaptation of life to the cyclic physical environment of the Earth, the circadian clock as endogenous and self-sustained timekeeping mechanisms plays modulatory roles in various fundamental life processes from molecular, biochemical, cellular, physiological, to behavioral levels. Circadian dysrhythmias lead to malfunctions of the body and numerous diseases. The zebrafish (*Danio rerio*) as an important animal model has recently become attractive for investigating regulatory mechanisms of vertebrate circadian clocks. In this chapter, we attempted to summarize the latest progresses of utilizing mutational analysis, transgenic technique, and transcriptome tools to delineate molecular genetic and genomic mechanisms underlying zebrafish circadian rhythmicity.

The Earth has the unique geophysical condition where environmental factors such as light and temperature change cyclically with a period of approximately 24 h. Through long-term adaption to this cyclic environment, organisms have evolved endogenous and self-sustained timing-keeping mechanisms, namely, the circadian clock. The circadian clock modulates almost all fundamental life processes from molecular, biochemical, cellular, physiological, to behavioral levels and allows for organisms to anticipate environmental changes and coordinate physiological and metabolic homeostasis. In mammals, the core oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus drives circadian rhythms, such as hormonal production and activities of neural circuits [1]. In nonmammalian vertebrates including zebrafish, the pineal gland is sensitive to light, secretes melatonin rhythmically, and regulates the circadian system [2].

In almost all animals studied, the core mechanism underlying the circadian clock is based on transcription/translation negative feedback loops. In mammals, PAS (PER-ARNT-SIM) domain-containing Clock and Bmal as positive regulatory elements form a complex that binds to E-boxes in the promoters of *Per* and *Cry* genes. PER and CRY proteins form another complex that enters to the nucleus and

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represses Clock-Bmal-mediated transcriptional activities through protein-protein interactions [3]. Periodic interactions of these circadian molecules generate molecular oscillations that drive rhythmic expression of numerous genes involved in fundamental life processes, such as metabolism, physiology, and behavior [3].

As a nonmammalian vertebrate animal model, zebrafish have been widely used in developmental genetic and medical studies [4]. The characteristics, composition, and operational mechanisms of the zebrafish circadian system are highly similar to those of the mammalian circadian system but with many advantages, such as short generation time, easy to breed and maintain, transparent embryos, external development, and embryonic circadian regulation independent of the maternal influence [3, 5]. With the recent rapid development of TALEN (transcription activator-like effector nuclease) and CRISPR-Cas9 (clustered, regularly interspaced, short palindromic repeats-CRISPR-associated (Cas) systems) genome-editing technologies, it becomes relatively easy to perform genetic manipulations such as generation of transgenic, knockout, knock-in zebrafish lines. Taking advantage of zebrafish as an attracting vertebrate model and new genome-editing methods, the zebrafish circadian field has developed rapidly in recent years, especially in mutational, transgenic, and transcriptome analyses of zebrafish circadian rhythmicity. This chapter primarily reviewed important progresses concerning molecular genetic and genomic analyses of the zebrafish circadian clock.

## 8.1 The Zebrafish Circadian System and Its Physiological and Behavioral Rhythms

The zebrafish circadian system has positive regulatory elements, Clock and Bmal, and negative regulatory elements, Per and Cry. Clock and Bmal form a complex that recognizes and binds to the E-box motifs in promoters of *per*, *cry*, and other downstream genes, activating their transcription. Per and Cry proteins form a complex that is translocated to the nucleus and interacts with the Clock-Bmal complex to inhibit its transcription activities (Fig. 8.1). In addition, another feedback loop composed of Rev-erbα/Nr1d1 and Rorα modulates *bmal* expression and stabilizes the core loop (Fig. 8.1) [6].

Zebrafish underwent the third round of the teleost genome duplication (TGD) [7]. As such, many zebrafish genes have extra copies compared with other vertebrates [8]. During evolution following the TGD, some extra copies may be lost and some retained [7]. Some of these multiple genes retain conserved functions; others appear diverged and function differently [7]. In zebrafish, there are three *bmal* genes [9], three *clock* genes [8], four *per* genes [10], and six *cry* genes [7] (Fig. 8.1). The existence of extra copies of circadian clock genes is thought to facilitate evolution of a complex and sophisticated circadian regulatory system in zebrafish.

The zebrafish pineal gland has typical photoreceptor cells and gene expression profiles similar to those in the retina [11] and possesses an inherent circadian clock

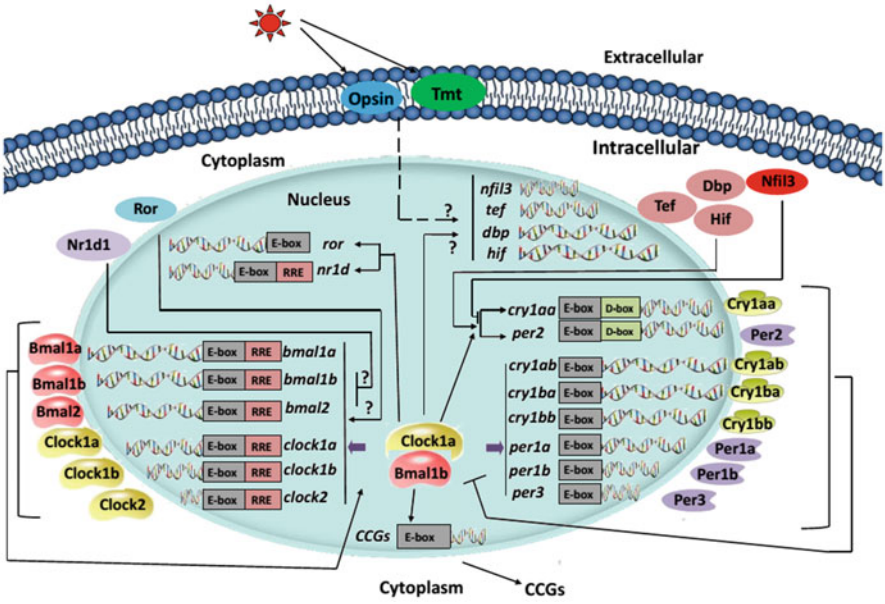


**Fig. 8.1** A zebrafish clockwork model. There are three *cloc*k genes, three *bmal* genes, four *per* genes, and six *cry* genes in zebrafish. Transcription-/translation-based negative feedback loops composed of these circadian clock genes and their proteins control the zebrafish circadian clock. Clock and Bmal form heterodimers that activate transcription of *per* and *cry* genes, while Per and Cry form a complex that turns off their own transcription by interacting with Clock-Bmal heterodimers. Clock-Bmal heterodimers also activate transcription of *nrd1*/*rev-erb*, *ror*, *nfil3*/*e4bp4*, *pdp*, *hif*, *tef*, and circadian controlled genes (CCGs) via E-boxes in their promoter regions. Nr1d1α/Rev-erbα and Rorα in turn regulate transcription of *bmal* genes through binding the RRE motif, while Nfil3/E4bp4 and Dbp in turn regulate expression of *cry1aa* and *per2* via binding the D-box. External light cue can be received by opsins and/or TMT opsins and induces expression of *cry1aa* and *per2* through Tef via D-box. RRE-/RORE-, Rev-erb-, and Ror-responsive element-binding site

that drives periodic melatonin synthesis. The Clock-Bmal heterodimer can regulate transcription of *arylalkylamine-N-acetyltransferase* (*aanat*), encoding the rate-limiting enzyme of melatonin synthesis, resulting in the level of melatonin lower during the day but higher during the night [11]. Although *aanat* is modulated by the circadian clock, its protein can be quickly suppressed and degraded with illumination during the night [12]. Environmental light-dark information is translated into neural signals and/or neuroendocrine signals through the pineal gland, which is regarded as the central circadian pacemaker in zebrafish [13].

An important output of the circadian clock is rhythmic locomotor behaviors. Mice and fruit flies display significant behavioral rhythms [14, 15]. Gregory Cahill and colleagues first demonstrated that larval and adult zebrafish display locomotor rhythms, and the average period of wild-type zebrafish larvae is 25.6 h [16, 17]; they also observed that exposure to light in the second day of development is critical for activation and entrainment of behavioral rhythms, and in particular

exposure to light-dark condition during the first 4 days of development greatly enhances the rhythms of zebrafish larvae [17]. More than 65 % of adult zebrafish display approximately 24-h locomotor rhythms only at 21 °C, but lower temperature or higher temperature significantly reduces percentages of adult zebrafish displaying locomotor rhythms [16].

Different from mice but the same as humans, zebrafish are diurnal animals, highlighting an advantage of zebrafish for studying the circadian clock. Small-size zebrafish larvae are readily suitable for high-throughput behavioral assays in 96-well plates. A 96-well plate can be placed in a closed box, on the top of which an infrared camera can be installed, a separate module can adjust the brightness of the light source, and a controller can adjust temperature (Fig. 8.2) [18]. Zebrafish larvae placed in a 96-well plate can be continuously recorded in terms of trajectory, distance, and speed for several days (Fig. 8.2). Zebrafish larvae display significant locomotor rhythms under LD (light/dark) and DD (constant darkness) conditions. Using the behavior assays, Yoav Gothilf and colleagues reported that CK1δ inhibitors, rather than CK1ε inhibitors, can disrupt locomotor rhythms of zebrafish larvae [19]; and melatonin treatment can significantly reduce locomotor rhythms of zebrafish larvae [20]. Alexander Shier and colleagues have conducted large-scale high-throughput behavioral assays and discovered hundreds of small compounds that affect zebrafish locomotor rhythms and sleep [21].

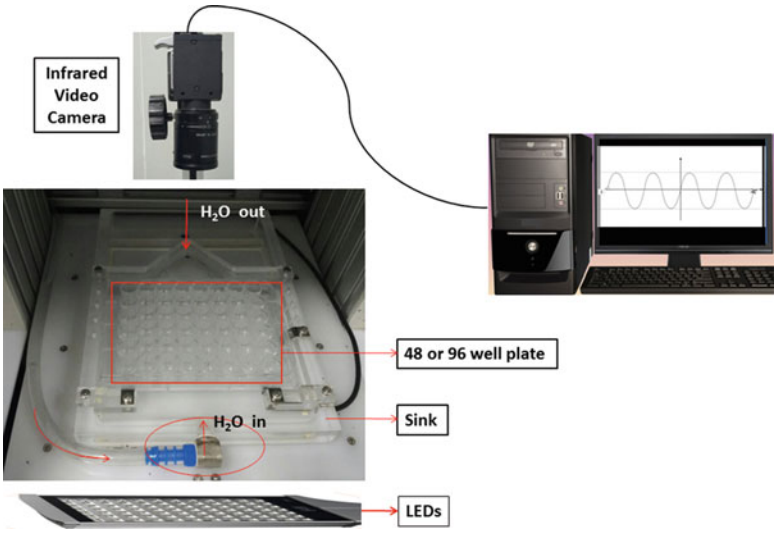


**Fig. 8.2** Zebrafish larval locomotor assays. Zebrafish larvae are placed in single well of 48- or 96-well plate. The plate is placed in a box that is light controlled by LEDs and has a temperature control unit. Locomotor activities are continuously monitored and recorded by an infrared camera and analyzed by DanioVision (Noldus) or ZebraLab (ViewPoint) software

## 8.2 Mutational Analysis of Zebrafish Circadian Rhythmicity

Mutants with gene deficiencies or deletions are useful for studying gene functions. ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis and then forward genetics that starts with phenotype screens [22] or reverse genetics such as TILLING (targeting-induced local lesions in genomes) that screens the mutated genes [23] have been employed in traditional zebrafish studies. Gregory Cahill and colleagues conducted ENU mutagenesis and screened 6,500 $F_2$ generation larvae using behavioral arrays and found eight zebrafish mutants that showed 1–2.5-h period differences compared with wild types, including seven mutants with shortened period and one with lengthened period [24]. They characterized a mutant, named $lag^{dg2}$, found that the locomotor period was 24 h for the homozygous mutant and 24.7 h for the heterozygous mutant, whereas the locomotor period was 25.3 h for the wild-type zebrafish, and there was no obviously morphological defects in homozygous mutant zebrafish [24]. They then examined melatonin secretion of ex vivo pineal glands of the homozygous, heterozygous, and wild-type adult fish at 21 °C and found that the period of melatonin secretion of homozygous mutant zebrafish was 0.7 h shorter than that of wild-type and heterozygous mutant fish pineal melatonin secretion. To determine the genetic nature of the mutant, they mapped the mutation with microsatellite markers and determined that the mutation was located on chromosome 7 wherein the only known clock gene is *per1b*. However, DNA sequencing did not detect any mutation in the *per1b* coding sequence [24]. Therefore, there may be an undiscovered clock gene that was mutated for the observed phenotype. Gregory Cahill and colleagues also reported a second circadian mutant – P36. The locomotor period was 24.3 h for the homozygous mutant and 24.8 h for the heterozygous mutant, while the locomotor period was 25.3 h for the wild-type zebrafish. Further, melatonin secretion period was 24.1 h for the homozygous mutant and 24.9 h for the heterozygous mutant. Molecular genetic analysis revealed that the 254th amino acid of Clock1a was mutated from isoleucine to asparagine in this mutant, which is located in the important PAS B functional domain [25].

However, the ENU mutagenesis method has disadvantages, such as time-consuming and tedious, often requiring screening thousands of lines and also cloning mutation sites by genetic mapping [26]. Generation of mutations by ENU mutagenesis is random such that ENU can hit multiple genes. Therefore, several generations of outcrossing are needed to obtain a single-gene mutant. Behavioral assays used in the Cahill screen were also limited in that small period differences make subsequent genetic mapping extremely difficult [24]. The alternative approach is retroviral insertion mutagenesis. Compared with ENU mutagenesis, retroviral insertion mutagenesis can quickly identify the virus-inserted loci and obtain mutants of interest [27]. We have obtained *per1b* mutant zebrafish by retroviral insertion mutagenesis [18]. The retrovirus was inserted into the first intron of the *per1b* gene, resulting in termination of *per1b* transcription [18]. Real-time quantitative PCR and in situ hybridization showed that expression

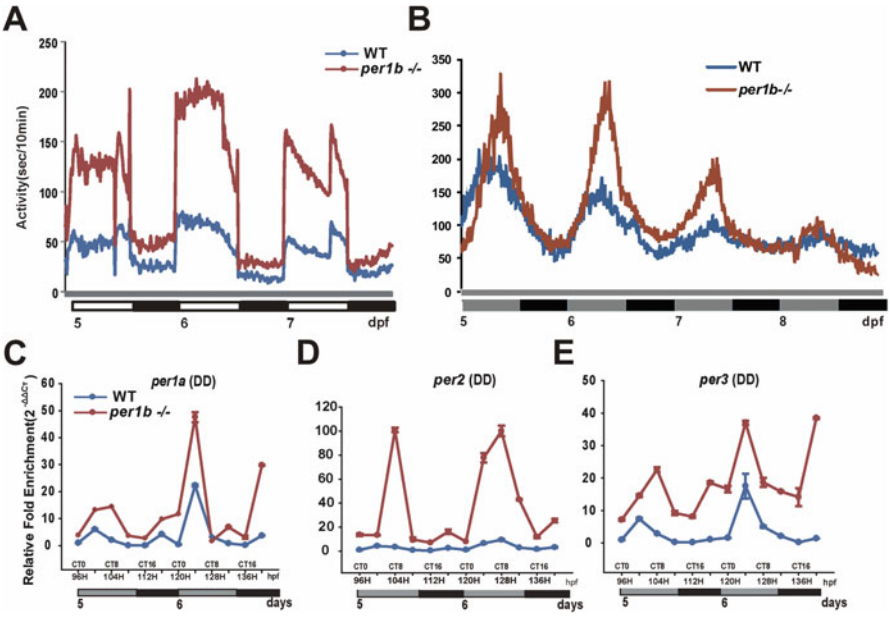


**Fig. 8.3** Characterization of retroviral insertion *per1b* mutant zebrafish. (**a**) The *per1b*−/− mutant larvae display hyperactive locomotor behaviors under LD. (**b**) The *per1b*−/− mutant larvae exhibit an approximately 1.2-h shortened period and an approximately 2-h advanced phase under DD. (**c**–**e**) Under DD, *per1a* (C), *per2* (D), and *per3* (E) are all upregulated in *per1b*−/− mutant zebrafish during both subjective daytime and nighttime as shown by quantitative RT-PCR, suggesting Per1b plays a repressive role in zebrafish circadian regulation. *hpf* hours postfertilization, *dpf* days postfertilization, *CT* circadian time, *DD* constant darkness (see Huang et al. [18] for details)

of *per1b* is nearly undetectable in the insertion mutant, and immunohistochemistry using Per1b antibody did not detect any signals in the mutant brain and eyes, indicating this is a *per1b*−/− null mutant. Behavioral assays showed that the locomotor period of the *per1b* −/− mutant larvae was approximately 1.2 h shorter than that of wild types with advanced phase and enhanced amplitude under DD (Fig. 8.3a, b). All three other *per* genes are significantly upregulated in the *per1b*−/− mutant (Fig. 8.3c, d), indicating Per1b plays a negative role in the zebrafish circadian clock [18]. We also demonstrated that *per1b*−/− fish resembles human attention-deficit/hyperactivity disorder (ADHD), displaying distinct behavioral characteristics such as hyperactivity, learning and memory deficits and impulsivity, and reduced levels of endogenous dopamine [18].

The recent advent of versatile genome-editing technologies, TALEN and CRISPR-Cas9, has made the generation of zebrafish mutants relatively easy [28, 29]. In the TALEN system, the two TALE domains are the DNA-recognizing component and the two FokI domains are the DNA cleavage component; a pair of TALEN as an engineered nuclease can target genes of interest [30]. Since its first use in yeast in 2010, TALEN has been used widely in human

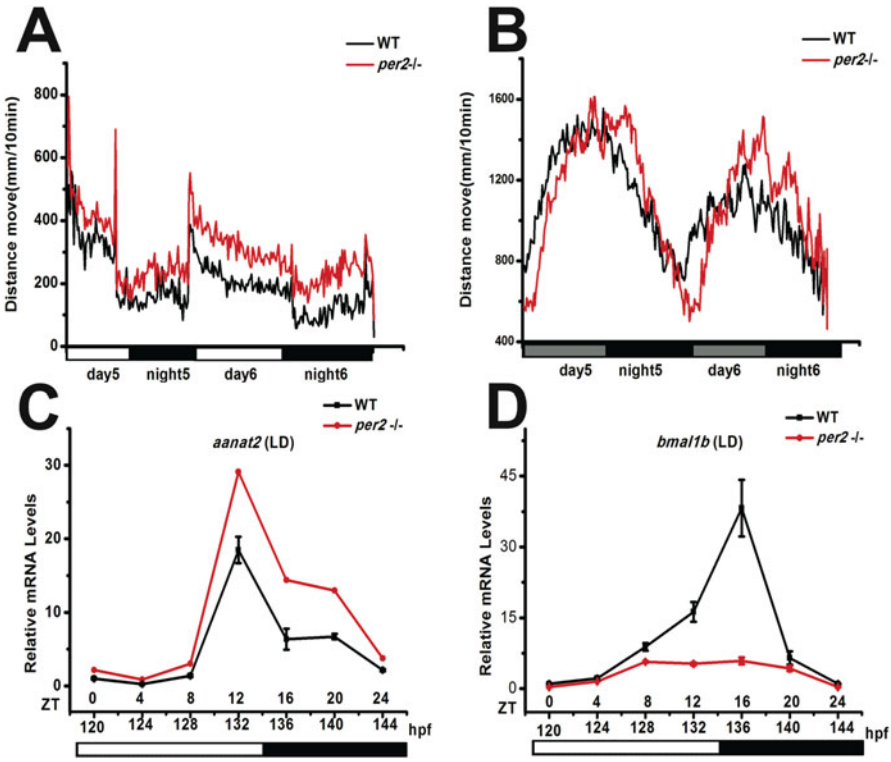


**Fig. 8.4** Characterization of TALEN-generated *per2* mutant zebrafish. (**a**) The *per2*−/− mutant larvae display lower locomotor activities under LD. (**b**) The *per2*−/− mutant fish show an approximately 1.2-h lengthened period and an approximately 2-h delayed phase under DD. (**c**–**d**) qRT-PCR analysis showed that *aanat2* is upregulated but *bmal1b* is downregulated in *per2*−/− mutant fish, indicating that Per2 plays dual roles in zebrafish circadian regulation. *hpf* hours postfertilization, *LD* light-dark condition, *ZT* Zeitgeber time (see Wang et al. [39] for details)

cells, mouse, rat, cow, pig, frog, zebrafish, medaka, fruit flies, silkworm, rice, and other organisms [30–36]. Numerous laboratories have optimized the TALEN method for improving its efficiencies [37, 38]. We used TALEN to generate *per2* mutants [39] and determined that the locomotor period of *per2*−/− mutant larvae was lengthened approximately 1.1 h with delayed phase and reduced amplitude under DD (Fig. 8.4a, b); Per2 can inhibit the *aanat2* expression through the E-box but enhance Rorα-mediated *bmal1b* expression via RORE (Fig. 8.4c, d), whereby playing dual roles in the zebrafish circadian clock [39].

In the CRISPR-Cas9 system, the single guide RNA (gRNA) is in charge of DNA-recognizing and the Cas9 endonuclease in charge of DNA cleavage. One single gRNA along with the Cas9 protein suffices to generate site-specific DNA double-strand breaks (DSBs) that trigger the endogenous nonhomologous end joining (NHEJ) DNA repair pathway to induce indel mutations in targeted genes in numerous species including zebrafish [29]. CRISPR-Cas9 has been used in a wide range of different

**Table 8.1** Zebrafish circadian clock mutants generated in the Wang laboratory

| Gene name | Ensemble ID | Mutated method |
|---|---|---|
| *per1a* | ENSDARG00000056885 | CRISPR-Cas9 |
| *per1b* | ENSDARG00000012499 | Retroviral insertion |
| *per2* | ENSDARG00000034503 | TALEN |
| *per3* | ENSDARG00000010519 | TALEN |
| *cry1aa* | ENSDARG00000045768 | TALEN |
| *cry1ab* | ENSDARG00000011583 | TALEN |
| *cry1ba* | ENSDARG00000069074 | ENU and TILLING |
| *cry1bb* | ENSDARG00000091131 | CRISPR-Cas9 |
| *cry2* | ENSDARG00000024049 | TALEN |
| *cry3* | ENSDARG00000011890 | TALEN |
| *clock1a* | ENSDARG00000011703 | TALEN |
| *clock1b* | ENSDARG00000003631 | ENU&TILLING |
| *clock2* | ENSDARG00000016536 | CRISPR-Cas9 |
| *bmal1a/arntl1a* | ENSDARG00000006791 | CRISPR-Cas9 |
| *bmal1b/arntl1b* | ENSDARG00000035732 | ENU&TILLING |
| *bmal2/arntl2* | ENSDARG00000041381 | TALEN |
| *timeless* | ENSDARG00000078497 | TALEN |
| *nfil3-5/e4bp4-5* | ENSDARG00000094965 | TALEN |
| *rev-erbα* | ENSDARG00000033160 | TALEN |

species as quickly as TALEN [40–44] and has developed a number of applications – such as multiple gene knockout simultaneously and deletion of long-fragment DNAs or miRNAs – allowing for examining phenotypes in $F_0$ generation with high mutation efficiencies [37, 45, 46]. Many zebrafish clock genes possess multiple copies, which have been difficult for generating mutants. CRISPR-Cas9 is readily available for tackling such a problem. Our laboratory has generated a full set of zebrafish mutants for core clock genes with TALEN, CRISPR-Cas9, and other approaches (Table 8.1), which should facilitate advancement of the zebrafish circadian field.

## 8.3 Transgenic Analysis of Zebrafish Circadian Rhythmicity

External fertilization and development, large clutch size, and ease to work with make it relatively easy to generate transgenic zebrafish lines [47]. Transgenic zebrafish have played important roles in developmental genetics, such as studying the expression patterns and functions of one particular protein, migration of various cells, and occurrence of disease processes [48]. Likewise, transgenic technology also has been useful for studying the zebrafish circadian clock.

The pineal gland as a photoreceptive organ serves as the central oscillator in zebrafish [49]. Gothilf et al. generated zebrafish *Tg*(*aanat2*: *EGFP*)$^{y8}$ transgenic

line displaying pineal gland-specific expression of GFP (green fluorescent protein) [50]. Through crossing this transgenic line with two known pineal gland mutants *flh* (*floating head*) and *mib* (*mindbomb*), they observed that the pineal GFP fluorescence intensity is reduced in the *flh*−/− background but elevated in the *mib*−/− background [50]. Yoav Gothilf and colleagues used this pineal transgenic line to demonstrate that expression of light-induced *per2* is crucial for *aanat2* rhythms established in zebrafish pineal gland [51]; and the pineal fluorescence intensity of this transgenic fish was weaker in *otx5*-knocking down larvae than in the control group, indicating *otx5* is upstream of *aanat2* [52].

Igor Dawid and colleagues used microarrays to analyze the pineal gland dissected out from this transgenic line and revealed approximately 500 differentially expressed genes between noon and midnight [53]. They found that *unc119* is rhythmically expressed [53]. Yoav Gothilf and colleagues used microarrays and bioinformatics tools to survey major *cis*-acting elements in the pineal gland and found that TAATC is the most important Crx/Otx binding site, while CAATC is another binding motif specifically in the pineal gland [54]. Lei Li and colleagues generated *Tg*(*Gnat2*: *gal4-VP16*/*UAS*: *nfsBmCherry*) transgenic zebrafish lines and showed that both retinal and pineal photoreceptor cells are required for maintaining circadian rhythms of visual sensitivity, even though retinal photoreceptor cells alone can sense the light signals and entrain circadian rhythms of visual sensitivity [49].

The luciferase reporter system also has been important for studying the zebrafish circadian clock. Cahill and Kaneko generated the transgenic zebrafish luciferase line driven by the *per3* promoter and observed that bioluminescence intensities of this transgenic fish exhibit a strong rhythm under LD for 6 days at 22 °C [55]. They also examined ex vivo organs of this transgenic line and found that bioluminescence intensities have strong circadian rhythms in the heart, kidney, and gall bladder under DD and LL (constant light) conditions, while bioluminescence expression shows a strong rhythm in the retina at higher temperature [56], consistent with previous studies using RNA expression data [57–59]. Steve Kay and colleagues also used this transgenic zebrafish to verify a small compound *longdaysin* that can prolong the circadian cycle [60].

Thomas Dickmeis and colleagues generated *Tg*(*4xE-box*:*Luc*) transgenic lines harboring luciferase driven by a small promoter and four E-boxes and observed that the transgenic fish show strong bioluminescence rhythms under LD and DD conditions [61].

## 8.4 Transcriptome Analysis of Zebrafish Circadian Rhythmicity

The advent of new DNA sequencing technologies should help discover new genes. Several studies employed DNA microarray and/or RNA sequencing (RNA-seq) analyses to dissect zebrafish circadian rhythmicity. Yoav Gothilf and colleagues used both

microarrays and RNA-seq to analyze the adult pineal glands [62]. They dissected out the pineal gland from the *Tg*(*aanat2*:*EGFP*)$^{y8}$ transgenic zebrafish under fluorescence microscopy. Total RNAs were extracted from 12 samples of the pineal glands at 4-h interval for 2 consecutive days under DD [62]. Microarray analysis revealed 36 rhythmically expressed genes, while RNA sequencing analysis uncovered 279 rhythmically expressed genes, including 30 overlapping genes revealed by both the methods. Among these 30 overlapping rhythmically expressed genes, nine are known core clock genes or clock-controlled genes; they also used independent quantitative real-time PCR or whole mount in situ hybridization to reconfirm rhythmic expression of randomly selected eight genes, indicative of validity of the two methods [62]. The authors also characterized pineal gland-specific gene *camk1gb* (*calcium/calmodulin-dependent protein kinase IGb*). The locomotor amplitude of the morphant larvae generated by microinjecting morpholino against *camk1gb* was significantly reduced, which can be rescued by *camk1gb* mRNAs; and knocking down *camk1gb* also significantly downregulated expression of *aanat2* but not key circadian clock genes such as *per1b* [62]. Therefore, *camk1gb* appears to be a downstream gene of the circadian clock, and facilitates melatonin secretion by regulating expression of *aanat2*, whereby playing a role in zebrafish locomotor rhythms [62].

Many clock genes or clock-controlled genes are expressed rhythmically only in particular tissues/organs, which has been difficult for identifying them. Because of small-size zebrafish larvae, they have been used as whole-animal for transcriptome analysis [63]. Jun Yan and colleagues used microarrays to analyze zebrafish larvae at 4-h interval from day 5 to 6 postfertilization, revealing that 2, 856 genes were rhythmic under both LD and DD conditions, accounting for approximately 17 % of the total expressed genes in zebrafish larvae. The vast majority of these genes are expressed in the retinal photoreceptor layer and intestinal bulb; 94 % of them displayed phase differences less than 4 h under both LD and DD conditions [63]. Intriguingly, they observed that expression levels of 233 genes are lower under DD than under LD, while expression levels of 21 genes are higher under DD than LD, indicating that these genes may be affected significantly by light [63]. Among these light-affected genes, *mitfa* (*microphthalmia-associated transcription factor a*) controls melanin formation. In zebrafish, *mitfa* is rhythmically expressed primarily in pigment cells and retinal pigment epithelial cells [63].

Light is the most important external signal affecting the circadian clock. Nicholas Foulkes, Thomas Dickmeis, and colleagues used microarrays to analyze light-treated zebrafish larvae, ex vivo cardiac tissues, and cell lines that were raised under DD and revealed 117 light regulatory genes, including 90 light-inducible genes and 27 light-repressive genes, which function largely in circadian regulation, stress responses, retinal photoreception, metabolism, or DNA repair [64]. Among light-inducible genes, *per2* expression levels were raised 30 times in larvae and 23 times in cell lines after 3-h illumination, while *cry1aa* and *cry1ba* were significantly upregulated in larvae and cell lines with light treatment [64]. In addition, *bmal2* in larvae, *clock1b* in ex vivo heart tissue and *cry2* in cell lines were also upregulated [64]. They observed that the promoters of these light-inducible genes harbor D-box and E-box motifs, which can be bound by circadian proteins [64].

## 8.5 Perspectives

Zebrafish are an attractive model for studying circadian rhythmicity. Using transgenic, mutational, and transcriptome approaches, zebrafish circadian studies have made rapid strides in recent years. However, zebrafish circadian regulatory mechanisms remain largely unexplored. With the advent of versatile genome-editing tools [29, 30], new generation of DNA sequencers [62], and in particular single-cell sequencing technology [65], the zebrafish circadian field is ushering in "a golden era."

In-depth characterization of zebrafish mutants for known circadian clock genes (Table 8.1) will uncover important functions of these genes in the circadian clock and also reveal their roles in fundamental life processes such as development, growth, reproduction, sleep, and immunity as well as in circadian misalignment-derived diseases, providing new targets and scientific basis for prevention, diagnosis, and treatment of these diseases. Using knock-in technology based on TALEN and/or CRISPR-Cas9 [66, 67], zebrafish strains harboring GFP or luciferase driven by promoters of endogenous circadian clock genes will be generated for investigating how these genes are regulated, potentially revealing their novel regulatory functions. Establishing zebrafish lines of tissue-specific conditional knockout or overexpression of circadian clock gene-based CRISPR-Cas9 technology [68] will help reveal novel circadian regulatory mechanisms in specific organs or cells such as the pineal gland, the liver, or retinal photoreceptor cells.

With the emergence of next-generation sequencers, DNA sequencing technology has become increasingly economical and fast. This will help to discover new circadian clock genes and circadian clock-controlled genes, especially noncoding RNAs including miRNAs and lncRNAs, leading to novel insights into how the circadian clock acts through these new genes and/or noncoding RNAs to modulate fundamental life processes. Single-cell DNA sequencing technology will also help reveal novel features of circadian regulation in circadian-associated cells such as pineal gland cells or retinal photoreceptor cells.

Finally, as zebrafish are readily applicable for high-throughput whole-animal drug screens, zebrafish circadian study should help set the stage for screening and developing drugs for dysrhythmia-derived diseases.

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## Key Questions of Interest

What are advantages and disadvantages of zebrafish as a circadian model?
What are roles of zebrafish Per1b in maintaining circadian rhythms?
What are roles of zebrafish Per2 in maintaining circadian rhythms?
What are roles of zebrafish Clock1a in maintaining circadian rhythms?

## Suggested Readings

### *Zebrafish Physiological and Behavioral Rhythms*

Cahill GM, Hurd MW, Batchelor MM (1998) Circadian rhythmicity in the locomotor activity of larval zebrafish. Neuroreport 9:3445–3449

Liu C, Hu J, Qu C, Wang L, Huang G, Niu P, Zhong Z, Hong F, Wang G, Postlethwait JH, Wang H (2015) Molecular evolution and functional divergence of zebrafish (Danio rerio) cryptochrome genes. Sci Rep 5:8113

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Vatine G, Vallone D, Gothilf Y, Foulkes NS (2011) It's time to swim! Zebrafish and the circadian clock. FEBS Lett 585:1485–1494

### *Mutational Analysis of Zebrafish Circadian Rhythmicity*

DeBruyne J, Hurd MW, Gutierrez L, Kaneko M, Tan Y, Wells DE, Cahill GM (2004) Isolation and phenogenetics of a novel circadian rhythm mutant in zebrafish. J Neurogenet 18:403–428

Huang J, Zhong Z, Wang M, Chen X, Tan Y, Zhang S, He W, He X, Huang G, Lu H, Wu P, Che Y, Yan YL, Postlethwait JH, Chen W, Wang H (2015) Circadian modulation of dopamine levels and dopaminergic neuron development contributes to attention deficiency and hyperactive behavior. J Neurosci 35:2572–2587

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### *Transcriptome Analysis of Zebrafish Circadian Rhythmicity*

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# Chapter 9
# The Amphibian Clock System

**Massimiliano Andreazzoli and Debora Angeloni**

**Abstract** The variety of experimental approaches possible in amphibians, in particular, in the anuran species *Xenopus laevis*, have been crucial in discovering key regulators of circadian rhythms, such as melatonin and melanopsin. Differently from mammals, amphibians are characterized by the peculiar presence of multiple anatomical structures and cell types that feature photosensitive and self-sustained circadian activities. In particular, in amphibians, both the retina and the pineal complex are photosensitive and display circadian melatonin secretion. Furthermore, skin melanophores are light responsive and represent an exclusive model to study a peripheral circadian clock. In this chapter, we will review (1) the cellular and molecular mechanisms regulating circadian rhythms in amphibian retina, (2) the molecular bases of pineal circadian rhythms and its link to cell differentiation and cell proliferation, and (3) the *Xenopus* melanophore system as an example of a well-described peripheral, light-sensitive, clock.

## 9.1 Introduction

The presence in *Xenopus laevis* of multiple photoreceptive tissues including opsin-positive neurons of the hypothalamic magnocellular preoptic nucleus [1] and skin melanophores, in addition to the retina and the pineal complex, raised the question of the existence of a master circadian pacemaker in amphibians. Physiological experiments aimed at identifying circadian oscillators in *Xenopus*, and based on the ablation of specific structures, have shown that the pineal gland, the frontal organ, and the eyes are all required to maintain the normal period of free-running activity rhythms. However, light-entrainable circadian oscillators were still active even after removal of all three structures [2]. On the contrary, an intact suprachiasmatic area was found to be absolutely required for free-running

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behavioral rhythmicity, suggesting that the hypothalamic oscillator is the main regulator of circadian rhythms, which are in turn modulated by the pineal and retinal activities [3]. More recently, the majority of work performed in amphibians has focused on the identification of the molecular modulators of the circadian clocks in the retina, pineal gland, and melanophores, while the suprachiasmatic nucleus has not been further characterized in molecular terms. As for many other aspects of amphibian biology, the model of choice for these studies has been *Xenopus laevis* because it offers a variety of experimental approaches ranging from functional screens aimed at isolating novel genes, to the generation of cell-type-specific transgenesis [4].

## 9.2 The Retinal Circadian System in *Xenopus*

Autonomous circadian rhythms have been demonstrated in the retina of all vertebrate classes, indicating the presence of a local circadian clock. The easy accessibility of *Xenopus* retina, together with the large size of its cells and its high survival rate in culture, has made it a well-suited system to study retinal development and physiology. The mature amphibian retina displays the typical vertebrate organization into three main cell layers: outer nuclear layer (ONL), composed of rods and cones photoreceptors; inner nuclear layer (INL), containing different types of interneurons (amacrine, bipolar, and horizontal cells); and ganglion cell layer (GCL), whose axons fasciculate to form the optic nerve and send nervous signals to the brain (Fig. 9.1). Many of the factors that concur to retina development have been initially isolated and functionally described in *Xenopus* and subsequently

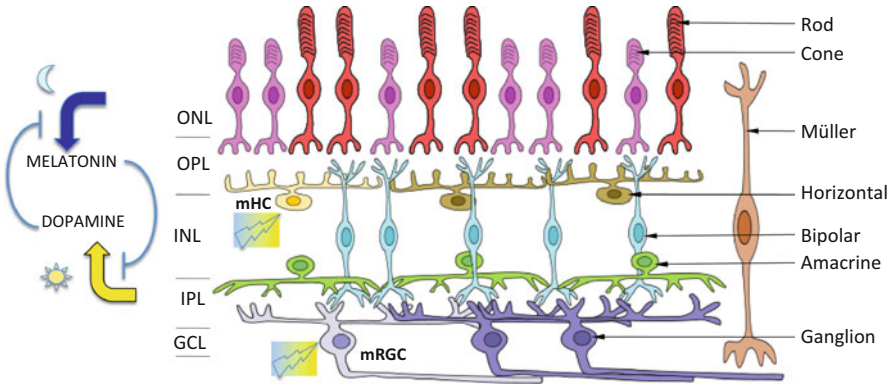


**Fig. 9.1** Organization and neurochemical outputs of the retinal circadian system in *Xenopus laevis*. The mutual antagonism between melatonin secreted by photoreceptors (*blue arrow*) and dopamine released by amacrine cells (*yellow arrow*) is shown on the *left*. mHC and mRGC indicate photosensitive melanopsin-expressing horizontal cells and retinal ganglion cells, respectively. *GCL* ganglion cell layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *ONL* outer nuclear layer, *OPL* outer plexiform layer

demonstrated to be extremely conserved among vertebrates [5]. In particular, in this animal model, it was shown that the circadian clock controls several parameters of retinal physiology, including melatonin and dopamine synthesis, rod disk shedding, cone elongation, and light sensitivity [6, 7].

Taking advantage of the features of *Xenopus* retina, Besharse and Iuvone [8] were able to isolate eye cups and maintain them in culture. This allowed the authors to investigate, in isolated conditions, the circadian activity of retinal arylalkylamine N-acetyltransferase (AANAT), the penultimate enzyme in the melatonin biosynthetic pathway. As they showed, in cultured eye cups, AANAT activity is regulated rhythmically, increasing during the night and decreasing during the day. This rhythmicity is also observed in constant darkness and can be reset by light, indicating that the retina harbors an autonomous circadian oscillator.

In isolated eye cups, transcription of the tryptophan hydroxylase (TPH) gene, coding for the rate-limiting enzyme of melatonin biosynthesis, is also cyclic reaching a peak in the early night. Therefore, also TPH mRNA is regulated by the circadian retinal clock, although, differently from AANAT activity, it is not acutely affected by light [9].

Gene expression studies in *Xenopus* have shown that a number of known clock genes and clock-regulated genes, including AANAT, are expressed in the retina already during early neurogenesis and in some cases are even maternally inherited in the oocyte. However, their expression appears to become rhythmic only in advanced phases of retinogenesis [10–12].

Cultures of isolated photoreceptor layers from *Xenopus* retina showed that these cells produced melatonin rhythmically, with high levels at night and low levels during the day. The average period is 24.3 h, consistently with the cyclic phase of AANAT activity and TPH transcription. The same rhythms are maintained also in constant darkness and are resettable by light cycles or by cyclic treatment with quinpirole, an agonist of D2-like (D2, D3, and D4) dopamine receptors [13]. In the retina, light induces amacrine cells and interplexiform neurons to secrete the neurohormone dopamine, which, in turn, increases *per2* levels specifically in photoreceptors [14, 15]. Both quinpirole and light treatments reset the oscillator toward a daytime phase. However, while quinpirole was shown to activate a signaling pathway that leads to reduced levels of cyclic AMP, light appears to act independently of this molecule [16, 17]. Furthermore, melatonin and dopamine inhibit each other's release, thus, suggesting the existence of a dual-oscillator mechanism within the retina [18].

These data suggested for the first time that photoreceptors are circadian clock cells capable of generating melatonin rhythms, although for their light resetting, they appear to require a feedback from dopaminergic amacrine cells. However, to formally rule out the possibility that the clock properties of photoreceptors may depend from other cells in the retina, it was necessary to await for the generation of transgenic protocols in *Xenopus*. Using this approach, Green and collaborators inserted a dominant negative form of the key circadian transcription factor CLOCK (XCLΔQ) under the control of mouse interphotoreceptor retinoid-binding protein (IRBP) promoter, which drives expression specifically in the photoreceptors

[19]. The obtained transgenic tadpoles carried photoreceptors with inactivated circadian clocks, and culture of isolated eye cups displayed an arrhythmic melatonin production, thus indicating that the clock in these cells is necessary for this rhythmic output. It is worth noting that inactivation of the circadian oscillator in photoreceptors does not affect average total melatonin levels, suggesting that CLOCK acts in a more complex manner than simply turning on melatonin synthesis at night.

The *Xenopus* retina contains approximately equal numbers of rods and cones that interact with each other via gap junctions. More recently, to further determine the distinct contribution to the circadian rhythmicity of each of the two photoreceptor cell types, Green and collaborators generated transgenic tadpoles in which XCLΔQ expression was driven by either a rod (rod opsin)- or a cone (cone arrestin)-specific promoter [20]. By analyzing melatonin release in isolated eye cups, the researchers observed that while rod-specific expression of the XCLΔQ clearly alters melatonin rhythms in the retina, a significantly lower effect is observed when XCLΔQ is expressed in cones. Considering that rods are able to respond to dim light at night, when melatonin is secreted, it is reasonable to think that this cell type may play a predominant role in increasing melatonin release at night. However, as rods and cones are electrically coupled, they are likely to interact to coordinate circadian clocks in individual photoreceptors and orchestrate the circadian release of melatonin.

Three melatonin receptor subtypes (Mel1a, Mel1b, and Mel1c) have been identified in *Xenopus laevis* [21, 22]. The *Xenopus* Mel1a receptor is the ortholog of the mammalian MT1 receptor, and the *Xenopus* Mel1b receptor is the ortholog of the mammalian MT2 receptor. Mel1c is the homologue of mammalian melatonin-related receptor GPR50 and does not bind melatonin [23]. The retinal expression of Mel1a and Mel1b receptors was found to correlate with cone circuits, in particular, with Mel1b receptors being expressed by OFF-bipolar cells and Mel1a receptors by horizontal cell processes specifically at cone terminals. This selective localization of Mel1a and Mel1b receptors has suggested a role in reducing noise from signaling by cone terminals in darkness, when visual function is dominated by rods [24].

It should be noted that, although the retinal melatonin has a role in controlling a number of cellular and physiological function within the retina, it is rapidly metabolized within the *Xenopus* retina [25], and it is therefore likely to provide a paracrine and/or intracrine rather than an endocrine, circadian signal.

A first attempt to understand the molecular regulation of rhythmically expressed retinal genes was addressed by experiments that linked the advantage of the large, easily dissectable *Xenopus* retina, to the use of appropriate molecular biology strategies.

This was the case for *Nocturnin* (*Noc*), a gene isolated through a high-stringency differential display screen aimed at selecting mRNAs that exhibit rhythmic expression in the photoreceptors. *Noc* mRNA displays high-amplitude, rhythmic expression, reaching the highest level during the night in isolated *Xenopus* eye cups in cyclic light, as well as in conditions of constant dark or constant light. *Noc*, which in the *Xenopus* retina is specifically expressed in photoreceptors, shares sequence

similarity with the C-terminal deadenylase domain of the yeast carbon catabolite repressor 4 (yCCR4). The ability of Noc to remove 3′ polyA tails from mRNA was indeed confirmed in cell culture-based assays, indicating that Noc belongs to the family of magnesium-dependent family of nucleases. Although specific substrates for Noc in the photoreceptors have not been identified yet, the circadian expression of this deadenylase suggests that its targets could be mRNAs related to rhythmic processes within these cells. The extent of such regulation is not known; however, an estimate was determined in mouse liver, where it was shown that the poly (A) tails of 2.3 % of all expressed mRNAs cycle in length daily and that these rhythmic poly(A) tail lengths correlate strongly with the circadian protein expression profiles of this tissue [26].

Besides the canonical photopigments present in cone and rods, a screen originally performed in *Xenopus* photosensitive melanophores unraveled the presence of an atypical photopigment that was named melanopsin ([27], see below). Melanopsin is expressed in several tissues and displays a peak of light absorption at blue light wavelengths. Intriguingly, it is more closely related to invertebrate rhabdomeric opsins than to the vertebrate ciliary opsins typically expressed in cones and rods. Similarly to other nonmammalian vertebrates, *Xenopus* has two melanopsins: opn4x (also known as *Xenopus*-like melanopsin) and opn4m (also known as mammalian-like melanopsin). In mammals, only opn4m is present and its retinal expression is restricted to a small subset of photosensitive retinal ganglion cells (RGCs) that convey photic information to the "non-image-forming" (NIF) visual system, resulting in appropriate regulation of pupillary light reflex, circadian photo-entrainment, and sleep [28]. In *Xenopus* embryonic retina, opn4m and opn4x are mostly co-expressed in the outer half of the INL, in correspondence to the soma of specific horizontal cells (melanopsin-expressing horizontal cells, mHCs), and in a restricted number of retinal ganglion cells (melanopsin-expressing retinal ganglion cells, mRGCs. Fig. 9.1). Marker analysis identified mHCs as $Prox1^{+}/Otx2^{-}/Isl1^{-}/Pax6^{-}$ and mRGCs as $Pax6^{+}/Isl1^{-}$. About 80 % of these melanopsin-expressing cells are found in the peripheral retina, with mHCs located mainly in the dorsal retina and present in a ratio of 6:1 compared to mRGCs. To monitor the activation of embryonic NIF retinal circuit, Bertolesi and collaborators [29] assessed the expression of c-fos, an immediate early gene activated in second-order neurons following transduction of the light stimulus from photosensitive cells, after 30 min of light exposure. These experiments show that, as is the case for cones and rods in the classic image-forming (IF) circuit, light-sensitive mHCs do not express c-fos, which is instead activated in mRGCs, as well as in BCs, RGCs, and in a population of dopaminergic, tyrosine hydroxylase-positive amacrine cells (TH + ACs). A further analysis aimed at investigating c-fos activation by light with different wavelengths and possibly at discriminating between classic photoreceptor pathways and melanopsin-driven circuits. As a result, a situation more promiscuous than expected was revealed. In fact, photoreceptors of the IF circuit were shown to respond to blue light, while red and green light appeared as effective at turning on c-fos expression in mRGCs as blue light. Intriguingly, TH + ACs display preferential c-fos induction by blue light, suggesting that these cells participate to a

melanopsin-triggered circuit. A possible connection to this circuit might involve interaction of TH + ACs with (i) On-BCs via the rod/cone pathways; (ii) mRGCs, through a retrograde signal; and (iii) mHCs; in this case, TH + ACs would connect mHCs to mRGCs [29]. Differently from mammals, which develop an active retinal NIF system much earlier than vision, in *Xenopus* both the classic visual pathway and melanopsin NIF circuit become functional at the same developmental time, corresponding to the tadpole stage 37/38, concomitant with the establishment of retinal layers and synaptic connections and innervation of brain target by RGC axons. However, overall maturation of the NIF system in *Xenopus* still appears to precede that of the IF system because of the activity of the photosensitive pineal organ, which rhythmically secretes melatonin already at early tailbud stage (see below).

## 9.3 The *Xenopus* Pineal Circadian System

The amphibian pineal organ is an epithalamic vesicle connected to the roof of the diencephalon by a slender stalk. The pineal epithelium surrounds a central lumen, which is opened to the third ventricle and is thus filled with cerebrospinal fluid. The frog pineal organ is associated with the frontal organ, a more superficial extracranial photoreceptive structure located underneath an area of pigmentless skin, which shares similarities to the reptilian parietal eye [30]. The pineal organ of amphibians is primarily composed of photoreceptors, structurally similar to retinal cones, projection neurons, and interstitial glial cells. Pineal photoreceptors are highly polarized, with an outer segment in the apical part protruding in the pineal lumen and a basal extremity that establish synaptic contacts with the projection neurons whose axons enter the brain through the pineal stalk and innervate several brain areas. Pineal and retinal photoreceptors share a similar phototransduction machinery. Thus, the amphibian pineal organ resembles a simplified retina, mainly lacking interneurons, whose structure clearly indicates its role as a luminance detector [31]. Despite these remarkable similarities, the pineal organ cannot discriminate between rapid light changes, as the retina does, but rather acts as a mediator of gradual light intensity changes. This is consistent with the idea that the pineal is an indicator of day length and may provide input to the circadian clocks.

Integrated with inputs from the retina, the photosensitive activity of the pineal organ functions in resetting its circadian clock. A major output of this entrainment is the rhythmic activity of AANAT, which, as in the retina, results in the cyclic production of melatonin. This timekeeping hormone is secreted by pineal photoreceptors into the cerebrospinal fluid and blood at high level during the night and at low level during the day. Therefore, while the retinal melatonin appears to act and be catabolized locally [25], pineal melatonin is supposed to play a systemic role, synchronizing circadian rhythms in the entire organism.

Experiments based on in vitro cultured *Xenopus* pineal organs revealed that they are capable of synthesizing melatonin in a rhythmic pattern soon after they

evaginate from the diencephalon at early tailbud stage (stage 26). When kept in constant darkness, cultured pineal organs produce melatonin, although with low amplitude rhythms, suggesting the presence of a circadian clock in this tissue. However, after the third day of culture in constant darkness, melatonin release quickly damps to very low levels, indicating that cyclic light exerts a positive effect on melatonin synthesis in the pineal organ. In these in vitro organ culture assays, melatonin production by the pineal organ precedes of 1–2 days melatonin synthesis observed in dissected optic vesicles. However, when established, retinal melatonin secretion is maintained rhythmic both in cyclic light and in constant darkness [32]. Studies performed in the Japanese newt (*Cynops pyrrhogaster*), a urodele amphibian, indicate that, although the pineal organ plays a major role in generating the circadian locomotor activity, the pineal clock is not entrainable by extrapineal photoreception [33, 34]. These data suggest that, at least in this species, but possibly also in other amphibians, there is little or no mutual coupling between the pineal clock and the putative brain oscillator.

A relatively recent addition to the cellular events controlled by the circadian clock is the generation of daily rhythms of cell cycle progression [35], which is now documented virtually in every analyzed adult cell type [36]. Particularly, synchronized circadian oscillations of both the cell cycle [37] and cell cycle regulatory genes have been demonstrated to occur in zebrafish also during embryonic development [38].

In *Xenopus*, photoreceptor precursors of developing pineal organ expressing Crx-b (previously known as Otx5) undergo themselves a circadian-controlled entry in S-phase, reaching a peak in the second half of the light phase [39]. In this context, the homeodomain-containing transcription factor Bsx was found to display a rhythmic pineal expression with the highest levels during the night. Bsx rhythmicity is lost in embryos kept in constant darkness, suggesting that the transcription of this gene is controlled by the alternation of light and dark rather than by the circadian oscillator. Bsx expression inversely correlates with cell proliferation. In fact, besides displaying a cyclic expression profile that is complementary to the daily rhythms of S-phase entry, Bsx is expressed in non-proliferating pineal photoreceptor precursors, and its expression is repressed by cell proliferation induced by cyclin A2/cdk2 overexpression. Furthermore, its knockdown leads to increased S-phase entry during the night, whereas overexpression of a hormone-inducible Bsx protein causes a flattening of the cell proliferation profile to the lowest level [39]. In addition, Bsx plays an instructive role in pineal photoreceptor determination. Indeed, pineal organs of Bsx knocked-down tadpoles feature a decrease of differentiated photoreceptors, while Bsx overexpression leads to an increase of pineal photoreceptors. This effect of Bsx is specific to pineal photoreceptors, as projection neurons are not affected either by gain- or by loss-of-function experiments.

Although the transcriptomes of retina and pineal organ are extremely similar, Bsx is one of the few examples of transcription factor that are expressed in the pineal organ but not in the retina. Bsx expression in *Xenopus* pineal photoreceptor precursors follows the activation of Crx-b, Not2, and Rx1/Rax and is likely to act

downstream of these transcription factors, which specify the early pineal territory. Therefore, Bsx appears to play a later role, functioning as a link between daily circadian cell cycle and pineal photoreceptor fate determination. Bsx target genes remain to be identified. However, the enrichment of putative Bsx-binding sites in the flanking genomic regions of eight long noncoding RNAs (lncRNAs) displaying a circadian expression with nocturnal peak suggests that Bsx might specifically control the expression of nocturnal genes [40].

## 9.4 The *Xenopus* Melanophore Circadian System

*Xenopus* melanophore response to light has been an important paradigm to study how peripheral, photosensitive cells adjust to circadian light/dark cycles and to identify the molecules responsible for this phenomenon.

Skin cells respond to light variations by adjusting their color, as a consequence of specific pigment migrations. In fact, when the black pigment melanin disperses in the cytoplasm of melanophores, it causes darkening of the skin, while its aggregation around the melanophore cell nucleus results in a pale aspect of the skin. Two different mechanisms have been described: the "primary color response," which is due to a direct response to light by chromatophores [41], and the "secondary color response," which is mediated by the eyes and is controlled by the neural and/or endocrine system [42].

### *9.4.1 Primary Color Response*

In the primary color response, chromatophores independently transduce light energy into the mechanical forces required for the processes of pigment and guanine crystals movements, which, in turn, induce color changes in the skin. A direct response to light is shown by melanophores of the posterior part of the tail fin of *Xenopus* late tadpoles (stage 51–56), which are fully aggregated under normal illumination, leading to the paleness of the tails. Conversely, in darkness pigment disperses, causing the tail to become dark. These effects are not mediated by the eyes or by the central nervous system [43–45]. Little is known about the molecular mechanisms mediating melanosome aggregation as a response to illumination, except that a rhodopsin-like molecule, distinct from melanopsin, expressed in cells of *Xenopus* tadpole tail fin is likely to be involved [46].

Differently from tail melanophores of late-stage tadpoles, in melanophores derived from *Xenopus* embryos, an opposite behavior is observed, with pigment dispersion under illumination and aggregation in the darkness. Working with cultured melanophores isolated from stage 30–35 tailbud embryos, Daniolos and collaborators [47] observed that melanin aggregation, induced by melatonin treatment in darkness, is reversed leading to dispersion when cells are irradiated with

white light. Furthermore, melanin photodispersion reached a peak when melanophores were stimulated with a 460 nm light, and this effect was found to be dependent on vitamin A, indicating the involvement of an opsin photopigment. This phenomenon was actually at the basis of melatonin discovery. Indeed, already in1917 McCord and Allen [48] reported that *Rana pipiens* tadpoles fed with a crude acetone extract of bovine pineal glands displayed lightened skin. It was only later, in 1958, that Lerner isolated the molecule responsible for melanin granule aggregation, a pineal indole, which he named melatonin [49].

Within the primary color response, the best characterized phenomenon is the one that occurs in cultured *Xenopus* melanophores, originally obtained from late tailbud embryos, which respond to light by pigment dispersion. The search for opsins expressed in dermal melanophores of *Xenopus laevis* led to the isolation of melanopsin, a photopigment that was shown to be synthesized not only in melanophores but also in other tissues displaying photosensitivity in amphibians, such as the hypothalamus, the iris and horizontal cells of the retina (see above; [27]). Photoactivated melanopsin initiates a phosphoinositide signaling pathway, similar to that found in invertebrate phototransduction, which causes the dispersion of melanosomes [50]. A first in vivo demonstration of melanopsin activity showed that melanophores of transgenic *Xenopus* constitutively overexpressing melanopsin are 100-fold more sensitive to light than control melanophores and induce a remarkable melanin dispersion [51]. This experimental evidence clearly indicates that melanopsin mediates the primary color response.

*Xenopus* embryonic dermal melanophores express the melanopsin gene *Opn4x* and *Opn4m* and display rhythmic expression of the circadian clock genes *Per1*, *Per2*, *Bmal1*, and *Clock*. The concomitant presence of the molecular core of the circadian clock and of photopigments suggested that these cells possess the main characteristics to be considered peripheral clocks. However, the neurohormonal signals potentially required to reset the melanophore clock by the central oscillator have not been clearly identified yet. In fact, although cycling light conditions are able to reset and synchronize *Xenopus* melanophores, neither melatonin nor α-melanocyte-stimulating hormone (α-MSH), which act as the main melanin-dispersing agent and master regulator of skin darkness, appear to significantly affect *Per1* cyclic expression. These data suggest that the presence of melanopsin might be sufficient to trigger melanin dispersion and modulate gene expression in these cells [52, 53], unless these hormones regulate the transcription of other clock genes such as *Per2* or *Bmal1* or control some later phases of gene expression, such as translational or posttranslational regulation.

### *9.4.2 Secondary Color Response*

In the secondary color response, the link between skin pigmentation and circadian rhythms has remained elusive for a long time. In amphibians, α-MSH secretion is mediated by a simple neuronal circuit in which RGCs project to dopaminergic

suprachiasmatic melanotrope inhibitory neurons that innervate cholinergic α-MSH-releasing cells [42]. Recently, Bertolesi and collaborators [54] made an important step forward by demonstrating that melanopsin activation in mRGC is required to inhibit α-MSH secretion, resulting in skin lightening due to melanin aggregation in melanophores. This effect, which is relevant to correlate skin pigmentation both to background adaption and to circadian light/dark cycles, implicates retina-generated melanopsin as a trigger for the secondary color response.

## 9.5 Concluding Remarks

Studies performed in *Xenopus laevis* have been pivotal in identifying important molecules regulating amphibian circadian rhythms both in the central nervous system and in peripheral tissues. Future experiments in amphibians, exploiting also the genetically amenable *Xenopus tropicalis* system, as well as the recently developed genome editing techniques based on the CRISPR/Cas system, are expected to continue to break new ground in our understanding of vertebrate circadian rhythms.

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# Chapter 10
# The Reptilian Clock System: Circadian Clock, Extraretinal Photoreception, and Clock-Dependent Celestial Compass Orientation Mechanisms in Reptiles

**Cristiano Bertolucci, Elena Frigato, and Augusto Foà**

**Abstract** Among vertebrates, reptiles are one of the most interesting taxa to understand the evolution of the circadian system to different ecological niches. Here we summarize the current knowledge about the circadian system of reptiles. In detail, paragraph 3 analyzes studies concerning the existence of peripheral and central circadian oscillators in reptiles, with complementary data gathered using molecular, physiological, and behavioral approaches, particularly, the role of SCN and the reactions of both central and peripheral oscillators to drastic changes in ambient temperatures. In paragraph 4, as it is peculiar to other nonmammalian vertebrates, also lizards, behavioral and hormonal rhythms (particularly melatonin) can be entrained by extraretinal and deep brain photoreceptors, whose position in the brain seems to vary somewhat in different lizard species. In paragraph 5, the seasonal changes in circadian organization are analyzed in fine detail in the ruin lizard *Podarcis sicula*, a species in which most research on seasonality has be done. Paragraph 6 reports some data on the role of ambient light irradiance in the circadian organization. Paragraph 7 deals with problems of orientation in space, with particular interest in celestial compass mechanisms which need a functional circadian clock to work properly. In this context recent results are reported on the functioning of both sun and sky polarization compasses and the pivotal role played by the parietal eye in those mechanisms of orientation. Paragraph 8 reports conclusion and perspectives.

## 10.1 Introduction

The endogenous circadian clock leads daily rhythmicity of a number of biological processes. It allows organisms to anticipate time of the day and season permitting them to adapt to cyclic environmental changes [1]. A fundamental feature of the

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circadian oscillators is their capacity to synchronize (*entrain*) rhythms with daily changes in environmental signals (*zeitgeber*), such as light, temperature, and food availability [1–3]. Besides circadian rhythms, many animals also display seasonal and circannual rhythms. Animals living in temperate zone experience drastic changes in behavior, reproduction, and metabolism in function of seasonal changes in climate conditions. For instance, photoperiodic animals have alterations in behavioral activity patterns in response to changing in day length.

## 10.2 Central and Peripheral Circadian Oscillators

In vertebrates, the molecular mechanism underlying self-sustained circadian oscillations involves transcription factors that act within transcriptional autoregulatory feedback loops, composed of positive and negative elements [4–6]. The positive loop is formed by *Clock*s and *Bmal*s, two basic helix-loop-helix (bHLH)/PER-ARNT-SIM (PAS) domain transcription factors. CLOCKs and BMALs establish heterodimers that activate the transcription of *Per*s, *Cry*s, and *Rev-Erb* genes, main components of the negative loop [7, 8]. Subsequent cycles of activation and repression in the expression of clock proteins generate circadian oscillations. The molecular circadian clock of reptiles has been well characterized only in a species, the ruin lizard *Podarcis sicula* [9, 10]. In this species homologues of the mammalian and bird clock genes *Cry1*, *Per2*, and *Clock* have been cloned. Analysis of the predicted ruin lizard clock protein showed high levels of identity (>76 %) with vertebrate orthologs and a close phylogenetic relationship with avian counterparts [10].

As in other vertebrates, daily and circadian clock gene expression has been found in the suprachiasmatic nucleus (SCN) of the hypothalamus, the master clock generating circadian rhythms, and in peripheral oscillators located in several organs and tissues [11–14]. In ruin lizard SCN *Per2* mRNA levels change across the 24 h both in light–dark (LD) cycles and in constant darkness (DD) (Fig. 10.1; [9]). Moreover, the existence of peripheral oscillators in lizards has been confirmed by the circadian expression of *Per2*, *Cry1*, and *Clk* in the eyes, heart, and liver of *P. sicula*, with profiles comparable to their mammalian and avian counterparts [10, 15]. The hypothesis that lizard SCNs contain the primary circadian pacemaker driving locomotor rhythms has been confirmed using different experimental approaches. Firstly, neuroanatomical and immunocytochemical evidences support the homology between SCN of lizards and mammals. Indeed, as in mammals, lizard SCNs are located dorsal to the optic chiasm and adjacent to the third ventricle, in the region of transition from the preoptic area to the hypothalamus [16, 17], and bind antibodies raised against vasoactive intestinal polypeptide (VIP), tyrosine hydroxylase (TH), serotonin, arginine–vasopressine, neuropeptide Y, and substance P [18, 19]. Furthermore, a prominent daily rhythm was found for neurotransmitters, such as VIP and TH, and for the transcription factor c-FOS [19, 20].

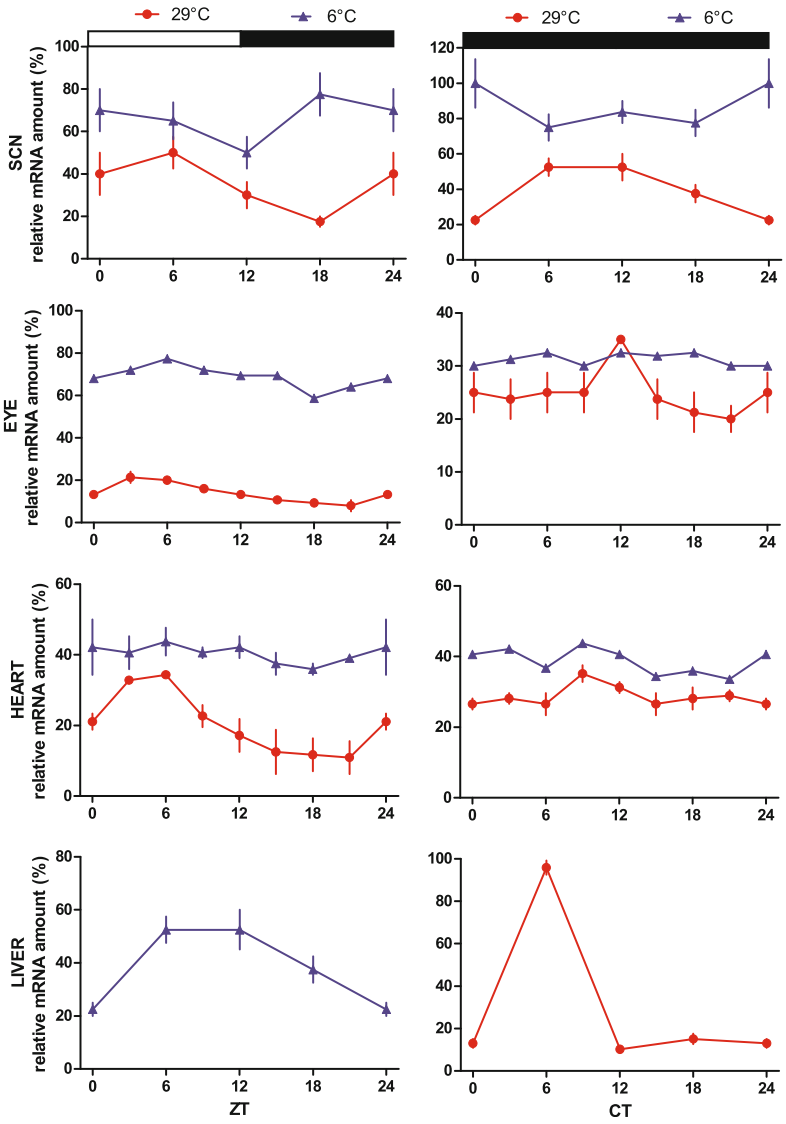


**Fig. 10.1** *Per2* mRNA expression in lizard tissues in LD cycle (*left panels*) and DD (*right panels*) at 29 °C (*red line* and *circles*) and 6 °C (*blue line* and *triangles*). On the *x*-axes are indicated the sampling times (zeitgeber time and circadian time; ZT and CT). *White* and *black bars* represent light and dark periods, respectively. Each point represents the mean ± SEM. Results obtained at 29 °C and 6 °C are plotted on the same graph to show the increase in basal *Per2* expression at lower temperatures (Modified from [9, 10, 15])

The functional demonstration of the role of the SCN as master clock in reptiles came from the abolition of behavioral circadian rhythms after electrolytic lesions to the SCN (*Dipsosaurus dorsalis* [16]; *P. sicula* [17]). Furthermore, lizard SCNs are

necessary to mediate entrainment to different zeitgeber. Indeed, total lesion of the SCN impaired the entrainment of locomotor activity rhythms to daily injections of exogenous melatonin and to LD cycles [20, 21]. Also in *Iguana iguana* results suggest a role of the SCN in the circadian system, since removal of all the known circadian components (retinas, the pineal gland, and the parietal eye) does not abolish circadian rhythms of locomotor activity [22].

Because reptiles living in temperate climate are ectotherm and are naturally exposed to a wide range of temperatures during the year, they are ideal models to investigate the effects of temperature on the vertebrate circadian clock [23]. For instance, low-amplitude (0.9–3 °C) daily temperature cycles entrain behavioral circadian rhythms, and hypothermia has a profound effect on clock gene expression both in central (SCN) and in peripheral (eye, heart, and liver) oscillators of the ruin lizards [2, 23] (Fig. 10.1). Indeed, *Per2* mRNA level in the SCN, which is rhythmic at 29 °C, becomes constantly elevated at low temperatures (6 °C), in both LD and DD conditions [9]. Similar results have been found in peripheral organs of ruin lizards exposed to 6 °C: *Per2*, *Cry1*, and *Clk mRNA* are expressed at high levels and loss the rhythmicity (Fig. 10.1 [10, 15]).

## 10.3 Retinal and Extraretinal Photoreceptors

The retinas of reptiles participate in the circadian clockwork not only as photosensory input to the SCN but also as a part of the circadian timekeeping mechanisms. In constant bright light, bilateral ocular enucleation induces a marked shortening in the free-running period ($\tau$) in the iguanid lizards *Sceloporus olivaceus* and *S. occidentalis* [24, 25]. Also in *P. sicula* the lesion of the optic nerves at the level of the optic chiasm and the bilateral retinalectomy in DD induce the same behavioral effects [17, 26]. Although the retina of *I. iguana* isolated in culture drives circadian rhythms of melatonin synthesis [22], the influence of the retinae on the circadian system of reptiles seems to be neurally mediated.

The retinas play also a role in the entrainment of circadian rhythms to light. However, differently from mammals, retinas are not requested for the entrainment of the behavioral rhythms of reptiles. In nine species of lizards, representing four different taxonomic families (Iguanidae, Gekkonidae, Xantusiidae, Lacertidae), the locomotor rhythms are entrainable to LD cycles after enucleation of lateral eyes [24, 27–30]. These results confirmed existence of the deep brain photoreceptors (DBP), extraocular photoreceptors located deep in the brain. Different locations of the DBP were found in different lizard species. For instance, in the American iguanid lizards *Anolis carolinensis* and *I. iguana*, cone-opsin immunopositive cells have been exclusively detected in the basal region of the lateral ventricles, while in the Japanese grass lizards, *Takydromus tachydromoides*, neurons expressing rhodopsin have been localized in the posterior pallial commissure and median eminence [31–33]. In all these reptilian species, DBP appear to be of similar shape as cerebrospinal fluid-contacting neurons. In contrast, in ruin lizards the DBP

are localized in the periventricular area on the hypothalamus and look to be of similar shape as neurosecretory cells [34]. Several studies have predicted that DBP play a role both in the regulation of seasonal and circadian physiology [35]. In ruin lizards posttranscriptional inactivation experiments demonstrate, for the first time in a vertebrate, the crucial role of a brain opsin in the photic entrainment of behavioral rhythms [34].

## 10.4 The Pineal and the Seasonality

Investigations in the Iguanid lizard *A. carolinensis* and *Tiliqua rugosa* showed that daily cycles of light and temperature entrain the pineal melatonin rhythm and that differences in length of photoperiod or thermoperiod affect this rhythm [30, 36, 37]. Hence, the current ambient lighting and temperature conditions (and their seasonal changes) are readily translated into an internal cue in the form of the pineal melatonin rhythm and can be used to regulate both daily and annual physiology of lizards [30]. Several studies demonstrated that *P. sicula* is a well-suited model to explore seasonal changes of circadian locomotor rhythms in reptiles [38, 39]. In the natural environment, the daily pattern of locomotor activity of ruin lizards changes dramatically throughout the year. In spring and autumn, activity is unimodal (one activity peak per day, without interruptions). In summer, activity becomes bimodally distributed, with an early or mid-morning peak and a late afternoon peak, separated by a time period during which activity is drastically reduced [40, 41]. Such seasonal changes in activity patterns have an endogenous component: ruin lizards kept in constant temperature (29 °C) and darkness (DD) retain the locomotor pattern they have in the field during the same season (Fig. 10.2a, b [42]). Again, while bimodal locomotor pattern in constant conditions observed in most lizards during summer is typically associated with a short $\tau$ and a long circadian activity ($\alpha$), the unimodal locomotor pattern observed in the remaining months showed long $\tau$ and short $\alpha$ (Fig. 10.2a, b [42]). In constant temperature and DD, pinealectomy induces a fast transition from the bimodal circadian locomotor behavior typical of summer to the unimodal circadian locomotor behavior typical of spring and autumn [43]. Chronic administration of exogenous melatonin (melatonin implants) has the same behavioral effects as pinealectomy in summer: the abolition of the bimodal pattern after application of the implants is always associated with a lengthening of $\tau$ and shortening of $\alpha$ [44]. These data suggest that the pineal and melatonin are crucially involved in establishing and maintaining summer bimodality.

Other investigations also demonstrate that the impact of melatonin and the pineal on the circadian organization of ruin lizards changes noteworthy with the season: (1) the effects of pinealectomy and melatonin implants on circadian locomotor rhythms are strong in summer and weak in the other seasons [38, 45];

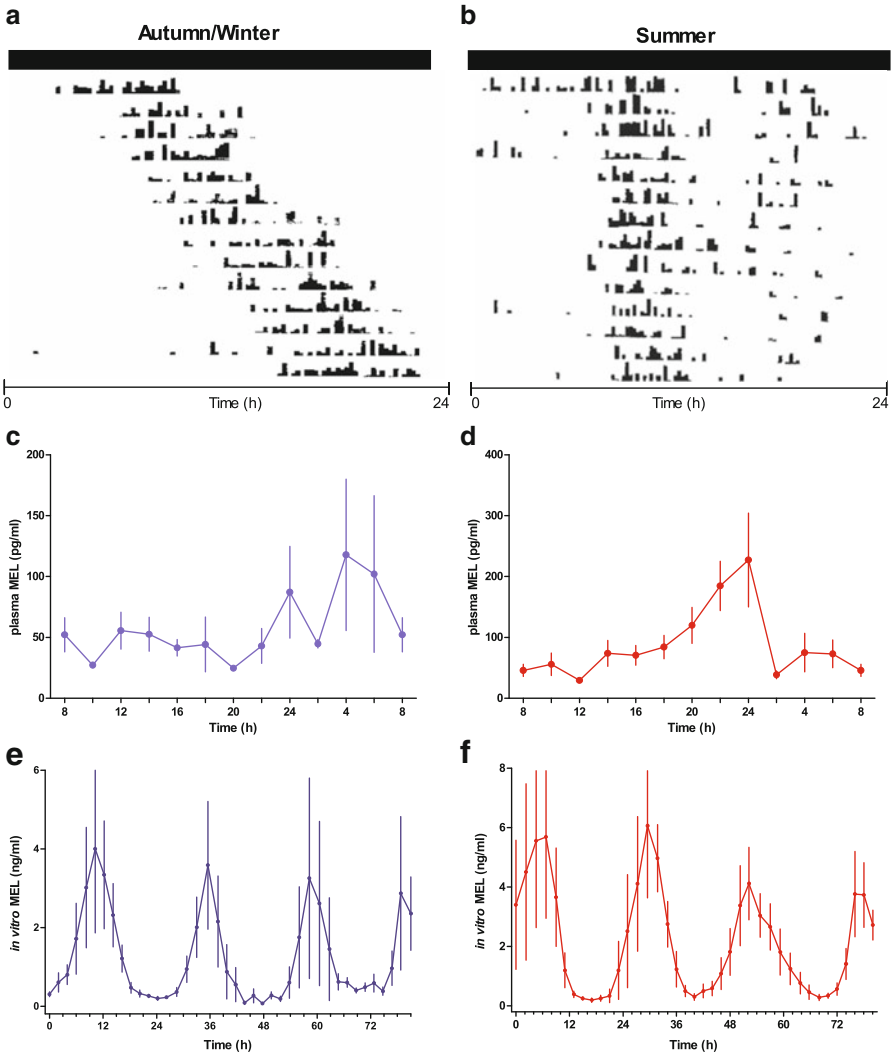


**Fig. 10.2** Circadian locomotor activity and melatonin profile in different seasons. (**a**, **b**) Locomotor activity records of two ruin lizards kept in constant temperature (29 °C) and DD in autumn–winter (**a**) and summer (**b**). Each horizontal line is a record of the activity over a single day, with consecutive days mounted, below each other. The examples are representative of the seasonal differences in the free-running activity: unimodal pattern, long τ and short α in autumn–winter and bimodal pattern, short τ, and long α in summer. (**c**, **d**) Seasonal variation of 24-h plasma melatonin profiles in ruin lizards in DD during autumn–winter (**c**) and summer (**d**). (**e**, **f**) In vitro circadian melatonin release from cultured pineal glands of ruin lizards in autumn–winter (**e**) and in summer (**f**). Each point represents mean ± SEM (Modified from [2, 47, 48])

(2) daily melatonin injections entrain locomotor rhythms only in summer [21]. Also daily injections of luzindole, a melatonin antagonist, alter locomotor rhythms in summer, but not in other seasons [38]; (3) a phase response curve for melatonin is generated in summer, but not in winter [46]; (4) a robust pineal-dependent circadian rhythm of plasma melatonin exists in summer, but not in spring or autumn (Fig. 10.2c, d [47]).

Thus the data of many investigations support the view that the pivotal role of the pineal for circadian organization in summer and its marginal role in spring or autumn are due to the presence (summer) or absence (spring and autumn) of a circadian melatonin signal in the blood circulation [47]. Surprisingly, *P. sicula* in vitro pineal culture investigations show a circadian rhythm of melatonin in constant temperature and DD, not only in summer but also in autumn–winter (Fig. 10.2e, f [48]). Differences between in vivo and in vitro data suggest that signals transmitted to the pineal via its innervation may, besides the intrapineal oscillator, play a role in regulating melatonin production. For instance, an oscillator located in the ruin lizard SCN (see previous paragraph) may suppress rhythmic melatonin synthesis in winter by signals transmitted via the pineal innervation. Interestingly, during summer the exposure of in vitro pineals to alternating summer and winter photoperiods does not cause significant changes in the daily melatonin rhythm [39]. Seasonal differences in the amplitude of the in vitro melatonin production were found, suggesting that photoperiodic information in ruin lizards may be encoded by the amplitude rather than by the duration of the melatonin signal [48].

Cassone and Menaker [49] hypothesized that the circadian system of birds functions as a *neuroendocrine loop*: pineal oscillators being influenced by rhythmic sympathetic input driven by the SCN and the SCN in turn being influenced by the rhythmic melatonin signal from the pineal gland. A similar neuroendocrine loop model was suggested to explain the circadian system of *I. iguana* [22]. To confirm this model in the circadian system of ruin lizard is necessary that each component of the loop undergoes remarkable seasonal changes in function: (1) the pineal plays a central role in the circadian organization in summer and marginal in other seasons [38, 45]; (2) the SCNs, the primary target sites of rhythmic blood-borne melatonin of pineal origin, are subjected to dramatic seasonal changes in responsiveness to the melatonin signal, with high responsiveness in summer and low or no responsiveness in other seasons [21, 38]. Due to the seasonal changes in structure of the ruin lizard circadian system outlined above, it was further examined whether retina of the lateral eyes expresses seasonal changes in function. Studies by Foà et al. [39] did not indicate any difference between summer and autumn–winter in the effectiveness of both RET-X and OC-X in altering circadian locomotor rhythms. Hence, the retinae do not seem to participate in the seasonal reorganization of the circadian system that occurs in ruin lizards, at least at for what concerns behavior.

## 10.5 Evolutionary Effects of the Photic Habitat on Circadian Organization

In a different context, some recent investigations were aimed at testing whether the photic habitat in which lizards live would affect in some way their circadian organization. A good model is proposed in five species of the genus *Anolis* among which there are marked differences in the photic habitat, i.e., the relative amount of sun and shade of their ecological niche [50–52]. All *Anolis* species showed temperature-compensated circadian rhythms of melatonin from pineals cultured in vitro. Light caused significant phase delays of the melatonin rhythm and the effect varied among species. In fact data indicate that pineals of shade-dwelling species are significantly more sensitive to photic resetting than species living in more brightly illuminated areas [50]. Other investigations tested the effects of differences in the photic niche at the behavioral level. The experiment consisted in measuring the photic-induction of locomotor activity in four species of *Anolis* (*A. cristatellus*, *A. gundlachi*, *A. pulchellus*, *A. krugi*) living in Puerto Rico (Greater Antillean island), whose microhabitats differ in solar irradiance. A series of 2-h light pulses of five different irradiances (from $10^{-3}$ to $10^{2}$ W/cm$^{2}$) were administered during the dark phase of a 12:12 LD cycle. The results actually showed that light causes an irradiance-dependent increase in locomotor activity in all four species. The behavioral responses at the highest irradiance were significantly greater in species occupying relatively more shaded habitats (*A. gundlachi*, *A. krugi*; 51). Altogether these data suggest that within the same genus, the circadian system is significantly more sensitive to light in shade-dwelling species than in those species adapted to live in more brightly illuminated areas.

## 10.6 Clock-Dependent Celestial Compass Mechanisms

Lizards, as most other vertebrate, are equipped with orientation mechanisms that allow them to find their nests or feeding grounds. Compass bearings can be computed by using the sun's azimuth (the point where the solar meridian – a line dropped vertically from the sun's position – intersects with the horizon). Obviously, since the continuous apparent change of the sun azimuth across the sky, such a compass needs to be time compensated, and such a time compensation is accomplished by the circadian clock. Time compensation can be demonstrated by exposing animals to LD cycle shifted of 6 h fast or slow and by observing deviations of orientation of about 90° counterclockwise (fast clock shift) or 90° clockwise (slow clock shift), with respect to the expected direction of orientation (e.g., home direction [53–55]).

A homing capacity was documented in several lizard species, but the underlying orientation mechanisms are mainly unknown in these reptiles [56]. Some investigations showed that lizards can use the above mentioned time-compensated sun

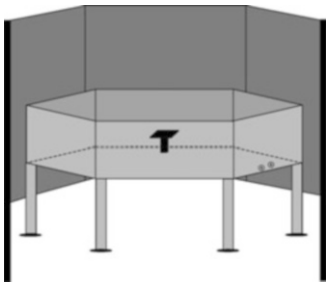

**Fig. 10.3** Schematic representation of the hexagonal Morris water maze used in the ruin lizard orientation experiments. Figure points out the goal platform mounted upon its pedestal located at the periphery of the maze in the center of a side wall and the fence that surrounding completely the water maze to prevent lizards from seeing landscape is partially shown (Modified from [61])

compass: both the spiny lizards *S. jarrovi* and the sleepy lizards *T. rugosa* use celestial cues to determine the compass bearing in homing experiments in the field [57, 58]. Furthermore, in outdoor arenas after directional training, the fringe-toed lizard *Uma notata* [59] and the ruin lizard *P. sicula* were shown to orientate using a sun-compass mechanism [59, 60]. The ruin lizard has been tested in a modified Morris water maze [61]. For this purpose, ruin lizards were individually trained to swim from the center of the water maze onto a hidden platform (the goal), positioned at the periphery of the maze in a given compass direction (Fig. 10.3). To test the use of a time-compensated sun compass, lizards reaching learning criteria were subjected to 6-h clock shift (fast or slow). Their directional choices deviated as expected on the basis of the clock shift demonstrating that *P. sicula* uses a time-compensated sun compass (Fig. 10.4 [60]).

It was suggested that the parietal eye plays a role in lizard orientation. The parietal eye has a lens, cornea, and retina. The retina is very simple (it is made of photoreceptors and ganglion cells only), and the photoreceptors synapse directly onto the ganglion cells, the axon of which forms the parietal nerve. The parietal eye nerve innervates several areas of the brain, but does not project to the visual part [62]. It synthesizes melatonin, but in small quantities, that may simply fulfill a local role [22, 63]. Field work in the lizards *S. jarrovi* and *T. rugosa* examined whether the parietal eye of lizards is involved in homing orientation: lizards of both species orientated at random when the parietal eye was entirely covered with a patch [57, 59]. Recent investigations in *P. sicula* tested whether an intact parietal eye is necessary for goal orientation in a Morris water maze under the sun. Ruin lizards with parietal eye covered or ablated were randomly orientated inside the Morris water maze [61]. This strongly suggests that only the parietal eye – and not other brain photoreceptors [34, 35] – is involved in lizard orientation. Since in all these experiments lizards with fully covered parietal eyes were disoriented in spite of the fact that their lateral eyes were unobstructed, the data so far available strongly support the view that contribution of the lateral eyes to sun-compass orientation of lizards is quite irrelevant.

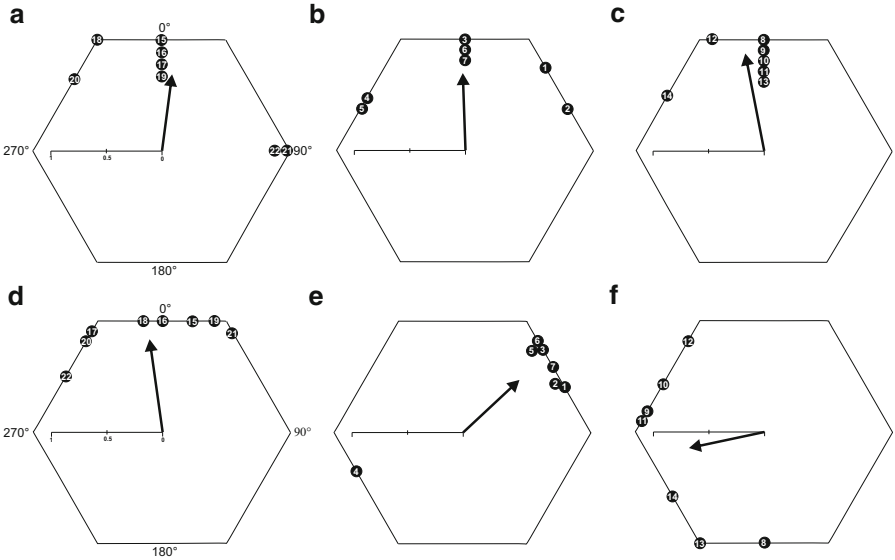


**Fig. 10.4** Clock-shift experiments in *P. sicula*. Each symbol indicates the directional choice of a single lizard identified by its number. In each hexagon the *inner arrow* represents the mean vector of the group, the length of which can be read from the scale. The goal direction is 0°. Control lizards: (**a**) directional choices in the last trial of the last training session and (**d**) in the single orientation test carried out 7 days later. Slow clock-shift lizards: (**b**) directional choices in the last trial of the last training session and (**e**) in the single orientation test carried out after the 6-h slow clock shift. The mean vector of slow clock-shift lizards deviated clockwise with respect to training direction, as expected on the basis of the treatment. Fast clock-shift lizards: (**c**) directional choices in the last trial of the last training session and (**f**) in the single orientation test carried out after the 6-h fast clock shift. The mean vector of fast clock-shift lizards deviated counterclockwise with respect to the training direction, as expected (Modified from [61])

It was suggested that the lizard parietal eye could also mediate a sky polarization compass sense, which may provide advantage over a sun azimuth compass, since it continues to work even when the sun's position is obscured by vegetation and clouds [57–59, 64]. Hence, it should be taken into account that sunlight passing through the atmosphere becomes partially polarized, as a function of the scattering angle. The e-vector of each scattered ray exhibits a prevailing vibration direction, which is perpendicular to the plane in which the ray was deflected (Rayleigh scattering [65]). This results in a symmetrical e-vector pattern, which is fixed with respect to the solar and antisolar meridians and can be used as a reference direction. The compass described above was defined as a sky polarization compass [66]. Often small lizards find themselves in conditions where they have to hide themselves under vegetation and cover to avoid predation, and, at the same time, the need to orientate within or toward their territories or burrows or toward known food sources may render the use of a sky polarization compass adaptive.

The hypothesis that the parietal eye may mediate a sky polarization compass is supported so far by its anatomical organization. The photoreceptors in the parietal

eye are arranged in a pattern that would theoretically allow for analysis of the e-vector of linearly polarized light [64, 67]. Results of experiments carried out in *U. notata* [58] and in *T. rugosa* [64] support the hypothesis that lizards can potentially use the e-vector direction of polarized light in the form of a sky polarization compass, but the effective role of the parietal eye in detecting sky polarization was not tested directly in these lizards. In *P. sicula*, laboratory experiments confirm the capability of using the e-vector direction of polarized light in orientation (Fig. 10.5a) [68] and further demonstrate the central role of the parietal eye in the functioning of a sky polarization compass (Fig. 10.5b) [68, 69]. Finally, in *P. sicula* outdoor experiments showed that the sky polarization compass is truly time compensated [70]. To do this the training and the orientation tests of lizards were performed under clear skies, with the sun disk not in view, before and after a 6-h clock shift. In such a situation, the directional choices of lizards were bimodally distributed (Fig. 10.6) [70]. The striking bimodal orientation – toward/away from the goal platform with the sun disk not visible – is explained by assuming that lizards orientate themselves by using sky polarization alone. In fact the e-vector pattern alone does not allow to discriminate between solar and antisolar meridian, and such a 180° ambiguity imposes an axial – bimodal – orientation instead of a unimodal one [71]. Six hour clock-shifted ruin lizards showed a bimodal distribution of directional choices, which was oriented perpendicularly to the training axis, as it was expected on the basis of the clock shift (Fig. 10.6). The axial nature of the observed orientation response does not allow to distinguish between the behavioral effects of a fast clock shift (expected counterclockwise deflection) and those of a slow clock shift (expected clockwise deflection). These data demonstrate for the first time that the only known celestial diurnal compass mechanism which does not need a direct vision of the sun disk, i.e., the sky polarization compass, is a time-compensated compass [70].

A time-compensated sky polarization compass is a very high-level task, since it requires knowledge of the time of day throughout the year and the capability of detecting the e-vector of polarized light. The data gathered so far in *P. sicula* showed that this lizard has all these capabilities: (1) it possesses a circadian clock allowing to exactly determine the time of day and a circannual clock to determine seasons throughout the year [38, 72, 73]; (2) it can use the e-vector direction of polarized light for compass orientation [68, 69].

## 10.7 Conclusions and Perspectives

The data available so far support the existence of a complex circadian organization in reptiles with the capability of the system to adapt to seasonal changes in environmental conditions, not only light but also ambient temperature. As regards light, there are extrapineal, extraretinal photoreceptors which warrant entrainment of behavioral rhythms in all lizard species tested so far after ablation of the lateral eyes and pinealectomy. Due to the actual impact of global warming on animal

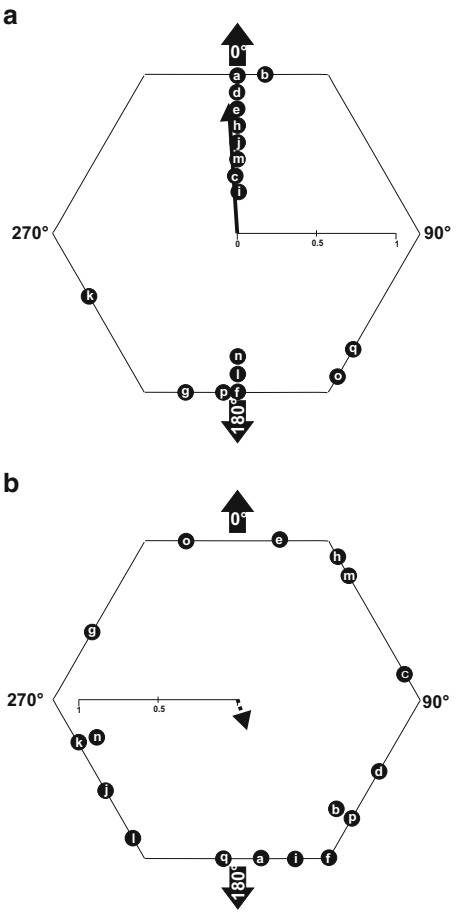


**Fig. 10.5** Indoor experiment with a Morris water maze in *P. sicula*. Orientation behavior of lizards trained and tested under an artificial light source producing plane polarized light with a single e-vector, a condition in which lizards are expected to show bimodal orientation. Two *outer solid arrows* show the expected axis of orientation under polarized light. In each hexagon the *inner arrow* represents the mean vector of the group calculated after doubling the angles. Solid line mean vector: the bearings distribution deviated from uniform; dotted line means vector: the bearings distribution did not deviate from uniform. (**a**) Intact lizards display the expected bimodal orientation under the single e-vector of polarized light. (**b**) These lizards were further tested under the single e-vector of polarized light after their parietal eyes were painted black. The directional choices of these lizards did not deviate from uniform, hence showing that these lizards are not capable of correct orientation after their parietal eyes were covered (Modified from [68])

physiology and adaptations, all studies concerning the role of seasonality and changes in ambient temperatures on circadian organization become more important. The peculiar role of the pineal discovered in our model animal, the ruin lizard *Podarcis sicula* consists in the presence in winter of a melatonin rhythm in vitro but not in vivo. Since this supports the view that the SCN suppresses melatonin rhythm

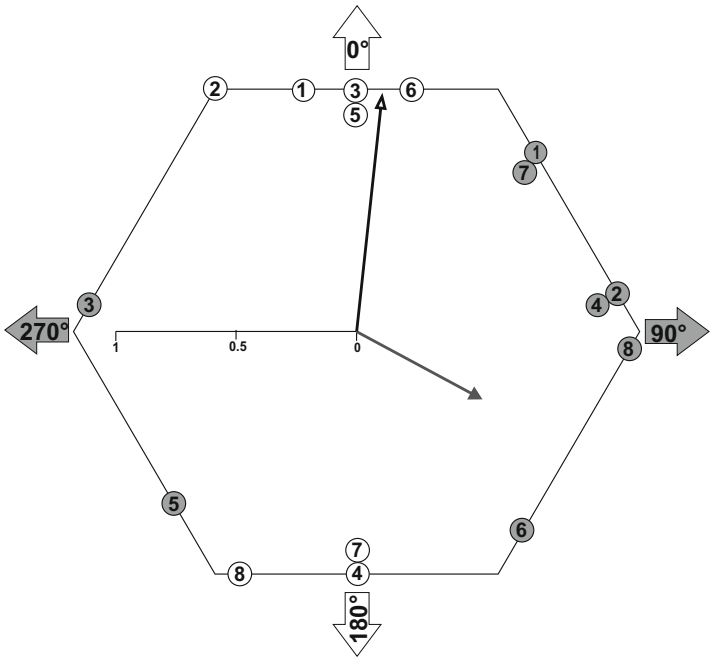


**Fig. 10.6** *P. sicula* tested outdoor in a Morris water maze under clear skies with the sun disk not in view. Each *empty circle* identified the directional choice of a lizard before clock shift and each *solid circle* with the same number the directional choice of the same lizard after 6-h clock-shift treatment. Empty *outer arrows* and *solid arrows* indicated the expected axis of orientation before and after clock shift, respectively. Inner *empty* and *solid arrows* represent the mean vectors of orientation obtained after doubling the angles before and after clock shift, respectively. The axis of orientation after clock shift was rotated perpendicularly with respect to the training axis, as expected on the basis of the clock shift. Other information are in Figs. 10.4 and 10.5 and in the text

in winter via its innervation, future research should investigate the effectiveness in vitro of various neurotransmitters eventually involved in changing or suppressing the pineal melatonin rhythms via innervation through the SCN. Another possibility that has to be taken into account is that the lizard pineal may be able to detect the changes in season by decoding thermoperiod. To test this, future in vitro pineal studies should be devoted to test pineal melatonin profiles under temperature cycles of different amplitudes and durations and in different seasons.

As regards the studies on celestial compass orientation mechanisms, new functional/molecular studies may be crucial to better understand the role of the parietal eye in orientation. Molecular investigations in *P. sicula* already identified pinopsin, a photopigment, which is expressed inside the pineal complex, including the parietal eye, but not in the rest of the brain [74]. Future research should be aimed at testing whether spatial distribution of previously identified photopigments expressing pinopsin within the parietal eye [74] were functionally and anatomically suited to accomplish the "matched filtering" of the e-vector pattern in the sky which

was already shown to occur in ommatidia (specifically the POL area) of desert ants and honeybees [75].

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# Chapter 11
# Avian Circadian Organization

**Vincent M. Cassone, Jiffin K. Paulose, Clifford E. Harpole, Ye Li, and Melissa Whitfield-Rucker**

**Abstract** This chapter details the organization of the avian circadian clock systems starting with the systemic levels of circadian organization that perceives the external cue (light) and then generates the timing. It has been shown that unlike the mammals, there are three different master clocks that regulate the timing and they are functionally organized in such a way that they produce synchronized events in daily life in birds. The molecular mechanism that generates the timing and then the mechanism by which the circadian clock is entrained to the external environment are discussed. Finally, the chapter explains the role of circadian clocks in the regulation of physiology and behavior of birds.

## 11.1 Introduction

Biological timekeeping is pervasive in avian biology [1]. On both daily and seasonal bases, birds phenologically express complex patterns of biochemistry, physiology, and behavior that are species and niche specific [2]. For example, the diurnal black-capped chickadee, *Poecile* (*Parus*) *atricapillus*, forages for insects and seeds during spring and summer days by hopping along tree branches [3]. They will store seeds in caches located in tree cavities, under bark, or among leaves and needles, where they will retrieve some time later [4]. Chickadees are highly vocal, with at least 13 distinct types of songs and calls. In the morning, male chickadees sing a nuptial song of two whistles, usually in isolation, but there are many other types of calls, including the mnemonic *chickadeedee* call that is so familiar to American bird watchers. During summer nights, chickadees sleep in thick vegetation or in cavities, sometimes huddling with other chickadees. In the winter, these nonmigratory birds continue to forage during the day, supplementing their diet by retrieving cached food, while at night, they undergo torpor, reducing their body temperature by 10–12 °C to conserve energy. In contrast, the nocturnal eastern

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screech owl, *Megascops asio*, is an ambush predator during the night, hunting from perches in semi-open wooded lands and pouncing on small rodents, bats, other birds, reptiles, and insects [5]. Often, they will carry their prey to secluded areas to protect themselves from larger predators. Screech owls are not as vocal in repertoire as are chickadees, although they are known for their series of territorial trills (the so-called screech). The males also express a courtship song, which is essentially a lower key warble than the territorial call. During the day, these birds sleep in cavities or huddled against a tree trunk, taking advantage of its cryptic coloration. As disparate as these two phenologies appear, the temporal organization of the nearly 20,000 species of birds is likely many times more diverse, so it may be perilous to generalize circadian organization among all birds.

Even so, basic principles of circadian organization can be divined from model organisms with the caveat that variations in temporal niches may have profound effects on circadian organization. Certainly, the formal properties of avian circadian rhythms are similar among all species studied in any detail [1], but there are differences in physiological detail. For example, the role of the pineal gland in the circadian organization of nocturnal owls is much reduced, since this organ is reduced or vestigial in most species [6], with reduced secretion of its hormone melatonin in at least one species, *Strix uralensis* [7, 8]. Yet, coding sequences of genes associated with circadian clock function (see below) are highly conserved among barn owls, *Tyto alba*; several species of *Buteo* hawks; passerine birds, such as the house sparrow, *Passer domesticus*; and galliform species such as Japanese quail, *Coturnix coturnix*, and domestic fowl, *Gallus domesticus* [9–14].

## 11.2 System-Level Organization

### *11.2.1 Extraocular Photoreceptors*

Benoit [15] first showed that domestic ducks, *Anas platyrhynchos*, continued to exhibit reproductive responses to changing photoperiod even after blinding by enucleation (EX). In the 1960s and 1970s, Menaker and his students showed that passerine birds clearly did not require the eyes for circadian entrainment [16, 17]. Menaker's group later showed that EX house sparrows entrained to a series of LD cycles of dimmer and dimmer illuminances. At a low level of dim light, birds no longer entrained to the light cycle, free-running with a circadian period. The authors then showed that the responsible photoreceptor resided inside the head by plucking feathers on the top of the birds' heads, reinstating entrainment. Entrainment was then blocked by injection of India ink beneath the scalp [17].

At least four distinct structures within the brain are functionally photoreceptive, containing several opsin-based photopigments and photoisomerases [18–20]: the pineal gland, which expresses a pineal-specific opsin, pinopsin [12, 21, 22], as well as melanopsin (OPN4) [12, 23, 24] and iodopsin (OPN1) [25, 26]; the preoptic area,

which expresses VA (vertebrate ancient) opsin [27, 28] and projects to the tuberal hypothalamus; the tuberal hypothalamus itself, which expresses OPN4 and neuropsin (OPN5) [20, 29]; and the lateral septal organ which expresses rhodopsin-like immunoreactivity [30]. The specific function of each of these photoreceptive organs is not at this stage completely known, although they likely subserve overlapping processes.

## *11.2.2 Pacemakers*

Circadian rhythmicity is widely distributed among tissues and cells in multicellular organisms [33]. "Pacemakers" have the capacity for sustained circadian rhythmicity and the ability to influence rhythmicity of tissues downstream. In birds, these structures are the pineal gland, the retinae, and the hypothalamic suprachiasmatic nuclei (SCN), whose mutual interactions are critical for overt circadian organization.

### 11.2.2.1 Pineal Gland as Pacemaker

Pinealectomized (PINX) house sparrows [34] become arrhythmic when placed in DD, demonstrating that the pineal gland is necessary for self-sustained circadian rhythmicity. However, the birds were still able to entrain to LD cycles. Zimmerman and Menaker [35] transplanted pineal glands from two groups of house sparrows into the anterior chambers of the eye of PINX, arrhythmic sparrows maintained in DD. The first group of donor birds were entrained to an early LD cycle, with lights on at midnight, while the second set of donors were entrained to a late LD cycle, with lights on at 11 AM. Transplantation restored circadian rhythms to both groups of recipients within 1 day. Moreover, birds that received pineal glands from early donors exhibited an early phase ($\phi_i$), while the recipients of late donor pineal glands exhibited a late $\phi_i$. Thus, the pineal gland is both responsible for circadian rhythmicity but also confers time of day to birds.

Because rhythmicity was restored almost immediately, before reinnervation could occur, it is believed that the pineal gland's action is due to its endocrine nature. Studies by a large number of researchers have established that the pineal hormone is melatonin [37], whose biosynthetic pathway is well known in many species of vertebrates, including birds. Pinealocytes, the photoreceptive, secretory cells of the avian pineal gland, take up the essential amino acid tryptophan, which is converted to 5-hydroxytryptophan by tryptophan hydroxylase (TrH; EC 1.14.16.4; [38]) and then decarboxylated to produce serotonin (5HT) by aromatic L-amino acid decarboxylase (AAADC; EC 4.1.1.28). During the night in LD and subjective night in DD, 5HT is converted to N-acetylserotonin (NAS) by arylalkylamine (or serotonin)-N-acetyltransferase (AANAT; EC 2.3.1.87; [39]. NAS is then converted to melatonin by hydroxyindole-O-methyltransferase (HIOMT; EC

2.1.1.4; [40]. The genes encoding each of these enzymes have been isolated, cloned, and sequenced in several avian species. In the domestic chicken, at least, TrH, AANAT, and HIOMT are regulated by both the molecular clockworks within the pinealocytes and directly by light at the transcriptional, translational, and posttranslational levels, so that the enzymatic regulation of pineal melatonin is a dynamic, rhythmic process [36].

Rhythmic melatonin administration, like pineal transplantation, can restore rhythmicity in PINX house sparrows, European starlings, *Sturnus vulgaris,* zebra finches, *Taeniopygia guttata*, or to EX/PINX pigeons, *Columba livia* [41–46] (Figs. 11.1, 11.2, 11.3, and 11.4). Locomotor activity is inhibited in diurnal birds by melatonin administration, and locomotor activity rebounds following the daily dosage, suggesting that melatonin induces a soporific state and/or sleep. In addition, the synchronization of locomotor behavior by rhythmic melatonin administration entrains the circadian clockworks in the PINX bird, since melatonin administration to house sparrows and pigeons in T-cycles different from 24 h results in systematic changes in the phase relationship (ψ) of melatonin to the onset of locomotor activity [42, 47]. Further, when three different behaviors, locomotor behavior, call, and song, are measured simultaneously in zebra finches, a daily melatonin regime entrains these outputs at differential rates, suggesting the hormone is entraining separable oscillators [46].

Avian pineal glands contain the circadian clockworks and photoreceptors to generate circadian patterns of melatonin biosynthesis in vitro as well as in vivo, which can be entrained to LD cycles directly [26, 48]. Pineal tissue and cell cultures express circadian patterns of AANAT activity [49, 50], gene expression [51], and melatonin efflux [52, 53] such that levels are high during the night and low during the day in LD. However, the rhythmic outputs from these cultures are not self-sustained; they persist for 2–8 days in DD before damping to arrhythmicity [54].

#### 11.2.2.2 Ocular Retinae

Surprisingly, retinal photoreceptors also synthesize and release melatonin in many vertebrate species. In Japanese quail and domestic pigeon, the retinae release almost as much melatonin into the systemic circulation as does the pineal gland, and removal of this source in addition to PINX results in arrhythmic circadian locomotor behavior, similar to the effects of PINX alone in passerine birds [55–57]. Thus, there is a variability in the effects of PINX among birds. This may in part be due to this retinal component in some species and that it is not the pineal per se but rhythmic melatonin that is important for circadian locomotor behavior.

#### 11.2.2.3 Suprachiasmatic Nuclei

In birds, two sets of structures have been associated with SCN function: the medial suprachiasmatic nuclei (mSCN) and the visual suprachiasmatic nuclei

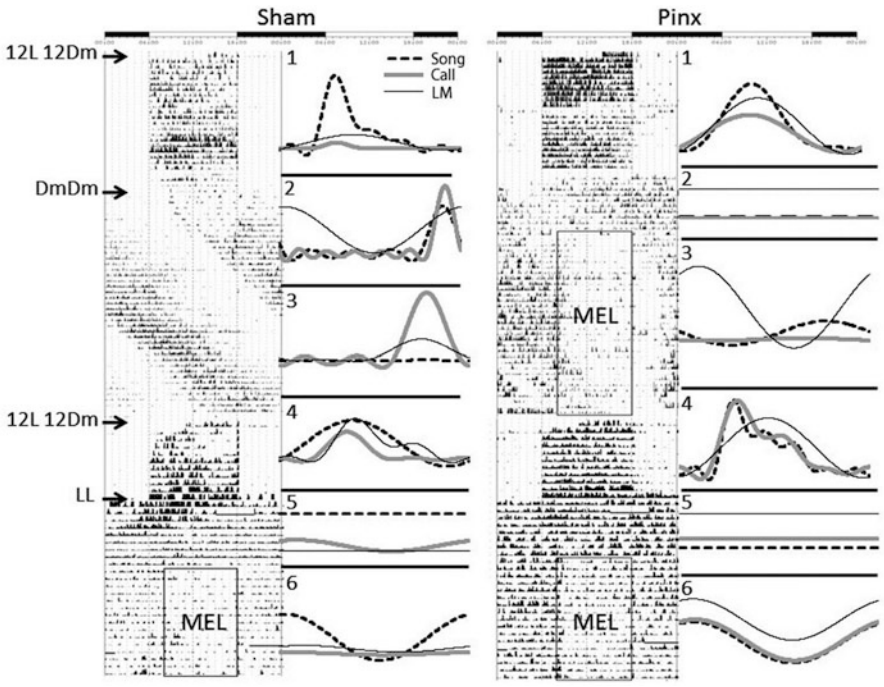


**Fig. 11.1** Effects of light conditions and rhythmic melatonin administration on the circadian rhythms of locomotor activity, singing, and calling in one SHAM (**a**) and one PINX (**b**) zebra finch. Single-plotted actogram on the left of each figure indicates locomotor activity, and the *blue rectangles* indicate the period of melatonin administration. The series of subfigures (*1–6*) on the right of each figure indicates the oscillation of locomotor activity (*green*), singing (*blue*), and calling (*red*) with cosinor-wave fitting in the conditions corresponding to the conditions to the left on the actogram. The *bar* on the *top* of each figure indicates the day (*white*) and night (*black*) phase in the 12L 12Dm conditions. *Abbreviations*: *DD* DmDm, *LD* 12L 12Dm, *Mel* melatonin administration, *PINX* pinealectomized, *SHAM* sham-operated [46]

(vSCN) [58–60]. Anatomical studies have shown these structures are connected via neuronal projections and are contiguous in terms of their cellular populations, especially in the distribution of astrocytes. The vSCN, but not the mSCN, expresses metabolic rhythmicity and electrical activity such that levels are high during the day and low during the night [43, 61–64]. Further, the vSCN, but not the mSCN, receives retinohypothalamic (RHT) input [58–60, 64, 65], and the vSCN, but not the mSCN, contains melatonin receptor binding [66, 67]. Administration of melatonin to chicks and house sparrows decreases glucose utilization within the vSCN [62, 64]. Finally, light activates *c-fos* expression in the vSCN, but not in the mSCN [68]. In quail, only the mSCN expresses clock gene rhythmicity [69, 70], while in the house sparrow, both structures rhythmically express the expression of *per2* [11, 71]. Importantly, lesions directed at the mSCN

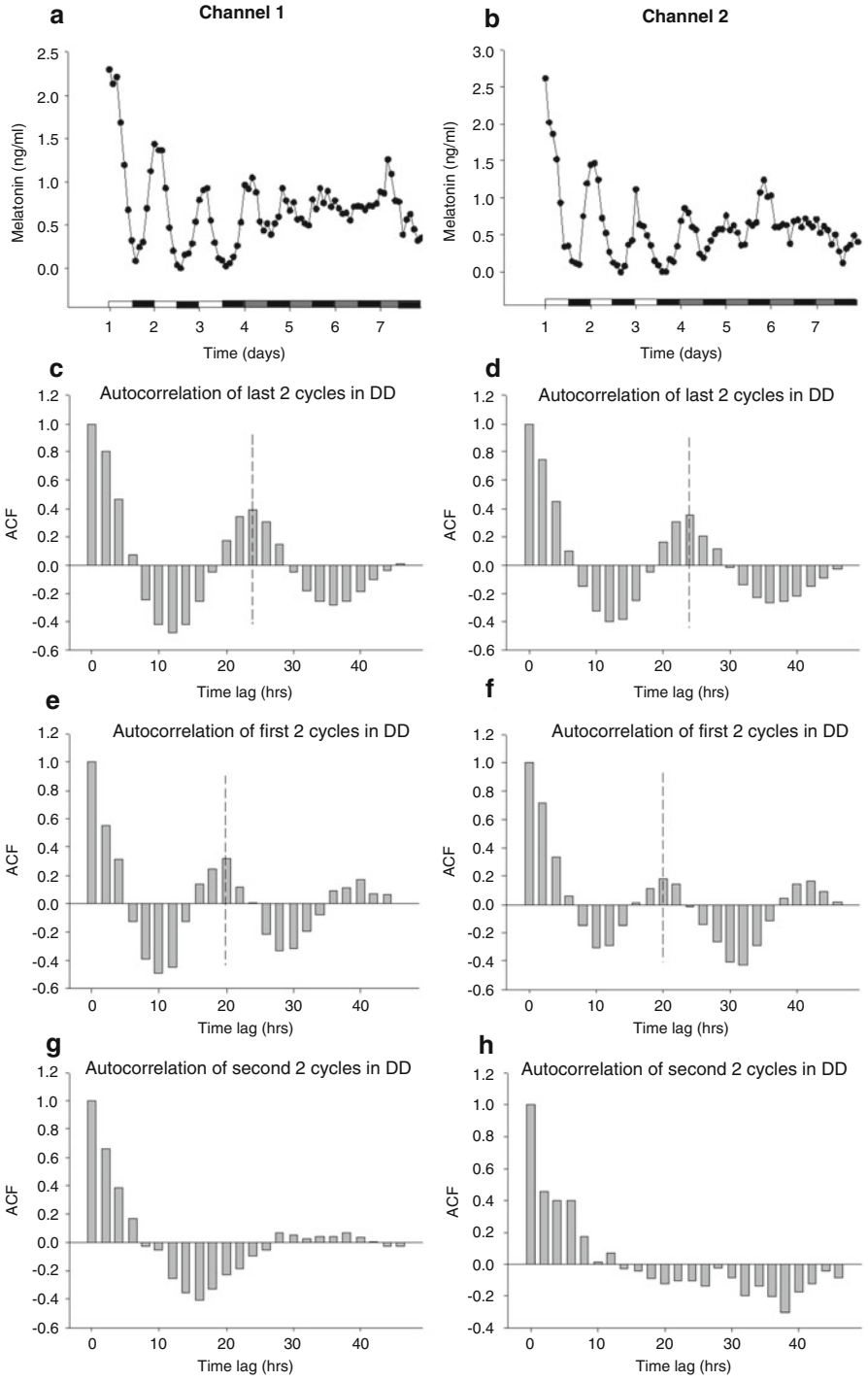


**Fig. 11.2** Melatonin rhythms from dispersed chick pineal cells in two individual channels. (**a**) Melatonin output from single channel 1 from dispersed pineal cells in 3 days LD and 4 days in constant darkness (DD). The X-axis indicates time in days. Day 1 is defined as the day when the perfusion system started (the same definition applies to all the x-axes in the following figures).

in Java sparrows, *Padda oryzivora*, and house sparrows result in arrhythmicity similar to that observed following PINX [72, 73].

### *11.2.3 The Avian Circadian System*

Circadian rhythms in birds are controlled by the multiple circadian pacemakers described above that are entrained by the multiple photoreceptive elements in the central nervous system. The relative importance of these pacemakers varies among the few species studied, but in each, the core of the circadian system can be identified as the pineal gland, the retinae, and the SCN (mSCN and vSCN).

There are two complementary theories for how the circadian system in birds is coordinated. In the "neuroendocrine loop model" for avian circadian organization, pineal and SCN pacemakers are damped circadian oscillators [74]. Damping may be due to individual cells within these structures and/or due to self-sustained oscillators within the structure(s) drifting out of phase from one another (Fig. 11.2). In this scenario, light inhibits the output of the pineal gland oscillators, passing through the skull of the bird and acting through photoreceptors within the gland themselves, and light activates SCN output as well as possessing the capacity to phase shift the clocks within each. During the night and subjective night in DD, pineal oscillators secrete melatonin and influence a wide array of downstream processes and structures. Among these are the vSCN, which are inhibited by melatonin. However, because the pineal is an endogenous oscillator, its output declines as dawn and subjective dawn in DD approach, disinhibiting SCN (vSCN and mSCN) output. The SCN in turn are active during the day and subjective day in DD, affecting a wide array of downstream processes and structures. Among these are the circadian rhythm in sympathetic outflow in NE turnover in the pineal gland at least, which are high during the day and low during the night. There, NE inhibits the biosynthesis and release of melatonin until, since the SCN are circadian oscillators, their output diminishes, and pineal oscillators are disinhibited. This mutually inhibitory relationship of SCN and pineal oscillators maintains stable phase relationships and enable each pacemaker to affect downstream processes singularly or in concert. Alternatively, Gwinner [75] proposed that the relationship

**Fig. 11.2** (continued) The *white bar* on the x-axis indicates daytime with light, the dark bar indicates nighttime in darkness, and the *gray bar* indicates the subjective daytime in constant darkness. (**b**) Melatonin output from single channel 2 from dispersed pineal cells in 3 days LD and 4 days in constant darkness (DD). (**c**–**h**) Represent the autocorrelation functions (ACF) corresponding to different phases of the melatonin output rhythms. The time lag (*dashed line*, indicating the period) of the last two cycles in LD is 24 h for both channels and ACF are 0.39 for channel 1 (**c**) and 0.35 for channel 2 (**d**); the time lag of the first two cycles in DD is 20 h for channel 1 (**e**) and channel 2 (**f**) with ACF as 0.25 and 0.18 for each. The time lag and ACF of the second two cycles in DD were not detected (**g** and **h**) [53]

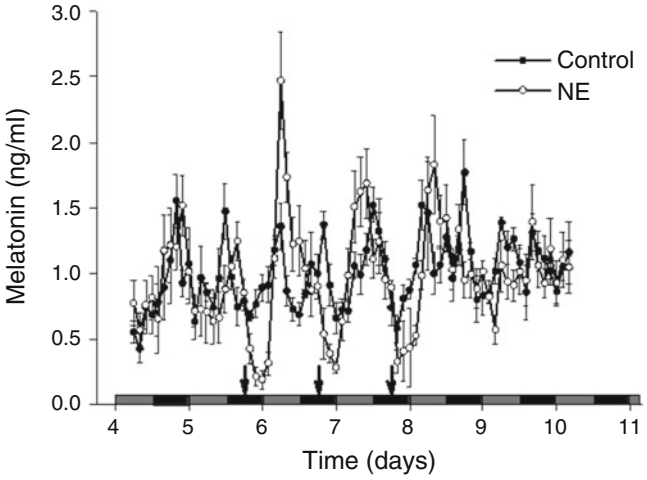


**Fig. 11.3** Effects of three consecutive NE administrations on chick pineal melatonin output. Dispersed pineal cells were cultured in a super-perfusion system for 3 days under LD and 7 days under DD. The plotting starts from CT 6 in day 4 under DD. At CT18 of day 5 to day 7 under DD, 100 μL of medium with NE (1 μM) or ascorbate was administered (the three *arrows*) to each channel of the treatment group (*white circles*) or control group (*black circles*) [53]

between pineal and SCN oscillators conveyed an "internal resonance" in which each oscillator increased the amplitude of its partner. These ideas are not mutually exclusive from the neuroendocrine loop model. Indeed, it is clear that application of NE to chick pineal glands increases the amplitude of melatonin output in vivo [76] and in vitro [53, 54] even as it inhibits melatonin output (Fig. 11.3). The pineal gland releases rebounding high levels of melatonin release following cessation of the NE administration.

Where do the retinae fit into this scheme? For columbiform and galliform species whose retinae secrete melatonin rhythmically, at least part of their role is to serve as a "second (and third) pineal gland" [77], secreting melatonin during the night, presumably influencing SCN and other downstream oscillators in parallel with pineal output. It is clear, however, that the retinae are the predominant pacemakers in galliform birds, but the nature of this function is not apparent at this stage [78].

The mechanism(s) by which the components of this system influences each other at the molecular level or by which the system influences downstream processes is not at all clear at this stage. However, new and exciting data pointing to distributed capacities for rhythmicity are becoming available, and these are described below in regard to peripheral oscillators. First, however, we must explore the molecular aspects of avian circadian clocks.

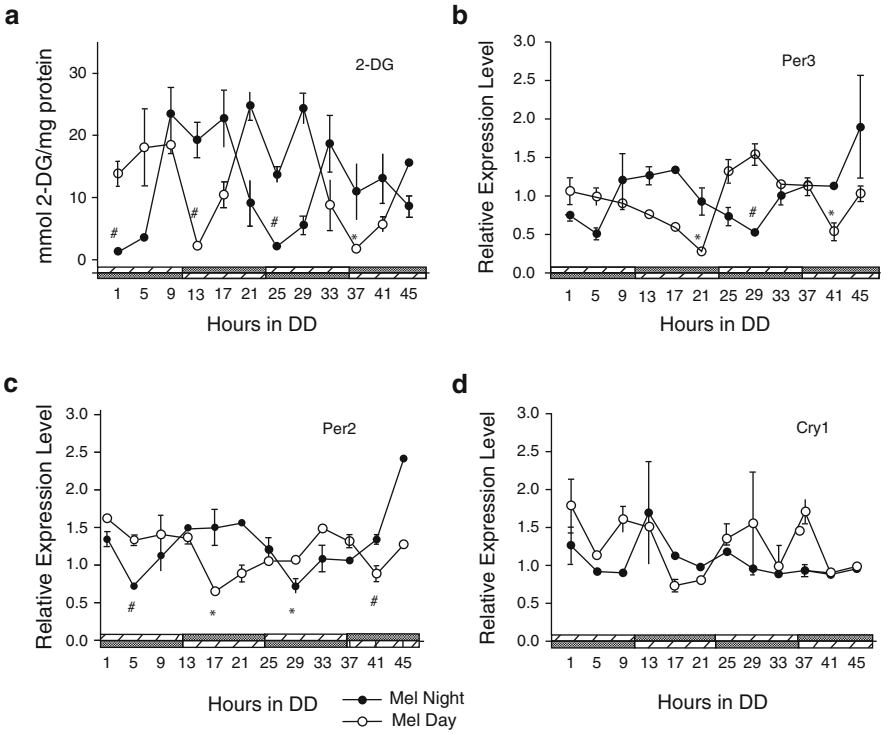


**Fig. 11.4** Exogenous melatonin imposes metabolic and clock gene rhythms in chick diencephalic astrocytes. (**a**) Under constant conditions, opposing cycles of rhythmic melatonin (nM) administration, either during the night or during the day, elicited rhythmic uptake of glucose in the cultures, such that 2DG uptake was higher during the time which melatonin was not present ($n = 4$). (**b**) The gPer3 mRNA rhythm under the MN cycle ($n = 3$ sample replicates) was 180° antiphase with respect to the rhythm generated under the MD cycle ($n = 3$ sample replicates). (**c**) The gPer2 mRNA rhythm under the MN cycle ($n = 3$ sample replicates) was 180° antiphase with respect to the rhythm generated under the MD cycle ($n = 3$ sample replicates). (**d**) No clear pattern of gCry1 expression was evident under either condition ($n = 3$ sample replicates each). For determined rhythmic cycles, significant differences between peak-to-trough values are indicated by # ($p < 0.05$) or * ($p < 0.001$). Comparisons were made between the first observed peak and trough for each day for each treatment [90]

## 11.3 Molecular Bases of Avian Circadian Clocks

At the molecular level, circadian rhythms are regulated by a highly conserved set of genes, collectively called "clock genes" in animals ranging from *Drosophila* to humans. The products of these genes dynamically interact to elicit rhythmic patterns of transcription, translation, biochemical and physiological processes, and behavior [31–33, 79, 80]. The central core of this gene network can be broadly characterized as "positive elements" *clock* and *bmal1* and "negative elements" *period 1* (*per1*), *period 2* (*per2*), *period 3* (*per3*) and the cryptochromes

*cryptochrome 1* (*cry1*) and *cryptochrome 2* (*cry2*). In contrast to mammals, birds do not express a *per1* and have been shown to only express only *per2* and *per3* [9, 12, 70, 81]. *Clock* and *bmal1* are transcribed and then translated in the cytoplasm, where they dimerize and reenter the nucleus and activate transcription of the negative elements through the activation of E-box promoter elements. The *pers* and *crys* in turn are transcribed and translated in the cytoplasm, where the PER proteins are targeted for proteasomal proteolysis by a series of protein kinases, most notably casein kinase 1ε (CK1ε) and CK1δ. This process slows the accumulation of the cytoplasmic PER and thereby increases the period of the molecular cycle. In the cytoplasm, PER and CRY proteins form oligomers that reenter the nucleus and interfere with the CLOCK/BMAL1-mediated activation. A secondary cycle involving two genes containing E-box promoters, *Reverbα* and *rorA*, amplify the cycle by activating and inhibiting *bmal1* transcription, respectively. Disruption and/or knockout of these genes' action has profound effects on the expression of circadian rhythms in animals in which these technologies are possible (i.e., mice and *Drosophila*) ranging from changes in period to arrhythmicity.

## 11.4 Avian Circadian System Revisited (Physiology vs. Molecular Clocks)

While clock genes are expressed by cells within pacemaker tissues [9, 12, 70, 81, 82], it was surprising to find rhythmic expression of clock genes in other parts of the brain as well as in peripheral tissues, such as the heart, liver, lungs, and gonads [83–86]. The question remains whether the pacemaker properties of the pacemaker tissues, which affect central and peripheral tissues physiologically, act through the expression of clock genes. Further, it is unclear whether the physiological effects of system components act on central and peripheral tissues at the transcriptional, translational, or enzymatic levels.

As stated above, PINX abolishes SCN metabolic rhythms [43], and administration of melatonin inhibits SCN metabolic activity in house sparrows and chickens [62, 64], and daily administration of the hormone entrains behavioral rhythms in house sparrows and zebra finches [46, 62]. However, in Japanese quail, at least, melatonin has little effect on mSCN clock gene expression [70]. Continuous administration of melatonin elevated blood levels of the hormone to supraphysiological levels, but did not affect the circadian patterns of *per2*, *per3*, or *clock* in the mSCN, nor did acute injection of the hormone. This lack of congruence between clock gene expression and physiology is not unique to birds, since melatonin administration in rats has little immediate effect on SCN clock gene expression [87], even though the hormone inhibits SCN metabolic and electrical activity [88, 89] to determine whether pacemakers in the pineal and eyes were responsible for peripheral clock gene rhythms. Karaganis et al. [85] showed that PINX or EX of chicks decreased the amplitudes of clock gene

expression and changed the $\phi_i$ of rhythms in *cry1*, *per3*, and *bmal1* rhythms but did not abolish them.

Similarly, it is also clear that sympathectomy disrupts circadian patterns of melatonin release from the chick pineal gland in vivo, and rhythmic NE administration decreases melatonin output and synchronizes a daily pattern [76]. Further, circadian patterns of melatonin in vitro damp to arrhythmicity after 3–10 days in DD, and administration of NE inhibits melatonin output and synchronizes a daily pattern [53, 54]. Yet, even as the rhythm of melatonin damps to arrhythmicity, clock gene expression patterns remain robustly rhythmic in the chick pineal gland, and NE has no effect on pineal clock gene expression [53].

Thus, much needs to be done to resolve the system-level properties of the avian (and other vertebrates) circadian clock with the putative molecular mechanisms underlying them. Perhaps, coupling among pacemaker tissues and between pacemakers and peripheral oscillations is not as strong as is coupling between zeitgebers and the system as a whole or between the distributed zeitgebers and individual pacemaker. One clue to this is evident from work in our lab on cultured hypothalamic astrocytes from embryonic chicks [90]. Chick astrocytes express functional $Mel_{1A}$ (MT1) and $Mel_{1C}$ melatonin receptors [91], and melatonin acutely affects astrocytic physiology. First, exogenous melatonin inhibits that metabolic activity, as measured by 2-deoxyglucose uptake [91]. Second, the hormone affects intercellular communication by increasing intracellular resting calcium levels and enhancing calcium waves, which spread from cell to cell [92]. Yet, a single melatonin administration of melatonin has no effect on clock gene expression [90]. However, when 1 nM melatonin is administered to astrocyte cultures for 12 h every day for 7 consecutive days, the hormone synchronizes a daily rhythm in 2DG uptake as well as *per2* and *per3* content (Fig. 11.4) [90]. The implication is that melatonin does affect clock gene expression, but it takes multiple cycles for entrainment to occur.

## 11.5 Conclusion

Avian circadian organization is a complex of mutually coupled, multiple pacemakers, each of which has direct and indirect photic input from multiple ocular and extraocular photoreceptors. The dynamics by which these pacemakers couple at the physiological/biochemical levels indicate that the SCN influences pineal oscillators via sympathetic inhibition of melatonin biosynthesis, and the pineal affects the SCN via melatonin-mediated inhibition of metabolic and electrical activity. However, the molecular mechanism by which these pacemakers influence (or do not influence) the molecular clockworks within the target pacemaker cells is not at all clear. Future research on the coupling of clock gene expression to the pacemakers' and system's physiological and behavioral outputs may require more detailed knowledge of the regulatory sequences of the avian clock genes themselves.

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# Chapter 12
# The Mammalian Neural Circadian System: From Molecules to Behaviour

**Beatriz Bano-Otalora and Hugh D. Piggins**

**Abstract** Circadian rhythms pervade all aspects of mammalian physiology and behaviour, providing a near 24 h temporal architecture for all major brain and body systems. Mammalian chronobiology research has focused on nocturnal laboratory rodent models, and this has yielded valuable insight into the neural basis of circadian timekeeping. This has identified the suprachiasmatic nuclei (SCN) as the dominant circadian pacemaker and characterised its neurophysiology, neurochemistry, and potential mechanisms of behavioural control. However, nocturnality is only one of four possible temporal niches, and understanding of the neural circadian system in diurnal, crepuscular, and cathemeral mammalian species is very much in its infancy. In this chapter, we review the fundamental properties of the neural circadian system in nocturnal rodents and then compare this with what is known about neural timekeeping in diurnal species. Through this, we identify gaps in our knowledge and key problems to investigate in order to gain a more complete understanding of circadian control of behaviour and physiology, particularly with regard to temporal niche preference.

## 12.1 Introduction to the Suprachiasmatic Nucleus as the Central Brain Clock

Since the early twentieth century, behavioural research has shown that many wild-caught as well as laboratory-bred rodent species have a propensity for voluntarily exercising in running wheels with the onset of this activity tightly linked with the time of lights off. Further, when assessed in the absence of a light-dark cycle, rhythms in locomotor activity persisted, with a period that deviated from the 24 h pattern seen under light-dark conditions. Based on this and a multitude of other findings, researchers in the 1970s surmised that the daily brain clock controlling these rhythms should receive visual information. Through mapping visual pathways and making discrete lesions in brain nuclei receiving retinal innervation, it

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was discovered in 1972 that the suprachiasmatic nuclei (SCN) of the hypothalamus contained the circadian pacemaker controlling daily rhythms in the physiology and behaviour of nocturnal rats [1, 2]. Subsequent demonstration that behavioural rhythms are rescued in SCN-lesioned adult rodents by implants of fetal SCN tissue cemented the SCN as the master pacemaker in mammals and catalysed modern neuroscience investigation of the biological timekeeping mechanisms.

Neuroanatomists in the late nineteenth and early twentieth century had identified the SCN as distinct structures in the mammalian hypothalamus [3], but their function was unknown until the 1970s. The SCN are readily identifiable in histological preparations, in part, because the density of cell packing in this structure is relatively high compared to adjacent anterior hypothalamic structures. Consequently, the SCN are now well-characterised, particularly in nocturnal rodent species such as rat (*Rattus norvegicus*), mouse (*Mus musculus*), Syrian hamster (*Mesocricetus auratus*) and Siberian hamster (*Phodopus sungorus*). The SCN are bilobed, situated on either side of the ventral floor of the third ventricle in the periventricular zone of the anterior hypothalamus. In the adult laboratory rat, they are ~0.7 to 1 mm in length along its rostrocaudal axis, ~0.5–0.6 mm on the dorsoventral plane and ~0.3–0.5 mm wide on the mediolateral plane. The SCN vary in morphology along their rostrocaudal axis (described as 'rugby ball shaped' in the rat) as well as between species, and consequently, these parameters are general estimates.

Cytologically, the SCN contain both neurons and astroglia with an estimated ratio of 7–8:1 in the rat SCN (reviewed in [4]). In rodents, SCN neurons are among the smallest of the brain, with cell body diameter of ~8–10 μm. The size of these cells in the medial SCN is smaller (~8 μm in diameter) than neurons in more lateral areas (~10 μm). Similarly, the density of cell packing is heterogeneous in the rat SCN with more cells per volume in the dorsomedial compared to lateral areas. It has been speculated that this dense cell packing and reduced extracellular space between neurons in the dorsomedial SCN may enable ephaptic communication between these cells, an idea that has yet to be experimentally scrutinised.

Physiologically, four key features define circadian timekeeping in the nocturnal rodent SCN: (1) The SCN exhibits daily changes in the uptake of 2-deoxyglucose, a marker of metabolic activity [5]. Since this is higher during the day than at night, this indicates that the SCN is metabolically most active during the animal's behavioural quiescent phase of the circadian cycle. (2) Consistent with the metabolic profile, *in vivo* and *in vitro* electrophysiological recordings show that SCN neurons of nocturnal rodents are spontaneously active and intrinsically generate ~24 h rhythms in the frequency of action potential (AP) discharge [6, 7]. This AP firing rate rhythm peaks during the behaviourally quiescent day, with the nadir during the active phase at night. (3) The 24 h variation in electrical activity does not depend on 'network' properties as dissociated SCN neurons isolated in culture also vary daily discharge of AP firing [8]. (4) SCN neuronal clocks are predisposed to synchronise their activity with another, and intercellular communication is necessary for this process. Thus, the SCN are composed of several thousand cell autonomous oscillators, and the ability of SCN neurons to maintain synchronous

activity in brain slices has enabled the development of 'clock-in-a-dish' preparations. With this *in vitro* model, the resetting actions of drugs on the firing rate rhythm can be readily assessed (see [9] for review).

Behaviourally, examination of nocturnal rodent wheel-running rhythms has allowed the description of several key properties of circadian rhythms. First, wheel running is synchronised or entrained to lights off such that the initiation of vigorous wheel running is coincident with lights off. Second, these rhythms are sustained in constant darkness, and their timing can be adjusted by exposure to short pulses of light. In nocturnal rodents, the onset of the active phase is defined as circadian time (CT12), and light pulses given in the early night (CT13-17) causes phase delays in the onset of the wheel-running rhythm, while the same stimulus given during the latter part of the active phase (CT18-22) causes phase advances. When given during the middle of the circadian day (CT4-8), light pulses are without phase-resetting effects. This phase response curve (PRC) to light is characteristic of many nocturnal rodent species (for review, see [10]).

Different types of stimuli that elicit increases in internal arousal, such as changing the cage or providing a novel running wheel, have very different phase-resetting actions [11]. During the subjective night, such stimuli have little phase-shifting effect, but, when given during the middle of the subjective day, they evoke phase advances in behavioural rhythms. As this PRC tends to be in anti-phase to that of light, such stimuli are called non-photic cues [12].

## 12.2 Connections of the SCN

### *12.2.1 Inputs*

Neuroanatomically, antero- and retrograde tracer compounds as well as viral tools have revealed SCN efferents and afferents. Three main input pathways have been extensively characterised (Fig. 12.1):

1. The retinohypothalamic tract (RHT) is a monosynaptic pathway from melanopsin-containing retinal ganglion cells to the SCN. In some rodent species, such as the rat, the ventral aspect of the SCN is more densely innervated than the dorsal [3], whereas in the others, such as the mouse, retinal innervation is dense in both dorsal and ventral aspects [13]. The main neurotransmitter of the RHT is glutamate, and it primarily acts on ionotropic glutamate receptors expressed by SCN neurons. Glutamate depolarises and increases action potential firing of SCN neurons [14]. In addition, the neuropeptide, pituitary adenylatecyclase-activating polypeptide (PACAP), is co-localised with glutamate in RHT terminals in the SCN where it acts to modulate the actions of glutamate [15]. This is the direct route via which environmental light information is conveyed to the SCN.

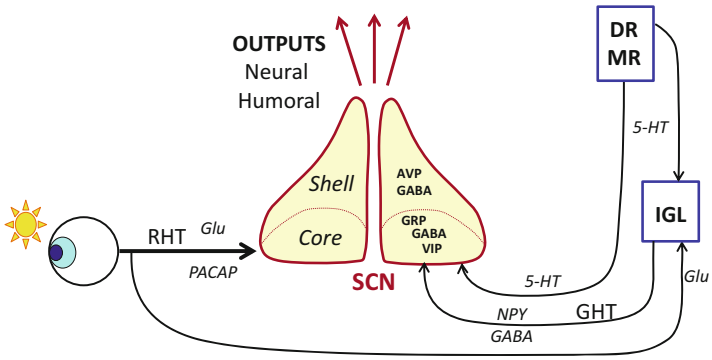


**Fig. 12.1** Mammalian circadian system: main inputs and outputs to the master circadian clock, the suprachiasmatic nucleus (SCN). Photic cues are conveyed to the SCN through the retinohypothalamic tract (RHT) with glutamate (Glu) and pituitary adenylate cyclase-activating polypeptide (PACAP) as main neurotransmitters. The SCN also receives photic information indirectly from the intergeniculate leaflet (IGL) via the geniculohypothalamic tract (GHT). The neurotransmitters used in this pathway are γ-aminobutyric acid (GABA) and neuropeptide Y (NPY). GABAergic and peptidergic projections from the IGL and serotonergic (5-HT) projections from the median raphe (MR) convey non-photic cues to the SCN. Master circadian clock is organised in two main subdivisions: the dorsomedial area or 'shell' characterised by the presence of neurons expressing mainly arginine vasopressin (AVP) and the ventrolateral part or 'core' where most neurons contain vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP). Virtually all neurons across the SCN express the neurotransmitter GABA. Timekeeping signals from the SCN are transmitted to the rest of the brain and body by neural outputs as well as by diffusible humoral signals

2. The geniculohypothalamic tract (GHT) mostly innervates the ventral and central aspects of the rodent SCN and originates from neurons in the intergeniculate leaflet (IGL) of the visual thalamus [16]. The IGL is also retinally innervated and thus represents an indirect route through which environmental light cues are communicated to the SCN. The characteristic neurochemical of the GHT is neuropeptide Y (NPY). This neuropeptide predominantly inhibits SCN neurons (see [9]), while functionally, activation of the GHT, or microinjection of NPY into the SCN [17], resets rodent behavioural rhythms at different times of the circadian cycle to light (non-photic PRC, phase advancing the SCN and circadian rhythms in wheel running during the subjective day, with much smaller effects during the subjective night). Stimuli that elevate internal arousal (such as cleaning a rodent's cage) activate the IGL, and the GHT signals this information to the SCN. In addition to NPY, GABA is also present in the GHT, reinforcing the inhibitory nature of this pathway.
3. The median raphe (MR) innervates the ventral and central SCN aspects, and the neurotransmitter serotonin (5-hydroxytryptophan or 5-HT) is the characteristic neurochemical of this pathway [18]. This neurochemical mostly suppresses SCN neuronal activity, although there is a range of pre- and postsynaptic 5-HT

receptors expressed in the SCN region, adding complexity to its actions. Similar to the GHT, input from the median raphe signals internal arousal state, although the magnitude of the resetting actions of 5-HT is less pronounced than those of the NPY. Nonetheless, current evidence indicates that both the GHT- and MR-SCN pathways are necessary for the resetting actions of non-photic stimuli to be fully realised [19].

In addition to these three pathways, there are also inputs arising from other hypothalamic structures. For example, the orexin neurons of the lateral hypothalamus provide a diffuse innervation to the SCN, with orexin-containing fibres in close proximity to clock gene-expressing neurons. Orexin alters SCN neuronal activity and acts to accentuate the resetting actions of NPY on the SCN, implicating this projection in non-photic entrainment of circadian rhythms [20].

In the SCN, the terminations of the RHT, GHT, and MR pathways overlap, particularly in the ventral aspects. Perhaps unsurprisingly, activation of non-photic pathways can limit the resetting effects of light pulses, while acute light exposure can reduce or eliminate shifts to non-photic stimuli [12]. Thus, SCN neurons actively integrate photic and non-photic cues to shape the phase of the molecular clock and the entrainment of the circadian system to the external world.

### *12.2.2 Major Projections*

From mapping of rat SCN efferents, the idea emerged that the dorsal and ventral aspects of the SCN may differ in their outputs. A crescent-shaped dorsal area, labelled the 'shell' by Moore and colleagues [3], was found to project mainly to the hypothalamus including the dorsomedial hypothalamus (DMH), the preoptic area (POA), the medial subparaventricular zone (mSPVz) and the paraventricular hypothalamic nucleus (PVH). In addition, the 'shell' efferents also targeted the paraventricular nucleus in the thalamus (PVT) and the bed nucleus of the stria terminalis (BST). By contrast, the more ventral 'core' SCN projected mainly to peri-SCN as well as the lateral SPVz and basal forebrain including the lateral septal nuclei. The implication of these anatomical studies is that different compartments of the SCN share and communicate different types of information.

## 12.3 Molecular Basis of Timekeeping

Single SCN neurons function as cell autonomous clocks as they contain the molecular clockworks. Building on key findings on the molecular basis for circadian timekeeping in *Drosophila melanogaster*, from the mid to late 1990s, several labs identified and characterised the interactions of fundamental molecular components of the mammalian circadian clock. These include the *period* (*Per1-2*),

*cryptochrome* (*Cry1-2*), *Clock* and *Bmal1* genes and their associated proteins [21, 22]. There is now a generally accepted model of this molecular clockwork with PER1-2/CRY1-2 constituting the negative limb and CLOCK and BMAL1 the positive limb of the clock. This molecular clock is named the transcriptional-translational feedback loop (TTFL) and conceptually is similar to the molecular circadian clock of flies, yeast and plants. Additional components, such as *Rev-Erbα* [23], *Chrono* [24], *dec1*/*dec2*, and others, function to fine-tune the molecular clock. Further, post-translational modulation of the clock function is provided by casein kinase 1δ/ε enzymes which influence PER-CRY complex stability and translocation to the nucleus to influence circadian clock speed [25]. Similarly, F-box proteins that alter protein ubiquitination and degradation influence CRY protein stability and clock speed in the SCN and other circadian oscillators [26–28]. In addition to this TTFL clock, a second non-TTFL oscillator is present in red blood cells and may be present in neurons [29]. Intriguingly, current evidence indicates that the circadian variation in SCN neuronal activity acts to facilitate the internal molecular clockworks such that chronic interference with electrical activity damps molecular oscillations and desynchronises SCN neurons [9].

### *12.3.1 Compartments of the SCN*

Visualisation of gene expression by *in situ* hybridisation indicates that not all regions of the SCN rhythmically express clock genes at the same phase or perhaps at all (for review, see [30]). In particular, a predominantly dorsal area that forms a crescent-like shape expresses detectable and robust rhythms in *Per1*/*Per2* which peak in the late day, while the ventromedial area that is largely enveloped by the dorsal area expresses rhythms that are weak or difficult to discern. These areas map well onto the ‘shell’ and ‘core’ regions previously described in relation to the differential regional pattern of SCN efferents. Similarly, imaging of luciferase reporters of *Per1* promoter activity or PER2 expression in living rodent SCN brain slices reveals ‘shell’ and ‘core’ regions that are strongly and weakly rhythmic, respectively. Through attempting to model these temporal patterns in bioluminescent signals, terms such as ‘waves’ and ‘tides’ have been used to capture the complex dynamic nature of spatio-temporal organisation of molecular activity in the SCN [30]. Subsequent studies have conceptualised that the dorsal SCN (roughly corresponding to the area designated the ‘shell’ on the basis of SCN extrinsic connections and intrinsic neurochemistry) as initiating the daily change in *Per1*/PER2 expression such that the peak in these signals in some occurs several hours after the peak in the ‘shell’. Thus, while the SCN as a whole functions as the mammalian brain’s master circadian clock, intra-SCN timekeeping is heterogeneous with some areas appearing to lead daily changes in molecular clock activity, while others follow.

## 12.4 Intrinsic Neurochemistry

Neurochemically, all SCN neurons contain GABA, but they can, to an extent, be distinguished by the neuropeptides that they synthesise. The prominent neuropeptides contained in SCN neurons include vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP), and arginine vasopressin (AVP) [31]. - VIP-expressing neurons appear anatomically partitioned from AVP-expressing neurons lending further credence to the idea of 'core' and 'shell' regions of the SCN (Fig. 12.1). As there are many studies of the organisation of the intrinsic neurochemistry of the SCN as well as the local neurophysiological actions of these substances, we direct the reader to recent comprehensive reviews on these topics.

### *12.4.1 VIP*

VIP-containing neurons are present throughout the ventral aspect of the nocturnal rodent SCN. In rat, it is estimated that VIP neurons constitute around 24 % of SCN neurons and these cells delineate the 'core' SCN. VIP neurons have extensive axonal arborisations throughout the SCN, and their axons form part of the SCN output to innervate other hypothalamic sites such as the dorsomedial hypothalamus. The cognate receptor for VIP, $VPAC_2$, is expressed throughout the dorsal and ventral aspects of mouse and rat SCN. Electron microscopy studies have indicated that some VIP neurons are directly retinorecipient and suggest that they form part of an interface with light input to the SCN. Consistent with this, the immediate early gene *c-fos* is induced by light in VIP neurons, while rat SCN firing rate rhythms are reset by VIP and VIP agonists in a pattern resembling the actions of light on behavioural rhythms (for review, see [32]). Further, microinjection of VIP into the SCN region evokes moderate phase shifts in wheel-running rhythms with a PRC resembling light [33].

The acute actions of VIP on SCN neurons are not well understood. Extracellular recordings from rat SCN neurons *in vitro* indicate that VIP evokes both activations and suppressions in the spontaneous firing rate of SCN neurons, with a larger portion of cells responding when tested during the night. The suppressive actions of VIP are mimicked by a $VPAC_2$ receptor agonist. Voltage clamp recordings indicate that VIP elicits the release of GABA, and, therefore, the mixed acute effects of VIP likely represent a combination of pre-and postsynaptic actions [33].

As an intrinsic signal, VIP-$VPAC_2$ communication plays a key role in synchronising SCN neurons; in transgenic mice lacking VIP or $VPAC_2$ expression ($Vip^{-/-}$ and $Vipr2^{-/-}$, respectively), coordination among SCN cellular timekeepers is dysfunctional [34], and these mice express severely disrupted behavioural activity patterns, ranging from arrhythmic to rhythmic with an abnormally short tau (~22 h) [33, 35, 36]. Some of the alterations in cellular rhythmicity and synchrony arising from VIP-$VPAC_2$ signalling deficits can be circumvented by treatment with

other non-VIP agonists [37, 38], revealing functional redundancy in intercellular communication within the SCN.

### *12.4.2 GRP*

Gastrin-releasing peptide (GRP) is synthesised by ~14 % of neurons in the central region of the SCN [3], and its cognate receptor, $BB_2$, is also expressed in the SCN. Similar to VIP neurons, some GRP neurons are retinorecipient, and GRP microinjected into the SCN causes light-like phase shifts in wheel-running behaviour [39]. Further, GRP resets rodent SCN firing rate rhythms *in vitro* with a temporal pattern of sensitivity resembling that of glutamate. GRP and associated peptides activate SCN neurons to elevate firing rate. A greater proportion of SCN cells respond to GRP when tested during the subjective night, and these actions are blocked by a $BB_2$ receptor antagonist. In mice, transgenic deletion of $BB_2$ expression does not affect ongoing rhythmicity but does reduce the phase-resetting actions of light [9].

### *12.4.3 AVP*

Arginine vasopressin (AVP) is arguably the most abundant of SCN neuropeptides (~37 % of rat SCN neurons [3]). AVP is expressed in neurons along the medial border of the SCN as well as in a small cluster of neurons near the ventrolateral pole of the SCN. This neuropeptide is often used as the neurochemical marker of the 'shell' compartment. AVP expression overtly varies over the 24 h light-dark (as well as constant dark) cycle, with high levels of mRNA/protein during the middle of the day and lower levels at night. AVP is a clock-controlled gene and its expression is predictably altered by clock gene mutations. Electrophysiological investigation reveals that AVP activates rodent SCN neurons [40]. These effects are mediated by AVP V1a and V1b receptors that are expressed in the SCN [41].

The role of AVP in the circadian rhythms is suggested to be noncritical since Brattleboro rats that do not express AVP continue to show rhythmic behaviour. However, recently, a study has indicated that AVP-V1a/1b receptor signals are necessary for effectively controlling the magnitude of resetting to changes in the external light-dark cycle [41]. In mice lacking both V1a and V1b expression, the SCN resets to large changes in the phase of the LD cycle much more quickly than in wild-type animals.

## 12.5 Outputs

The peptide prokineticin-2 (PK2) is synthesised in the mouse SCN and is implicated in conveying circadian information to the rest of the brain. Levels of PK2 mRNA in the SCN vary across the light-dark and circadian cycles. The main receptor for PK2 in the brain is PK2r. PK2r is expressed by distinct populations of the SCN including AVP- and GRP-containing neurons, and PK2 acting via the PK2r alters SCN electrical activity [42]. PK2 is predominantly excitatory in the SCN and appears to act by reducing GABA release. Further, injections of PK2 into the lateral ventricle of rats at night, when the level of PK2 in the SCN is very low, reduce motor activity. Since PK2 mRNA peaks during the day when rats are behaviourally quiescent, then this suggests that PK2 functions as an inhibitory output signal. It is unclear whether this represents a direct inhibition of motor centres or arises through indirect pathways. PK2 expressing cells in the SCN potentially innervate a number of extra-SCN sites including the dorsomedial hypothalamus and the habenula (Hb), and PK2r mRNA is also expressed in these structures. There has been very little examination of the influence of PK2 at the target sites of SCN efferents, but, in the lateral Hb, PK2 acts presynaptically to enhance GABA release and reduce neuronal activity [43]. The model that has emerged is that PK2 released from SCN efferents during the day acts to suppress motor centres of the brain and that as PK2 release declines during the late day, this disinhibits these motor centres and is permissive of the expression of locomotor activity. Unfortunately, there has been limited follow-up on PK2's role, and while this model remains an intriguing possibility, more insightful experiments are required to be fully confident that PK2 does indeed function as an SCN output signal.

There is limited evidence that other factors such as transforming growth factor-alpha (TGFα) [44] and cardiotrophin-like cytokine [45] are involved, but, aside from these early initial investigations, there is little additional evidence to support such a role for these chemicals.

## 12.6 Circadian Organisation in Diurnal Species

Most of our current knowledge of the biological timekeeping mechanisms in mammals arises from laboratory investigations focused on nocturnal rodent models, but studies in diurnal species are much more limited. One layer of complexity in understanding the neural mechanism underpinning diurnal temporal niche preference in mammals is that diurnalism has re-emerged in several independent evolutionary lineages [46]. Thus, comparative analysis of diurnal species from different taxonomic groups is necessary to identify convergent adaptations that are common to a diurnal niche and therefore more likely to be shared by most diurnal species, including humans [47].

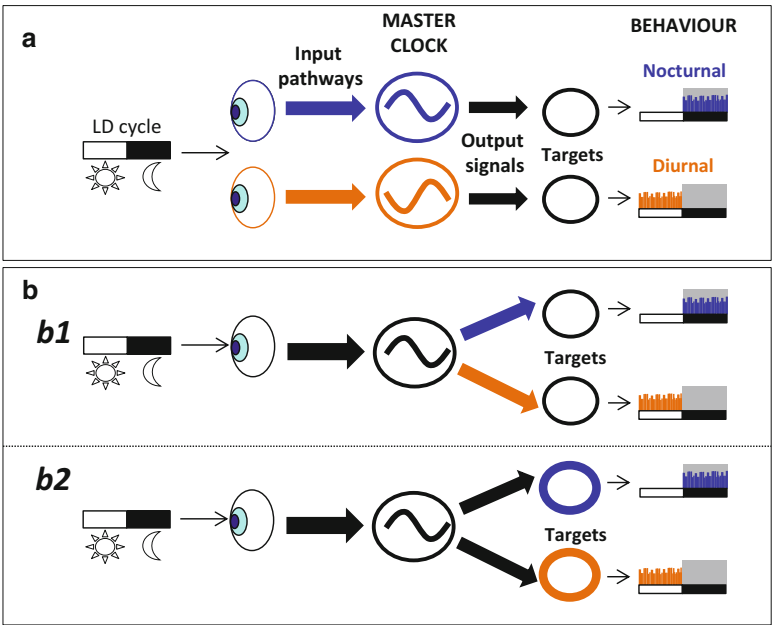


**Fig. 12.2** Schematic diagram of the possible neural mechanisms underlying temporal niche preference. Different behavioural outputs in diurnal and nocturnal animals could arise from (**a**) a reversed phase relationship between the SCN and the external light-dark cycle (LD) and, (**b**) alternatively, changes in the coupling between the SCN and the functions that it controls, including reversed expression and release of SCN timekeeping signals (***b1***) or altered responsiveness in target areas downstream from the SCN (***b2***) (Adapted from [81])

From a theoretical point of view, the different patterns of behaviour and physiology in diurnal and nocturnal species could be the result of (1) changes in the central master oscillator and in the mechanisms involved in its entrainment to the LD cycle, but similar coupling of the clock to the functions it controls, and (2), alternatively, a circadian master oscillator with similar fundamental features and entrainment to the LD cycle but with different coupling between the clock and the functions that it regulates (Fig. 12.2) [48].

### *12.6.1 Inputs to the Central Pacemaker*

In the natural environment, nocturnal and diurnal species differ considerably in the lighting conditions which they routinely experience. Indeed, diurnal species are exposed to light for longer durations and at higher intensities than nocturnal ones. It is therefore not surprising that evolutionary adaptation to a diurnal temporal niche has resulted in a visual system that is distinctly different from nocturnal animals. This includes, for example, lens pigmentation which confers strong filtering

properties and high cone density in the retina for colour vision processing [49]. In addition, anatomical studies have revealed that some diurnal species possess thicker optic nerves and more prominent optic chiasm and tracts than nocturnal animals [50, 51]. However, the significance of such differences, from a circadian point of view, is though unknown.

Adaptation to light-saturated environments by diurnal species could also account for the finding that the circadian systems of such species appear to be less sensitive to light than those of nocturnal animals. This is reflected in higher thresholds for inducing sustained photic responses in SCN neurons in diurnal rodents [52]. Intriguingly, differences between diurnal and nocturnal species are reported in the polarity of electrophysiological responses of their SCN neurons to photic stimulation. For example, the majority of photically responsive SCN neurons are suppressed by light in diurnal species, including the degu (*Octodon degus* [52]) and 13-lined ground squirrel (*Spermophilus tridecemlineatus* [53]), in contrast to predominantly excitatory responses observed in SCN neurons of nocturnal animals. This finding was also replicated *in vitro* by recording SCN responses to optic nerve stimulation [54]. Additional studies using other diurnal species are needed to confirm if these differences in the physiological effects of light inputs are a common feature of diurnalism.

A fundamental property of the circadian system is the PRC which describes the resetting effects of light on the SCN clock. As stated earlier, the shifting effects of light on the SCN clock depend on the time of day when light is applied. With pulses of light given during the night, the pattern of PRC appears to be quite similar across a wide range of diurnal and nocturnal species. Thus, a light pulse early and late in the subjective night causes phase delays and phase advances, respectively, regardless of the timing of activity. However, with light pulses given during the subjective day, differences between diurnal and nocturnal species emerge. For several hours during the subjective day, nocturnal animals are generally unresponsive to the phase-shifting actions of light pulses (the so-called dead zone), whereas in diurnal species, including degus and humans, the duration of photic insensitivity during daytime is much shorter or even absent [47, 55]. Indeed, this daytime sensitivity of the diurnal SCN to light is also mirrored when the activity of the immediate early gene, *c-fos*, is examined. Light pulses during the subjective day cause changes in *c-fos* expression in the diurnal SCN, but have no effect in nocturnal rodents [56]. However, these subtle variations in the shape of the PRC do not seem sufficient to explain the 180° phase reversal in the entrained activity pattern between nocturnal and diurnal species [47].

Anatomical pathways involved in conveying non-photic internal arousal to the SCN in nocturnal animals are conserved in diurnal species. However, it is unclear if their neurophysiological characteristics and the processing of information conveyed to the SCN are also conserved. As stated previously, arousal-promoting cues are conveyed to the SCN by the GHT, which co-releases NPY and GABA in the SCN, as well as via a brainstem pathway originating in the median raphe, which uses serotonin as its neurochemical signal. To date, studies comparing the role of GABA or serotonin in the SCN of diurnal and nocturnal animals are few, and the

limited evidence available suggests that these two neurochemicals elicit distinct behavioural effects in diurnal and nocturnal animals [57].

Microinjections of $GABA_A$ agonists, such as muscimol, directly into the SCN during the mid-subjective day cause phase shifts of behavioural activity in both diurnal and nocturnal animals. Interestingly, these shifts are in opposite directions, phase advance in nocturnal hamsters and large phase delay in diurnal Nile grass rat, *Arvicanthis niloticus*. These effects of muscimol are not prevented by blocking voltage-gated sodium channels with tetrodotoxin, indicating that they most likely represent direct actions of $GABA_A$ activation in the SCN region (for review, see [51]). These phase alterations have been associated with downregulation of both *Per1* and *Per2* expressions in nocturnal animals and only decrease of *Per2* in the SCN of *A. niloticus*. GABAergic stimulation reduces the shifting effects of light at night in both diurnal and nocturnal animals [58].

The circadian window of sensitivity to serotonin-resetting actions also appears to differ between the nocturnal and diurnal species studied to date [57]. In nocturnal species, such as hamster, *in vivo* administration of the 5-HT agonist (8-OH-DPAT) causes phases advances only during the mid-subjective day. By contrast, in the diurnal *Arvicanthis ansorgei*, serotonergic activation induces phase advances during the subjective night. Intriguingly, serotonergic stimulation potentiates photic-resetting effect at night in diurnal *A. ansorgei* [59], instead of reducing it as it occurs in nocturnal animals.

Similarly to nocturnal animals, orexin fibres are found in the SCN of diurnal species [60]. Unfortunately, how orexin influences SCN activity and its potential role in conveying/modulating non-photic cues to the SCN in diurnal species is unknown. Importantly, the temporal pattern of activation of orexin cells is phase reversed in nocturnal and diurnal animals, that is, higher activation at night in nocturnal animals and during the day in diurnal rodents [48]. Thus, it will be interesting to study if orexinergic cues would induce phase shifts of the SCN only at night and if such cues can potentiate light-induced phase shifts as is seen for serotonergic stimulation.

### *12.6.2 The Central Pacemaker*

Interspecific differences in the morphological organisation of the SCN at both the structural and neurochemical levels have been found; however, none seem clearly associated with diurnality. For example, the SCN in some diurnal species, including *O. degus* [61] and *Funambulus palmarum* [50], have an elliptical shape in the coronal plane, in contrast with the typical oval shape described for nocturnal rats and hamsters. However, this feature cannot be generalised to all diurnal species as diurnal Nile grass rats, for example, also have oval-shaped SCN [62]. The typical organisation of the SCN into ‘core’ and ‘shell’ described in nocturnal species seems to be present in some but not all diurnal species. For example, cell density in degus SCN does not appear greater in any part of the nucleus. Interestingly, AVP cells in

this species are found not only in the dorsomedial part but also in the central region of the SCN [61]. Recently, a study in capuchin monkeys has also reported the presence of AVP in both ventral and dorsal SCN regions [63]. Such pattern contrasts with the typical distribution of the AVP cell bodies limited to the dorsomedial SCN found in nocturnal species. Future studies are needed to explore the functional impact of these differences.

Despite the varied activity patterns across animal species, some features of the SCN are fundamentally conserved. For example, global SCN metabolic activity (assessed by 2-deoxyglucose) peaks during the day in both nocturnal and diurnal rodent species [5]. However, whether more subtle regional differences in SCN metabolism are related to temporal niche remains to be examined. Further, the possibility that the SCN output signal generated differs between nocturnal and diurnal species needs to be interrogated. For example, such output signals may vary in terms of their ratio of synthesis and release of inhibitory vs excitatory neurotransmitters [46].

Studies of electrical activity rhythms in diurnal species are scarce and the available data variable. *In vivo* multiunit activity SCN recordings made from the diurnal chipmunk (*Eutamias sibericus*) mirror the population-level metabolic rhythm. Thus, chipmunk SCN neurons generate daily rhythms in electrical outputs, being significantly more active during the day than at night [64]. However, a lack of significant daily variation in spontaneous firing was found in the degu SCN (in both light-responsive and light-unresponsive cells) [52]. At present, there is insufficient data to conclude on the nature of SCN neuronal rhythms in diurnal species, and many more such assessments from other diurnal species are necessary. Further, the possibility that temporal niche preference may be captured by one cell population but not another needs examining. Therefore, more detailed investigations are required to determine the SCN neuronal properties that contribute to the emergence of nocturnal and diurnal behavioural patterns.

At the molecular level, there do not appear to be any major alterations in the daily pattern of the SCN TTFL that can account for differences in temporal niche. Indeed, the phasing of core-clock gene expression, and their corresponding protein rhythms in the SCN, is remarkably similar in diurnal and nocturnal mammals despite major differences in their activity patterns (e.g. [65, 66]). Patterns of *Per1* and *Per2* expression, with high levels during the light phase and low levels at night, have been found in all diurnal rodent species studied so far including degus, barbary-striped grass mouse, ground squirrel, *A. ansorgei* and *A. niloticus* [58, 65, 67–70] and in typical nocturnal species, such as rats, mice and hamsters. In addition, recent studies comparing individuals with different activity patterns (e.g. day active vs night active) within the same species have found no differences in the phases of *Per1* and *Per2* clock gene expression or in their protein rhythms [66, 67].

Interestingly, however, the expression patterns of these core-clock genes outside the SCN appear to peak in phase with the animal's activity pattern. Indeed, in extra-SCN brain regions (e.g. the hippocampus and cortex), *Per1*/*Per*2 rhythms in nocturnal animals peak at night, which is nearly 180° out of phase with the peaks

seen in diurnal species [65, 67, 69]. Together, this suggests that the different temporal niche preferences in most diurnal or nocturnal species should result from different coupling between the clock and functions that it regulates.

Two cases are noteworthy to mention where changes in the pattern of coupling between the SCN and the LD cycle potentially influence an animal's activity phase preference: (i) Mice lacking the inner-retinal photopigment melanopsin (OPN4) and RPE65, a key protein used in retinal chromophore recycling, exhibit a switch from nocturnal to diurnal activity. The phase of clock gene expression rhythms is also reversed in the SCN of these animals suggesting that neural responses to light upstream of the SCN can produce acute temporal-niche switching. In the same way, a switch from nocturnal to diurnal phenotype also occurs when wild-type mice are transferred from an LD cycle with standard light intensity to an LD cycle with scotopic levels of light during the 12 h light phase [71]. (ii) In the case of the subterranean mole rat *Spalax ehrenbergi*, most individuals are active during the light phase, but a subset of them can switch from being active during daytime to becoming active at night. Interestingly, the molecular clock gene, *Per2*, also shifts to exhibit a peak during the dark phase in the SCN, while *Per1* oscillates in a biphasic manner [72].

### *12.6.3 Circadian Outputs*

The SCN convey temporal information to other brain regions and to peripheral organs through rhythmic release of output signals (potentially including AVP, VIP, TGFα, PK2, GABA). Oppositely phased activity rhythms in nocturnal and diurnal species may result from reversed expression and release of these output signals (Fig. 12.2(b1)). However, to date, comparative analyses of PK2 and TGFα expression in diurnal and nocturnal species have revealed similar rhythmic profiles regardless of their pattern of activity [73, 74]. In the same way, the daily pattern of expression of VIP mRNA under LD conditions in the SCN of diurnal and nocturnal animals seems overtly similar [75]. AVP expression is under clock control. Measurement of its concentration in cerebrospinal fluid, as well as AVP mRNA expression in the SCN, reveals circadian rhythms in AVP peaking during daytime. Hence, both transcription and release of AVP appear to peak during daytime regardless on the animal's behavioural pattern. Collectively, these studies suggest that the fundamental mechanisms for diurnality should be located downstream the SCN. Thus, target areas would show reversed responses to SCN output signals (Fig. 12.2(b2)). An interesting example supporting this idea is the control of corticosterone rhythms by AVP-containing projections from the SCN to the paraventricular nucleus of the hypothalamus (PVN). Plasma corticosteroids (corticosterone in rats and mice; cortisol in degus, humans, and non-human primates) show daily variations with maximal values at dusk in nocturnal species and at dawn in diurnal mammals. This prepares the organisms for the forthcoming period of increased activity (night or day, respectively). Intriguingly, PVN neurons of diurnal

and nocturnal rodents show different responsiveness to AVP administration. Indeed, AVP infused in the PVN of the diurnal grass rat (*A. ansorgei*) stimulates release of plasma corticosterone. By contrast, the same treatment has a strong inhibitory effect on corticosterone levels in nocturnal rats [76]. The authors propose that changes in the phenotype of the neurons targeted by the SCN efferents (i.e. glutamatergic instead of GABAergic) could underlie these reversed responses to AVP in animals with different activity patterns.

## 12.7 Summary and Questions of Interests

- SCN neurons exhibit intrinsic circadian variation in molecular, metabolic, and electrophysiological characteristics.
- Regional differences in neurochemical and timekeeping characteristics in the SCN are pronounced in some species.
- SCN molecular clock does not appear to differ between nocturnal and diurnal species.

1. What processes and mechanisms make an animal diurnal?
2. How do SCN output signals influence activity in specific target areas?
3. Why are 'core' and 'shell' compartments more discernable in some species and not others?
4. What are the mechanisms underlying temporal niche switching within the same species?

**Suggested Further Reading**

Very recent studies indicate new roles for known neuropeptides in the SCN [77, 78] and identify a new G-protein-coupled receptor and its function in the SCN [79]. Further, interactions between the SCN and other hypothalamic regions shape diurnal temperature preference [80].

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# Part III
# Human Circadian Rhythms: Entrainment and Sleep Regulation

# Chapter 13
# Circadian Rhythms Versus Daily Patterns in Human Physiology and Behavior

**Josiane L. Broussard, Amy C. Reynolds, Christopher M. Depner, Sally A. Ferguson, Drew Dawson, and Kenneth P. Wright Jr.**

**Abstract** The endogenous circadian timekeeping system modulates human physiology and behavior with a near 24 h periodicity conferring adaptation to the ~24 h solar light-dark cycle. Thus, the circadian timekeeping system times physiology and behavior so that it is prepared for environmental changes. The term *circadian* implies an endogenous "clock-driven" process. However, not all observed daily patterns in physiology and behavior are clock driven and instead may be due to environmental or behavioral factors. For example, the barren rock on the top of a mountain shows a daily temperature oscillation that is not endogenous to the rock but instead is caused by the sun heating the rock during the day and radiative heat loss after sunset. Other factors such as wind, rain, and cloud cover impact the observed daily temperature oscillation of the rock. Similarly, some of the daily patterns observed in physiology and behavior are driven by external factors, while others arise from the interaction between circadian and behavioral processes (e.g., sleep-wake, fasting-feeding). To improve understanding of the mechanisms underlying observed daily patterns in physiology and behavior in humans, a variety of circadian protocols have been implemented (Tables 13.1 and 13.2). These protocols will be reviewed in the following pages, and the strengths and limitations of each will be discussed. First, we review markers of the endogenous clock in humans.

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**Table 13.1** Comparison of common experimental procedures for circadian protocols

| | Constant routine | Ultrashort sleep-wake schedule | Forced desynchrony | Shift of sleep to daytime |
|---|---|---|---|---|
| Ambient light | Constant dim light (e.g., 1.5 lx in the angle of gaze) | Alternating rapid LD cycle (e.g., 20 min, 60 min, or 90 min day) | Alternating dim LD cycle (e.g., 20 h, 28 h, or 42.85 h days) | Shift of LD cycle on a 24 h day |
| Ambient temperature | Constant thermoneutral | Controlled yet alternating due to changes in activity and bed microclimate during sleep opportunity | Controlled yet alternating due to changes in activity and bed microclimate during sleep opportunity | Controlled yet alternating due to changes in activity and bed microclimate during sleep opportunity |
| Food intake/ meals | Continuous IV feeding or miniature meals divided into isocaloric hourly snacks | Snacks | Typical BLDS | Typical BLDS |
| Posture | Bed rest with head of bed raised to 35–45° | Alternating ambulatory during wakefulness and supine during sleep | Alternating ambulatory during wakefulness and supine during sleep | Alternating ambulatory during wakefulness and supine during sleep |
| Wakefulness-sleep | Continuous wakefulness | Alternating wakefulness and sleep | Alternating wakefulness and sleep | Alternating wakefulness and sleep |
| Duration | Day to days | Days | Days to weeks | Days |

*LD* light-dark, *BLDS* breakfast, lunch, dinner, snack, *IV* intravenous

**Table 13.2** Outcomes derived from circadian protocols

| | Constant routine | Ultrashort sleep-wake schedule | Forced desynchrony | Shift of sleep to the daytime |
|---|---|---|---|---|
| Circadian phase | Yes, gold standard | No, except for melatonin phase | Not ideal, except for melatonin phase | Not ideal, except for melatonin phase |
| Circadian amplitude | Yes | No | Yes | No |
| Circadian period | No | Yes | Yes, gold standard | No |
| Circadian oscillations in physiology and behavior | Yes | Yes | Yes | Yes |
| Circadian versus sleep-wake modulation of physiology and behavior and interactions | No | No | Yes, gold standard | Yes |

## 13.1 Markers of Circadian Rhythms in Humans In Vivo

The master clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus [1, 2]. Peripheral cell-autonomous clocks have also been observed in tissues outside the brain, such as fibroblasts, red blood and mononuclear cells, adipose tissue, pancreatic islet cells, skeletal myotubes, hepatocytes, and cardiomyocytes, and in immortalized cancer cell lines [3–14]. Unlike nonhuman models, scientists do not have direct access to the SCN in humans and instead use marker rhythms driven by the SCN to indicate phase, amplitude, and period of the circadian clock. Estimations of circadian timing of the internal biological clock (i.e., phase) and the strength or robustness of the observed oscillation (i.e., amplitude) are informative when determining changes in response to environmental or physiological perturbations. Phase represents the time within the circadian cycle at which a particular event occurs (e.g., minimum, maximum, onset, offset, midpoint), whereas amplitude is commonly defined as the magnitude between the mesor and the maximum of a rhythm. The mesor is defined as the value midway between the maximum and minimum of a fitted rhythm or time series, and thus amplitude is approximately half of the minimum to maximum range of the rhythm.

### *13.1.1 Melatonin*

The most commonly used circadian marker rhythm in humans is the melatonin rhythm. Melatonin is easily measured in saliva, blood, and urine and is the most

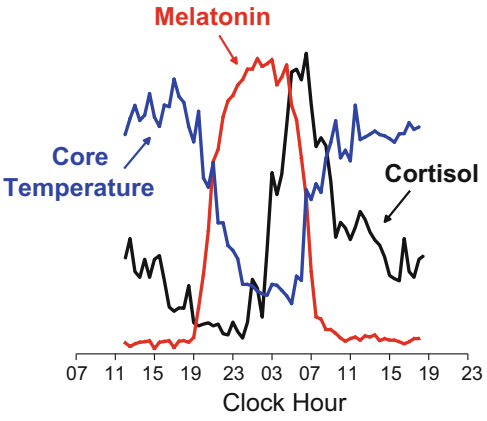


**Fig. 13.1** Phase relationships between the primary circadian phase marker rhythms driven by the SCN in humans. High melatonin and low core temperature levels represent the biological night, whereas low melatonin and high core temperature levels represent the biological day. Cortisol levels are lowest in the first part of the biological night, peak near the end of the biological night, and decrease across the biological day

precise marker of circadian phase and period [15–19] when exposure to light is controlled. High melatonin levels are considered to be a marker of the biological night (Fig. 13.1). During entrainment when the circadian clock is synchronized to the 24 h day, melatonin levels rise on average ~2 h prior to habitual bedtime [20], peak during the nighttime, and return to low levels shortly after habitual wake time [21]. Note that there are large individual differences in the timing of the melatonin rhythm, which are larger in the modern environment versus after exposure to the natural light-dark cycle [21]. Further, there are large individual differences in peak melatonin levels [22]. A common misperception is that exposure to darkness increases melatonin levels. Rather, the SCN controls the melatonin circadian rhythm via a multisynaptic pathway. This pathway includes an efferent projection to the paraventricular nucleus of the hypothalamus (PVN) and a descending projection to sympathetic preganglionic neurons in the upper thoracic spinal cord which, in turn, project to the superior cervical ganglion (SCG). Postganglionic nerve fibers from the SCG then release norepinephrine to stimulate beta- and alpha-adrenergic receptors on the pineal gland [23]. Beta-adrenergic receptor activation signals the pineal gland to synthesize melatonin from tryptophan via several enzymatic steps. If maintained in constant conditions, the melatonin rhythm continues to rise and fall independent of light exposure. If exposure to light occurs during the biological night, melatonin levels will be acutely reduced as photic input from the retina to the SCN results in inhibition of the multisynaptic SCN-pineal circuit, removing the rate-limiting step of melatonin synthesis. Thus, to accurately assess melatonin levels, samples must be collected every 30–60 min under dim light conditions (e.g., <8 lx maximum).

A variety of markers have been developed to quantify the timing of melatonin rhythm. Changes over and under arbitrarily defined thresholds are often used. For example, melatonin onset is most commonly defined as when melatonin levels rise above 10 pg/ml in plasma, or 3–4 pg/ml in saliva, as salivary levels are 30–40 % of plasma levels [19, 24]. Other thresholds for melatonin onset are when melatonin levels rise two standard deviations above a stable low daytime baseline [19] and the time of a threshold change is calculated by linear interpolation. If the entire melatonin rhythm is assessed, individualized thresholds can be computed to determine the thresholds of when melatonin levels reach 25 % of the three-harmonic fitted peak-to-trough melatonin amplitude [18] and the 50 % mean crossing [15], for example. Melatonin offset is considered the time at which melatonin levels return to low daytime levels, falling below these thresholds, and the melatonin midpoint is the time midway between the onset and offset. The fitted melatonin peak has also been used as a phase marker, and comparisons of the various markers show similar variability in their estimates of circadian phase [15].

### *13.1.2 Body Temperature and Cortisol*

Two other commonly used circadian marker rhythms in humans are body temperature and cortisol (Fig. 13.1). The SCN controls rhythms in body temperature via

multisynaptic projections to the preoptic temperature control center of the hypothalamus, and through direct effects of melatonin on peripheral vasodilation. The SCN also controls rhythms in cortisol via input into the endocrine hypothalamic pituitary adrenal (HPA) axis, as well as through a multisynaptic neural pathway to the adrenal glands that bypasses the HPA axis [25]. Core and distal skin (e.g., hands and feet) body temperatures show approximate inverse rhythms with high core and low distal skin temperatures during the daytime and low core and high distal skin temperatures at night. The fitted minimum of the core body temperature rhythm is the most common circadian temperature phase marker. Accurate assessment of the circadian rhythms in body temperatures requires control of posture, activity, and sleep, as changes in these factors alter the observed temperature rhythm (e.g., physical activity acutely increases core temperature and sleep reduces core temperature). Cortisol levels peak in the morning, decrease across the day, are low near habitual bedtime, and rise throughout the night (Fig. 13.1). Given the pulsatile nature of cortisol, accurate assessment of the cortisol rhythm requires frequent sampling (e.g., every 20–30 min).

## 13.2 Protocols to Evaluate Circadian Phase and Amplitude in Humans

### *13.2.1 Constant Routines*

The constant routine protocol, a modification by Czeisler and colleagues (discussed in [26]) of the Mills test [27], can be used to assess the phase and amplitude of the clock immediately upon release from entrainment into "constant conditions" (Table 13.2). The constant routine protocol controls for factors that influence circadian variables of interest by making constant or equally distributing factors such as ambient light and temperature, physical activity and posture, nutrition intake, and sleep-wake state across the circadian cycle (Fig. 13.2, Table 13.1). Constant environmental conditions include dim-ambient light commonly maintained at ~1.5 lx in the angle of gaze (~0.6 W/m$^2$), ambient temperature in the thermoneutral range (e.g., 22–24 °C), and a light bed sheet pulled up to the waist to maintain a constant temperature microclimate. Constant behavioral conditions include continuous wakefulness to control for sleep-induced changes in physiology and constant posture (i.e., bed rest with the head raised to a 35–45° angle) to control for posture or activity-induced changes (e.g., participants remain in bed and use bedpans/urinals). Continuous monitoring by research staff is required to ensure constant wakefulness and consistent posture. Concurrent brain wave recording is also recommended to maximize continuous wakefulness, as microsleeps will occur and unattended subjects will fall asleep. Nutrition intake is often in the form of miniature snacks distributed equally across the circadian cycle (e.g., hourly) or via less commonly used continuous enteral feeding or venous glucose infusion (Table 13.1). Meals are typically identical across the constant routine (e.g., one-fourth of a ham sandwich, room-temperature juice and water), isocaloric, and total daily calories consumed are increased by ~5 % to account for the higher energy needs associated with extended wakefulness [28]. At a

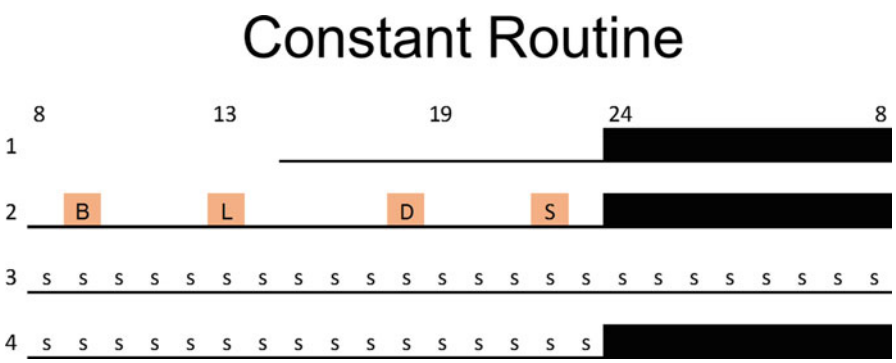


**Fig. 13.2** The constant routine protocol is typically preceded by subjects maintaining their habitual and consistent wake-sleep schedule in the home environment. The first one to two nights in the laboratory may consist of the habitual wake and sleep schedules with typical meals. The constant routine protocol on days 3–4 is composed of miniature hourly snacks (s), constant posture, wakefulness, dim light, and ambient temperature. Participants are continuously monitored during the constant routine to ensure wakefulness and compliance with procedures. A 40 h constant routine permits assessment of multiple measure of circadian phase such as body temperature, melatonin, and cortisol and recovery sleep at the habitual sleep time. Constant routines also commonly precede and follow exposure to a phase-shifting stimulus to determine the change in circadian phase. *B* breakfast, *L* lunch, *D* dinner, *S* snack, *orange boxes* habitual meals, *black boxes* scheduled sleep, *underline* scheduled wakefulness

minimum, ~24 h is required to assess a full circadian cycle if melatonin is used as a circadian phase marker, and more time (i.e., 1.5–2 days) is needed if body temperature is used, given residual effects of prior sleep on temperature. Constant routines are often ~40 h in duration and thus recovery sleep occurs at habitual bedtime.

Comparisons between the constant routine and typical sleep-wake conditions demonstrate that the circadian rhythm in melatonin is minimally influenced by sleep-wake state [29], assuming that ambient light is maintained at dim levels. Circadian rhythms in body temperature and cortisol are, however, influenced by sleep-wake state [21, 29, 30]. The sleep-induced decrease in core temperature [31] (increase in distal skin temperature) and posture-/activity-induced increase in core temperature (decrease in distal skin temperature) can mask the circadian temperature rhythms, resulting in imprecise estimates of circadian phase and amplitude. Similarly, there is a sleep-induced decrease in cortisol levels shortly after the beginning of the sleep episode and a wake-induced increase [32, 33].

Limitations of the constant routine procedure include constant wakefulness, which requires a degree of sleep deprivation that may influence outcomes of interest (i.e., circadian time is not the only factor changing); time (i.e., minimum of 24 h); protocol costs; and a tightly controlled environment which cannot be easily performed outside of the laboratory. Additionally, the constant routine cannot be used to assess circadian period nor interactions between circadian phase and wake-sleep influences on physiology and behavior (Table 13.2). Furthermore, like most laboratory protocols, findings may not translate directly to real-world conditions.

Variations on the constant routine include constant posture protocols that either permit sleep and typical meals [34] or modified constant routines that permit sleep and some changes in posture (e.g., bathroom breaks). Constant posture and modified constant routine protocols still include control for dim-ambient light and thermoneutral ambient temperature and can therefore still be used to determine circadian timing of the melatonin rhythm. The constant posture protocol has been used to measure the melatonin rhythm during wakefulness and sleep from blood samples taken via an indwelling catheter with an extension tubing that exits a room porthole to allow blood to be assessed with minimal disruption of sleep [34]. Individuals can also be awakened from sleep to obtain saliva samples from which to assess melatonin levels. When assessing the melatonin rhythm using saliva instead of blood sampling, food and fluid intake are typically proscribed ~30 min prior to collecting saliva samples, and the mouth is rinsed 30 min prior to sample collection to reduce the risk of food contamination of saliva samples. Furthermore, if posture is not constant, seated posture is typically maintained for ~15 min prior to sample collection. If sleep or changes in posture are permitted, circadian body temperature rhythm phase cannot be precisely determined [35, 36].

Limitations of constant posture and modified constant routines include time (i.e., minimum of 24 h) and protocol costs, and require a tightly controlled laboratory environment. These routines cannot be easily performed outside of the laboratory. Additionally, when daytime sleep opportunities are allowed, total sleep time is reduced relative to nighttime sleep opportunities [37], and such differences in total sleep time could influence outcomes of interest.

### *13.2.2 Assessment of the Dim-Light Melatonin Onset*

The phase of the melatonin rhythm can also be assessed without performing a constant routine. As noted, melatonin, as opposed to temperature and cortisol, is less impacted by posture and meals. As long as light is maintained at dim levels and food and posture are controlled prior to sample collection (e.g., food proscribed 30 min and posture consistent 15 min immediately prior to the sample), saliva and blood samples can be used to accurately assess melatonin levels. Generally, the dim-light melatonin onset (DLMO) can be determined from samples obtained starting ~7 h prior to and ending ~1 to 2 h after habitual bedtime, assuming that the subject is stably entrained. Saliva sampling can also be easily performed outside of the laboratory when measuring and controlling light levels [38–40].

Limitations of the DLMO assessment include the inability to assess melatonin amplitude or other melatonin markers such as the midpoint and offset and melatonin duration. If the subject is not entrained, 24 h sampling may be required to obtain the melatonin onset, which would permit assessment of melatonin offset, midpoint, and amplitude.

## 13.3 Protocols to Evaluate Circadian Period and Amplitude in Humans

Accurate assessment of circadian period in sighted humans requires assessment in the absence of external synchronizers, or under tightly controlled exposure to synchronizers, which ensures their even distribution with respect to circadian phase.

### *13.3.1 "Free-Running" Temporal Isolation Protocol*

In the 1960s Aschoff and Wever began performing their classic bunker studies in an isolation unit free from natural time cues [41]. Subjects lived in isolation, typically self-selecting their own meal, sleep-wake and light-dark schedules. These and related studies provided information on fundamental concepts of circadian physiology. For example, findings showed circadian rhythms in body temperature and associations between body temperature and sleep, e.g., subjects most commonly chose to initiate sleep on the downward curve of the body temperature rhythm [42, 43] and slept the longest when sleep was initiated near the body temperature minimum. Observed periods for urine corticosteroids and rectal temperature were on average ~25 h [44], and shorter periods were observed in subjects who napped versus those who maintained one primary sleep episode per "day" [45]. Limitations of these "free-running" temporal isolation protocols include subjects self-selected environmental (e.g., light) and behavioral (sleep and meals) events at a limited number of circadian phases. Thus, the nonuniform distribution of zeitgebers, or synchronizers, with respect to circadian phase likely had an impact on the outcomes observed [17, 46]. Experiments by Middleton et al. [47, 48] performed in constant dim light with self-selected sleep-wake schedules, but with knowledge of clock time, revealed circadian periods closer to 24 h (e.g., 24.3 h).

### *13.3.2 Forced Desynchrony in Laboratory Conditions*

In 1938, Nathaniel Kleitman and Bruce Richardson performed a month-long study ~30 m underground in Mammoth Cave, Kentucky. During their study, they pioneered the forced desynchrony protocol. Kleitman and Richardson lived on a 28 h day and compared circadian temperature rhythms on 28 h versus 24 h day for 1 week each [49]. Regardless of the day length, ~24 h body temperature rhythms were observed, indicating that the ~24 h circadian rhythm in humans is not dependent upon the environmental light-dark cycle. Modern versions of the forced desynchrony protocol established by Czeisler and colleagues [17, 18, 31, 34, 44, 50–55] scheduled individuals to live in the laboratory in dim light-dark, wake-sleep cycles that are outside the range of entrainment of the human circadian clock (e.g., 20 h, 28 h, or 42.85 h days; Fig. 13.3, Tables 13.1 and 13.2). These laboratory

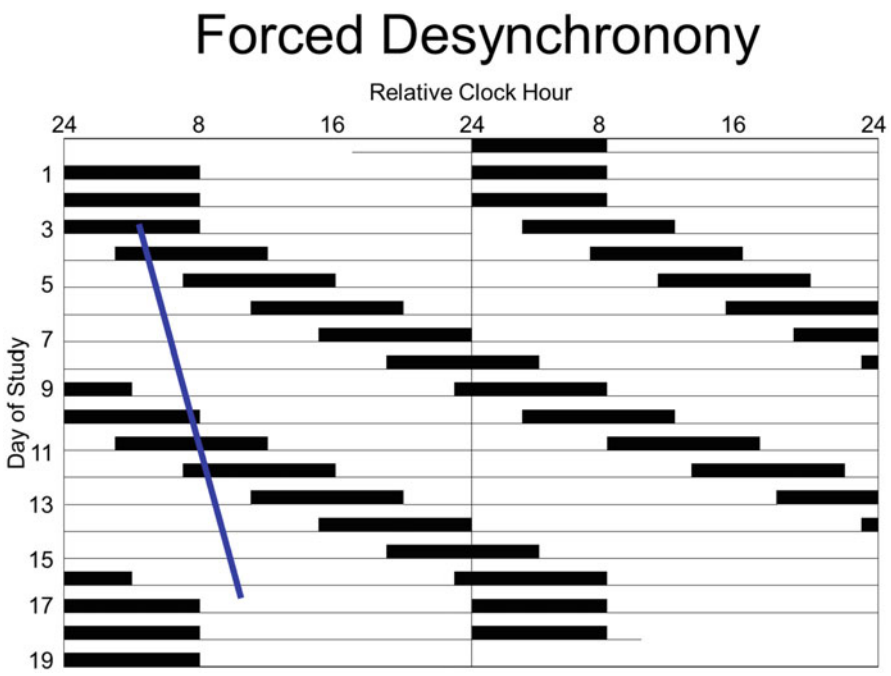


**Fig. 13.3** Double raster plot of the 28 h forced desynchrony protocol during which subjects initially start off on a 24 h day ($T = 24$ h). Typically, participants first undergo a 40 h constant routine (not shown here; but see Fig. 13.2) and then transition to a non-24 h day length that is outside the range of entrainment of the near 24 h period of the human circadian clock under controlled dim light conditions. Common forced desynchrony day lengths include $T = 28$ h (shown), 20 h, and 42.85 h. The example 28 h day length shown includes a 2:1 wake to sleep ratio of scheduled 18 h and 40 min wake and 9 h and 20 min sleep. The forced desynchrony is also often followed by a second constant routine protocol to assess circadian phase. The *blue line* illustrates the trajectory of a core body temperature minimum with a period longer than 24 h

studies last for at least 1 week on the non-24 h day length and often 2–4 weeks in order to obtain precise estimates of circadian period upon release from entrainment and revealed circadian period estimates close to 24 h (i.e., ~24.15 h) [17, 18, 53]. In these studies, participants live in an environment free of time cues (i.e., no access to timepieces, sunlight, or electronic devices), and light-dark exposure, physical activity, and food intake are all controlled. The specific day lengths noted above were chosen as their harmonics do not overlap with 24 h (i.e., the sought-after period) nor do their harmonics share any harmonics with a ~24 h period [17].

The traditional forced desynchrony protocol maintains the habitual 2:1 wake to sleep ratio (e.g., 18 h and 40 min scheduled wake and 9 h and 20 min scheduled sleep for the 28 h day), although modifications have been made to examine interactions between sleep homeostasis and circadian phase [56–62]. When scheduling wake-sleep for a 2:1 ratio of the imposed day length, sleep duration is not equal at all circadian phases and will be shorter during the biological day [50, 63]. Furthermore, the forced desynchrony protocol assumes a symmetrical phase response curve for any photic and non-photic phase shifts that may persist despite the minimization of these

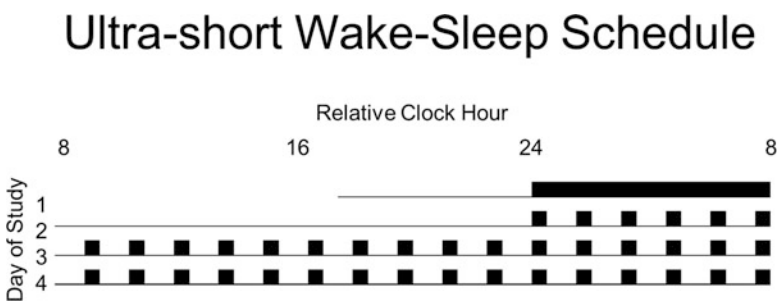


**Fig. 13.4** The ultrashort wake-sleep schedule may include a typical night of sleep in the laboratory followed by a day (shown) or 24 h or more of wakefulness (not shown), followed by an alternating schedule of wake and sleep (60 min wake and 30 min sleep, in this example) for 1 or more days

influences. Relative coordination between observed rhythms—for example, between the melatonin rhythm and wake-sleep cycle—may result and thus influence the observed melatonin rhythm. However, this does not appear to affect the estimation of period, providing that the data train is sufficiently long [17]. The traditional forced desynchrony protocol requires a facility with maximal control over environmental conditions and has a high cost in both subject and experimenter time (Table 13.1). Related recruitment of participants for such longer term in-laboratory studies is laborious, and large demands are placed on laboratory resources for such studies.

Variations on the forced desynchrony protocol include ultrashort sleep-wakefulness cycles. Ultrashort sleep cycles schedule sleep and wake in brief segments across one or more circadian cycles. Versions include the 90 min day with scheduled 60 min wake and 30 min sleep opportunities [64] and the 20 min day with scheduled 13 min wake and 7 min sleep opportunities [65] (Fig. 13.4). The ultrashort versions of the forced desynchrony protocol are more cost-effective and provide a similar overall circadian period estimate as the traditional forced desynchrony protocol [66–68]. However, the precision and the error in the estimate for individual participants have not been reported but are likely larger than the traditional forced desynchrony estimates. Limitations of the ultrashort forced desynchrony protocol include an inability to assess circadian phase and amplitude (with the exception of melatonin phase if light is dim during scheduled wake) (Table 13.2). Further, this approach results in an inability to assess interactions between circadian phase and changes in the duration of wakefulness or sleep on outcomes. Like the traditional forced desynchrony protocol, sleep duration is not equal at all circadian phases.

### *13.3.3 Forced Desynchrony in Free-Living Conditions*

Another approach to examine circadian period has been to study visually impaired individuals living outside of the laboratory. Studies of blind individuals in the real world provide circadian period estimates that are similar, but longer, to those found

in forced desynchrony protocols, but with less precision due in large part to the infrequent sampling of urine (e.g., every 4–8 h) for circadian phase markers in these studies [69]. Other limitations include the long duration of such field studies and uneven distribution of non-photic time cues in the blind (e.g., activity, sleep-wake cycle, meal times, working hours, social interactions, alcohol, caffeine, medication) that may induce daily advances or delays of the circadian system, effectively shortening or lengthening the measured period. Some blind individuals also maintain photic input into the SCN. Another difference from most laboratory studies is that the observed period in the blind is likely assessed long after the "release" from entrainment and therefore may have different properties.

### *13.3.4 Analysis of Data from Forced Desynchrony Protocols to Estimate Circadian Period*

Frequently sampled melatonin, temperature, and cortisol data from forced desynchrony protocols are often analyzed with harmonic regression models using an exact maximum likelihood fitting procedure [17]. Data can be fitted with periodic components of the imposed sleep-wake cycle and the sought-for circadian period, together with their harmonics. Such techniques utilize the frequently sampled data available in the dataset and are thus robust and provide the most precise estimates of circadian period and amplitude. Alternatives include fitting linear regression through daily circadian phase estimates, which are less precise as they include error in the phase estimate as well as show higher variance in the period estimate. As with all circadian protocols, the derived estimates of phase, amplitude, and period are only as accurate as the robustness of the analytic techniques and degree of experimental control over factors impinging on the outcome of interest. Masking effects may influence the observed rhythms without impinging upon, and thus not representing, the phase, amplitude, and period of the master clock in the SCN.

## 13.4 Protocols to Evaluate Circadian Rhythms in Physiology and Behavior

Beyond using the above circadian protocols for assessment of phase, amplitude, and period of the master clock in humans, other physiological and behavioral data can also be collected and aligned to the known circadian phase makers (e.g., melatonin or core body temperature) to provide evidence for circadian variation in such parameters (Table 13.2). For example, constant routine protocols have shown circadian variation in blood pressure and thyroid-stimulating hormone (TSH) [29, 70], whereas daily patterns in prolactin, human growth hormone (hGH), and parathyroid hormone (PTH) are sleep-wake dependent [29]. In addition, many aspects of cognitive function, including reaction time, cognitive processing speed,

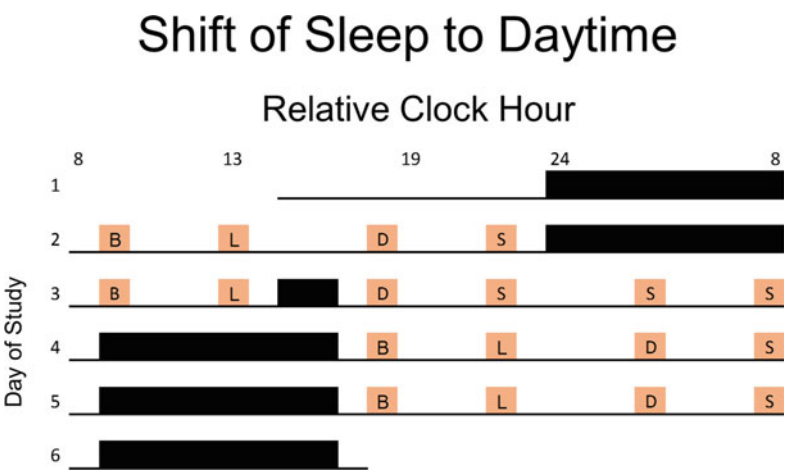


**Fig. 13.5** The shift of sleep to the daytime is a common simulation of a shift work schedule with 1 to 2 days of sleep at night followed by a transition day with a daytime nap and 1 or more days with daytime sleep and evening/overnight wakefulness

math processing speed and accuracy, visual search abilities, executive function/decision-making skills, as well as alertness, sleepiness, mood, hunger, and appetite are influenced by time awake and/or circadian time of day [50, 55, 71–75]. When considering sleep-wake versus circadian-dependent variables, it is important to consider whether altered sleep duration is playing a role in the observed changes. For example, it is known that hunger and appetite hormones leptin and ghrelin, as well as free fatty acids, are altered during sleep restriction [76, 77]. Therefore, sleep duration must be taken into account when considering the effects of these circadian protocols on outcome variables.

Another circadian protocol used to examine circadian versus wakefulness-sleep patterns in physiology is to shift sleep to the daytime (Fig. 13.5) [37, 78], reflective of patterns found in night work, to see whether daily patterns remain synchronous with the melatonin circadian rhythm, move with the wake-sleep cycle, or are abolished. Using such protocols, findings provide evidence that hGH and peptide-YY (PYY) are wake-sleep/feeding-fasting driven, whereas diet-induced thermogenesis is primarily influenced by the circadian clock [79, 80]. Other manipulations may then be necessary to determine the specific non-circadian factors driving such patterns that are altered by a shift in wake-sleep (e.g., wake-sleep, feeding-fasting, physical activity-inactivity).

Forced desynchrony protocols with a reasonable amount of "daily" wake time have been use to describe interactions between circadian and wake-sleep-driven processes. Findings from forced desynchrony protocols have shown many physiological and behavioral variables which are under circadian control and/or interact with wake-sleep factors. For example, findings from forced desynchrony protocols have shown circadian rhythms in physiology such as blood pressure [70], epinephrine, norepinephrine, heart rate, platelet aggregability [81], glucose tolerance in response to meals [82–84], EEG activity during wakefulness and sleep,

susceptibility to presyncope [85], and periodic limb movements [86]. Some of these outcomes, including EEG and performance, show changes that are dependent upon the levels of sleep homeostasis and circadian phase and their interaction. For example, in many of the papers cited above, the amplitude of the circadian variation in performance builds with time awake.

Building on this, recent research has focused on the effects of sleep and circadian manipulations on human metabolomics and transcriptomics [87–91] in an effort to elucidate altered mechanisms and biochemical pathways by which these manipulations confer increased risk for disease states and to identify potential health and disease biomarkers.

## 13.5 Summary and Conclusions

The circadian protocols reviewed above have been used to elucidate much information about physiology and behavioral outcomes that are controlled and/or modulated by circadian timing. In the end, when interested in determining mechanisms that contribute to daily patterns in physiology and behavior, it is important to recognize that the integrated timing of circadian clock, wake-sleep, feeding-fasting, physical activity-inactivity, and light-dark cycles likely serve to enhance daily oscillations and promote robustness. A well-tuned system that has robust and coordinated oscillations in these parameters is likely to promote maximal cognitive and physiological health outcomes. Understanding the mechanisms underlying daily patterns in physiology and behavior also permits the development of countermeasure strategies for when these daily patterns are unavoidably disrupted, such as during shift work, jet lag and circadian rhythm, and/or sleep-wake disorders. Additional research is needed to understand the interacting factors that produce robust daily patterns in physiology and behavior, some of which will be circadian and others that will be driven by other factors. For example, manipulation of meal timing, physical activity, and environmental conditions in the above circadian protocols will add to our understanding of biological mechanisms controlling daily patterns in physiology.

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# Chapter 14
# Light Resetting and Entrainment of Human Circadian Rhythms

**Joshua J. Gooley**

**Abstract** Light is the most important synchronizer of human circadian rhythms. In this chapter, we review the resetting effects of light on the human circadian system. First, we describe the importance of circadian entrainment and how the circadian system is organized to fulfill this purpose. Then, we discuss factors that influence the magnitude of circadian light responses, including the circadian phase of light exposure and characteristics of the light stimulus such as intensity, duration, and wavelength. Finally, we review the effects of electrical lighting and sunlight on the timing of circadian rhythms in laboratory studies and under real-world conditions. The main summary points of the chapter are:

1. Exposure to light in the early biological night induces a phase delay shift of human circadian rhythms, whereas exposure to light in the late biological night induces a phase advance shift.
2. Circadian responses to light can be enhanced by increasing the intensity or duration of the light stimulus, using short-wavelength light, or exposing oneself to dim light prior to the resetting stimulus.
3. The phase-resetting effects of light on circadian rhythms are greatest near the early part of a continuous light stimulus, as compared to the later part.
4. The human circadian system can be reset and entrained by exposure to electrical lighting including ordinary room light.
5. The circadian timing of sleep and other rhythms is modulated by exposure to electrical lighting and natural lighting under real-word conditions.

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## 14.1 The Human Circadian System

### *14.1.1 Why Is Entrainment in Humans Important?*

Behavioral and physiologic rhythms are normally synchronized to the 24-h solar day. Even in the absence of periodic environmental time cues, humans exhibit daily rhythms of sleep and other behaviors that are close to, but not exactly, 24 h. Hence, similar to other organisms, there is an internal timekeeping system in humans that needs to be adjusted daily in order to align with the rising and setting of the sun. The circadian system provides an internal representation of day and night, which allows the body to anticipate daily changes in the environment. When a person is normally entrained to the solar day, the circadian system therefore facilitates the transition to and from sleep, ensuring that a consolidated period of sleep occurs at night [1]. If the circadian clock becomes misaligned with light-dark cues, impaired cognitive function and sleep disturbances can arise [2]. The circadian system also temporally coordinates metabolic activity and organ function, thus ensuring appropriate internal synchrony with rest-activity patterns and feeding cycles [3]. The process of circadian entrainment is therefore critical for maintaining normal human performance, sleep behavior, and energy balance.

### *14.1.2 Organization of the Human Circadian System*

The master circadian clock in humans is thought to be located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus. As demonstrated in monkeys and other mammalian species, lesions that include the SCN abolish circadian generation of behavioral and endocrine rhythms [4]. Similarly, sleep-wake and melatonin secretion patterns are altered in patients with pituitary tumors that compress the optic chiasm [5], which is situated just ventral to the SCN. Postmortem tracing techniques in humans have revealed a direct retinal projection to the region of the SCN [6]. As in other mammals, the retinohypothalamic tract is thought to be the sole pathway by which light resets the phase of clock neurons in the SCN [7]. Additionally, blind individuals with complete loss of retinal function do not show light-induced suppression of melatonin or phase-shift responses [8], suggesting that the eyes are necessary for SCN responses to light.

The retinal photoreceptors that mediate light resetting of circadian rhythms are distinct from those that mediate pattern-forming vision. In humans, this was first suggested by the observation that some blind individuals with total loss of rod and cone function exhibit melatonin suppression responses to light and can entrain normally under natural conditions [9]. It was later discovered that the retinohypothalamic projection to the SCN originates from a small subset of retinal ganglion cells that expresses the photopigment melanopsin [10]. The melanopsin cells are intrinsically photosensitive and respond preferentially to blue light, but can be

activated indirectly by rods and cones, as shown in rodents and in macaques with trichromatic vision similar to humans [11, 12]. It is therefore likely that both rod-cone photoreceptors and melanopsin contribute to circadian light responses in humans.

To entrain to the imposed solar day length, the phase of the SCN clock must be reset by a small amount each day. The SCN rhythm, in turn, coordinates behavioral and physiologic rhythms with diurnal changes in environmental lighting. In humans, the most commonly used markers of circadian clock function are core body temperature, melatonin, and cortisol, which exhibit clear circadian variation when measured under conditions that minimize the effects of exogenous influences on these rhythms [13]. The SCN clock can synchronize clocks in other tissues of the body either through rhythmic activation of neural and endocrine pathways or indirectly through its effects on rest-activity and feeding cycles. This hierarchical structure allows the circadian system to integrate light information and non-photic cues to regulate diverse physiologic processes.

## 14.2 Effects of Light on Resetting of Human Circadian Rhythms

### *14.2.1 Circadian Phase-Dependent Resetting*

The magnitude and direction of resetting responses to light are circadian phase-dependent. Exposure to light in the early part of the biological night elicits a phase delay shift of the human circadian system, whereas exposure to light in the late biological night elicits a phase advance shift. The phase response curve (PRC) in humans therefore closely resembles PRCs observed in other species (Fig. 14.1). The crossover point between phase delay and phase advance shifts occurs near the nadir of the core body temperature rhythm, within a few hours of usual wake time. Both the phase and amplitude of the circadian clock determine the type of resetting response that occurs [14]. Type-0 resetting is characterized by large phase shifts of up to 12 h and occurs via prior reduction of circadian clock amplitude. By comparison, Type-1 resetting is characterized by small phase shifts of only a few hours with little or no reduction in amplitude of the circadian pacemaker. A light stimulus of intermediate strength, if given during the biological night at the transition point between phase delay and phase advance shifts, can reduce the amplitude of the circadian clock to zero. This is termed critical resetting, in which phase cannot be determined and behavioral circadian rhythms are absent.

The first human PRC was constructed in response to a 5-h bright light stimulus (~10,000 lx of white light) administered on 3 consecutive days [15]. Phase shifts of up to 12 h could be achieved when the light stimulus was centered in the biological night, whereas smaller shifts were observed when the light stimulus was given at other circadian phases (Fig. 14.1). Resetting responses for this light stimulus

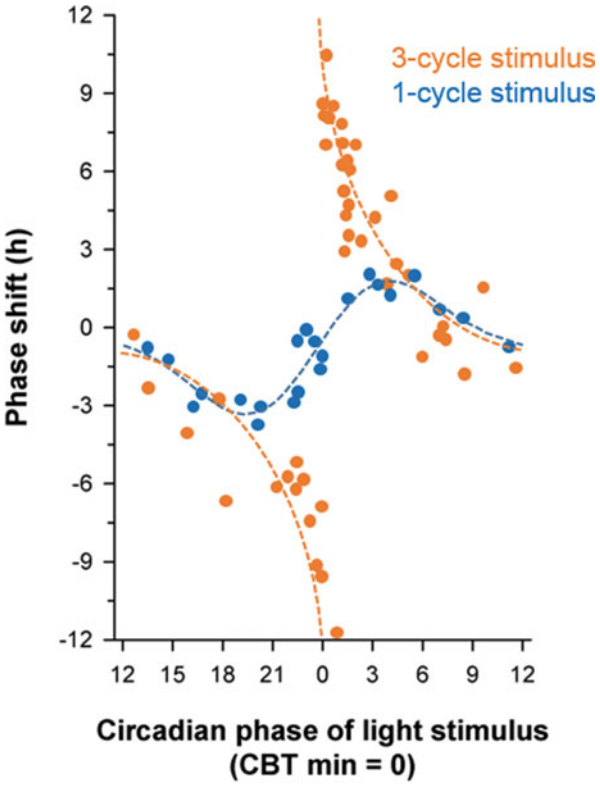


**Fig. 14.1** Circadian phase-dependent resetting. The human circadian system shows Type-0 resetting in response to three cycles of exposure to bright white light (5 h, ~10,000 lx), whereas Type-1 resetting is observed for a single exposure to bright white light (6.7 h, ~10,000 lx). The circadian phase of light exposure is plotted relative to the minimum of the core body temperature (CBT) rhythm, defined as initial phase zero. By convention, phase delay shifts are indicated by negative values on the phase response curve (Figures are reproduced with permission, from Czeisler et al. [15] (© AAAS) and from Khalsa et al. [17] (© Physiological Society))

therefore matched the Type-0 PRC described in other organisms. Reducing the light exposure protocol from three cycles to two cycles resulted in a marked reduction in the amplitude of the core body temperature rhythm [16], suggesting that Type-0 resetting for the three-cycle stimulus occurred through prior reduction of circadian pacemaker amplitude. Further reduction of the light stimulus to a single cycle of light exposure (6.7 h, ~10,000 lx) resulted in weak Type-1 resetting [17], with maximum phase delay shifts of about −3 h and maximum phase advance shifts of about 2 h (Fig. 14.1).

In subsequent PRC studies, Type-1 resetting was also observed in response to a 1-h pulse of bright white light (~8000 lx) [18] and in response to 6.5 h of narrow-bandwidth 480-nm light [19]. Similar Type-1 resetting responses were found in older versus younger participants (age ranges of 18–31 years and 59–75 years) exposed to a three-cycle stimulus of 3 h of moderately bright light (~3000 lx) [20]. In all of the aforementioned PRC studies, the magnitude of phase delay shifts was greater than for phase advance shifts, and the inflection point occurred in the later part of the usual sleep period. In addition, the human PRC did not exhibit a "dead zone" during the biological daytime, suggesting that the human circadian system is sensitive to light across all circadian phases.

### 14.2.2 Dose-Dependent Effects of Light

The magnitude of circadian responses to light can be enhanced by increasing the intensity of the stimulus. The effects of different illuminance levels on human circadian resetting were first examined using a three-cycle light stimulus, in which white light was administered for 5 h during the phase advance region of the PRC (centered 1.5 h after the core body temperature minimum) [21]. A nonlinear increase in phase advance shifts was observed with increasing light intensity, such that ~100 lx of white light elicited a half-maximal phase-resetting response [22]. A similar dose-response relationship was shown for a single cycle of exposure to 6.5 h of white light administered during the phase delay region of the PRC [23]. In that study, half of the maximal phase-delaying response to light was achieved for ~120 lx of room light, with a saturating phase delay shift of about −3 h in response to ~550 lx of white light (Fig. 14.2). In older individuals (aged ≥65 years) exposed to similar experimental procedures, the half-maximal phase-resetting response occurred for exposure to ~260 lx [24], raising the possibility that circadian sensitivity to light might be reduced in aging. However, the magnitude of phase-shift responses was similar in younger versus older participants at higher light intensities [24–26]. The aforementioned studies demonstrate that circadian resetting responses are strongly dose-dependent, and the human circadian system is exquisitely sensitive to ordinary room light.

Another approach for enhancing the magnitude of phase resetting is to increase the duration of the light pulse. For example, a duration-dependent increase in phase delay shifts was observed in a study that used 1-h, 2-h, and 3-h pulses of bright white light (~2000 lx, ~4000 lx, or ~8000 lx) administered in the early biological night [27]. In another study, a nonlinear increase in phase delay shifts was observed as the duration of light exposure was increased (~10,000 lx for 12 min, 1 h, 2.5 h, or 4 h) [28]. Per minute of exposure to light, shorter-duration light stimuli were more effective than longer-duration light stimuli at resetting circadian rhythms (Fig. 14.2). A similar result was obtained in a previous study that compared PRCs to 1 h versus 6.7 h of bright white light (~10,000 lx). In that study, the 1-h light stimulus induced maximum phase-shift responses that were nearly 40 % of responses to the longer-duration light stimulus, despite representing only 15 % of the stimulus duration [17, 18]. These results suggest that the early part of a continuous light stimulus has greater resetting effects than the later part of the same light stimulus.

### 14.2.3 Effects of Intermittent Light Exposure

Exposure to intermittent bright light represents an alternative and perhaps more efficient approach for resetting human circadian rhythms than exposure to continuous light. This was first examined by comparing the phase-advancing effects of exposure to 5 h of continuous bright light (~10,000 lx) over 3 consecutive days, relative to the

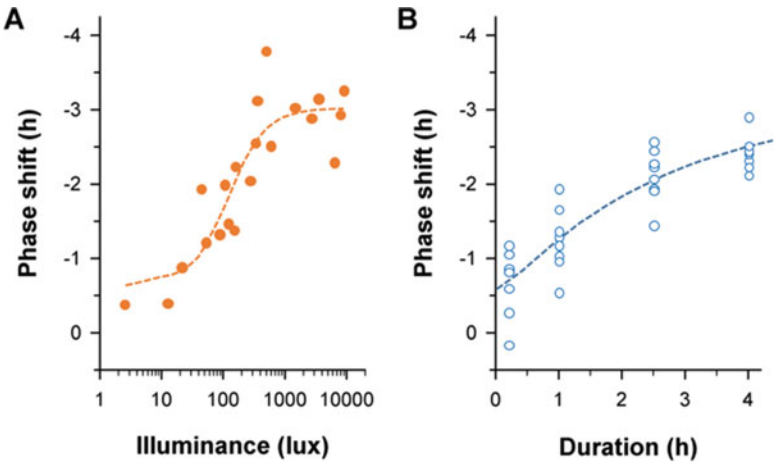


**Fig. 14.2** Dose-dependent resetting of the melatonin rhythm. (**a**) An illuminance-response curve shows phase delay shifts in individual subjects following exposure to different intensities of polychromatic white light during the early biological night. (**b**) A duration-response curve is shown for circadian resetting following exposure to bright white light (~10,000 lx) given during the early biological night (Figures are reproduced with permission, from Zeitzer et al. [23] (© Physiological Society) and from Chang et al. [28] (© Physiological Society))

effects of exposure to repeated administration of alternating bright light and darkness (either ~5 min of lights on and ~20 min of lights off or ~46 min of lights on and 44 min of lights off) [29]. Despite having a much-reduced total exposure time to bright light, the intermittent stimuli elicited phase-shift responses that were nearly as large as for continuous exposure to bright light. Similar results were obtained in another study in which subjects were exposed to a single cycle of 6.5 h of continuous bright light or intermittent bright light (~10,000 lx for 15 min, <3 lx for 60 min), administered during the phase delay region of the PRC [30]. Despite representing only 23 % of the exposure time in the continuous light condition, the intermittent light stimulus elicited phase-resetting responses that were similar in magnitude (about −2.5 h) (Fig. 14.3). These results further demonstrate that short light pulses are more efficient (on a per-minute basis) than longer light pulses at resetting circadian rhythms.

The phase-resetting effects of intermittent light have been examined as a potential treatment for shift work and jet lag in combined laboratory and field studies. In participants who were maintained on an advancing sleep-wake cycle, exposure to 3.5 h of alternating bright light and dim room light (3000–5000 lx for 30 min, <60 lx for 30 min) on 3 consecutive mornings elicited a phase advance shift of about 1.5 h, which was similar to the effects of continuous exposure to bright light [31, 32]. Additionally, when administered over several consecutive simulated night shifts, exposure to intermittent bright light (~4100 lx for 15–20 min, <50 lx for 40–45 min) facilitated partial circadian adaptation to a night shift schedule [33], similar to the effects of continuous bright light reported previously [34]. Such

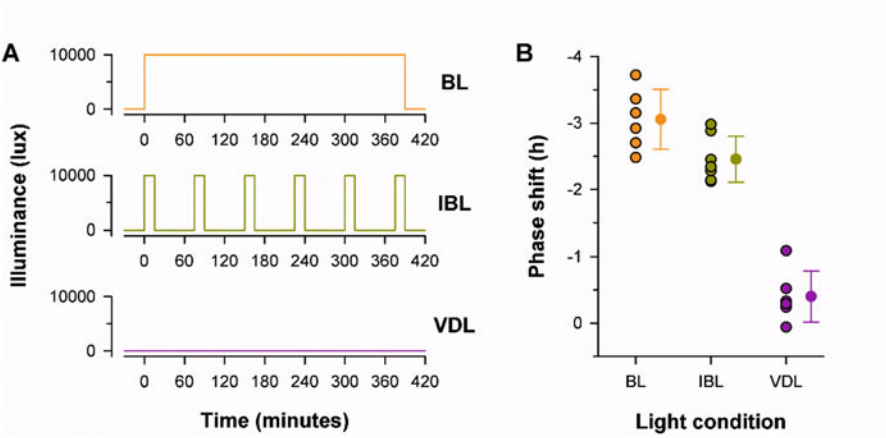


**Fig. 14.3** Intermittent light is highly effective at resetting human circadian rhythms. (**a**) Subjects were exposed to 6.5 h of bright polychromatic white light (BL, ~10,000 lx), intermittent bright light (IBL, 15 min of ~10,000 lx alternating with 60 min of ~1 lx), or very dim light (VDL, ~1 lx) during the early biological night. (**b**) Phase delay shifts of the melatonin rhythm following exposure to IBL were nearly as great as resetting responses to continuous BL. Individual phase-resetting responses and the mean ± SD are shown for each light exposure group (Figures are reproduced with permission, from Gronfier et al. [30] (© American Physiological Society))

studies suggest that circadian alignment to shift work is possible if exposure to bright light, dim light, and darkness is carefully planned [35, 36].

### *14.2.4 Spectral Sensitivity of Circadian Resetting*

In mammals, light resetting of circadian rhythms is mediated by melanopsin-containing retinal ganglion cells. Melanopsin-dependent responses exhibit peak sensitivity to ~480-nm light, which corresponds to the blue portion of the visual spectrum. By comparison, the photopic visual system in humans, which is responsible for mediating color vision, is most sensitive to 555-nm green light. The contribution of melanopsin versus cone photoreceptors to circadian photoreception can therefore be inferred, at least to some degree, by differences in the magnitude of circadian responses to shorter versus longer wavelengths of light. In a study that used 460-nm and 555-nm light stimuli that were matched for photon density ($2.8 \times 10^{13}$ photons $cm^{-2}$ $s^{-1}$) [37], the phase-delaying effects of 6.5 h of exposure to blue light were about twice as great relative to the effects of green light, with a maximum phase-shift response of about −3 h (Fig. 14.4). Using similar experimental procedures, a blind patient without detectable rod and cone function exhibited a substantial phase delay shift for the blue light stimulus (about −1.2 h), whereas little or no response was observed for the green light stimulus [38]. These findings and others suggest that the primary pathway by which light

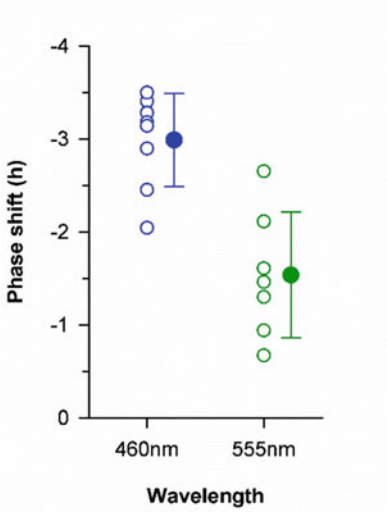


**Fig. 14.4** Circadian responses are short-wavelength sensitive. Phase delay shifts of the melatonin rhythm are shown following exposure to 6.5 h of narrow-bandwidth *blue* light (460 nm) or *green* light (555 nm) matched for photon density (6.5 h, $2.8 \times 10^{13}$ photons $cm^{-2}$ $s^{-1}$). Individual phase-resetting responses and the mean ± SD are shown for each light exposure group (The figure is replotted using data from Lockley et al. [37] (© Endocrine Society))

resets human circadian rhythms is through activation of melanopsin, rather than cone photoreceptors [32, 39].

There is substantial evidence, however, that classical visual photoreceptors contribute to circadian resetting responses in mice and humans. In fact, circadian phase-shift responses remain intact in melanopsin null mice and are eliminated only when all photoreceptor types are rendered dysfunctional [40, 41]. A role for cones in light resetting of human circadian rhythms was first suggested by a study in which exposure to a red light stimulus over 3 consecutive days (~220 lx) elicited an average phase advance shift of ~1 h [42]. More recently, the phase-delaying effect of a single cycle of exposure to 6 h of narrow-bandwidth red light (631 nm, $1.0 \times 10^{13}$ photons $cm^{-2}$ $s^{-1}$) was examined, and the average phase delay shift was close to an hour [43]. A role for the photopic visual system in mediating circadian responses is further supported by studies comparing irradiance-response curves to 460-nm light versus 555-nm light [44]. At lower irradiances ($<1.0 \times 10^{12}$ photons $cm^{-2}$ $s^{-1}$), the phase-delaying effects of the 555-nm light stimulus were too large relative to the 460-nm light stimulus to be explained solely by the activation of melanopsin. In addition, the sensitivity of melatonin suppression to 555-nm light decayed exponentially relative to 460-nm light during the 6.5-h exposure. These results suggest that cone photoreceptors might contribute substantially to circadian responses at lower irradiances and during the early part of a light exposure.

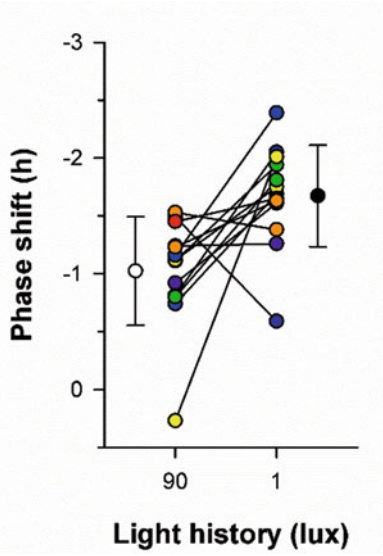


**Fig. 14.5** Light resetting of human circadian rhythms is modulated by photic history. Phase delay shifts are shown in participants who were exposed to 90 lx of polychromatic *white* light during the early biological night, after being exposed to 90 or 1 lx of light throughout the usual wake period. Individual phase-resetting responses and the mean ± SD are shown for each light exposure condition (The figure is reproduced with permission, from Chang et al. [45] (© Physiological Society))

### *14.2.5 Effects of Lighting History on Resetting*

Even though it is well established that visual responses show dark adaptation and light adaptation, little is known about the effects of photic history on circadian light responses. In many of the studies that characterized basic properties of human resetting responses, subjects were exposed to dim light or darkness for several hours or days prior to the light intervention. Recent work suggests that the magnitude of light resetting depends not only on the light stimulus itself but also on the level of light an individual is exposed to beforehand. The phase-delaying effects of exposure to 6.5 h of ordinary room light (~90 lx) have been compared following exposure to very dim light (~1 lx) versus room light (~90 lx) throughout the preceding wake period [45]. Prior exposure to dim light increased circadian responses to room light by about 60–70 % relative to prior exposure to room light (Fig. 14.5), demonstrating that a person's recent history of light exposure modulates the sensitivity of the circadian system to light.

## 14.3 Entrainment of Human Circadian Rhythms

### *14.3.1 Limits of Entrainment*

For stable entrainment to occur, the phase of the circadian clock ($\varphi$) must be reset by an amount that is equivalent to the difference between the endogenous period length ($\tau$) and the imposed period of the environmental synchronizer ($T$), i.e., the 24-h solar day under natural conditions. Stated in the form of an equation, entrainment occurs when $\Delta\varphi = \tau - T$. It therefore follows that a person with a circadian period longer than 24 h would require a net phase advance shift to synchronize with the solar day, whereas a person with a shorter circadian period would require a net phase delay shift to entrain. The circadian system aligns with the T-cycle such that the distribution of exposure to light in the phase advance and phase delay regions of the PRC results in a net phase shift that satisfies the aforementioned equation, ensuring that the observed period of the clock equals the period of the solar day. Given that most individuals have a circadian period within the range from 23.5 to 24.5 h [46], only a small adjustment of the circadian clock is usually needed for circadian entrainment to the natural light-dark cycle.

In early human chronobiology experiments performed in underground bunkers, it was demonstrated that the human circadian system can synchronize to artificial light-dark cycles close to 24 h, whereas most subjects could not entrain to cycle lengths that were substantially shorter (22 h 40 min) or longer (26 h 40 min) than 24 h [47]. Hence, the range of entrainment of the human circadian pacemaker is limited to T-cycle lengths that are relatively close to 24 h. More recently, the minimum light intensity required for entrainment was explored in laboratory experiments in which several participants were first entrained to a 24-h light-dark cycle with exposure to bright indoor room light (450 lx) during wake periods, followed by 3 weeks of exposure to the same schedule but with the light dimmed to about 1.5 lx [48]. The dim light exposure condition was sufficient to maintain entrainment of the melatonin rhythm to the 24-h T-cycle, but not to longer (24.6 h) or shorter (23.5 h) day lengths. It has since been shown that exposure to room light (~100 lx) can entrain human circadian rhythms to a T-cycle equivalent to $\tau + 1$ h [49]. Hence, exposure to ordinary room light can adjust the circadian clock by an hour each day (Fig. 14.6), which is consistent with earlier work on the dose-dependent resetting effects of white light on human circadian rhythms [23].

It might seem counterintuitive that exposure to dim light does not elicit detectable phase-resetting responses [22, 23], and yet the circadian system can maintain entrainment to a 24-h cycle with exposure to dim light during wake periods [48]. This could be explained in part by aftereffects of entrainment, in which circadian period is modulated by prior entrainment conditions. For example, it has been shown that the period of the human circadian pacemaker is longer if preceded by entrainment to a 24.65-h day, as compared to entrainment to a 23.5-h day [50]. Plasticity in circadian period could therefore help to reduce the phase shift

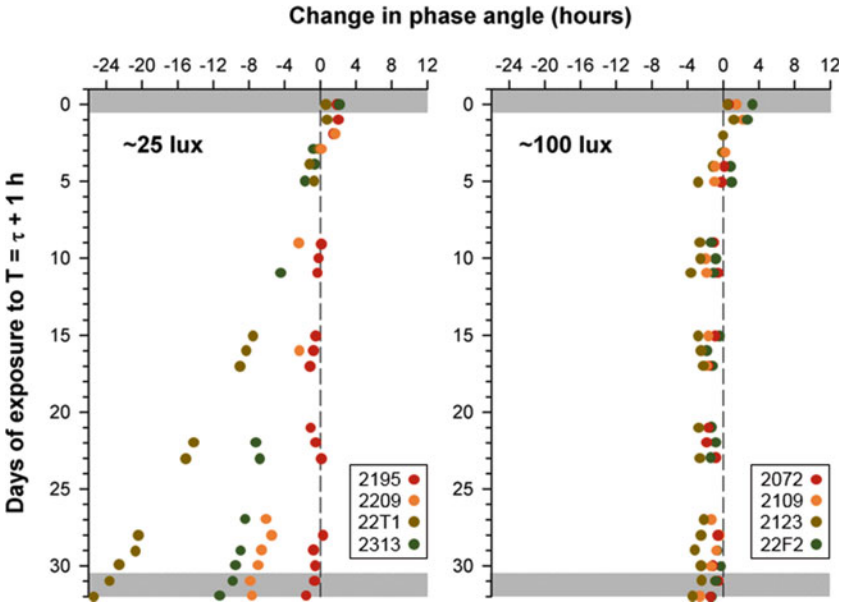


**Fig. 14.6** Entrainment of the circadian system to ordinary room light. Subjects were exposed to a T-cycle equivalent to $\tau + 1$ h over the course of a month while being exposed to either dim light (~25 lx, *left panel*) or ordinary room light (~100 lx, *right panel*) during wake periods. The melatonin offset is plotted with respect to phase measured on day 3 of the protocol, and *colored circles* correspond to different subjects (The figure is reproduced with permission, from Gronfier et al. [49] (© National Academy of Sciences))

required for entrainment, hence making it easier to maintain synchrony to a given T-cycle when light levels are decreased.

### *14.3.2 Phase Angle of Entrainment*

The temporal relationship between circadian phase of a rhythm and environmental time is referred to as the phase angle of entrainment. For example, the onset of melatonin secretion usually begins a few hours before a person's habitual bedtime, but there is substantial individual variation in this time interval. As shown in young healthy subjects, brighter light exposure is associated with a narrower range of phase angles across individuals [49, 51]. The phase angle of entrainment correlates strongly with circadian period in humans, such that plasma melatonin levels rise later (i.e., closer to bedtime) in individuals with a longer circadian period. Therefore, individual differences in circadian period are thought to contribute to differences in chronotype. Morning

types wake up at an earlier clock time but at a later biological time than evening types [52]. It is therefore easier for morning types to wake up feeling refreshed, as their wake time occurs further from the peak of the circadian drive for sleep. It should be highlighted, however, that circadian period does not fully account for individual differences in phase angle. For example, laboratory studies suggest that the age-related advance in circadian phase is not due to a shortening of the circadian period [53], but might be explained by other factors including differences in the distribution of light exposure across the circadian cycle, or reduced sensitivity to light [24].

### *14.3.3 Entrainment Under Real-World Conditions*

Under free-living conditions, people are exposed to a large host of periodic and nonperiodic environmental factors that can influence their patterns of behavior and exposure to light [54]. To date, however, few studies have examined human circadian entrainment under real-world conditions. A recent study compared

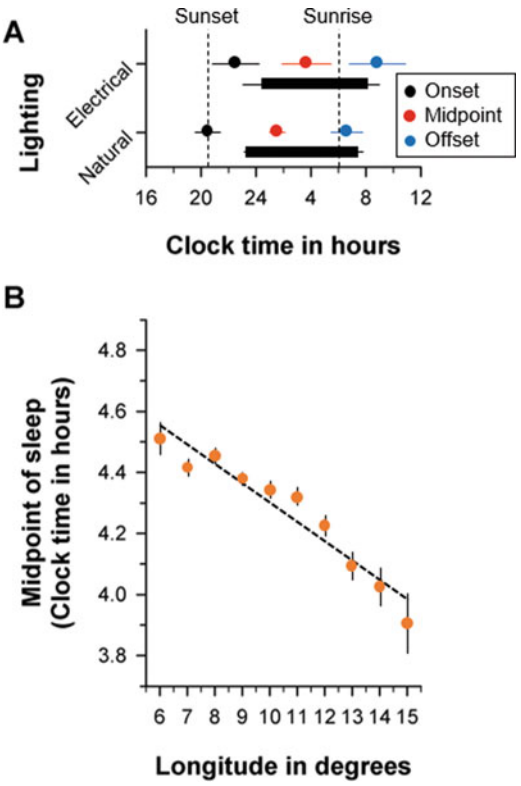


**Fig. 14.7** Regulation of circadian phase by electrical lighting and sunlight. (**a**) The timing of the melatonin rhythm and sleep (*black bars*) is shown for subjects exposed to electrical lighting as part of their usual routine versus natural lighting while camping. The mean ± SD is shown for each marker of the melatonin rhythm. (**b**) The midpoint of self-reported nighttime sleep is plotted against longitude (from west to east on the abscissa) in participants residing in areas of Germany with up to 300,000 inhabitants. The mean ± SEM is shown for the midpoint of sleep in 1° bins (Figures are reproduced with permission, from Wright et al. [55] (© Cell Press) and from Roenneberg et al. [56] (© Cell Press))

circadian phase in a group of subjects who were exposed only to natural lighting (i.e., sunlight and firelight) as part of a camping trip in the Rocky Mountains versus free access to electrical lighting under modern conditions in the same set of individuals [55]. Based on laboratory assessments of circadian phase after exposure to each of these conditions, the onset of melatonin secretion occurred much closer to sunset after camping (Fig. 14.7). In addition, participants who were later chronotypes showed a larger advance in circadian phase when exposed to natural lighting, bringing them closer to early types. Access to electrical lighting was associated with reduced exposure to sunlight during the daytime and increased exposure to light after sunset. These findings suggest that modern-day use of electrical lighting has altered the phase angle of entrainment and the timing of biological night in humans.

It might be expected that social time, which is strongly influenced by work schedules and lifestyle choices, has replaced environmental time as the primary factor influencing the timing of sleep-wake behavior. Exposure to natural light remains a strong synchronizing stimulus in the modern world, however, as demonstrated in a study that examined self-reported sleep-wake behavior in 21,600 individuals living in Germany within a single time zone [56]. If the circadian clock is synchronized with solar time rather than social time, there should be a small but systematic difference in the timing of sleep-wake behavior from east to west. Consistent with this prediction, it was found that the timing of sleep varied with differences in longitude (Fig. 14.7). Additionally, spending more time outdoors was associated with an earlier chronotype. Exposure to natural lighting therefore influences the timing of sleep-wake in humans, despite our ability to manipulate our own lighting environment using electricity.

## 14.4 Summary and Future Directions

Over the past few decades, remarkable progress has been made in understanding the role of light in regulating human circadian rhythms. Laboratory studies have revealed that humans exhibit circadian phase-dependent resetting, with the magnitude of responses determined by the intensity, duration, and wavelength of the light stimulus. The interaction of these light parameters on resetting of human circadian rhythms is still poorly understood, however, because most studies have manipulated one parameter while keeping the others constant. The magnitude of circadian responses is also modulated by light levels experienced prior to the light stimulus, but the dose-dependent effects of daytime light exposure on subsequent light resetting responses have not been explored. In general, little is known about how the spectral composition of light, which changes throughout a typical day under real-world conditions, modulates the circadian phase angle of entrainment.

As we learn more about how exposure to light regulates circadian phase, it is possible that this knowledge can be applied to improve treatments for circadian rhythm sleep disorders [57]. A growing number of studies have examined light

therapy and/or light avoidance for treating circadian misalignment associated with shift work, jet lag, and delayed sleep-phase disorder. Given that laboratory conditions and real-world conditions differ in a substantial number of ways, it is important to explore further the combined effects of exposure to electrical lighting and natural lighting on human circadian rhythms. There is also a need to evaluate the degree to which electronic devices (e.g., laptops, tablets, and smartphones) contribute to light exposure patterns and sleep timing and their potential effects on circadian phase [58]. In many individuals, there is also a marked difference in their sleep schedule on weekdays versus weekends, but the impact of this behavior on light exposure patterns, circadian entrainment, and long-term health requires further investigation. In conclusion, light exerts a powerful influence on the human circadian system, with important implications for the timing of behavioral and physiologic rhythms. Exposure to natural lighting normally synchronizes the human circadian system such that sleep occurs primarily at night, but in the modern age, it is possible to reset the clock using electrical lighting – this can be either advantageous or disadvantageous, depending on the circumstances.

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# Chapter 15
# Delayed Sleep Phase Disorder: Mechanisms and Treatment Approaches

**Jade M. Murray, Tracey L. Sletten, Michelle Magee, and Shantha M.W. Rajaratnam**

**Abstract** This chapter will introduce the basic characteristics of delayed sleep phase disorder and provide an overview of the current methods for diagnosis. Current theories on the etiology and mechanisms of the disorder are described, and a review of treatment options is presented. In summary:

- DSPD is characterized by a delay in the timing of the sleep-wake cycle, such that sleep onset is difficult to achieve at a desired or required time. Difficulty waking is also experienced, particularly when sleep is attenuated to maintain required schedules.
- DSPD is associated with a number of negative health consequences, including a high prevalence of comorbid depression.
- Significant functional impairments are associated with DSPD, including poor school or job performance, dysfunctional relationships, and negative health behaviors such as smoking and excessive alcohol use.
- Prevalence rates vary between cultures and between adolescents and middle-aged adults.
- It is likely multiple factors, such as genetic, environmental, and physiological, contribute to the etiology of the disorder, although the underlying basis of the disorder has not been fully elucidated.
- Assessment of circadian phase is an important diagnostic tool and may improve treatment outcomes.
- Exogenous melatonin and bright light therapy, both separately and combined, are emerging as effective treatments for DSPD, while further research is required for other promising pharmacological approaches.

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## 15.1 Clinical Characteristics of Delayed Sleep Phase Disorder

The endogenous circadian pacemaker, in addition to peripheral oscillators, is responsible for regulating the timing of a number of physiological and behavioral features, including the sleep-wake cycle. The propensity for sleep and wake fluctuates rhythmically through an interaction between the circadian and homeostatic processes that regulate sleep and wakefulness [1]. In healthy sleepers, the circadian rhythm of sleep propensity occurs at appropriate times relative to an individual's desired sleep-wake schedule, referred to as optimal phase alignment [2]. Optimal alignment between the circadian drive for sleep and the desired sleep-wake schedule is conducive to sleep, that is, of sufficient duration and good quality, with minimal awakenings and a feeling of being refreshed on waking. However, there are instances in which the circadian pacemaker is not aligned to the desired sleep-wake schedule, with sleep and wake occurring earlier or later than desired. This desynchrony is referred to as phase misalignment and is likely to compromise sleep quality and duration, with extended sleep-onset latency or early awakenings, depending on whether circadian phase is advanced or delayed, as well as reduced sleep efficiency [3].

Delayed sleep phase disorder (DSPD) is a manifestation of phase misalignment. According to the International Classification of Sleep Disorders, Third Edition (ICSD-3) [4], DSPD is characterized by a delay in the timing of the major sleep episode, relative to desired clock time, resulting in symptoms of sleep-onset insomnia or difficulty in awakening at the desired time, and is thought to arise due to a delay in the endogenous circadian pacemaker. Figure 15.1 shows actigraphically recorded rest-activity patterns of a DSPD patient, most notably illustrating the delayed timing of the major sleep episode.

In a seminal study, DSPD was first described as a chronobiological disorder marked by sleep-onset insomnia, after 30 out of 450 "insomnia" patients were identified as having symptoms that differed slightly from those of primary insomnia [5]. It was noted that while insomnia patients usually have a disturbed sleep pattern, with a distinct difficulty maintaining sleep, patients with DSPD have normal sleep quality and duration when bedtime and sleep initiation were delayed to a time that aligns with the endogenous circadian phase and adequate sleep opportunity is permitted. Similarly, unlike insomnia patients, when patients with DSPD attempt sleep at a later clock time and thus a later circadian phase, sleep-onset latency (SOL; the time it takes to fall asleep) is significantly reduced.

As such, DSPD is most commonly characterized by difficulty initiating sleep when attempting sleep at a desired time necessary to achieve sufficient sleep to attend school or work or to meet social obligations. Difficulty waking is also experienced by DSPD patients, particularly when sleep is attenuated to maintain required schedules that comply with societal norms. Because of the discrepancy between desired sleep and wake times and the endogenous rhythm of sleep propensity, there are often substantial differences between bed and wake times during

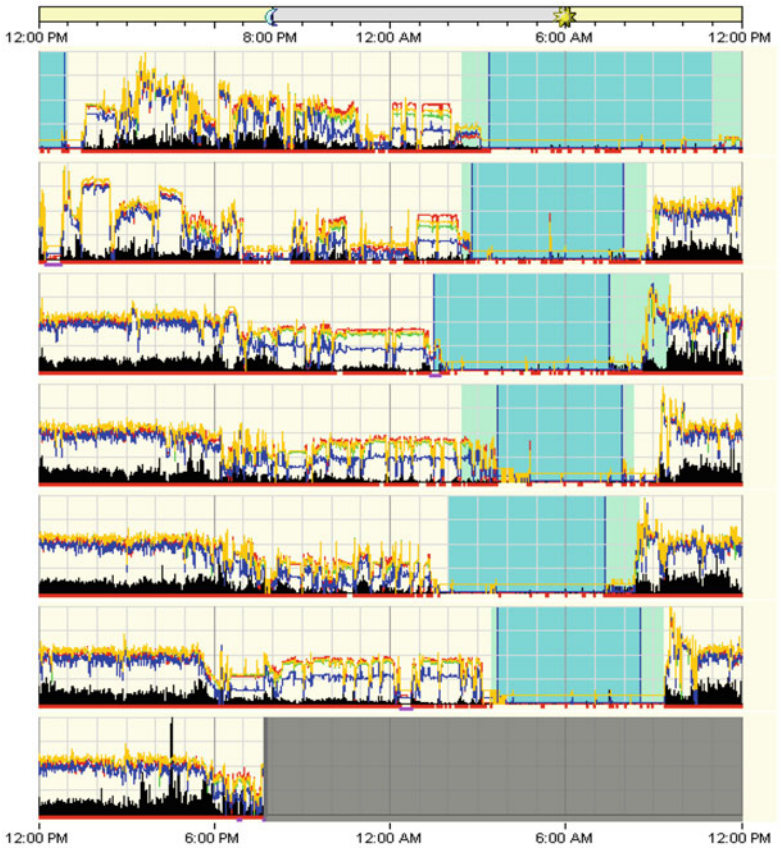


**Fig. 15.1** Actigraphically recorded rest-activity pattern in a typical DSPD patient. Consecutive days are shown on the vertical axis. Clock time is represented on the horizontal axis. Data are *double plotted* (48 h on the horizontal axis). This patient, a male aged 26 years, shows delayed bed and wake times, on average 0230 h and 1000 h, respectively. Average sleep duration is only 5.5 h. *Black vertical lines* represent activity level per minute; the *blue bars* represent sleep episodes, with the *lighter blue portion* indicating sleep-onset latency and wake time in bed after sleep offset

the school term or working week compared with vacation or weekends, as patients attempt to maintain a schedule suitable for attending work or school but revert back to a less restricted, and, later, schedule on weekends or on vacation. On week nights, bedtime is often advanced to an earlier desired time, and sleep-onset latency (SOL) is likely to be increased. However, on vacation or weekends, when bed and wake times are likely less restricted, bedtime occurs at a later clock time that better aligns with the delayed circadian timing [5].

DSPD is also associated with significant functional impairments. Adult DSPD patients report significant impairments to job performance, financial difficulties and marital problems [6], and also greater medication use, particularly hypnotics [7]. Adolescents with DSPD show poorer school performance [8], dysfunctional

school behaviors, and underachievement [9] and are more likely to engage in negative health behaviors such as smoking [10] and excessive alcohol [8] and caffeine use [11]. Rajaratnam, Licamele, and Birznieks [12] also reported significantly increased absenteeism and reduced productivity in survey respondents at high risk of DSPD, compared with respondents who were not at risk. Furthermore, they found that daytime sleepiness, associated with the increased sleep deficit on nights before work or school, was increased in those at high risk of DSPD. Other risks include increased likelihood of depression. Depressive symptoms are commonly reported in DSPD patients with one study showing that 64 % of patients with DSPD had a comorbid diagnosis of depression [13]. Others have reported similar prevalence of comorbidity with depression [14].

## 15.2 Prevalence of DSPD

Determining the true prevalence of DSPD is difficult, due in part to the nature of the diagnostic criteria, as they are almost exclusively based on self-reported symptoms. Additionally, the variability in beliefs and attitudes about sleep, particularly with regard to the social norms of bed and wake times among different countries and cultures, adds to the difficulty in estimating global prevalence. In addition to this, methods and criteria used for diagnosing the disorder vary greatly in the literature. For example, while some studies use only the criteria set out in diagnostic manuals such as the ICSD-3 [4], others combine this with circadian phase assessments to more precisely detect the presence of a circadian phase misalignment. Another factor making it difficult to determine the true prevalence of DSPD is the potential for DSPD to be misclassified as insomnia. It has been estimated that up to 10 % of insomnia diagnoses are misdiagnosed cases of DSPD [15].

The prevalence of DSPD in teenagers and young adults is greater (7–16 %) [15] than middle-aged individuals (3.1 %) [16]. A comprehensive Norwegian study showed a general population prevalence of 0.17 % [17], while an Australian study has reported a prevalence of 1.1 % in an adolescent population [14]. This estimate is substantially less than a study of 191 American college-aged students, in which 11.5 % were reported to meet criteria for DSPD [18], and a study by Saxvig et al. [8] in a sample of 1285 high school students, reporting that 8.4 % met criteria for DSPD.

## 15.3 Mechanisms

The following section describes several key theories relating to the etiology of the delayed sleep pattern observed in DSPD. Although discussed separately, it is likely that multiple factors contribute, likely, with heterogeneity attributed to specific individual vulnerabilities.

### 15.3.1 Circadian Phase Delay

Phase misalignment observed in DSPD is thought to be largely due to a delay in the endogenous circadian pacemaker, as measured by rhythms of melatonin and core body temperature (CBT) [13]. This delay in the timing of circadian rhythms is also associated with a delay in the wake maintenance zone (WMZ), an interval of between 2 and 3 h occurring prior to melatonin secretion onset and habitual bedtime, in which alertness is increased and propensity for sleep is reduced [19, 20]. Delayed timing of the WMZ means that it coincides with the desired time of sleep, making sleep onset difficult [21]. While it is well documented that DSPD arises from a delay in the timing of the internal clock of an individual [13], the exact causal mechanisms of this delay have not been fully elucidated.

### 15.3.2 Circadian Period (Tau)

In non-disordered humans, the average circadian period is 24.18 h [22]. The circadian pacemaker is synchronized or entrained to the 24-h solar day by zeitgebers, most notably the light/dark cycle. Differences in circadian period between individuals are associated with differences in the phase of entrainment to the light/dark cycle.

Individuals with DSPD may have a longer circadian period than those who do not have DSPD. If the circadian pacemaker oscillates more slowly, taking longer to complete a cycle, this would lead to a strong tendency for circadian rhythms to be delayed relative to the 24 h day/night cycle. Indeed a difference of just 6 min in period length can equate to an hour difference in entrained phase in healthy young individuals [2]. Campbell and Murphy [23] compared a DSPD patient with three healthy controls under free-running conditions and found that circadian period was almost 1 h longer in the patient than in controls. A more recent study that compared circadian period in DSPD patients using a modified 78-h ultradian constant routine protocol also showed that circadian period was 25 min longer than that of normal sleepers [24]. Indeed the sleep characteristics common to DSPD, such as difficulty falling asleep, later circadian phase, and a diurnal evening preference, are all associated with a longer circadian period [14], lending weight to this explanation as a causal mechanism for the disorder.

### 15.3.3 Phase Relationship Between the Circadian Pacemaker and Sleep and the Phase Response Curve

It is well established that the circadian (phase-resetting) response to light depends on circadian timing of light exposure, described by the phase response curve (PRC)

for light, in addition to the duration, intensity, and wavelength [25]. Light administered shortly after the core body temperature minimum will cause a phase advance. In young healthy adults, core body temperature minimum occurs, on average, 2 h before habitual wake time [26]. Ozaki and colleagues [27] postulated that masking of this phase advance region of the PRC occurs in DSPD patients, showing that DSPD patients have a longer interval between the core body temperature minimum and sleep offset, than controls. Specifically, core body temperature minimum occurred 4 h before wake time in DSPD patients, compared with 2.5 h before wake time in controls [27], indicating an altered phase angle of entrainment. This was subsequently confirmed by others [28, 29]. This suggests that DSPD patients may be missing exposure to light in the early biological morning. Others, however, report that there is little difference in the phase of the core body temperature minimum relative to sleep offset between DSPD patients and controls [30]. Studies, however, consistently report delayed circadian timing, as well as later sleep-onset and sleep-offset times, in the DSPD patient groups. To date, therefore, it is unclear whether altered phase relationships between the circadian pacemaker and sleep timing may be a causal mechanism of DSPD, resulting in altered light/dark exposure in particular during the critical phase-advance portion of the PRC.

### *15.3.4 Hypersensitivity to Light*

Further to reduced light exposure in the early biological morning, DSPD patients may experience increased sensitivity to evening light exposure, during the delay portion of the light PRC. Aoki et al. [31] have reported that melatonin suppression was significantly greater in DSPD patients compared to controls, suggesting circadian hypersensitivity to light, which would increase the phase-delaying effect of the evening light exposure. In this study, DSPD patients and aged-matched controls were administered 1000 lux of light 2 h before the time of peak melatonin secretion, as determined in a previous laboratory visit. After 2 h of exposure to light, maximum suppression in DSPD patients averaged 71 %, while maximum suppression reached only 56 % in controls. Further more comprehensive studies are needed to confirm this hypothesis, particularly given that patients are often exposed to longer durations of evening light as a result of their later bedtimes.

### *15.3.5 Homeostatic Process Dysfunction*

While DSPD is a circadian rhythm disorder, there may be an underlying basis for the disorder relating to a dysfunction of the homeostatic process. During pubertal maturation, there is a decline in slow-wave activity [32], a marker of accumulated homeostatic sleep pressure. This decline in slow-wave activity during puberty

occurs during the critical period in which DSPD is likely to emerge and has therefore been theorized as playing a role in the pathophysiology of DSPD [14]. In other words, slower accumulation of homeostatic sleep pressure would result in later sleep-onset times and thus may contribute to DSPD. Uchiyama et al. [33] made the observation that propensity for sleep differed between DSPD patients and controls following sleep deprivation and suggested that reduced accumulation of sleep pressure may explain the underlying pathophysiology of the disorder. While the propensity for sleep in the control group was high after extended wakefulness, even throughout the day when melatonin secretion was minimal, DSPD patients had minimal propensity for sleep.

It has been well established that slow-wave sleep is a marker of homeostatic sleep pressure [34]. Polysomnography findings from Watanabe et al. [29], showing less slow-wave sleep despite greater overall total sleep time in DSPD patients compared to controls, suggest that altered function of the homeostatic drive for sleep may contribute to the etiology of the disorder. The reduction in slow-wave sleep indicates, in line with others [32, 33], that there is a slower accumulation of homeostatic sleep pressure, contributing to the delayed sleep timing typical of DSPD patients.

Reminiscent of these differences between DSPD patients and controls are differences seen between morning and evening chronotypes. As evidenced by polysomnographically measured slow-wave sleep, sleep pressure builds up more slowly in evening chronotypes, and the time course for dissipation of sleep pressure differs between chronotypes [35]. Even when circadian timing is controlled for, morning and evening chronotypes differ in the dissipation of homeostatic sleep pressure, such that the morning types have a higher initial level and faster rate of slow-wave sleep decay [36].

### *15.3.6 Genetic Mechanisms*

Several genes have been identified in mammals that are relevant to the circadian pacemaker, including PERIOD (*Per1/Per2/Per3)*, *CLOCK*, and *BMAL1 genes*. These genes are expressed in the suprachiasmatic nuclei (SCN) and are thought to play a role in the mechanisms controlling the pacemaker [37, 38]. Particular mutations of these genes are known to modulate circadian rhythms in hamsters and mice, and therefore it could be possible that mutations of these clock genes could also generate abnormal circadian rhythms in humans [38, 39]. For example, the 4-repeat allele of *Per3* has been shown to be more frequent in DSPD patients and strongly associated with extreme eveningness preference, while morningness preference is associated with the 5-repeat allele [40]. Conflicting findings have been reported, however, relating to the association of *Per3* with DSPD and diurnal preference. In one study almost 30 % of DSPD patients were homozygous for the 5-repeat allele of *Per3* [41], while another study found no association between diurnal preference and *Per3* polymorphisms [42]. Given these discrepancies, and

the limited understanding of gene function and expression of circadian clock genes, including *Per3* in humans, it is difficult to draw definitive conclusions about the genetic basis for DSPD [42].

While much of the genetic focus for DSPD has been on the PERIOD gene and other genes associated with the circadian pacemaker, other genetic mechanisms, such as the association of DSPD with the human leukocyte antigen DR1 [43] and the arylalkylamine N-acetyltransferase gene [44], have been postulated that require further future investigation.

## 15.4 Current Methods for Diagnosis

### *15.4.1 International Classification of Sleep Disorders (ICSD)*

Current diagnostic measures for DSPD are based largely on criteria set out in the International Classification of Sleep Disorders, Third Edition (ICSD-3) [4], which focus heavily on the sleep behaviors. To be diagnosed with DSPD, patients must meet criteria relating to circadian rhythm sleep disorders in general (see Box 15.1, reproduced from the ICSD-3), as well as specific criteria for DSPD (see Box 15.2, reproduced from the ICSD-3). In addition to the specific criteria for circadian rhythm sleep disorders and DSPD, the ICSD-3 manual provides detailed subsections to further describe the nuances of the disorder, including associated features of the disorder, predisposing and precipitating factors, familial patterns, pathophysiology, and the onset and likely course of DSPD [15].

**Box 15.1 ICSD-3: General Criteria for Circadian Rhythm Sleep-Wake Disorder**

Criteria A–C must be met:

A. A chronic or recurrent pattern of sleep-wake rhythm disruption primarily due to alteration of the endogenous circadian timing system or misalignment between the endogenous circadian rhythm and the sleep-wake schedule desired or required by an individual's physical environment or social/work schedules.
B. The circadian rhythm disruption leads to insomnia symptoms, excessive sleepiness, or both.
C. The sleep and wake disturbances cause clinically significant distress or impairment in mental, physical, social, occupational, educational, or other important areas of functioning.

Source: Reproduced from the ICSD-3 (permission granted)

**Box 15.2 ICSD-3: Diagnostic Criteria (DSPD)**

Criteria A–E must be met:

A. There is significant delay in the phase of the major sleep episode in relation to the desired or required sleep time and wake-up time, as evidenced by a chronic or recurrent complaint by the patient or a caregiver of inability to fall asleep and difficulty awakening at a desired or required clock time.
B. The symptoms are present for at least 3 months.
C. When patients are allowed to choose their ad libitum schedule, they will exhibit improved sleep quality and duration for age and maintain delayed phase of the 24-h sleep-wake pattern.
D. Sleep log and, whenever possible, actigraphy monitoring for at least 7 days (preferably 14 days) demonstrate a delay in the timing of the habitual sleep period. Both work/school days and free days must be included within this monitoring.
E. The sleep disturbance is not better explained by another current sleep disorder, medical or neurological disorder, mental disorder, or medication use or substance use disorder.

Source: Reproduced from the ICSD-3 (permission granted)

### *15.4.2 Diagnostic and Statistical Manual of Mental Disorders (DSM)*

The current version of the Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-V) [45] outlines both the criteria for diagnosis of DSPD and a diagnostic overview for circadian rhythm disorders in general. This overview states that there should be a present persistent or recurrent pattern of sleep disruption as a result of a variation in the circadian system or a misalignment between the endogenous circadian rhythm and the desired or required sleep-wake schedule required of an individual. More specifically, the DSM-V states that patients should present with a delay of usually 2 or more hours in the timing of the major sleep episode relative to the desired or required sleep-wake schedule. Additionally, this delay should cause difficulty awakening, with increased daytime sleepiness, particularly in the earlier part of the day. One of the defining features of the disorder is that when patients can set their own schedule, such as on vacation or on weekends, sleep initiation is improved, and quality and duration of sleep is normal. The DSM-V recommends that confirmation of diagnosis should occur via sleep diaries or actigraphy and that this information should be collected on weekends, as well as during the week, to ensure that the patient consistently displays a delayed pattern of sleep and wake.

## 15.5 Methods for Evaluating Circadian Phase, Sleep, and Impairments

### *15.5.1 Dim Light Melatonin Onset (DLMO)*

To accurately diagnose, and subsequently treat DSPD, circadian phase assessments are recommended [14, 46]. Endogenous melatonin is a pineal hormone secreted during the biological night, involved in the regulation of the sleep-wake cycle, and the effect of which is mediated by specific receptors located in the SCN [47]. The rhythm of melatonin is widely used as a marker of the timing of the endogenous circadian pacemaker [47]. A common reference point for the melatonin rhythm is the onset of the nocturnal increase in circulating melatonin levels, defined as dim light melatonin onset (DLMO), which can be measured in saliva or plasma, or inferred from urinary melatonin metabolite 6-sulfatoxymelatonin (aMT6s), via serial sampling under dim light conditions. DLMO has high specificity and sensitivity in differentiating patients with DSPD, as compared to sleep diaries and polysomnographic features such as sleep-onset latency [48]. A method for salivary DLMO assessment in laboratory settings has been described [49] and details the requirements for accurate phase assessment. The optimal protocol begins 6.5 h prior to habitual bedtime, and throughout the laboratory protocol, participants remain seated and awake in dim light conditions (<5 lux). After 30 min in dim lighting, saliva sampling begins, with participants given a cotton swab to roll around in the mouth for up to 5 min before participants spit the swab back into a tube. Samples are taken every half hour until 2 h after habitual bedtime. Throughout the protocol, food intake and movement are controlled, with small snacks provided and movement allowed only for toileting, except in the 10 min before a sample, during which time they must remain seated. If food is consumed, participants are required to brush their teeth with water only [49].

The light levels under which DLMO is assessed should be low [50], ideally less than 5 lux [49] to minimize acute suppression by light. Gooley et al. [51] showed that on average, melatonin onset occurred 1 h and 57 min before scheduled bedtime in participants exposed to dim light (<3lux), compared to 23 min before scheduled bedtime in participants assessed under normal room light conditions [51], highlighting the importance of maintaining low light intensity during the assessment. Current limitations of implementing DLMO in clinical settings include cost [21] and lack of insurance reimbursement, suitable facilities with controlled lighting environment, and training of health practitioners, in particular primary care practitioners.

### *15.5.2 Sleep Diaries and Actigraphy*

Sleep diaries and wrist actigraphy are often used in place of the gold standard polysomnography, as they provide a more practical, less invasive method for

assessing sleep over extended durations, which is necessary for diagnosis of DSPD. Diaries and actigraphy are recommended by ICSD-3 (see Box 15.2) to document the delay in the timing of the habitual sleep episode (preferably for 14 days). Sleep diaries and actigraphy should not, however, be relied upon as a proxy measures of circadian phase, because there are substantial individual differences in habitual sleep-onset and sleep-offset times relative to the timing of the circadian pacemaker, even in healthy individuals [52, 53].

In the 2002 update of the practice parameters for the role of actigraphy in the study of sleep and circadian rhythms, Littner et al. [54] suggested that although actigraphy is not advised to be used in isolation, it may serve a role in the assessment of sleep disorders. In 2007, this was updated to include its utility in specific disorders, including DSPD [55].

### *15.5.3 Diurnal Preference*

The Horne-Ostberg Morningness-Eveningness Questionnaire (MEQ) [56] is a subjective measure of diurnal preference, which may be used to identify those with a preference for delayed sleep timing, but is not a measure of endogenous circadian phase. The questionnaire consists of 19 items relating to an individual's preferences of times to complete mental and physical activity and is rated on either Likert-type or timescale responses. The outcome is a score indicating one of five preferences: extreme evening, moderately evening, neither type, moderate morning, or extreme morning. A number of studies, in both DSPD patients and healthy sleepers, have reported a negative correlation between MEQ score and circadian phase, such that a lower MEQ score is associated with a later circadian phase [57]. As a preliminary screening measure, this information can be useful in determining whether further investigation, for example, sleep diary, actigraphy, and DLMO testing, may be required to determine whether there is a circadian disorder.

## 15.6 Treatment

There are a number of treatment strategies available for effective management of DSPD, although these options vary in efficacy. Currently, timed melatonin administration is recommended for adults and adolescents, with and without depression or other psychiatric conditions [58]. The burden of disease for sleep disorders in general is high, and prescription of hypnotics is often used as a first-line approach by general practitioners. Described below are methods of treatment aimed at realigning circadian phase with an individual's desired sleep-wake cycle.

### 15.6.1 Phase Resetting

Phase resetting refers to the adjustment of the timing of circadian pacemaker via perturbations such as bright light administration. Phase resetting, particularly the magnitude and direction of response, is dependent upon several factors: the timing at which the perturbation is administered, the amplitude of the circadian rhythm being measured, and the dose of the stimulus (for light, intensity, duration, and wavelength) [25].

### 15.6.2 Chronotherapy and Phototherapy

Used more broadly now to refer to any treatment aimed at shifting circadian rhythms, chronotherapy was initially described by Czeisler and colleagues [59] as a method that involves scheduling of sleep episodes several hours later each day, to further delay patients until they reach their desired bedtime. Once desired bedtime is attained, patients are instructed to strictly adhere to the desired sleep-wake cycle. This method was established on the observation that DSPD patients have difficulty phase advancing to an earlier bedtime, but little problem delaying. While there have not been any clinical trials conducted to determine the efficacy of this treatment, observational studies have reported positive results. However, relapse has been reported to be high when therapy is ceased [57].

Phototherapy is a form of phase resetting involving administration of light to shift circadian phase and has been investigated as a treatment of DSPD. Timing of light administration treatment is based on the light PRC in humans [60], such that it is appropriately scheduled to achieve the necessary phase advance. Because of the large degree of interindividual variability in phase relationships between the sleep-wake cycle and the circadian pacemaker [52], and the conflicting reports from previous studies as to whether these phase relationships are altered in DSPD, it is particularly important to measure circadian phase prior to treatment administration, to optimize treatment outcomes [48].

In a controlled trial, administering 2 weeks of timed early morning bright light (2500 lux, 2 h duration within a 3-h period between 6:00 and 9:00 a.m.), with evening light restriction (dark goggles from 16:00 to dusk and only bedside lamps from dusk to bedtime), phase advanced the core body temperature minimum [61]. Similarly, the melatonin rhythm was phase advanced by 1 h and 20 min in a study in which patients with sleep-onset insomnia were administered either bright morning light for 1 week (2500 lux, 1 h duration beginning 1–1.5 h prior to baseline wake time) or a dim red light control condition [62]. The phase advance in the bright light group was associated with greater decreases between pre- and posttreatment measures for sleep-onset latency, as well as an earlier sleep-onset time and increased total sleep time. In a further study, 1 week of early morning administration of blue light (blue light LEDs with 470 nm peak wavelength with

irradiance of 65 μW/ $cm^2$), coinciding with a 30 min advance of wake time to 06:00, advanced the timing of melatonin onset by 2.53 h, although there were no changes in sleep times [63].

Despite the literature expounding the efficacy of phase resetting with phototherapy and its potential for treatment of DSPD, there are several limitations to its employment in clinical settings. Patient compliance is a limiting factor, as commitment is required to follow the scheduled timing of treatment, usually timed before a patient's desired wake time. Given the difficulties DSPD patients experience with waking at the desired time, this poses a potential problem for the utility of light therapy as a treatment option [64]. Also, there are limited guidelines for the use of phototherapy, particularly with regard to dose and timing [65].

## *15.6.3 Pharmacological Interventions*

### 15.6.3.1 Exogenous Melatonin

Timed melatonin administration has been investigated for its phase-shifting properties in DSPD patients, and there is increasing evidence that exogenous melatonin can be an effective treatment for DSPD [66]. PRCs for melatonin administration have been described [67, 68] (see Fig. 15.2), with the most recent showing that administration of 0.5 mg of melatonin approximately 2–4 h before DLMO or 9–11 h after the mid-sleep point induces a maximal phase advance, while maximal delays occur when administration occurs approximately 12–15 h after DLMO or up to 4 h after wake time [68].

Although the few controlled studies examining exogenous melatonin treatment for DSPD have reported generally positive results [66], there is considerable variability in the efficacy, most likely due to the variability in the methodologies employed. An early study by Nagtegaal et al. [46] showed administration of 5 mg of exogenous melatonin 5 h before DLMO advanced DLMO, while the timing of core body temperature minimum was not shifted. In contrast, Mundey et al. [69] later observed an advance in both core body temperature minimum and DLMO following administration of either 0.3 or 3.0 mg of exogenous melatonin that ranged between 1.5 and 6.5 h prior to individual DLMO. A meta-analysis [66] of melatonin treatment in both adults and children with DSPD revealed that overall, there is a significant advance in circadian timing, as well as an earlier circadian and clock time of sleep onset.

Exogenous melatonin also has a direct, sleep-promoting effect [70], particularly when administered at a time when endogenous melatonin levels are low [71]. Additionally, Kayumov et al. [72] and Rahman et al. [73] showed that after treatment with 5 mg of exogenous melatonin, sleep-onset latency improved significantly. The sleep-promoting effect of melatonin may benefit DSPD patients by improving sleep when the circadian drive for wakefulness is high (i.e., wake maintenance zone).

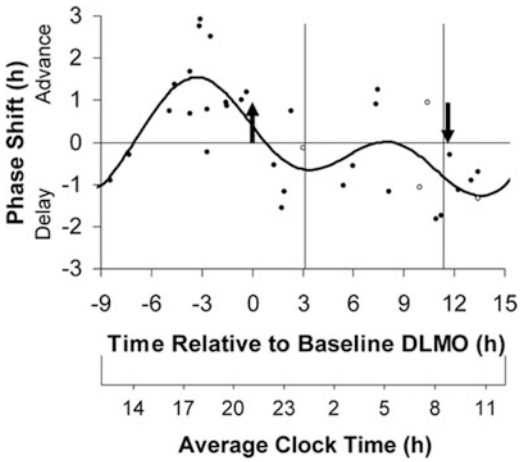


**Fig. 15.2** Melatonin PRC generated from 0.5 mg exogenous oral melatonin with subjects under 3-day free-running ultradian light/dark cycle (LD 2.5 h wake, 1.5 h sleep). Phase shifts of the circadian clock, measured by changes in the timing of the DLMO, are plotted against the time of administration of the melatonin pill relative to the baseline DLMO (top x-axis). The average baseline DLMO is represented by the *upward arrow*, the average baseline DLMOff by the *downward arrow*, and the average assigned baseline sleep times at home from before the laboratory sessions are enclosed by the vertical lines. Each *dot* represents the phase shift of an individual subject, calculated by subtracting the phase shift during the placebo session (free-run) from the phase shift during the melatonin session. The *open circles* represent the four women who had varying hormone levels during the study due to the use of hormonal birth control. The *curved line* illustrates the dual harmonic curve fit. The average clock time axis (*bottom x*-axis) corresponds to the average baseline sleep times at home (Adapted from Burgess et al. [68])

#### 15.6.3.2 Melatonin Agonists

Other potential pharmacological interventions for DSPD include melatonin agonists, such as tasimelteon and ramelteon, although these agonists have not been specifically investigated in the treatment of DSPD. In two randomized controlled trials in normal sleepers, tasimelteon was shown to improve polysomnographically recorded sleep outcomes, including sleep-onset latency and wake after sleep onset, in participants in whom transient insomnia was induced by shifting sleep time 5 h earlier. Significant improvements in sleep efficiency, sleep-onset latency, and total sleep time were reported with tasimelteon treatment (10, 20, 50, or 100 mg) compared to placebo. Additionally, treatment with 100 mg produced a significant phase advance of dim light melatonin onset (DLMO) after 1-day treatment, compared with the placebo, although all treatment groups showed an advance in DLMO [64]. Tasimelteon has subsequently been shown to ameliorate the symptoms of non-24-h sleep-wake disorder (N24SWD) and result in entrainment of endogenous circadian rhythms in totally blind patients [74]. The demonstrated phase-shifting and sleep-promoting effects of tasimelteon in healthy individuals and N24SWD patients strongly suggest that the drug may be efficacious in the treatment of DSPD.

Tasimelteon now has FDA approval for use in the treatment of N24SWD, and a 20 mg dose, taken before bedtime, is recommended. Ramelteon may also have therapeutic potential for DSPD, as previous studies have demonstrated improvement in sleep-onset latency and total sleep time, as well as a phase advance in the melatonin rhythm in a healthy adult population [75].

### *15.6.4 Combined Therapies*

There is a rationale for melatonin and phototherapy to be combined for treatment of DSPD, given that the melatonin PRC is approximately 12 h out of phase with the light PRC [76]. Theoretically, one would expect complementary action of light and melatonin such that combined treatment may produce larger effects on sleep and circadian phase compared to individual treatments alone. A recent study investigated the efficacy of combining exogenous melatonin with phototherapy treatment, with a focus on adolescents and younger adults. Researchers compared four different conditions: dim light and placebo capsule, bright light and placebo capsule, dim light and melatonin capsule, or bright light and melatonin capsule. During the treatment phase, rise times were also experimentally advanced by 1 h per day until preferred rise time was achieved. Regardless of treatment, there was a 1 h advance of bedtime, a 2 h advance of DLMO, and a 2 h advance of rise time, indicating that advancing rise time was the precipitating factor for a circadian phase advance [77]. Further research is required to determine whether combined exogenous melatonin and bright light has clinical utility in DSPD.

Other researchers have investigated the efficacy of combining exogenous melatonin with cognitive behavioral therapy for insomnia (CBTi). CBTi is a non-pharmacological treatment approach, most commonly employed for the treatment of insomnia, and focuses on changing behavior and habits, as well as misconceptions about sleep that may be perpetuating the disorder [78]. It is therefore intuitive that improving behavior, particularly regarding light exposure at night, coupled with timed morning bright light will improve sleep outcomes and advance circadian phase. Adolescents with DSPD who undertook 8 weeks of bright light therapy, with an advance of rise time and six sessions of CBTi, had earlier sleep-onset times, reduced sleep-onset latency, and wake after sleep onset [79]. However, because circadian phase was not assessed, it is difficult to draw conclusions regarding whether CBTi was targeting the underlying delay in the endogenous circadian pacemaker and whether phase advances were achieved.

## Questions of Interest

1. How might criteria for diagnosing DSPD change if circadian phase assessments were included?

2. How might you recommend a treatment program for a patient presenting with the symptoms of DSPD?
3. Given that the circadian pacemaker is responsive to light even at levels commonly experienced through artificial lighting, and recent reports that light levels comparable to those emitted by computerized, hand-held devices is sufficient to induce a circadian response [80], how should light exposure be considered in the diagnosis of DSPD?

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# Part IV
# Clock Interactions Within and Between Individual and the Natural World

# Chapter 16
# Interaction Between Central and Peripheral Clocks in Mammals

**Ueli Schibler**

**Abstract** Nearly all physiological processes in mammalian organisms undergo daily oscillations. These rhythms are not just driven by environmental changes created by the earth's rotation around its own axis, but are coordinated by a sophisticated, hierarchically organized timing system. In fact, self-sustained and cell-autonomous clocks are ticking in nearly all body cells and even in cells kept in tissue culture. When monitored in individual cells, the period lengths of the cycles vary from cell to cell and, over time, even within the same cell. In animals, however, the cellular clocks are synchronized within and between organs by numerous signaling pathways governed by cyclic signals controlled by the master circadian pacemaker in the brain's suprachiasmatic nucleus (SCN) and the environment. This chapter reviews some of our knowledge on how phase coherence is established in the body. Obviously, only synchronized circadian clocks can produces overt rhythms in physiology and behavior.

## 16.1 Purpose and Architecture of the Circadian Timing System

### *16.1.1 Circadian Oscillators: Transcriptional/Translational or Posttranslational?*

The earth rotation around its own axis generates daily light-dark cycles, the so-called photoperiod, and cycles in ambient temperatures. Depending on geographic latitude and altitude, these cycles undergo seasonal changes with respect to the relative length of the light and dark phases and the temperature differences between day and night. In the course of time, organisms have evolved timing

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devices that allow them to anticipate daily or annually recurrent events, such as food availability, predator abundance, light intensity, and oscillations in ambient temperature. Remarkably, the circadian timing system of mammals regulates daily rest-activity rhythms – and the corresponding cycles in physiology – in a highly dynamic, season-dependent manner [1, 2]. Thus, it cannot only predict the time of day but can anticipate the season-dependent shortening and lengthening of the light and dark phases.

Most mammalian organisms divide their day into active and resting phases. In diurnal and nocturnal species, the active phase is restricted to the day and the night, respectively. The rest-activity cycles are driven by the circadian clock and the sleep homeostat, two-timers with different design principles [3]. While the circadian clock is an oscillator, the sleep homeostat resembles an hourglass timer, building up sleep pressure during the activity phase and reducing sleep pressure during the resting phase. These two timing systems are connected, but, as revealed by the famous "bunker experiments" conducted by Jürgen Aschoff during the 1960s, they can become uncoupled in human subjects living for extended time periods without external timing cues. Curiously, some human volunteers examined in these experiments displayed sleep-wake cycles of up to 50 h after a few weeks, but maintained approximately 24-h rhythms in core body temperature and urine production [4]. During the past decades, impressive progress has been made in deciphering the cellular architecture of the circadian timing system [5, 6]. We now know that circadian clocks function in a self-sustained and cell-autonomous manner and that virtually all body cells harbor such oscillators. Moreover, we start to learn how the numerous cellular timekeepers are orchestrated in a hierarchical manner by the master clock in the brain's suprachiasmatic nucleus (SCN) and by environmental cycles, so as to maintain phase coherence. Perhaps even more importantly, the physiological functions of circadian clocks in several tissues have been elucidated [7].

With regard to the molecular clockwork circuitry, the field is still in the hunter and gathering era, and this is not meant in a pejorative sense. Genetic and biochemical work has revealed dozens of important transcription factors, protein kinases, and other regulatory proteins with essential roles in circadian timing [8]. Moreover, three-dimensional structures of mammalian core clock components could be resolved at the atomic levels [9–11], and large multisubunit complexes containing some of these polypeptides could successfully be purified to near homogeneity [12, 13]. Nonetheless, it is fair to say that we are far from understanding how the molecular circadian clockwork circuitry generates 24-h rhythms. As Richard Feynman, the father of quantum electrodynamics, pointed out, "What I cannot create, I do not understand." In chronobiology terms, Feynman's "create" would mean to assemble the circadian molecular clockwork in a test tube with purified components. If, indeed, circadian oscillations were generated by transcriptional/translational feedback loops (TTFLs), as most of us assume today, there is little hope to establish a cell-free biochemical clock system within the near future. Even if all the essential components

were known and available in pure form, such a system would at the very least require sustained in vitro transcription and translation, balanced kinase and phosphatase activities, functional RNA and protein degradation machineries, and two synthetic compartments resembling the nucleus and cytosol. Alas, the repertoire of "molecular cogwheels" is still incomplete, and the reactions described above either do not function at all in vitro or do not persist for extended time periods in the test tube. In contrast, a fully functional cyanobacteria circadian oscillator generating approximately 24-h cycles in protein phosphorylation and dephosphorylation could be assembled with just three proteins (KaiA, KaiB, and KaiC) and ATP. Cyanobacteria produce most of their energy by photosynthesis, and during the night, the ATP levels fall below the threshold concentration required for the maintenance of transcription and translation. Yet, Takao Kondo and his coworkers observed that circadian KaiC phosphorylation and dephosphorylation continue in constant darkness, that is, in the absence of RNA or protein synthesis [14]. Encouraged by this observation, they purified the three cyanobacteria core clock proteins from *Escherichia coli* strains overexpressing them and mixed them with ATP. To their delight – and to the astonishment of the entire research field – temperature-compensated KaiC phosphorylation and dephosphorylation cycles persisted in the test tube for at least 10 days (see [15] for review). The rhythmic KaiC phosphorylation is, however, not the entire story on circadian timekeeping in cyanobacteria. Kondo and his colleagues found that circadian KaiC accumulation persisted in cells in which KaiC was locked in the phosphorylated state by the overexpression of KaiA. Hence, a transcriptional/translational feedback loop (TTFL) can be operative in the absence of cyclic KaiC phosphorylation. Moreover, at 20 °C, a suboptimal temperature for cyanobacteria, circadian oscillations are only generated when both the KaiC "phosphorylator" and the TTFL are functional [16].

Is it conceivable that the TTFL of mammalian cells also cooperates with another, perhaps simpler and more basic molecular oscillator not requiring gene expression? The answer is "probably yes." Recently, Reddy and colleagues [17] and Rhee and colleagues [18] have discovered circadian oscillations in the hyperoxidation of peroxiredoxins (PRXs) in red blood cells from humans and mice, respectively. As mature erythrocytes are devoid of nuclei and ribosomes [19], they are incapable of transcription and translation. Therefore, the circadian hyperoxidation of PRXs must be accomplished by a posttranslational mechanism. Only a tiny fraction (~1 %) of total cellular peroxiredoxin is hyperoxidized in the course of a day, and this hyperoxidized peroxiredoxin is selectively degraded by proteasomes. In red blood cells, the rhythmic PRX hyperoxidation is probably the consequence of $H_2O_2$ production through hemoglobin auto-oxidation. However, the underlying "metabolic oscillator" rendering this process rhythmic is still unknown. Nonetheless, daily PRX hyperoxidation cycles have been observed in a wide variety of organisms encompassing cyanobacteria, microalgae, Archaea, fungi, green plants, nematodes, insects, mice, and humans. Yet, some of these organisms harbor TTFLs with very

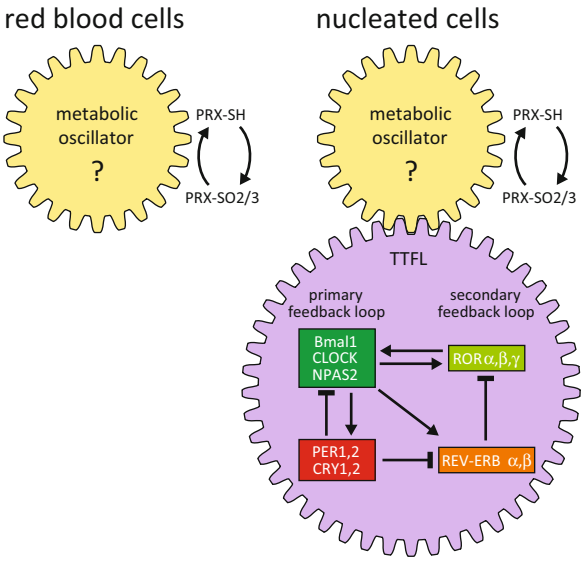


**Fig. 16.1** Coupling of metabolic oscillations with the transcriptional translational feedback loop (TTFL) in mammalian cells. Cultured red blood cells, which are devoid of nuclei and ribosomes and thus incapable of transcription and translation, display circadian peroxiredoxin (PRX) redox cycles (see text). These are probably outputs of a metabolic oscillator whose core clock components have yet to be identified. Since daily rhythms of PRX oxidation have been observed in a wide variety of organisms, the underlying metabolic clock may be the basic circadian timing device in all organisms and cells. If this conjecture were correct, the basic metabolic oscillator would have to be tightly coupled with the transcriptional translational feedback loop (TTFL; see Fig. 16.3 for details) in nucleated cells, since mutations in some clock genes cause a shortening or lengthening of the period

different molecular makeups [20], and although nematodes possess an ultradian oscillator probably involved in the gating of molting cycles [21], they appear to lack a circadian TTFL. Given its wide phylogenetic distribution, the metabolic oscillator driving PRX hyperoxidation cycles has been proposed to be the prototype version of circadian timekeepers. It may have evolved as a consequence of the Great Oxidation Event (GOE) 2.3 billion years ago. Thereafter, additional layers may have been added, aimed at reinforcing the redox oscillator and its gating of different cellular processes to appropriate time windows. These layers, initially serving as clock outputs and subsequently becoming essential TTFL components of the clock, would be expected to have different functions in different phyla. This would explain why the TTFL constituents share little, if any, sequence similarities between cyanobacteria, fungi, plants, and metazoans. Clearly, if a metabolic oscillator were the basic cellular clock, it would have to be tightly coupled with the TTFL. Otherwise, it would be difficult to rationalize how mutations in canonical core clock components such as PERs and CRYs could provoke changes in the period length (Fig. 16.1).

### *16.1.2 Master and Slave Oscillators: A Clock in Every Cell*

Initially, the suprachiasmatic nucleus (SCN) in the ventral hypothalamus was thought to be the only mammalian structure capable of circadian rhythm generation. Reppert and colleagues have demonstrated that individual SCN neurons kept in culture can generate circadian rhythms in firing frequency [22], emphasizing the cell-autonomous nature of circadian oscillators. Working with cultured retinas from golden hamsters, Tosini and Menaker were the first to challenge the exclusiveness of the SCN in producing circadian rhythms in mammals. They observed circadian cycles of melatonin secretion in experiments with superfused retinas. Moreover, the phase of these fluctuations could be entrained by light-dark cycles [23]. Subsequently, my own research group demonstrated circadian rhythms in clock gene expression in Rat-1 cells and H35 hepatoma cells, two cell lines established several decades earlier from immortalized fibroblasts and hepatocytes of rats [24]. Some years later, the cell-autonomous and self-sustained nature of these oscillators was revealed by real-time fluorescence and luminescence microscopy of individual fibroblasts expressing fluorescent proteins or firefly luciferase, respectively, under the control of promoters and enhancers of core clock genes [25, 26]. The identification of circadian clocks in cultured peripheral cell types of different origins opened the possibility that circadian clocks might reside in most body cells. Indeed, cultured tissue explants from transgenic rats and mice expressing firefly luciferase under the control of cis-acting regulatory elements of the core clock genes *Per1* and *Per2*, respectively, produced robust daily bioluminescence cycles [27, 28]. In the meantime, circadian clock gene expression has been recorded in virtually all organs and tissues, with the notable exception of the testis and thymus [29, 30]. It is not yet clear whether the cells of these organs are devoid of functional circadian clocks or whether their oscillators are not synchronized within the tissue.

Circadian oscillators can fulfill cell-autonomous and body-wide tasks. One important cell-autonomous function of such biological clocks is their capability to gate chemically incompatible reactions to different time windows. For example, it would make sense to replicate DNA at a time when mitochondrial activity, i.e., oxidative metabolism, is minimal, so as to reduce the chance of oxidizing guanylyl residues while the replication fork moves along the DNA. 8-oxo-guanine is copied into adenine instead of thymine, and letting mitochondria produce reactive oxygen species during the S-phase of the cell cycle would thus dramatically increase the mutation rate. In yeast, metabolic oscillations indeed sequester DNA synthesis and mitochondrial activity to different time windows [31], and I anticipate that circadian oscillators may do the same in mammalian cells. Clearly, cell division cycles synchronize circadian oscillators in dividing cells [32], and the reduction of the mutation load may be one of the purposes of coupling cell division and circadian cycles.

There is no need to synchronize the phases of oscillators for cell-autonomous functions, but circadian physiology depends mainly on processes that are coordinated within the entire organism. For example, the timing of metabolic activities has to be adapted to the needs of the organism during the absorptive and

postabsorptive phases, and the numerous cellular clocks have to be synchronized for this purpose [33]. In the following sections, I will review some of our knowledge on how the SCN and daily environmental cycles establish phase coherence within the body. Before comparing the molecular makeup of cellular circadian oscillators, I will briefly summarize our current knowledge on their interactions in the SCN and peripheral tissues.

### *16.1.3 Cellular Clocks Within the Suprachiasmatic Nucleus Master (SCN)*

In mammals the suprachiasmatic nucleus has been identified as the circadian master pacemaker by elegant lesion and transplantation experiments. Stephan and Zucker showed in 1972 that in rats kept in constant darkness, the bilateral ablation of the SCN resulted in arrhythmic locomotor activity and drinking [34]. These lesion experiments pointed to an important function of the SCN in biological timing, but they could not ascertain whether the SCN was the generator of circadian behavior or a "relay station" transmitting rhythmic information from higher brain areas to the periphery. This issue was solved by Ralph and Menaker, who lesioned the SCN of wild-type and *Tau* mutant hamsters. When SCN-lesioned hamsters received implants of fetal SCN tissue, they always reconstituted rhythmicity with a period length of the donor genotype, irrespective of the host genotype. Thus, SCN-lesioned wild-type hamsters receiving implants from heterozygous and homozygous *Tau* mutant animals resumed wheel-running activity with period lengths of 22 and 20 h, respectively. Conversely, heterozygous and homozygous *Tau* mutant hamsters receiving fetal SCN grafts from wild-type donors reconstituted locomotor activity cycles with a period length of approximately 24 h [35]. Since the SCN is indispensable for behavioral rhythmicity and since it dictates the period length in an autonomous manner, it must contain the central pacemaker.

The SCN is composed of about 10,000 neurons in mice and about 100,000 neurons in humans [36]. Laboratory rodents remain behaviorally rhythmic for unlimited time spans when kept under constant conditions. Therefore, the SCN must keep phase coherence throughout the life span of an animal. The robustness of SCN rhythms can also be observed in organotypic brain slice cultures, in which clock gene expression and firing frequency oscillate for many days with little, if any, damping in amplitude. Yet, the period length of these cycles varies greatly in dispersed neurons of enzymatically dissociated SCN tissue. Therefore, the phases of individual SCN neurons must be tightly coupled in the intact SCN [37–39]. The coupling circuits involve synaptic and paracrine signaling and, to a lesser extent, GAP junctions. They not only ensure phase coherence, but render the clocks in SCN neurons resilient to mutations in clock genes. For example, SCN neurons homozygous for *Per1* or *Cry1* null alleles display robust rhythms in *Per2* expression in SCN organotypic cultures, but not in enzymatically dissociated SCN neurons. In vivo, synaptic or paracrine communication routes between SCN neurons can be

attenuated by genetic loss-of-function experiments, for example, by inactivating *Vipr2*, the gene encoding the vasoactive intestinal peptide (VIP) receptor VPAC2 [37]. Although such animals still contain functional cellular oscillators, they become arrhythmic, supposedly because phase coherence between individual SCN neurons is lost. The signaling pathways involved in intercellular coupling SCN neurons are schematically depicted in Fig. 16.2.

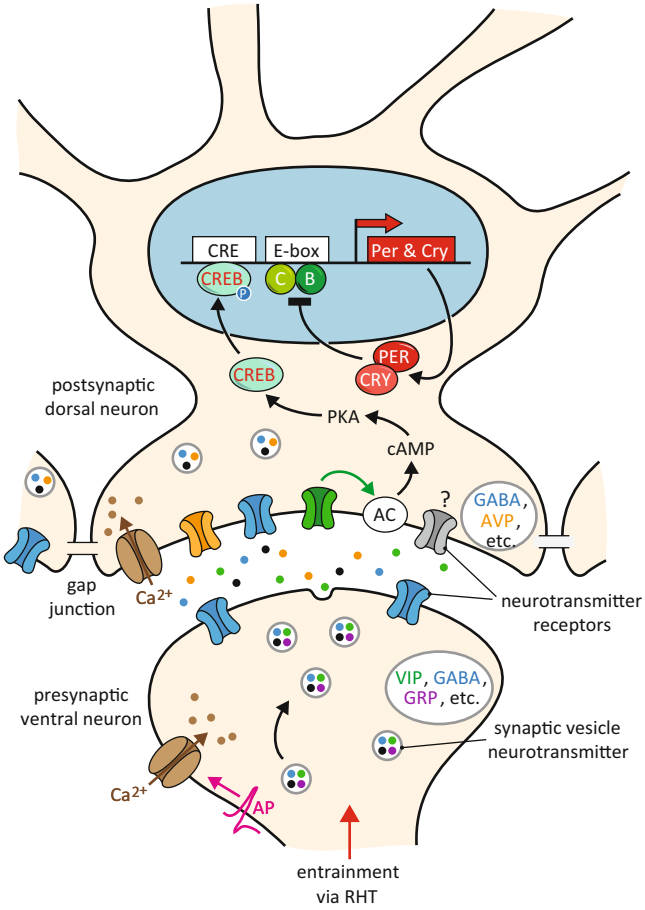


**Fig. 16.2** Intercellular coupling of SCN neurons. During photic entrainment, neurons in the SCN ventral core communicate with dorsal neurons, and neurons within subdivisions are also synchronized with each other. Chemical synapses are essential for synchronization. The main neurotransmitters include VIP, GRP, and GABA. More than 50 % of SCN neurons require rhythmic VIP-VPAC2 signaling to maintain robust rhythms. Action potentials (AP) are required to trigger neurotransmitter release from presynaptic neurons. For instance, VIP-VPAC2 signaling activates adenylyl cyclase (AC), cAMP, protein kinase A (PKA), and CREB phosphorylation (CREB-p). The acute induction of Per expression by phospho-CREB is followed by repression of PER and CRY in the following cycle, reestablishing rhythms with a new phase. In addition, gap junctions may contribute to circadian precision by electrically coupling neighboring neurons (The cartoon and part of this figure legend are adapted from reference [98], with permission from McMillan Publishers Ltd.)

### *16.1.4 Cellular Clocks in Peripheral Organs*

Are cellular circadian clocks phase-coupled within peripheral organs as well? Work with PER2::luc mice, which express a functional PER2-luciferase fusion protein from the endogenous *Per2* locus, suggests that this may indeed be the case. Thus, explants of the cornea, liver, pituitary glands, kidney, and lung from SCN-lesioned PER2::luc mice displayed rhythmic PER2-luciferase expression during several days, albeit with different phases. Hence, phase coherence appeared to persist within, but not between tissues of behaviorally arrhythmic mice [28]. Using an RT-Biolumicorder circadian gene expression in peripheral tissues (e.g., the liver) can now be recorded for weeks to months in freely moving mice. This device contains a photon-proof, ventilated cage equipped with a photomultiplier tube counting the photons emitted by organs expressing a circadian luciferase reporter gene. In addition, it is equipped with a computer programmable feeding machine, a passive infrared sensor (PIR) recording spontaneous locomotor activity, and a computer programmable lighting device, permitting the synchronization of the SCN (as manifested by locomotor activity cycles) by skeleton photoperiods [40]. Using RT-Biolumicorder technology, the internal synchrony between cellular oscillators in the liver was confirmed in vivo by real-time monitoring of circadian Rev-erbα-luciferase expression. Thus, the livers of behaviorally arrhythmic SCN-lesioned mice kept in constant darkness produced robustly circadian oscillations in bioluminescence weeks after the surgical intervention (Pascal Gos, André Liani, and U.S., unpublished results). Moreover, *Vipr2* knockout mice with severely dampened locomotor activity rhythms displayed high-amplitude circadian clock gene expression in the liver, although with a significantly advanced phase when compared to wild-type animals [41]. The phase coupling in peripheral tissues, unlike that operative in SCN explants, can obviously not be assisted by synaptic signaling pathways, and in contrast to cultured SCN slices, explants of peripheral tissues or cultured fibroblasts progressively lose phase synchrony. Even the hepatocyte clocks, whose phase coherence in vivo has been convincingly documented, desynchronize relatively rapidly in cultured liver explants. So is synaptic signaling always a prerequisite for the maintenance of intercellular synchrony in tissue culture? At least in one examined biological oscillatory system, the ultradian segmentation clock, this does not appear to be the case. Remarkably, intercellular phase coherence can be reproduced in quasi-monolayer cultures in this system [42]. During embryogenesis the formation of somites, metameric structures giving rise to the vertebral column, ribs, skeletal muscles, and subcutaneous tissues, is regulated by the segmentation clock, an ultradian oscillator driving negative feedback loops in the expression of the helix-loop-helix transcriptional repressor HES7 [43]. In mouse embryos, a wave of Notch and FGF signaling initiating in the posterior presomitic mesoderm propagates into the anterior region approximately every 2 h. In presomitic mesoderm explants, which grow as quasi-monolayers in the culture dish, ultradian concentric waves of HES7-driven gene expression can be observed. Notch signaling is locally constrained by the nature of Notch ligands

(e.g., Delta1), which are proteins with a transmembrane domain. This may explain why phase coherence can be reproduced in tissue culture for the ultradian somite oscillator but not for circadian clocks, which may communicate through diffusible rather than membrane-bound ligands. In tissue culture such diffusible cues are likely to be diluted below the threshold concentration required for binding to their receptors.

### *16.1.5 Comparison of Molecular Clockwork Circuitries in SCN Neurons and Peripheral Cell Types*

The major purpose of circadian molecular timekeepers is to drive the oscillating transactivation potential of one or multiple transcription factors, which govern the rhythmic expression of clock-controlled genes and thereby orchestrate overt cycles in physiology and behavior. The PAS domain helix-loop-helix transcription factors BMAL1, CLOCK, and NPAS2 and the nuclear RAR-related orphan receptors RORα, RORβ, and RORγ are the central cogwheels of the molecular oscillators. The circadian activity of these transcription factors is regulated by interactions with coactivators (e.g., CBP/p300, PGC1), corepressors (e.g., PER-CRY complexes, NURD, NCoR1), posttranslational modifications (e.g., phosphorylations, acetylations, sumoylations, poly(ADP)-ribosylation, and sumoylation), and binding ligands (e.g., nicotinamide dinucleotide cofactors, heme, cholesterol sulfate) [13, 33, 44].

As mentioned above, we cannot claim to understand how the molecular clockwork circuitry of mammals generates cycles in gene expression of approximately 24 h. Nonetheless, research conducted during the past two decades by many groups has revealed genes that are essential for proper circadian timing. Functional redundancies have made the genetic dissection of clock genes challenging. Indeed, to this date there is only a single gene, *Bmal1*, whose disruption renders mice immediately arrhythmic when they are kept in constant darkness [45]. For all of the other core clock genes, there are at least two functionally redundant isoforms, and strong phenotypes are only observed in mice with multiple loss-of-function alleles [8]. Detailed knowledge about the current state of understanding of mammalian circadian oscillators can be gathered from several excellent review articles (e.g., [6, 8, 46]). The cartoon of Fig. 16.3 depicts skeleton models of circadian oscillators in SCN neurons and peripheral cell types, with focus on the clock components that are relevant for the remaining part of this article. For reasons of simplicity, posttranslational modifications and possible ligands of core clock transcription factors are not included in this model. In both SCN neurons and peripheral cell types, the molecular oscillators consist of two coupled feedback loops. In the canonical feedback loop, protein complexes with a molecular mass of about 2 megadaltons containing cryptochromes (CRY1 and CRY2), period proteins (PER1 and PER2), and about 30 additional polypeptides bind to and repress

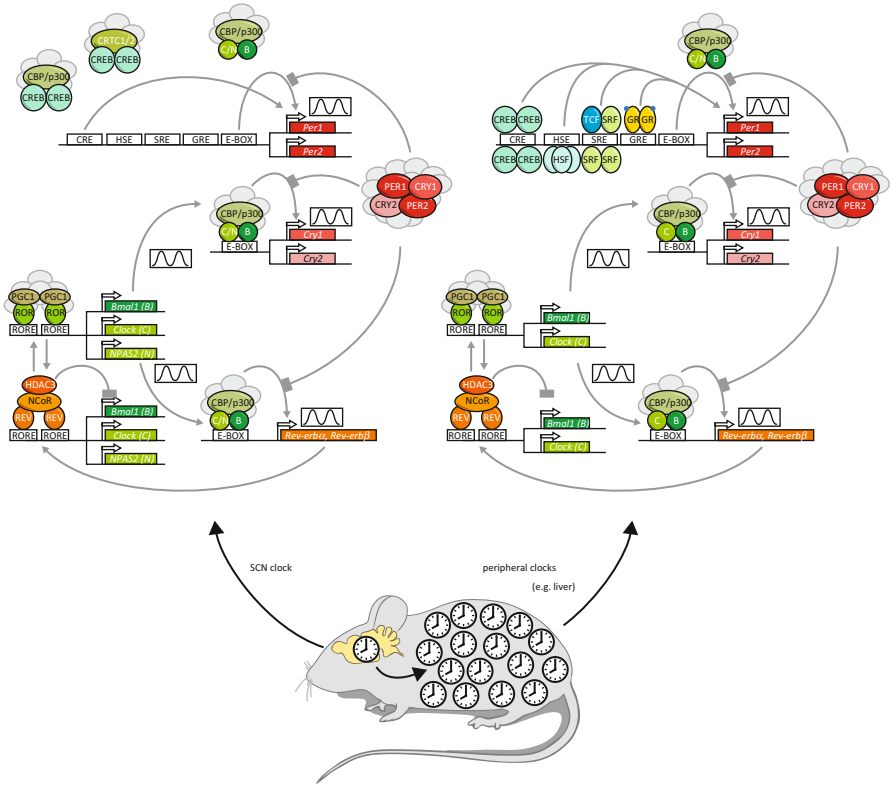


**Fig. 16.3** Circadian oscillators in SCN neurons and peripheral cell types. Circadian oscillators have a similar makeup in SCN neurons and peripheral cell types. However, there are some interesting differences in how they function in the SCN and peripheral organs, such as the liver (see text). In the SCN, the core clock transcription factors CLOCK (or its paralog NPAS2, which can functionally replace CLOCK in the SCN) and BMAL1 are absolutely required for circadian, the expression of the core clock repressors PER1 and PER2. Moreover, cyclic AMP response element-binding protein (CREB)-related immediate early transcription factors (IETFs) and their coactivator complexes are probably the major transcriptional regulators synchronizing the SCN oscillators (see Fig. 16.2). In contrast, clocks in peripheral tissues are mainly driven by IETFs such as CREB (and paralogs), heat shock transcription factor 1 (HSF1), serum response factor (SRF), and glucocorticoid receptor (GR), which sense various systemic signals (represented by an arrow pointing from the brain to the periphery). For example, in mice hepatocyte clocks continue to tick in the absence of CLOCK and BMAL1, as long as they receive systemic signals from the SCN or environmental cycles. Only when tissue slices are kept in culture, i.e., in the absence of cyclic systemic cues, do the endogenous clocks depend on CLOCK-BMAL1 heterodimers (DeBruyne et al. 2007b; Kornmann et al. 2007a) (Reprinted from Ref. [44] with permission from Cold Spring Harbor Laboratory Press)

activator complexes harboring heterodimers of the basic helix-loop-helix transcription factors CLOCK and BMAL1 [12, 13]. The latter stimulate transcription of *Per* and *Cry* genes, and when PER-CRY complexes reach a critical concentration, they shut down their own expression by removing the CLOCK-BMAL1 complexes from

the E-box sequences within promoters and enhancers of *Per* and *Cry* genes [47]. A second feedback loop, orchestrated by nuclear orphan receptors of the REV-ERB (NR1D) and ROR (NR1F) families, drives the rhythmic transcription of *Bmal1*, *Clock*, and *Npas2*. In this transcriptional circuitry, REV-ERBα (NR1D1) and REV-ERBβ (NR1D2) repressors compete with RORα (NR1F1), RORβ (NR1F2, neuron-specific), and RORγ (NR1F3) for the binding to RORE elements in the *Bmal1* and *Clock* promoters. As *Rev-erbα* and *Rev-erbβ* are direct targets of CLOCK-BMAL1 complexes, the canonical and secondary feedback loops are tightly coupled. While the two linked feedback loops are operative in SCN neurons and peripheral tissues, there are several noteworthy differences between central and peripheral clocks:

1. CLOCK depletion abolishes circadian rhythm generation in peripheral organ slices kept in vitro or in cultured cells [48], but not in cultured SCN slices, in which NPAS2, a closely related CLOCK homologue, can substitute for CLOCK [49, 50].
2. In the SCN of BMAL1-deficient neurons *Per1* and *Per2* transcription is frozen at nadir values [45]. In contrast, in the livers of mice with BMAL1- or CLOCK-depleted hepatocytes, PER2 expression continues to oscillate with amplitudes and magnitudes similar to those observed in hepatocytes of wild-type animals, and rhythmic PER1 accumulation only shows a moderate diminution in magnitude [49, 51]. Therefore, circadian *Per2* transcription in the livers of intact animals must be driven mostly by systemic cues, sensed by transcription factors other than CLOCK-BMAL1 complexes. Some of these transcription factors have yet to be identified, but I propose that they functionally compete with CLOCK-BMAL1 complexes.
3. The expression of the neuron-specific RORβ isoforms in the SCN constitutes a further difference between the central pacemaker and nonneuronal peripheral clocks [52].
4. Cellular circadian oscillators are tightly coupled within the SCN through electrical (synaptic) and paracrine signaling, and this intercellular phase coupling is preserved in organotypic brain slice cultures (see above). As aforementioned, intercellular phase coupling may also be operative in some peripheral organs, but the phase coherence is lost in tissue explants.
5. The synchronization pathways are obviously different in SCN and peripheral cells. Light-dark cycles are clearly the major *Zeitgebers* for the central pacemaker, while feeding-fasting rhythms are the dominant phase resetting cues for most peripheral organs [53, 54].

In addition, glucocorticoid signaling, daily polymerization cycles of the actin cytoskeleton, and body temperature rhythms – all directly or indirectly depending on the SCN – participate in the synchronization of peripheral clocks (see below). Remarkably, the SCN is largely insensitive to the signals it uses to phase-entrain peripheral clocks. Thus, it is weakly influenced, at best, by feeding-fasting rhythms [53], temperature cycles [55, 56], or glucocorticoid signaling [57]. With regard to the latter, SCN neurons are among the few cell types in adult mice and rats that do

not express the glucocorticoid receptor [57, 58]. Interestingly, the glucocorticoid receptor does accumulate in the SCN of newborn and young animals, which are not yet rhythmic.

## 16.2 Synchronization of Peripheral Clocks

### *16.2.1 Synchronization of Circadian Oscillators in Cultured Cells*

Circadian oscillators in cultured cells have been discovered owing to the ability of serum to transiently synchronize them. Before serum treatment, the numerous cellular oscillators were all in different phases, leading to apparently arrhythmic expression of core clock and clock-controlled genes in the population. The transient synchronization of the fibroblast clocks by a serum shock implied that they all could be reset to the same (or a similar) phase, persisting in the population at least for a few cycles. This is compatible with a phase transition curve (PTC) for a type zero phase response curve (PRC-0). In such a PTC, the slope of the new phase is nearly zero when plotted against the old phase [59]. In contrast, the SCN, as manifested by locomotor activity cycles, is synchronized according to a type one (PRC-1). In a PTC for a PRC-1, the slope of the new phase only shows slight deviations from the diagonal, when the new phase is plotted against the old phase [59]. The two different types of phase response curves (PRCs) and phase transition curves (PTCs) are illustrated in Fig. 16.4.

Let us come back to the synchronization of circadian oscillators of cultured cells by a serum shock. Serum contains a wide variety of signaling components, such as hormones, growth factors, metabolites, and cytokines. This begged the question of what blood-borne factors could have participated in the synchronization of circadian clocks. Several research groups tried to elucidate this issue by trying a wide variety of defined signaling molecules. Surprisingly, many different components activating a bewildering array of signal transduction cascades were capable of synchronizing clocks in cultured cells. These included agonists for nuclear hormone receptors (dexamethasone, retinoic acid), activators of tyrosine kinase receptors (e.g., FGF, insulin [60]) and G-protein coupled receptors (e.g., thrombin, PARs; Ka Yi Hui and U.S., unpublished results), signaling molecules stimulating cAMP signaling (butyryl-cAMP, forskolin) [61], angiotensin II [62], calcium ionophores (e.g., calcimycin [61]), drugs affecting cytoskeleton dynamics (see below), and glucose [63]. All of these substances probably reset the phase by acting through immediate early transcription factors (IETFs) inducing (or repressing, in the case of glucose) the expression of the period genes *Per1* and/or *Per2* [64]. These two genes are likely the clock genes whose expression serves as a "state variable" of mammalian circadian oscillators. In circadian parlance, a state variable is the parameter determining the position in time of an oscillation.

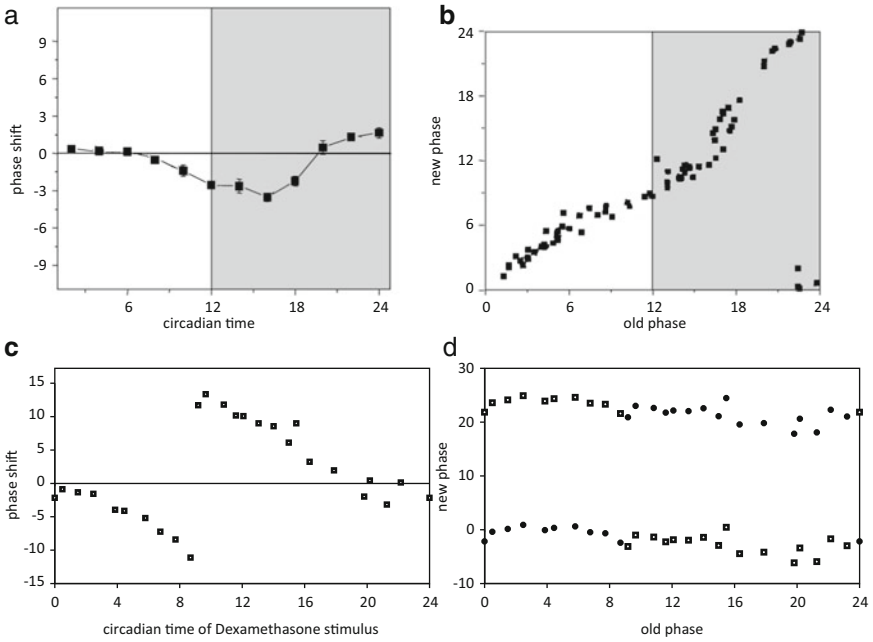


**Fig. 16.4** Phase response curves (PRCs) and phase transition curves (PTCs). (**a**, **b**) Individual wild-type mice (strain C57BL/6) were kept in constant darkness in cages equipped with running wheels for at least 2 weeks before they were exposed to light pulses at different circadian times (see [99]. (**a**) In order to obtain a PRC, the phase shifts (*Y*-axis) in circadian locomotor activities observed after the light treatments were plotted against circadian time (*X*-axis). Positive values and negative values represent phase advances and phase delays, respectively. Note that the light-induced phase shifts are small during the subjective day, negative during the first half of the subjective night, and positive during the second half of the subjective night. (**b**) The PTC was constructed by plotting the new phase (after light-induced phase shifts, such as the ones depicted in panel (**a**) against the old phase (i.e., the phase before the light pulses were delivered) (for details, see [99]). Note that the magnitudes of the phase shifts are relatively small in the PRC (panel **a**). As a consequence, the deviations of the new phase from the old phase are also small, and the overall slope of the PTC is roughly 1. Hence the observed PRC is called "PRC type one" or "PRC-1" (the diagrams in panels (**a**) and (**b**) were adapted from reference [99], with permission from SAGE press). (**c**, **d**) NIH3T3 cells harboring a luciferase reporter gene whose expression is driven by the *Bmal1* promoter were synchronized by a serum shock, and circadian bioluminescence cycles were recorded in real time. At the indicated circadian times (during the second luminescence cycle), parallel cultures were treated for 15 min with 100-nM dexamethasone and the resulting phase shifts were determined. Circadian time 0 is defined as the time at which minimal bioluminescence was observed. (**c**) Phase response curve (PRC). (**d**) Phase transition curve (PTC). The data presented in (**c**) are represented as a phase transition curve (PTC), in which the new phase is plotted against the old phase. *Black dots* and *open squares* can be considered as phase shifts measured on two consecutive days. For simplicity all points are plotted on both days. Note that all new phases are nearly identical irrespective of when cells were treated with dexamethasone. Hence, the slope of the PTC is near 0, and the corresponding phase response curve is therefore called "PRC type zero" or "PRC-0" (for details, see [25]. The diagrams of panels (**c**, **d**) are reprinted from [25], with permission of Elsevier press)

How relevant are these mechanisms for the synchronization of peripheral clocks in vivo? An argument in favor of their importance is the presence of functional cis-acting response elements for several IETFs implicated in these pathways within the enhancer and promoter sequences of *Per1* and *Per2* genes. These IETFs include the cyclic AMP response element-binding protein (CREB) and its cousin activating transcription factor 4 (ATF4), serum response factors (SRF), glucocorticoid receptor (GR), and heat shock factor 1 (HSF1) [44, 65]. Furthermore, the activities of these IETFs follow a robust diurnal rhythm in peripheral tissues, reaching zenith values at the light-dark transition (ZT12) or during the night. However, the minute concentrations at which sera from several species can synchronize the clocks of serum-starved fibroblasts pose a conundrum. For example, rat serum provided at 1 % of the culture medium efficiently resets the circadian oscillators of Rat-1 cells, NIH3T3 cells, or primary fibroblasts. Yet, cells are exposed to "100 %" serum throughout the day in animals. It must thus be the daytime-dependent differences in the levels of blood-borne signals, rather than their absolute concentration, that set the phase of circadian gene expression in vivo. Alternatively, it could be argued that fibroblasts may be an inappropriate experimental system to study synchronization. Reassuringly, however, mouse embryonic fibroblasts (MEFs) efficiently synchronize to diffusible rhythmic signals controlled by the SCN when implanted under the skin of mice [66]. It would be interesting to examine whether cultured cells can be stably synchronized by perifusing them with serum samples kept at the same concentration, but harvested at different time points. In such experiments cells could be perifused for 2 h with medium containing 10 % serum collected at ZT00, then for 2 h with medium containing 10 % serum collected at ZT02, then for 2 h with medium containing 10 % serum collected at ZT04, and so on (for several days). If the cells synchronized to such reconstituted daily oscillations of blood-borne signaling cues, the hypothesis postulated above – namely, that concentration differences, not absolute concentrations of serum factors, set the phase – would be experimentally verified.

Thus far, sustained synchronization of clocks in cultured cells over many days could only be accomplished by simulated body temperature rhythms. HSF1 plays an important role in this process [67, 56], but there must be additional temperature-sensing devices communicating temperature-dependent signals to the cellular clocks. In fact, HSF1-deficient cells still synchronize to altered temperature cycles, albeit more sluggishly than wild-type cells [67]. HSF2, another HSF isoform, is also expressed in the examined fibroblasts, but its depletion has no effect on the phase resetting by imposed body temperature cycles. Conceivably, temperature-dependent transient receptor potential channels, such as TRPV [68], may assist HSF1 in the synchronization of circadian gene expression. Interestingly, pyrexia, a thermo-TRP, is required for the temperature-dependent synchronization of fruit fly circadian rhythms in the low-temperature range [69]. In addition, the two closely related cold-inducible RNA-binding proteins CIRBP (also known as CIRP) and RBM3 may influence the phase resetting by affecting the amplitude of core clock gene expression [70, 71] (see below).

## *16.2.2 Synchronization of Peripheral Oscillators In Vivo*

### 16.2.2.1 Cyclic Zeitgeber Cues Are Gated by the SCN and Environmental Cues

Under constant conditions, i.e., in the absence of external timing cues, the SCN is clearly required for the maintenance of phase coherence in the body (see above). It can establish synchrony in peripheral organs by indirect and more direct mechanisms. As aforementioned, feeding-fasting rhythms are the dominant *Zeitgebers* for peripheral clocks in most tissues. Obviously, animals don't absorb food when they are asleep, and feeding-fasting rhythms are thus controlled by rest-activity cycles, which are themselves driven by the SCN. When food is offered to mice or rats only during the day, the phase of circadian clock gene expression becomes completely inverted in most peripheral organs after about 10–14 days, but it remains nearly unaffected in the SCN. Hence, under these conditions the phase of peripheral oscillators becomes uncoupled from that of the SCN [53]. How do we know that the SCN also emits more direct synchronization cues? The evidence for the existence of signals depending on the phase of the SCN is supported by the following observations. If food is offered ad libitum to day-fed animals, the original phase, supposedly dictated by the SCN, is established after only 2–3 days (i.e., much faster than it takes to uncouple peripheral clocks from the SCN by daytime feeding). Therefore, in the absence of feeding- or fasting-dependent signals, the SCN rapidly reassumes its master role [53]. In keeping with this observation, SCN-lesioned mice adapt the phase of their peripheral timekeepers to inverted feeding rhythms within a couple of days, most probably because cyclic signals directly depending on the SCN are no longer counteracting feeding-fasting-dependent signals in these animals [40]. The phase-shifting kinetics after inversion of the feeding regimen can actually be explored in determining whether a given phase-entrainment pathway depends on feeding-fasting- or SCN-dependent signaling cues. As insinuated by the experiments with cultured cells described above, a major problem in searching for individual synchronization pathways relevant in vivo is the large redundancy of signaling mechanisms participating in the phase entrainment of peripheral clocks. Consequently, the disruption of a single signaling pathway has minor effects at most on the steady-state phase, and this renders the assessment of its significance difficult. However, the phase-shifting kinetics is quite sensitive to the inactivation of a single signaling mechanism, and measuring the time required for peripheral oscillators to adapt to the newly imposed feeding-fasting regimen can provide valuable information on whether or not an examined pathway is relevant in vivo. If the disruption of a given pathway accelerates phase-shifting kinetics, this pathway is likely to be controlled by the SCN. If, in contrast, the inactivation of a signaling route slows down phase-shifting kinetics, the investigated synchronization mechanism is probably participating in the phase entrainment by feeding-fasting cycles.

Before describing the admittedly fragmentary knowledge on individual pathways implicated in the phase setting of peripheral clocks, it should be mentioned that light-dark cycles can partially substitute for the master pacemaker in the SCN. Using two different mouse model systems for the conditional disruption of *Bmal1* expression in SCN neurons, Husse et al. and Izumo et al. demonstrated that circadian gene expression can be rescued in peripheral tissues by light-dark cycles [72, 73]. Hence, the SCN and the photoperiod appear to conduct partially redundant functions in the establishment of phase coherence in the body. At least in nocturnal rodents, both drive rest-activity cycles – and thereby feeding-fasting and body temperature rhythms, which are well established *Zeitgebers* for peripheral clocks.

#### 16.2.2.2 Signals Depending on Feeding-Fasting Cycles

How can feeding and/or fasting synchronize peripheral clocks? Our knowledge on the involved molecular mechanisms is scrappy at best and entirely based on candidate approaches. Among the culprits one would expect metabolites and their sensors, feeding-dependent hormones and their receptors, and, perhaps, neuronal signals transmitted from nutrient-sensing brain areas to peripheral organs via the peripheral nervous system. Several nutrient state-dependent signal sensors belonging to these classes have been proposed, including AMP-dependent kinase (AMPK) [74], insulin-phosphatidylinositol 3-kinase (PI3K) [75], insulin [76], and the two NAD+-sensing enzymes Sirtuin 1 (SIRT1) [77, 78] and PARP-1 [80]. The phase-shifting kinetics outlined above have been compared for *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice, and these experiments have confirmed the role of PARP-1 in the phase entrainment of liver oscillators by feeding-fasting cycles [79]. Intriguingly, PARP-1 and its cofactor CTCF have been reported to mediate the interaction of rhythmically transcribed genes with the nuclear lamina in a cyclic fashion, thereby contributing to the circadian transcription of these genes through oscillating repression mediated by LADs (lamina-associated domains) [80].

Are the *Zeitgeber* signals for peripheral clocks generated during the feeding phase or the fasting phase? The final word is still out, but there are observations supporting roles of both in the synchronization of liver clocks. For example, ultradian feeding (i.e., offering multiple meals at regular intervals throughout the day) has little influence on the phase of hepatic clock gene expression in mice [81] and rats [82]. Hence, prolonged fasting periods appear to be required for the phase resetting. In rats starved for a day, a single meal offered during 30 min can induce a burst of *Per2* gene expression and change the phase of hepatocyte clocks [83]. As described in the same study, food intake increases the magnitude of *Per2* mRNA accumulation in starved rats irrespective of daytime. The available evidence suggests therefore that the phase entrainment of liver clocks requires both an extended time span of little or no food ingestion and a time window of feeding. It will be interesting to examine whether feeding-fasting rhythms with an overall period length shorter or longer than 24 h can drive cycles in clock gene expression of corresponding durations against the 24-h *Zeitgeber* signals emitted by the SCN. If

so, these would represent an ultimate demonstration of the dominance of feeding rhythms over the SCN in the synchronization of peripheral clocks.

#### 16.2.2.3 Blood-Borne Signals: Glucocorticoid Hormones and Signaling Proteins Modulating Actin Cytoskeleton Dynamics

Elegant parabiosis experiments with intact and SCN-lesioned mice indicated that blood-borne signals can synchronize clocks at least in some peripheral organs [84]. The plasma levels of glucocorticoid hormones – cortisol in humans and corticosterone in laboratory rodents – have long been known to accumulate with an about tenfold daily amplitude. Moreover, dexamethasone, a potent glucocorticoid receptor (GR) agonist, efficiently synchronizes circadian oscillators in cultured fibroblasts, probably mainly through the immediate early activation of *Per1* [57]. As shown in experiments with mice with a liver-specific *Gr* disruption, glucocorticoid signaling also participates in the synchronization of peripheral clocks in vivo. Thus, the phase of circadian gene expression adapted more rapidly to an inverted feeding regimen in GR-deficient than in GR-proficient hepatocytes [85]. Hence, signaling through the GR is likely to participate in the synchronization of hepatocyte oscillators by cues controlled by the SCN rather than by feeding-fasting rhythms. Glucocorticoid hormones not only synchronize clock gene expression in the liver, but regulate about 60 % of rhythmically expressed genes, and hepatocyte nuclear factor 4 (HNF4) has been identified as a mediator in this process [86].

Gerber and colleagues used synthetic tandem repeat promoter (STAR-PROM) screening in an unbiased search for IETFs activated by diurnally active blood-borne signals [87]. This resulted in the identification of the RhoA-MRTF-SRF pathway as a rhythmically controlled signal transduction pathway. In this pathway, discovered by Treisman and coworkers [88], the activation of the RhoA GTPase promotes the polymerization of globular actin (g-actin) into filamentous actin (f-actin). Since g-actin acts as a stoichiometric repressor of MRTFs (myocardin-related transcription factors), actin polymerization releases MRTFs, which are then recruited as coactivators to prebound SRF and trigger transcription of SRF target genes. *Per2* is among these genes [87, 89], and this offers a plausible mechanism for the synchronization of peripheral clocks by the MRTF-SRF pathway. Indeed, drugs preventing the association of MRTF with g-actin (cytochalasin D) or promoting actin polymerization (Jasplakinolide) efficiently resynchronize circadian clock in vitro [89, 90]. The STAR-PROM screening procedure was conducted with cultured cells, but consecutive experiments revealed that the MRTF-SRF pathway is also diurnally activated in vivo. Thus, both polymerization of actin and cytoplasmic-nuclear shuttling of MRTF follow a robust circadian rhythm [87]. Importantly, SRF-deficient hepatocytes synchronize their circadian clocks more rapidly than wild-type hepatocytes to a feeding regimen whose phase is in conflict with that of the SCN (Pascal Gos, Alfred Nordheim, and U.S., unpublished results). Therefore, the blood-borne signals

driving the circadian activity of the MRTF-SRF pathway depend on the SCN rather than on feeding-fasting cycles. This conclusion is supported by the observation that the phase of the relevant blood-borne signals is not affected by inverted feeding rhythms [87].

#### 16.2.2.4 Body Temperature Rhythms

In the liver several systemically driven genes, i.e., genes whose expression oscillates in the absence of hepatocyte clocks, encode temperature-related polypeptides, such as heat shock proteins (HSPs, also known as chaperones that fold nascent or denatured proteins) and cold-inducible RNA-binding proteins (e.g., CIRP/CIRBP). Moreover, heat shock factor 1 (HSF1) was identified as a transcription factor shuttling in the liver from the cytoplasm to the nucleus in a diurnal fashion, with a phase consistent with that of body temperature rhythms [91]. As outlined above, simulated mouse body temperature can efficiently synchronize circadian gene expression in cultured cells, and it is thus likely that they also participate in the phase resetting of peripheral oscillators in vivo. As already noticed for glucocorticoids and feeding-fasting rhythms, the phase of the SCN appears to be resilient to temperature rhythms, both in animals [55] and in organotypic cultures [56].

The cold-inducible RNA-binding protein CIRBP and its mRNA reach maximal levels in the liver during the resting phase, i.e., when body temperature is minimal. Rhythmic CIRBP accumulation can be mimicked in cultured cells exposed to simulated body temperature cycles [71]. As aforementioned, CIRBP may affect the phase-shifting velocity of circadian clock by modulating the amplitude and magnitude of circadian gene expression in a cell-type specific manner. While it increases the amplitude and magnitude of *Per2* expression in fibroblasts, it reduces these parameters in the liver. As a consequence, circadian liver gene expression synchronizes more slowly to signals from the SCN (Flore Sinturel, Pascal Gos, and U.S., unpublished results).

While the daily expression cycles of heat shock mRNAs are driven primarily by HSF1-dependent transcriptional mechanisms, the diurnal rhythm of *Cirp* mRNA accumulation is regulated at the posttranscriptional level, most probably by a temperature-dependent efficiency of *Cirp* pre-mRNA splicing [92].

#### 16.2.2.5 Synchronization via the Peripheral Nervous System

In an important study, Kalsbeek and coworkers have examined the impact of hepatic denervation on the phase of circadian liver gene expression in rats fed ad libitum, in rats given six meals evenly distributed over the 24-h day (i.e., in the absence of diurnal feeding rhythms), and in adrenalectomized rats (i.e., in the absence of glucocorticoid signaling) [82]. The hepatic phase resetting was still intact in adrenalectomized rats subjected to diurnal feeding cycles or in intact rats exposed to an ultradian feeding regimen. However, adrenalectomized rats subjected to an ultradian feeding schedule

were no longer capable of synchronizing their liver clocks, suggesting that either feeding rhythms or glucocorticoids are required for this process. In no cases did hepatic denervation have a significant impact on the phase of liver clocks, and it was thus concluded that neuronal inputs do not play a major role in the synchronization of these oscillators. Nevertheless, Menaker and colleagues have discovered a tissue, the submaxillary gland, which was unable to synchronize its clocks to an altered feeding regimen. In contrast to the liver, the submaxillary glands of rats either kept their phase or became arrhythmic in daytime-fed animals. When sympathetic innervation was surgically disrupted, however, the submaxillary gland behaved like the liver (and most other organs) and adapted the phase of its clocks to the new feeding schedule. Therefore, in the submaxillary gland, neuronal signals controlled by the SCN are at least equally strong *Zeitgebers* as feeding-fasting rhythms. When the signals transduced via the sympathetic nervous system and the feeding-fasting cycles were in conflict, the former won the competition and maintained the original phase in the submaxillary glands of 6 out of 12 rats. In the remaining six rats, the two *Zeitgebers* were similarly potent and led to arrhythmicity, probably by synchronizing approximately half of the gland's oscillators to nearly opposite phases. These studies published by the Kalsbeek and Menaker laboratories are important for two reasons. First, they show that different *Zeitgebers* contribute differentially to the phase resetting of circadian clocks in different organs, and, second, they reveal that the inactivation of a signaling mechanism can unmask the participation of another one. The cartoon in Fig. 16.5 schematically depicts the major pathways participating in the synchronization of circadian clocks in peripheral tissues.

## 16.3 Key Questions and Outstanding Issues

### *16.3.1 Characterization of the Metabolic Circadian Oscillator*

The discovery of circadian peroxiredoxin redox cycles in many different phyla from bacteria to mammals raises a fundamental issue: is there indeed a metabolic oscillator lying at the heart of all circadian timekeeping systems? Clearly, major research efforts should be undertaken toward the identification of the core components of this oscillator. As for any biological process of interest, this can best be accomplished through unbiased forward genetic approaches. While the activity of circadian core clock regulators is known to be influenced by metabolites and metabolic regulators in a variety of organisms, unbiased genetic screens for circadian phenotypes in cyanobacteria, fungi, plants, *Drosophila*, and mammals (or mammalian cells) have not yet revealed strong candidates for core components of the metabolic clock. It should be emphasized, however, that such basic regulators may well be encoded by essential genes, which obviously would escape genetic loss-of-function screens. Perhaps, genome-wide gain-of-function screens, in which

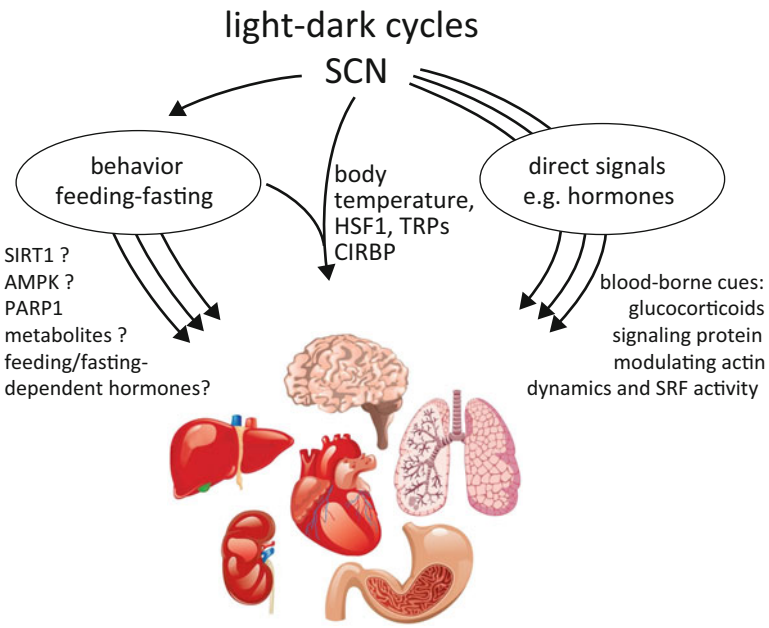


**Fig. 16.5** Synchronization of peripheral clocks by the SCN and light-dark cycles. The SCN and/or light-dark cycles drive rest-activity cycles and thereby feeding-fasting rhythms, which are dominant *Zeitgebers* for clocks in peripheral tissues. In addition, the SCN controls more direct signals involved in the phase entrainment of peripheral clocks. Note that the brain is listed among peripheral tissues, since the oscillators in brain regions other than the SCN are synchronized by feeding-fasting cycles and, supposedly, other signaling cues controlled by the SCN. Body temperature cycles, generated by the SCN and rest-activity cycles, also participate in the phase adjustments of peripheral clocks. The phase of the SCN is resilient to some of the synchronization pathways it controls, including feeding-fasting rhythms, body temperature cycles, and glucocorticoid signaling (see text) (Adapted from Ref. [103], with permission from John Wiley & Sons Ltd.)

genes are overexpressed throughout the day, might be more appropriate in the pursuit of metabolic core clock components. Even though such screens are more difficult to interpret than loss-of-function screens, they might provide promising candidates whose contribution to the generation of metabolic oscillations could then be scrutinized by conditional or partial loss-of-function. The discovery of a basic metabolic clock not requiring de novo gene expression might be a first step toward the reconstitution of a mammalian circadian oscillator in the test tube, as has been accomplished for the circadian "phosphorylator" of cyanobacteria.

### *16.3.2 Circadian Clocks and Fitness*

Circadian clocks exist in virtually all light-sensitive organisms, and there can thus be no doubt about their importance under natural conditions. Moreover, within mammals, the molecular makeup of the TTFL appears to be identical in mice and humans,

which diverged 65–110 million years ago [93]. This time span is much longer than necessary for loosing genes, whose functions had become superfluous. For example, olfactory gene repertoires diverged dramatically in chimpanzees and humans since they separated from their common ancestor between 6 and 7 million years ago [91]. Furnishing direct compelling evidence for the virtues of circadian clocks under laboratory conditions is another matter – quite arduous, as it turns out. Unfortunately, this cannot simply be achieved by examining the consequences of a clock gene disruption. Core clock genes encode transcription factors and protein kinases, which participate in many functions not related to circadian rhythm generation. For example, BMAL1 is required for normal male and female fertility [95, 96], but other core clock genes are not. In my opinion, "resonance experiments," such as the ones conducted with cyanobacteria [97, 98] and plants [99], provide the most convincing evidence for the beneficial properties of circadian clocks. In such experiments, the fitness of mutant and wild-type organisms harboring circadian clocks with different period lengths was examined when they were exposed to resonating or non-resonating light-dark cycles (T-cycles). The outcome was unequivocal. Cyanobacteria strains with resonating clocks rapidly outgrew strains with discordant oscillators, and plants with resonating clocks displayed more efficient photosynthesis and carbon fixation than their counterparts with dissonant circadian timekeepers. In "resonance experiments" the mutated gene is unlikely to be the culprit for a confounding phenotype unrelated to circadian rhythms, since depending on the length of the T-cycle applied, the same allele can improve or deteriorate the fitness of its possessor.

In a pioneering study, the survival of homo- and heterozygous *Tau* mutant mice free-running with period lengths of 20 h and 22 h, respectively, has been compared with that of wild-type mice under quasi-natural outdoor conditions [100]. In these experiments the frequency of the *Tau* mutant allele, carrying a dominant mutation in the gene encoding casein kinase 1ε, was rapidly decreasing in the population. This observation is in keeping with the expectation that clocks resonating with the T-cycle (here the natural photoperiod) also confer a selective advantage in mammalian species. However, the converse experiments, in which *Tau* and wild-type mice are exposed to short T-cycles (resonating with the period length of the mutant animals), are still outstanding.

### *16.3.3 Synchronization of Peripheral Clocks by Feeding-Fasting Cycles*

For most organs, feeding-fasting cycles are the dominant *Zeitgebers*. Yet, our knowledge on how they synchronize peripheral clocks is scanty at best. The functional redundancy of the involved mechanisms renders their dissection extremely challenging and precludes the application of simple forward genetic screens. I am afraid, therefore, that we will have to continue with "candidate approaches" to identify relevant signaling pathways. In parallel, it may be worthwhile to identify IETFs responding to signals elicited by feeding and fasting. Our

laboratory has recently adapted the STAR-PROM screening technology [87] to a bar-coded version, which allows the screening for virtually all transcription factors in parallel and in an unbiased fashion (Pauline Gosselin, Gianpaolo Rando, Fabienne Fleury-Olela, and U.S., manuscript in preparation). In future experiments the bar-coded STAR-PROM library may be delivered to the liver through hemodynamic injection into the tail vein and the relevant IETF binding sites identified by sequencing the bar codes. Clearly, chronobiologists will continue to enjoy a lot of fun and excitement for many decades to come.

## 16.4 Summary Points

- Different phyla have different circadian clocks, but it is possible that an ancient metabolic oscillator lies at the basis of circadian rhythms in all organisms from cyanobacteria to humans.
- The mammalian circadian timing system is composed of a master pacemaker in the brain's suprachiasmatic nucleus (SCN) and subsidiary clocks in virtually all body cells.
- Mammalian circadian oscillators tick in a self-sustained and cell-autonomous manner.
- The cellular clocks of the SCN master pacemaker are tightly coupled. They exchange phase information through synaptic and paracrine signaling mechanisms and, perhaps, through gap junctions. This phase coupling ensures that animals remain rhythmic through their entire life span, even when kept in the absence of external timing cues.
- The SCN master clock is synchronized daily by the photoperiod.
- The SCN master clock and the photoperiod synchronize circadian clocks in peripheral tissues mainly through feeding-fasting cycles (which are driven by rest-activity cycles), body temperature rhythms, and endocrine pathways.
- In peripheral cells circadian gene expression is driven by systemic cues and local oscillators.

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# Chapter 17
# Circadian Photoentrainment Mechanism in Mammals

**Yu Hsin Liu and Satchidananda Panda**

**Abstract** Proper alignment of an organism's behavior, physiology, and metabolism to the daily cyclical changes in light and dark is essential for health and well-being. This process is dependent upon a small subset of intrinsically photosensitive retinal ganglion cells (ipRGCs) that express the photopigment, melanopsin. The signaling pathways involved in melanopsin phototransduction are distinct from those used in rod and cone opsins and are more reminiscent of invertebrate opsins. Distributed throughout the retina, ipRGCs send monosynaptic projections from the retina to many regions of the brain to facilitate non-image-forming visual processes, but their glutamatergic inputs to the hypothalamic suprachiasmatic nucleus (SCN) are essential for entraining circadian rhythms in behavior and physiology to environmental lighting conditions. While nearly all cells in the mammalian body have cell-autonomous molecular clocks, the remarkable intercellular coupling among SCN neurons allows for the SCN to act as the master circadian oscillator and transduces light information into rhythmic electrical and chemical signals that facilitate photoentrainment. This chapter delves into the role of ipRGCs in circadian photoentrainment, the melanopsin signaling pathway, the interaction between ipRGCs and the heterogeneous SCN neurons, and the network properties and signaling pathways that underlie SCN function.

## 17.1 Introduction

Due to the infallible daily rise and set of the sun, nearly all organisms on Earth have evolved near 24-h rhythms of behavior and physiology that underlie their continued existence. One might imagine that single-celled organisms existing hundreds of millions of years ago evolved sunlight-dependent timing of DNA replication such that DNA was only uncoiled and replicated at night when protected from the sun's harsh UV radiation. In step with the predictable nature of geophysical time, nearly

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all organisms evolved an intrinsic circadian (from the Latin words "*circa*" and "*dian*" meaning "about a day") clock that allows an organism to anticipate and continue its normal patterns of behavior and physiology in the absence of light and facilitates the ability to shift its circadian rhythms in response to changes in lighting conditions. To that end, circadian rhythms are defined as rhythms in behavior or physiology that persist in constant environmental conditions with a period length of approximately 24 h.

Most organisms do not have exactly 24-h intrinsic circadian rhythms. Kept in conditions of 12 h of light followed by 12 h of dark (12 L:12D) with all other basic needs fulfilled (controlled temperatures and readily available food), mice show robust activity behavior that initiates with the fall of darkness and terminates with the onset of light. When the lighting conditions are changed to complete darkness (DD), the same mice exhibit similar cycles of activity and rest/sleep but with period lengths slightly shorter than 24 h. This experiment highlights the fact that circadian rhythms are indeed intrinsic and do not depend on external lighting cues but also that the circadian clock can be, and normally is, synchronized to the 24-h day by light. In humans, this intrinsic rhythm has a period length of slightly more than 24 h. The ability of circadian light input to modulate intrinsic rhythms, or photoentrainment, facilitates adaptation of physiology and behavior to variation in day length as a result of the changing seasons in nature.

Most mammalian cells have intrinsic molecular circadian clocks that are made up of transcription-translation-based feedback loops involving at least ten core clock genes that ultimately regulate the expression of genes critical for cell physiology and metabolism. An estimated 43 % of all protein-coding genes in the mouse exhibit circadian transcriptional rhythms somewhere in the body [1]. In order to ensure that the circadian changes in physiology and behavior driven by cyclical gene expression accurately reflect the anticipated changes in a 24-h day, the circadian clocks in individual cells are synchronized with one another and to the environmental lighting conditions by the hypothalamic suprachiasmatic nucleus (SCN). The SCN is a bilateral midline structure that consists of ~10,000 neurons per nuclei and acts as a master clock to orchestrate molecular oscillations in the brain and peripheral tissues through approximately 24-h cycles of gene expression and physiology [2]. The necessity of the SCN for normal circadian rhythmicity is clear where destruction of the SCN leads to loss of rhythmic changes in serum corticosterone [3], loss of circadian water drinking and activity rhythms [4], and impaired fertility [5] in rodents. However, the role of the SCN as the pacemaker or master conductor of body-wide circadian rhythms is revealed by experiments where transplantation of SCN tissue into the brains of rodents with ablated SCN can rescue locomotor activity rhythms [6] and where arrhythmic SCN-ablated hamsters adopt the donor-specific period length from the transplanted SCN of hamsters with genetically altered period lengths [7].

The SCN receives direct light input from intrinsically photosensitive retinal ganglion cells in the retina via the retinohypothalamic tract [8] and translates that light information into signals that drive circadian rhythms throughout the body via the secretion of distinct neuropeptides [9] and efferent connections to the many

regions of the brain, which include the preoptic area and the paraventricular nucleus of the hypothalamus [10]. These circadian rhythms play an important role in the regulation of neuronal, physiological, and endocrine rhythms in body temperature, cortisol levels, heart rate [11], melatonin secretion, physical ability [12], and cognitive ability [13].

In many organisms, the circadian photoreceptors and the clock are cell autonomous. Intracellular signaling from light-sensitive molecules in specific cells drives circadian responses mediated by those same cells. For example, cells in the pineal gland in birds and melanocytes in frogs contain photopigments that detect ambient light conditions and lead to the release of melatonin. However, in mammals the photosensitive molecules that detect ambient light to drive circadian rhythms are located in retinal ganglion cells in the eye that then send axons to the SCN which mediates circadian responses. Due to the modular nature of the mammalian master oscillator and photoreceptors, describing the mechanisms underlying circadian photoentrainment in mammals requires an understanding of the genetic underpinnings and signaling properties of each module and a study of the intercellular communication that connects them.

## 17.2 Paradigms in Studying Entrainment

From a behavioral standpoint, circadian photoentrainment, or the synchronization of an animal's sleep and activity rhythms to the light environment, is studied within a handful of paradigms:

### *17.2.1 12L:12D*

Kept in a cage with a running wheel and under 24-h lighting conditions of 12L:12D, a normal wild-type mouse will spend the majority of the 12 h of light sleeping or in a low-activity state. As soon as the lights turn off, the mouse will start running on a provided wheel with very little delay. In this scenario, the activity phase is aligned with the dark phase of the lighting conditions. Although mice have a free-running period length (the length of time necessary to complete one circadian cycle of sleep/rest and activity) of less than 24 h as determined by measuring time spent wheel running under constant dark conditions, in a 24-h light-dark environment, light signals the master oscillator in the SCN and synchronizes the clock to an exact 24-h period length. This paradigm establishes the phase relationship between light or dark onset and activity onset and shows whether photoreceptive and master oscillator systems are intact and synchronizable.

### 17.2.2 Phase Advance or Phase Delay

With an animal entrained to 12L:12D, advancing or delaying the time that lights come on, phase advance or phase delay, respectively, reveals the amount of time necessary for an animal to entrain to a different phase of light and reflects the strength of the coupling, or intercellular synchronization, within the SCN. Hypothetically, if a mouse had maximally weak intercellular SCN coupling, it would be arrhythmic, has no free-running period length, and would have circadian rhythms that were driven solely by external light cues. This animal would respond to an advance or delay in the time of lights on by instantaneously adapting to the new lighting regime. In the opposite hypothesis, if a mouse had an intrinsic circadian rhythm with infinitely strong coupling and hence had rhythms that were imperturbable by light, this animal would free run at its intrinsic period length regardless of lighting conditions. Given that intrinsic intercellular coupling is at neither extreme, a wild-type mouse entrained to 12L:12D will adjust to a phase advance by initiating wheel-running activity 1 h earlier each day and will adjust to phase delay by initiating wheel-running activity 2 h later each day. In other words, a wild-type mouse takes ~1 day to adjust to each hour of phase advance and ~0.5 day to adjust to each hour of phase delay. The difference in the amount of time necessary to adjust to phase advance or phase delay is due to properties of the SCN and will be discussed later.

### 17.2.3 Discrete Light Pulse During DD

In order to elucidate the acute effects of light stimulation on circadian activity rhythms, animals held under constant dark conditions are exposed to a discrete pulse of light during their active phase. In constant darkness, circadian rhythms run according to circadian time (CT) whereby the recurrent rhythms of sleep/rest and activity are driven solely by the master oscillator. Under this timing system, circadian time 12 (CT12) is aligned to the start of the active phase. Under DD lighting conditions, the spectral composition and irradiance of the light stimulus and the circadian phase at which a light stimulus is administered greatly affect the magnitude and direction of the activity phase shift [14]. In a wild-type mouse, a 15-min pulse of bright light given during subjective day (CT0-12) will not cause a significant phase shift. The same light pulse given during early subjective night (CT12–19) or late subjective night (at CT19–24) will result in a phase delay or phase advance, respectively, with light pulses at CT16 and CT20 resulting in the greatest changes in phase. Studying the effects of light pulses under DD conditions reveals the phase-dependent manner in which light affects circadian activity and allows for analysis of intrinsic activity rhythms such that effects of negative masking are readily identifiable.

### *17.2.4 Changes in Photoperiod*

Evaluating the effects of day length (photoperiod) on SCN rhythmicity reveals the interplay between regions of the SCN and also how light modulates the cell-autonomous clock. In entraining to a 16:8L:D photoperiod (long photoperiod), the peaks of anterior and posterior SCN activity are wider and less robust. Conversely, under 8:16L:D photoperiod, the peaks of anterior and posterior SCN activity have greater amplitude, are narrower, and are more tightly aligned. Looking globally at SCN activity, single-cell SCN neuron activity in SCN slice culture is equally robust during long or short photoperiods, but the peaks of single-cell SCN neuron activity are more tightly aligned during short photoperiods resulting in the increased amplitude of total SCN peak activity. At the level of clock gene expression, the peaks of *Per2* and *Per1* expression are further apart under conditions of long photoperiod and much closer together during conditions of short photoperiod. Changes in how an animal's SCN fires under different photoperiods ultimately speak to interactions between light and the molecular clock and intercellular coupling between SCN neurons.

## 17.3 A Specialized Photoreceptor Is Necessary for Photoentrainment

In the late 1890s, Ramon y Cajal elucidated and described the neurons in the retina and laid the groundwork for the axiom that rod and cone photoreceptors were the only light-sensitive cells in the retina. However, in the 1920s, Clyde E. Keeler, a graduate student at Harvard at the time, described a naturally occurring mouse that while lacking rod and cone photoreceptors and determined to be functionally blind [15] still exhibited appropriate constriction of the pupils to the presence of bright light [16]. Further functional and anatomical characterization of these animals decades later showed that while the retinas of afflicted newborn pups were largely indistinguishable from age-matched wild-type pups, the rod photoreceptors start to degenerate around 8 days after birth and that by 4 weeks postnatal, all rod photoreceptors and the vast majority of cone photoreceptors are lost leaving intact and normal-looking ganglion cell and inner nuclear layers. Named *rd* for the striking retinal degeneration phenotype, it was thus surprising that light-induced suppression of pineal melatonin levels [17], negative masking, and pupillary and circadian responses to light were intact despite the drastic loss of rods and cones. However, it was found that while cones are largely lost in the *rd*/*rd* mice, a small number of cone cell bodies persist, albeit with missing outer segments [18].

Mice have cones that are sensitive in the green and ultraviolet wavelengths. Both of these types of cones have been implicated in circadian photoentrainment based on action spectrum studies [19]. However, in transgenic animals that have significantly reduced numbers of cones but normal numbers of rods, circadian responses

are unattenuated. Transgenic mice that lacked rods and green cones also showed intact circadian responses [20]. At scotopic light levels where cones are not active, rods are sufficient for providing the light information necessary to entrain mice to circadian light cues [21]. Seemingly, neither rods nor cones are alone necessary to drive circadian responses, yet, all circadian responses are lost if the eyes are removed. All of this pointed to a photosensitive cell type in the retina that worked in addition to, and independent of, the canonical rod and cone photoreceptors.

Anterograde labeling of retinal ganglion cell projections using engineered pseudorabies virus in *rd* mutant mice showed that there were retinal ganglion cells in *rd* mice that projected to regions of the brain known to be critical for circadian rhythms: SCN, IGL, vLGN, and pretectum (including OPN) [22]. Retrograde transport of fluorescent microspheres injected into the SCN in the hypothalamus of wild-type rats labeled a subset of retinal ganglion cells that were revealed to be intrinsically photosensitive – exhibiting characteristic large depolarizations to light with superimposed fast action potentials that persisted despite the application of sufficient dosages of molecules known to inhibit rod and cone light responses. These photosensitive retinal ganglion cells were shown to have an average soma diameter of $14.7 \pm 1.2$ um and to predominantly stratify to the outer (OFF) sublamina of the inner plexiform layer [23].

## 17.4 Melanopsin-Expressing Retinal Ganglion Cells Are Necessary for Circadian Photoentrainment

Around the turn of the twentieth century, a novel opsin was identified in a cDNA library made from light-sensitive melanophores of *Xenopus laevis*. Like retinal photoreceptors, the dermal melanophores of *Xenopus laevis* are photosensitive. Light stimulation in dermal melanophores decreased melatonin levels and triggered melanosome dispersion to the cellular periphery causing the melanophores to appear darker. This response could be elicited with the application of GPCR activators or retinaldehydes and had an action spectrum very similar to opsin-based photopigments. Analysis of protein extracts from *X. laevis* melanosomes for opsin-like nucleotide sequences revealed a novel opsin named "melanopsin" for the melanophores in which they were discovered. Like all opsins, melanopsin is a G-protein-coupled receptor that shows greatest homology with invertebrate opsins. In *Xenopus*, melanopsin mRNA was shown to be present in the magnocellular preoptic nucleus, the SCN, and in the iris, retinal pigment epithelium, and inner retina in the eye [24]. The presence of melanopsin in deep brain structures, the iris, and the retina led to the suspicion that melanopsin might be involved in light-driven circadian systems.

Melanopsin (gene name *Opn4*) was successfully cloned from mouse retina cDNA and found to be 57 % identical to *Xenopus* melanopsin – differing significantly in sequence and length of cytoplasmic and extracellular tails. In the mouse

retina, melanopsin was shown to be expressed in a few cells in the ganglion cell layer and even fewer cells in the amacrine cell layer [25]. The localization and distribution of these melanopsin-positive cells were strikingly similar to the localization and distribution of the known retinal cells that project to the SCN [26, 27]. In fact, RGCs that project to the SCN were shown to be intrinsically photosensitive [23] and to be immunopositive for melanopsin [28].

To establish melanopsin as the critical photopigment for circadian photoentrainment, transgenic mice lacking melanopsin were created. Using various approaches to for examining photoentrainment, melanopsin-null (*Opn4–/–*) mice were found to be able to entrain to 12L:12D cycles and have free-running period lengths, total activity, and length of activity phases similar to wild-type control animals – suggesting that melanopsin was not crucial for core oscillator function. However, in response to a 15-min pulse of 480-nm light (the wavelength that elicited the greatest responses from melanopsin-expressing retinal ganglion cells) 3 h after the onset of activity (early subjective night) under constant dark conditions, *Opn4–/–* mice had greatly attenuated phase delays in activity onset in subsequent days compared to WT animals. Furthermore, under constant light conditions, *Opn4–/–* mice showed shorter period lengths than wild-type animals. These deficits in phase shifting suggested that melanopsin accounted for a significant portion, though not all, of light input to the core oscillator in the SCN and reinforce the value of rods and cones as contributors to the circadian visual system [8].

To determine whether the photoentrainment capabilities of *Opn4–/–* mice were due to canonical photoreceptors or to yet unidentified photopigments in the retina, mice that lacked melanopsin and rods and cones were created (*Opn4–/–*; *rd/rd*). *Opn4–/–*; *rd/rd* mice are unable to entrain to L:D lighting conditions, exhibit the complete absence of negative masking, have no pupillary constriction in response to bright light, have no photic inhibition of arylalkylamine*N*-acetyltransferase (the rate-limiting step in melatonin secretion), and exhibit free-running rhythms regardless of lighting condition [29]. Thus, all of the circadian visual system can be accounted for by three photopigments, of which, no one photopigment is essential for photoentrainment [8, 30]. Like the circadian systems of plants, invertebrates, and lesser vertebrates, the mammalian circadian system requires photic input from many different photopigments to adapt behavior to ambient light.

## 17.5 Retinal Circuitry

The mammalian retina is a thin structure that consists of three layers of cell bodies separated by layers of cell processes. The retina lines the back half of the eye and consists of five main cell types: photoreceptors, bipolar cells, horizontal cells, amacrine cells, and retinal ganglion cells. Briefly, working in the direction of phototransduction, rod and cone photoreceptors are the canonical light-sensitive cells in the retina and make up the outer nuclear layer (ONL), which is the

outermost layer of the retina and farthest from the pupil. Rods and cones hyperpolarize in response to light stimulation and only depolarize when light stimulation is removed. Hyperpolarization and depolarization in rods and cones lead to decreases or increases in neurotransmitter (namely, glutamate) release, respectively, around a baseline level. Rods and cones synapse onto bipolar cell and horizontal cell dendrites in the outer plexiform layer (OPL). The cell bodies of bipolar cells and horizontal cells are found in the outermost regions of the inner nuclear layer (INL). Bipolar cells can be split into two broad classes, ON or OFF. ON bipolar cells are depolarized in response to the presence of light, whereas OFF bipolar cells are hyperpolarized due to the presence of light. The opposite reactions of ON and OFF bipolar cells to the same glutamate released by photoreceptor terminals are due to the presence of cGMP-gated sodium channels in ON bipolar cells that cause ON bipolar cells to hyperpolarize in the presence of glutamate. Thus ON bipolar cell depolarization in the presence of light is due to the removal of inhibition when photoreceptors are exposed to light. Like rods and cones, bipolar cells generate graded potentials instead of action potentials. Each bipolar cell and horizontal cell receives input from many different photoreceptors and integrates the light stimulus signal. Horizontal cells summate the signals from many photoreceptors across a wide dendritic field and feedback to the photoreceptors, releasing GABA and hyperpolarizing them. Generally, cone bipolar cells receive input from all cone pedicles within their dendritic field and send their signals to axon terminals in the inner plexiform layer (IPL) where they synapse onto the dendrites of retinal ganglion cells. Rod bipolar cells receive input from many rod photoreceptors; however, rod signals are transmitted to ganglion cells via two indirect pathways. In scotopic light conditions, rod bipolar cells synapse with AII amacrine cells which in turn signal to ON and OFF cone bipolar cells that ultimately synapse with ganglion cells. In mesopic light conditions, gap junctions between rod and cone photoreceptors allow rods to signal to the ganglion cells via the cone pathway. Amacrine cells are interneurons that have somas in the innermost region of the INL. Amacrine cells are inhibitory neurons that work laterally in the IPL to modulate the signals for bipolar cells and retinal ganglion cells (RGC). RGC somas make up the ganglion cell layer (GCL), the layer closest to the vitreous humor and the pupil. RGCs receive modulated and processed light information from many bipolar cells and send those signals down their axons, which upon leaving the eye form the optic nerve, to various targets in the brain.

## 17.6 Photoreceptors in the Mammalian Eye

Rod and cone photoreceptors make up >70 % of the cells in the retina and detect all light information used for pattern/image-forming vision. Only 3 % of all photoreceptors are cones, the rest being rods. The mouse retina contains 6.4 million rods and 180,000 cones [31]. Of the light incident upon the eye, only 57 % is transmitted to the retinal surface – all other photons are either scattered or absorbed by the

cornea, lens, and macula. Of the photons that make it through to the retinal layer, only a small portion will be in the visible range and thus be absorbed by rod and cone photopigments. Of the photons absorbed by photopigments, only 67 % successfully photoisomerize a visual pigment molecule. All told, only about 0.6 % of the photons that are incident on the cornea will lead to photocurrents [32].

Rods show peak sensitivity at 498 nm and are insensitive to wavelengths of light longer than 640 nm. A single photon of light is enough to photoisomerize the 11-cis retinal in rod rhodopsin and activate the rod photoreceptor [33] resulting in a large photocurrent that slowly returns to baseline. Mice have two types of cone pigments, S cone and M cone pigments which are maximally sensitive to 360 nm and 508 nm wavelengths, respectively. Activation of cone photopigments by a single photon leads to a quick photocurrent surge that rapidly decreases to undershoot the baseline before rising back to the baseline. Hence, both rod and cone photoreceptors can respond to single photons of light, but cones are 100 times lower in sensitivity due to their miniscule single-photon response.

Rods and cones work together to facilitate vision over a wide range of luminance conditions. At scotopic light levels (very low light, i.e., starlight), only rods, with their robust responses to single photons, are active. At mesopic light levels (i.e., moonlight), both rods and cones are active and contribute to vision. However, as light levels increase to the point where 400–500 photoisomerizations are occurring per second in rods, rods are unable to increase their responses to further increases in light intensity and are considered saturated. In these photopic light levels (i.e., sunlight), only cones functionally contribute to vision. Cone photopigments will continue to respond to increasing light intensity, no matter how bright; however, the number of functional cone photopigments is limited such that greater than 1,000,000 photoisomerizations per second, cones will downregulate their photopigments such that the number of photoisomerizations maxes out at ~1,000,000/s.

In contrast to rods and cones, intrinsically photosensitive retinal ganglion cells (ipRGCs) express melanopsin which is structurally more similar to the rhabdomeric invertebrate opsins than to the ciliary opsins found in rods and cones [24]. Like other retinal ganglion cells, ipRGCs receive signals generated in rods and cones and send action potentials to the brain via their axons which meet at the optic disk to form the optic nerve. However, unlike other retinal ganglion cells, ipRGCs are photosensitive – exhibiting a peak sensitivity to 484-nm light and responding to capture of single photons of light with a large and extraordinarily prolonged photocurrent. The single-photon response of ipRGCs lasts 20 times longer than that of rods and more than 100 times longer than of cones [34]. IpRGCs will fire tonically in the presence of light and respond incrementally to changes in light intensity. Spiking is sluggish, with a long latency to reach steady-state firing rates in dim lighting and firing persists for minutes after termination of light stimulus [23]. Under saturating light conditions, mRGCs initiate spike trains after a short delay following light onset, increasing firing rate to a peak rate before setting at a steady-state firing rate about 50 % of peak rate. After lights off, activity

decreased to baseline over 5–30 s. These rates and timings coincide with the timing of circadian phase shifting [35].

While rod opsins, cone opsins, and melanopsin in an intact retina have been shown to individually be capable of facilitating circadian photoentrainment, ipRGCs are absolutely essential for the circadian visual system. Inducible ablation of ipRGCs with targeted expression of diphtheria toxin showed that mice without ipRGCs had normal stratification of the major cell layers, normal rod and cone responses, and normal pattern forming vision. However, in terms of non-image-forming (NIF) visual processes, mice with ablated ipRGCs had complete loss of pupillary light reflex, were unable to photoentrain (showing a normal/wild-type free-running period length regardless of lighting condition), and did not exhibit negative masking. In these mice, the master clock was unaffected, but light cannot reach NIF visual areas of the brain [36]. Thus, while melanopsin is an important source of light information for circadian photoentrainment and ipRGCs and canonical photoreceptors are each sufficient for conveying light information to the circadian system, all circadian light information is conveyed to the brain through ipRGCs.

## 17.7 Intrinsically Photosensitive Melanopsin-Expressing Retinal Ganglion Cells

IpRGCs make up approximately 2 % of all mouse retinal ganglion cells and are evenly distributed throughout the mouse retina. Melanopsin is present on the plasma membrane in the soma, dendrite, and axons, but axonal expression stops at the optic nerve head. To date, five distinct subtypes of intrinsically photosensitive melanopsin-expressing retinal ganglion cells have been described. These subtypes differ in their morphology, retinal connections, intrinsic photosensitivity, and physiology.

IpRGC subtypes can be identified based on their dendritic stratification, dendritic fiends, and soma size. Of the five subtypes of ipRGC (termed M1 through M5), subtypes M1, M2, and M3 are the most well studied due to their higher melanopsin expression which facilitates identification via immunohistochemistry. M4 and M5 ipRGCs were identified by crossing mice with Cre recombinase expression driven by the melanopsin promoter with a Cre-reporter line [36, 37]. M1 ipRGCs are the most well characterized with dendrites that stratify in the OFF or outer sublamina of the IPL, cell bodies ~14 um in diameter, and a dendritic field ~350 um in diameter that consists of long, tortuous, irregularly branched dendrites with pronounced mitochondria-laden varicosities. M2 ipRGCs stratify in the ON or inner sublamina of the IPL, have slightly larger cell bodies, and have similar size though more highly and evenly branched dendritic fields as compared to M1 ipRGCs. M3 ipRGCs stratify in both the ON and OFF sublamina and are otherwise similar to M2s in terms of soma size and size and complexity of

dendritic arbor. M4 ipRGCs stratify in the ON sublamina, have the largest soma diameters (~20 um), and have wider dendritic fields and more complex dendritic branching than M2 ipRGCs [37]. Lastly, M5 ipRGCs also stratify in the ON sublamina but are readily distinguishable from M2 and M4 ipRGCs by their smaller dendritic fields (~150 to 200 um) and highly branched, bushy dendritic arbor.

While all five ipRGC subtypes are intrinsically photosensitive, as determined by electrophysiology, their photoresponses vary greatly between subtypes with M1 ipRGCs displaying the greatest photoresponses and M5 ipRGCs displaying the smallest photoresponses to the same stimulation. This is likely attributable to their differential levels of melanopsin expression on the plasma membrane. Furthermore, though the distinctive stratification of each ipRGC subtype suggests different inputs and, hence, different functions, whether ipRGC subtypes play specific or unique roles in non-image-forming vision remains largely unknown.

| | Soma diameter | Dendritic field diameter | Stratification | *Opn4* immunoreactivity | Intrinsic photosensitivity |
|---|---|---|---|---|---|
| M1 | ~15 um | −350 um, irregular branching pattern, tiles | Outer | +++ | +++<br>Large photocurrent, but prone to depolarization block – so fires at low rates |
| M2 | ~17.4 um | ~324 um, radiate branching pattern, more uniform dendritic density; tiles | Inner | ++ | 10× less than M1, 10× smaller photocurrent, but can fire higher-frequency axn potentials |
| M3 | Like M2 | Diameter similar to M2. Do not tile | Inner and outer | + | Low input resistance, hyperpolarized resting membrane potential similar to M2.ON –predominant, large, and sustained |
| M4 | 17–22 um | 300–444 um, large, radiate dendritic arbor, more branch points and thicker axon than M2 | Inner | − | Weak, peak photocurrent $18.5 \pm 11.4$ pA |
| M5 | | 149–217 um compact highly branched arbor | Inner | − | Weak, peak photocurrent $12.9 \pm 4$ pA |

Refs. [37–39]

## 17.8 IpRGCs Convey Light Information from the Retina to the SCN

Rods and cones contribute to the light detection and dynamic range of ipRGCs. In transgenic mice that lack melanopsin, the RGCs that would normally express melanopsin still send projections to regions of the brain involved in non-image-forming (NIF) visual processes but are no longer intrinsically photosensitive. In these mice, PLR and circadian photoentrainment are reduced but not absent, showing that rod and cone photoreceptors can detect light for the purpose of NIF vision and that rods and cones signal that information to NIF brain regions via ipRGCs [8]. At levels of irradiance below the threshold of melanopsin but greater than the threshold of rods, cones drive the responses in pupil size when exposed to light. As pupillary light response is attributable to ipRGC function, it would seem that ipRGCs do receive input specifically from the cone pathway. However, cones do not normally support circadian photoentrainment. In mice lacking melanopsin and rods, but with functional cones (*Opn4*−/− *Gnat1*−/−), even the brightest intensities of light do not allow all tested animals to entrain to 12 h:12 h LD cycles [40]. Mice that lack functional rods (*Gnat1*−/−) but retain cones and melanopsin phototransduction pathways show normal photoentrainment in bright light but fail to photoentrain at scotopic light conditions. Thus, ipRGCs must also receive input from the rod pathway as wild-type mice are consistently able to photoentrain at low light levels [21]. While many studies of rod pathways state as a rule that rod bipolar cells do not synapse directly with ganglion cells and instead signal to cone bipolar cells through AII amacrine cells or that gap junctions between rods and cones mediate rod signals to ganglion cells [41], confocal microscopy and 3D computer analysis have been used to show direct contacts between rod bipolar cells and ipRGCs [42].

Even though cones alone are not sufficient to facilitate photoentrainment, there is abundant evidence that bipolar cells (the vast majority of which receive cone photoreceptors inputs) do synapse with ipRGCs. Ultrastructural studies have shown ipRGC processes in direct apposition to bipolar and amacrine cell terminals [43]. Retrograde labeling studies corroborate and elaborate on that proposal by showing that M1-ipRGCs are downstream of dopaminergic amacrine cells and that M2-ipRGCs are downstream of type 8 cone bipolar cells and monostratified amacrine cells [44]. Functionally, the relationship between ipRGCs and bipolar cells has been confirmed in melanopsin-null animals where application of L-AP4 (a group III mGluR receptor agonist that selectively silences signaling at the photoreceptor-ON bipolar cell synapse) abolishes all light responses from M1-, M2-, and M3-ipRGCs [45]. Application of L-AP4 also leads to a reduction in light-induced *c-Fos* expression M1-ipRGCs [46] and a significant reduction in the maximal light-evoked depolarization of M3-ipRGCs [45] even though M1- and M3-ipRGCs stratify in the OFF sublamina. This suggests that synaptic input via the ON pathway is primarily responsible for shaping the light response of ipRGCs.

To facilitate photoentrainment, ipRGC axons extend dorsally from the optic chiasm and terminate with fine unmyelinated fibers in the SCN [47]. IpRGC projections to SCN are resolvable at P0 [48] and light-induced c-*fos* in retinorecipient regions of the SCN are present at P1 [49]. While many ipRGC axons ultimately terminate in the SCN, some projections to the SCN are collaterals of projections that ultimately terminate in the ventral lateral geniculate nucleus and the intergeniculate nucleus [50]. Regardless of their final site of termination, all RGCs retrogradely labeled by fluorescent dyes injected into the SCN were found to be intrinsically photosensitive [23], and ablation of ipRGCs in adulthood results in the absence of anterograde labeled axons from the retina to the SCN and the complete loss of circadian photoentrainment [36]. Thus, ipRGC projections to the SCN are the principal conduits for all light input to the SCN. The axons of ipRGCs also send projections to other regions of the brain such as the lateral geniculate nucleus, the lateral hypothalamus, the subparaventricular zone, and the olivary pretectal nucleus.

## 17.9 Melanopsin Signal Transduction

Melanopsin is a G-protein-coupled receptor (GPCR) that shares greater homology with invertebrate rhabdomeric opsins than with rod and cone ciliary opsins. Melanopsin differs greatly from rod and cone opsins in sequence and length. Unlike rod and cone opsins, melanopsin has a long cytoplasmic tail with multiple phosphorylation sites, the presence of a tyrosine instead of glutamate counterion (E113), and an elongation of the third cytoplasmic loop [24]. Like all other opsins, melanopsin uses 11-cis-retinal as its chromophore. In the presence of stimulating photons, 11-*cis*-retinal is photoconverted to all-*trans*-retinal and triggers G-protein activation which in turn leads to further signaling. In vertebrate opsins, conversion of all-*trans*-retinal back to 11-*cis*-retinal is an elaborate process that involves release of all-*trans*-retinal from the opsin for conversion back to 11-*cis*-retinal in the retinal pigment epithelium. Invertebrate opsins, on the other hand, are capable of converting all-*trans*-retinal back to 11-*cis*-retinal in an arrestin-dependent manner. Heterologous expression of melanopsin in *Xenopus* oocytes allows for stepwise analysis of the role of different molecules in melanopsin signal transduction. Heterologous expression of mouse melanopsin in *Xenopus* oocytes revealed that in response to light stimulation, photocurrents could be generated if the oocytes were cultured in the presence, but not in the absence, of 11-*cis*-retinal. This showed that melanopsin used 11-*cis*-retinal as a chromophore. When these oocytes were cultured in the presence of all-*trans*-retinal, a small light-evoked membrane current was observed in some cells. Culturing the melanopsin-expressing oocytes in the presence of 11-*cis*-retinal or all-*trans*-retinal and either mouse arrestin or fly arrestin elicited an even larger light-evoked membrane current further solidifying the similarity between melanopsin and invertebrate opsins and revealing the intrinsic photoisomerase ability of melanopsin. Using the light-evoked membrane

current elicited by melanopsin-expressing oocytes in the presence of retinal and arrestin as a baseline, individual injections of BAPTA; pertussis toxin; antibodies to $G\alpha_s$, $G\alpha_i$, and $G\alpha_q/G\alpha_{11}$; and inhibitors against PLC into the oocytes revealed that only injections of BAPTA, antibodies against $G\alpha_q/G\alpha_{11}$, and PLC inhibitors abolished (in the case of BAPTA) or significantly attenuated light-evoked membrane currents. This series of experiments established $G\alpha_q/G\alpha_{11}$ as the melanopsin's G protein and showed the necessity of PLC and calcium influx in melanopsin signal transduction. Since TRP channels are involved in invertebrate opsin signal transduction and the current-voltage relationships of light-activated ipRGC are very similar to those associated with TRP channels, mouse TRPC3 was co-expressed with melanopsin in *Xenopus* oocytes. In the presence of arrestin, TRPC3 was shown to produce a sustained component of the photocurrent that lasted the duration of light stimulation, could be blocked by application of TRP channel blockers and replacement of extracellular sodium with non-permeant cations, and was largely unaltered by application of BAPTA (suggesting that $IP_3$-mediated calcium release from the endoplasmic reticulum did not play a significant role). In the absence of arrestin, the photocurrent persisted for minutes after termination of light stimulus before returning to baseline. This implicates TRPCs in mediating melanopsin signal transduction, suggests that signal transduction is mediated by DAG, identifies sodium current as the inward current, and further emphasizes the role of arresting in signal termination [51].

To identify the exact type of TRPC and PLC involved in melanopsin signal transduction, immunohistochemistry for all known TRPCs was conducted in the mouse retina. In the mouse ganglion cell layer, TRPC6 and TRPC7 immunopositive cells were shown to colocalize with melanopsin immunoreactivity [52] suggesting that light-mediated calcium influx is mediated via TRPC6 and TRPC7 in ipRGCs. Also, single-cell RT-PCR of cultured ipRGCs showed that all analyzed cells expressed the PLCβ4 isozyme of PLCβ [53]. Functional studies involving transgenic mice that were *PLCβ4-null* or null for both TRPC6 and TRPC7 (*TRPC6−/−*;*TRPC7−/−*) showed that both *PLCβ4-null* and *TRPC6−/−*; *TRPC7−/−* mice were unable to elicit photocurrents in response to flashes of light [54] confirming the key roles of PLCβ4 and TRPC6 and TRPC7 in melanopsin phototransduction.

From experiments described above, the secondary messenger system involved in the melanopsin signal cascade is known to involve PLC. Activated PLC hydrolyzes phosphatidylinosotol bisphosphate ($PIP_2$) into membrane-associated diacylglycerol (DAG) and cytosolic inositol 1,4,5-triphosphate ($IP_3$). In the cytosol, $IP_3$ opens $Ca^{2+}$ channels in the endoplasmic reticulum which results in an increase in intracellular calcium. However, in dissociated ipRGC cultures, ipRGC photoresponses persisted in the presence of agents that disrupt $IP_3$-mediated release of $Ca^{2+}$, and depletion of intracellular calcium stores had no effect on the amplitude of ipRGC light response. The persistence of photoresponse in the absence of $IP_3$ signaling turns attention to DAG as the mediator of melanopsin signal transduction. However, administering membrane permeant and constitutively active DAG analogue OAG failed to block light responses in isolated mRGCs [53]. In *Drosophila* photoreceptors, which are

thought to depend on membrane-associated phosphoinositol cascade, application of DAG metabolite polyunsaturated fatty acids (PUFA) but not DAG itself resulted in significant currents through light-gated transient receptor potential (TRP) channels. However, this response was not reproducible in mRGCs [53].

While the general overview of melanopsin phototransduction has thus been elucidated, this picture is likely incomplete as multiple isoforms of melanopsin have been identified in mammals [54] and melanopsin is capable of signaling via many different $G_\alpha$ subunits from the $G_q/G_{11}$ subfamily [55].

## 17.10 Development and Differentiation of Melanopsin-Expressing Retinal Ganglion Cells

Melanopsin-expressing ipRGCs are the first functional photoreceptors in the mammalian eye. In the murine retina, melanopsin mRNA is detectable at embryonic day 10.5 (E10.5), but rod opsin and mouse green cone opsin mRNA are not detectable until postnatal day 5 and 7 (P5 and P7), respectively [56]. While *c-fos* mRNA is detectable in response to light stimulus at P0, discernible spike trains are not evident until P2, and characteristic sustained firing to light is not observed until P4. Between P4 and P6, major dendritic organization occurs within the retina, and ipRGC dendrites start to arrange themselves into ON or OFF sublamina in the IPL. By P6, as rod and cone photoreceptors are starting to develop, ipRGC photosensitivity increases dramatically [35]. Visual signaling is not thought to develop until P10 as classical photoreceptors come online/become functional at P10-P12 [57]. Light activation of the ipRGCs at the neonatal time points is functional in that it leads to activation of the SCN. Light-dependent c-fos expression is detectable in the wild-type P0 SCN, is clearly distributed throughout the core region of the P4 SCN, and shows very robust expression throughout the entire P14 SCN. In contrast, in melanopsin-null mice, c-fos expression is not detectable at the P0 or P4 time points, but shows robust expression at the P14 time point [58]. This illustrates the timing of functional activity in the three photosensitive cell types in the retina, as light detection in the melanopsin-null animal is facilitated by the rods and cones which are not active until after P10, and reemphasizes that rods and cones contribute to the light input of the SCN.

In mouse retinas, retinal ganglion cells are the first cells to be born [59] followed by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells, and, lastly, Muller glial cells. Among other vertebrate species, RGCs and Muller glia always appear first and last, respectively, but the order of appearance of the retinal cell types varies. The differentiation of these retinal cell types is driven by two transcription factor classes, basic helix-loop-helix (bHLH) and homeobox proteins. The five known retinal neuron-promoting bHLH genes, *Ath5*, *Ngn2*, *Ath3*, *NeuroD1*, and *Ash1*, are activated in the order listed and coincide with the peak of genesis for a particular cell retinal cell type [60]. The transcription factor *Ath5* is

essential for RGC development [61]. Expression of Mouse *ath5* (*Math5*) begins at E11 and disappears from the retina at early postnatal ages [60]. Compared to wild-type mice, mice with targeted deletions of transcription factor *Math5* lack RGCs and optic nerves and have thinner retinas, less rod bipolar cells, and muller glia and calretinin-positive amacrine cells but more cones and cholinergic amacrine cells [62, 63]. *Math5*-null mice cannot entrain to light due to the absence of RGCs innervating the SCN – in all lighting conditions, they free run with period length similar to wild-type littermates, suggesting that the intrinsic SCN clock is not affected [64].

*Math5* is required for the appropriate expression of Class IV POU domain-containing effector gene *Brn3b* which is necessary for RGC development [63]. *Brn3b*-null mice showed an 80 % loss of RGCs [65–67], have pronounced deficits in PLR, and do not phase shift to pulses of light during subjective night, but can still weakly photoentrain with free-running period lengths similar to control *Brn3b*-heterozygote animals [68] suggesting that some of the normally *Brn3b* negative RGCs in the remaining 20 % of RGCs in *Brn3b* null mice must be projecting to the SCN. In fact, the seemingly homogenous population of M1 mRGCs can be subdivided into *Brn3b*-positive or *Brn3b*-negative populations that appear to have specific brain targets and, hence, specific functions [69]. However, given the transgenic animals used in these experiments, it is not clear whether other mRGC subtypes were involved. To date, there is no concrete proof that subtypes of mRGCs have subtype-specific central projections or functions.

## 17.11 ipRGC Axons Release PACAP and Glutamate

Light information reaches the SCN via the retinohypothalamic tract (RHT) which originates with the ipRGCs. IpRGC axons project from the retina to the SCN and release glutamate and pituitary adenylate cyclase-activating peptide (PACAP) at synaptic contacts in SCN neurons. In the SCN, glutamate from ipRGCs acts at NMDA and AMPA glutamate receptors to cause an influx in calcium in retinorecipient SCN neurons and mediate photic regulation of the circadian system [70]. Direct application of glutamate to acute brain slices containing SCN shifts the endogenous phase of circadian SCN neuronal firing in a manner synonymous with light pulse-induced phase shifting of circadian wheel-running activity [71]. Also, in vivo intraperitoneal injections of NMDA and AMPA glutamate antagonists reduced light-induced *c-Fos* expression in SCN neurons [72].The crucial role of glutamate transmission in ipRGC-to-SCN signal propagation is illustrated by the fact that transgenic mice with mRGCs that cannot package glutamate into synaptic vesicles ($Opn4^{Cre/+}$; $VLGUT2^{fl/fl}$) elicit a much smaller activation of the immediate-early gene, *c-Fos*, in light-recipient SCN neurons than wild-type animals and are unable to entrain to light at moderate light levels. However, the fact that these mice can weakly entrain to light at very high light levels suggests that other factors might be involved in ipRGC activation of the SCN [73].

PACAP is colocalized with glutamate in the RHT [74]; activates two different receptors within the SCN, PAC1, and VPAC2; and enhances the effects of glutamate [75]. Since PACAP-deficient mice exhibit intact photoentrainment, albeit with weakened phase advance and delay, it is obvious that PACAP is not necessary for circadian photoentrainment. However, in vivo delivery of nanomolar concentrations of PACAP is enough to induce phase shifts in activity and *mPer1* and *mPer2* expression [76] and delivery of micromolar concentrations of PACAP enhance and delay glutamate-mediated phase delay or advance, respectively [77], and modulated glutamate-induced *mPer1* and *mPer2* gene expression [76]. Furthermore, PAC1 receptor-deficient mice (PAC1 –/–) had shorter period lengths in constant darkness. Light stimulation during early night led to longer phase delays in PAC1 –/– mice and remarkable reduction in light-induced *mPer1* and *mPer2* gene expression. While light stimulation during late night resulted in *mPer1* and *c-fos* expression levels similar to wild-type animals, PAC1 receptor-deficient PAC1 –/– mice exhibited phase delay (as opposed to phase advance) and a slight decrease in *mPer2* expression [78].

## 17.12 SCN Neuron Diversity

The SCN is a paired midline structure in the anteroventral hypothalamus just lateral to the third ventricle and immediately dorsal to the optic chiasm. The murine SCN contains a total of about 20,000 neurons (10,000 per unilateral nucleus) with two main subdivisions, the "core" and "shell," distinguished by neuropeptide content, efferent and afferent projections, and activation in response to light. The core is located in the ventrolateral aspects of the SCN, directly abuts the optic chiasm, receives input from the retina, and sends projections to the shell. The shell sits in the dorsomedial portion of the SCN and partially surrounds and receives input from the core but only sparsely projects back to the core.

SCN neurons in the core and shell differ in their neurochemical content. The core region consists of ~1100 vasoactive intestinal peptide (VIP)-, ~900 calretinin-, ~700 neurotensin-, and ~500 gastrin-releasing peptide (GRP)-containing cells. The shell region contains ~2100 arginine vasopressin (AVP)-, ~1100 angiotensin II-, and ~500 met-enkephalin-containing neurons [2]. Most cells in the SCN, regardless of location or neuropeptide expression, express GABA [79] which is used for intercellular synaptic communication among SCN neurons [80]. While many of the neurons in the SCN co-express neuropeptides, VIP and AVP are rarely, if ever, co-expressed in the same SCN neurons. Thus, VIP and AVP are often thought of as the neuropeptides that define and delineate core and shell regions, respectively. These two distinct, yet connected, cell populations have been shown to differ in their projection patterns [81], innervations [82], and rhythmicity [83].

Immuno-electron microscopy studies have shown that VIP neurons receive direct inputs from the retina in the ventrolateral regions of the SCN [84, 85]. Light induction is normally followed by near-immediate *c-Fos* and *per2*

expression in the retinorecipient ventrolateral core regions of the SCN when animals are exposed to light in early or late subjective night and is followed by phase delay or phase advance, respectively [86]. Immunohistochemistry showed co-localization of VIP and c-Fos in the ventral SCN [87]. This has led to the thought that the immediate-early gene *c-Fos* drives light-induced changes within the SCN. However, behavioral phase shift can still occur without expression of *c-Fos* – especially in the middle of subjective night between phase delay and advance periods where light stimulation induces maximum *c-Fos* gene expression but does not alter circadian activity rhythms [88]. Also, *c-Fos* knockout mice were still able to respond to light pulses but showed dampened phase-response curves [89].

## 17.13 Network Properties of the SCN

Like most other mammalian cells, individual SCN neurons have intrinsic molecular clocks that are made up of feedback loops of transcription and translation involving core clock genes that include *Per1* (period homologue 1), *Per2*,*Cry1* (Cryptochrome 1), *Cry2*, *Clock* (circadian locomotor output cycles kaput), *Bmal1*, *Rev-erbα*, *Rorα*, and *CK1ε* (casein kinase 1ε). Briefly, during the day, CLOCK interacts with BMAL1 to activate transcription of the *Per* and *Cry* genes. *Per* and *Cry* transcripts are translated into PER and CRY proteins which heterodimerize and translocate to the nucleus where they interact with the CLOCK-BMAL1 complex and inhibit their own transcription. This cycle takes approximately 24 h, and the turnover of PER and CRY proteins is tightly regulated by the *CK1ε*- and AMPK-mediated phosphorylation of PER and CRY, respectively, which target them for degradation by E3 ubiquitin ligase complexes. During the night, the PER-CRY repressor complex gets degraded, and CLOCK-BMAL1 can again activate a new cycle of transcription. In parallel, a second transcription-translation negative feedback loop involving CLOCK-BMAL1 activated transcription of *Rev-erbα* and *Rorα* where REV-ERBα and RORα feedback to inhibit *Bmal1* transcription resulting in an antiphase oscillation of BMAL1.

These oscillations of the molecular clock can be observed by using PER2::LUC fusion reporter mice which have firefly *Luc* (luciferase) gene fused to the terminal exon of the *mPer2* locus such that the expression of *Per2* drives the expression of *Luc*. When tissue or cells from PER2::LUC animals are cultured in the presence of luciferin, the light-emitting compound found in fireflies, the production of luciferase cleaves luciferin and releases light. Since the production of luciferase is driven by the production of Per2, which itself is tightly regulated by other clock genes, the detection of light from cell or tissue cultures of PER2::LUC animals can be used to study the oscillations of the molecular clock [90].

PER2::LUC assays of SCN explants have shown that the molecular clock in the SCN is highly synchronized, producing a robust increase and decreases in Per2 expression across the day. However, when intercellular coupling is disrupted, as in

the case of a culture of dissociated SCN neurons, the necessity of coupling between SCN neurons for normal SCN functioning becomes obvious as the robust synchronized SCN oscillations are lost despite the continued rhythmicity of individual SCN neurons. Similarly, when action potentials of SCN neurons in slice culture are blocked by TTX, *mPer2* expression becomes desynchronized [91]. The intercellular network coupling is essential to synchronize the individual cell-autonomous molecular oscillations of individual SCN neurons.

The core region is essential for synchronizing the expression of clock genes within the SCN. If a SCN slice is divided such that the dorsal aspect of the SCN is separated from the ventral, core-containing aspect of the SCN, PER1::LUC imaging shows that the piece containing ventral SCN remains synchronized, whereas the cells in the dorsal piece remain rhythmic but are no longer synchronized [91]. Furthermore, by looking at neuropeptide release from SCN slices, isolated dorsal SCN has shorter period lengths than both isolated ventral SCN and intact SCN slices that contain both core and shell. This suggests that the ventral SCN core synchronizes the dorsal SCN shell to its slower period length [92].

The network properties of the SCN allow the SCN to withstand perturbation and continue producing rhythmic circadian signals. Where single gene perturbations to the central clock in fibroblasts and isolated SCN neurons result in PER2::LUC arrhythmicity, when SCN cells are coupled, the effects of these mutations are non-cell autonomous. Strong intercellular coupling in the SCN rescues the cell-autonomous defect seen in individuals. For example, *Bmal1*-null mice are arrhythmic at the behavioral level, and dissociated SCN neurons from *Bmal1*-null mice show fluctuating *Per2* levels but no circadian oscillations. However, acute SCN slices from *Bmal1*-null mice show highly variable stochastic oscillations that had period lengths in the circadian range. These stochastic oscillations were abolished by inhibiting intercellular communication, suggesting that the unique intercellular coupling properties of the SCN network provide a feed-forward signal between SCN neurons that promote circadian-like oscillations [93]. Similarly, at the cell-autonomous level, both peripheral cells and dispersed SCN neurons are sensitive to temperature perturbations. However, in intact SCN explants, strong intercellular coupling preserves the robustness of SCN oscillations and renders the SCN resistant to temperature perturbations [94]. Ultimately, it seems that the network properties of the SCN smooth individual variations in cellular rhythms and thus coordinate a rhythmic timekeeping signal.

Under constant dark conditions (DD), mice have average circadian periods of ~23.3 h, and *mPer2* mRNA and mPer2 protein levels show robust circadian variation. The lack of light means that there are no external light cues that would induce acute transcription of clock genes; hence, the animal runs at its intrinsic period length as dictated by the kinetics of its molecular clock. However, in the light of long-term constant light conditions (LL), mice show behavioral circadian periods of ~24.5 h and mPer2 protein levels that remain constantly elevated, despite continued normal circadian variation in *mPer2* mRNA levels [95]. It is striking that despite mPer2 protein levels remaining constant, *mPer2* mRNA levels continue to cycle. This highlights the remarkable intercellular coupling characteristic of SCN

neurons which allows the oscillations of the molecular clock to be tightly synchronized in the SCN and able to withstand perturbations to the system.

Seasonal changes in day length, or photoperiod, ultimately result in changes to behavioral circadian patterns. Under short photoperiods, there is an increase in PER2::LUC rhythm in the anterior SCN slices and a narrower PER2::LUC peak in the posterior SCN slices. Under long photoperiods, the Per2 peak is wider but shows a decreased amplitude. Entrainment to different photoperiods also seems to skew the tight association between behavioral activity onset, lights off, and mPer2 peak expression seen in short photoperiods to a schema where mPer2 expression peaks approximately 6 h before lights off and activity onset [96]. Supporting the associations seen between behavior and mPer2 expression, under long photoperiod conditions, SCN neuron firing rhythms are much broader as a whole [97], even though single-cell firing peaks remain as narrow as during short photoperiods [98]. Thus it seems that even though the width across which electrical activity is happening during the long photoperiod increases, the amplitude is overall decreased. This decreased amplitude in response to long photoperiod (representative of summer days) and the narrower peaks and increased amplitude of Per2 expression during short periods (representative of winter days) may explain why phase shifting is enhanced under short photoperiod conditions. As individual cell rhythms of light sensitivity are more tightly aligned (as evidenced by the globally increased amplitude of Per2 expression) under the short photoperiod, there is an increased phase-shifting response to light.

The ability of the SCN to "remember" light conditions, as evidenced by acute SCN explants continuing to cycle with the period length of prior photoentrainment schemes, and the time it takes to re-entrain to new lighting conditions after acute phase advance or delay, is a manifestation of the strength of coupling within the SCN. Illustrating this property is the fact that mice with weak coupling, like mice that lack the receptor for VIP (*Vipr2*$^{-/-}$), are able to re-entrain to phase shifts much more rapidly than wild-type animals [99]. Surprisingly, applying low doses exogenous VIP to the SCN in vitro or to the SCN in vivo via cannula also reduces intercellular synchrony and results in more rapid re-entrainment [100].Therefore, light entrainment of the SCN must involve transient weakening of the network and resetting of the clock. In fact, PER2::LUC imaging shows that individual SCN neurons within an intact SCN slice are more widely distributed in phase after a 6-h advance of the light-dark cycle and require at least 8 days to become resynchronized [101].

## 17.14 Molecular Mechanisms of SCN Photoentrainment

Light at night is known to trigger chromatin remodeling in the mouse SCN [102] which probably corresponds to changes in the level of transcripts. However, the exact molecular mechanism through which light information is transduced into

changes in SCN rhythmicity is largely unknown. Some recent explorations of light-induced gene transcription in the SCN begin to uncover the intricate molecular pathways involved in photoentrainment.

Light stimulation at night leads glutamate and PACAP release from ipRGC axon terminals onto retinorecipient cells in the SCN. Activation of NMDA receptors on SCN neurons by glutamate causes calcium influx in SCN neurons [103]. Calcium-dependent gene expression is mediated by cAMP response element binding protein (CREB) which binds *cis*-regulatory elements (or cAMP response elements (CRE)) on the promoters of target genes. CREB gets phosphorylated into phospho-CREB via a plethora of signaling pathways (such as ERK, MAPK, PKA, and CaMK), and the interaction of phospho-CREB to CREB-binding protein (CBP) results in target gene expression [104].The promoter regions of many light-responsive genes, like *c-Fos*, *Nr4a1*, and *Dusp1*, and clock genes, such as *Per1* and *Per2*, all have CREs. Light-induced upregulation of *Per1* peaks 1 h after the initiation of light and then returns to baseline despite the continued presence of light. Since the transcription of *Per1* is dependent on CREB, this suggests that CREB transcription stops 1 h after being induced. In order to identify the negative-feedback mechanism that limits light's effect on SCN gene transcription, analysis of microarray data from the SCNs of wild-type animals that received a pulse of light during nighttime identified light-induced transcripts in the SCN. To pin down key players involved in CREB-mediated gene transcription, transcripts were enriched for the presence of CRE. Individual knockdown of the 108 light-induced CRE-site positive using RNAi in *Per2-luc*-transfected U2OS cells for changes to period length showed that knockdown of *Sik1* causes period lengthening. Light stimulus causes CREB-regulated transcription coactivator 1 (CRTC1) to coactivate CREB which leads to the expression of both *Per1* and *Sik1*. SIK1 phosphorylates and deactivates CRTC1 which then inhibits expression of *Per1*. Because knockdown of *Sik1* in the SCN results in extended light-induced phase shifts and decreased delay time to re-entrain to light advance, SIK1 likely suppresses the effect of light on the clock [105].

Microarray studies of mRNA from SCN and peripheral tissues of male mice entrained to 12:12L:D and collected every 2 h showed 1412 genes with circadian oscillations in transcript abundance. A separate group of animals exposed to 1-h light pulses during times that corresponded with subjective day, early night, and late night were sacrificed at 1, 2, and 4 h after the beginning of the light pulse. In these animals, 508 probe sets were identified with levels that changed in response to light (of which, only 84 were rhythmically expressed). Light-induced genes included circadian clock components like *Per1* and *Per2*; immediate-early genes such as *c-Fos* and *JunB*; and genes that involved intracellular signaling cascades, like CREB and MAPK signaling; kinases; phosphatases; and transcription factors, which likely are involved in converting light signals into signals that change the phase of the clock. Light-repressed genes included many transcripts that are involved in intercellular communication, such as *Vip* and *Avp1a*. This paints the SCN's phase shifting response to light pulse as dependent on resetting the cell-

autonomous clock while weakening intercellular coupling. Comparison of rhythmically expressed genes in the SCN to rhythmically expressed genes in 84 other mouse tissues revealed 213 SCN-enriched genes, of which 13 are transcription factors. Of the 13 transcription factors identified, *Lhx1* was identified as an SCN-enriched transcription factor that was suppressed by light. Despite normal expression and light-induced levels of *Per1*, *Per2*, *c-Fos*, and, *JunB*, mice that lack LHX1 specifically in the SCN ($Ror\alpha^{Cre}$;$Lhx1^{loxP}$) show decreased expression of *Vip* and *Avpr1a*mRNA and reduced VIP protein levels. These animals have normal SCN architecture, have normal innervation from ipRGCs, and are capable of entraining to 12:12L:D. However, when released into DD conditions, $Ror\alpha^{Cre}$;$Lhx1^{loxP/loxP}$ animals become arrhythmic after 3 weeks. They also take less time to re-entrain to phase advance and delay suggesting weakened SCN oscillations. Furthermore, multielectrode recordings from $Ror\alpha^{Cre}$;$Lhx1^{loxP/loxP}$ animals showed dampening of firing rhythm and lack of synchrony of peak phase between channels [106].These experiments establish that when intercellular coupling is disrupted by loss of *Lhx1*, the effects of light pulse on overt circadian rhythms are lost despite proper induction of immediate-early and clock gene transcription.

*Mutant mice with light entrainment phenotypes:*

| Gene | Mutant allele(s) | Aberrant light entrainment phenotype | Resources |
|---|---|---|---|
| *CHRNB2* | $\beta2^{V287L}$ | Decreased latency to peak activity after dark onset | [107] |
| *dexras1* | $Dexras1^{-/-}$ | Unable to entrain in low light. Attenuated phase delay in response to light pulse in early night | [108] |
| *Eif4ebp1* | *4e-bp1 KO* | Faster re-entrainment for phase delay and advance. Rhythmicity persists in LL | [109] |
| *Opn4* | $Opn4^{-/-}$ | Attenuated phase shift to light pulse during early night. Attenuated negative masking | [8, 29] |
| | $Opn4^{-/-}$; *rd/rd* | Free-running period length in all lighting conditions. No negative masking | [29] |
| *PAC1* | $PAC1^{-/-}$ | Attenuated phase shift response during early night but not late night. Delayed resetting. Negative masking impaired at low light intensity | [110] |
| *PACAP* | $PACAP^{-/-}$ | DD period is 0.3 h shorter. Attenuated phase shifts due to light pulses in early and late night. LL period is 0.3 h shorter | [111] |
| *rd* | *Rd/rd* | Reduced negative masking in low light* | [29] |
| *Rev-erbα* | $Rev\text{-}erb\alpha^{-/-}$ | Increased phase shift in response to light pulse in late night. No period lengthening in LL | [112] |
| *Rorα*, *Lhx1* | $Ror\alpha^{Cre}$;$Lhx1^{loxP}$ | Arrhythmic in DD. Rapid re-entrainment for phase advance and delay | [106] |
| *V1a*, *V1b* | $V1a^{-/-}V1b^{-/-}$ | Rapid re-entrainment for phase advance and delay | [113] |

(continued)

| Gene | Mutant allele(s) | Aberrant light entrainment phenotype | Resources |
|---|---|---|---|
| *Vip* | $VIP^{-/-}PHI^{-/-}$ | DD period is 1 hr shorter/arrhythmic. No phase shift in response to light pulse | [114] |
| | $Vip^{-/-}$ | Attenuated reduction of wheel-running activity in LL. Unable to entrain to skeleton photoperiod* | [100] [115] |
| *Vipr2* | $Vipr2^{-/-}$ | Weakly rhythmic/arrhythmic in DD. Re-entrains faster for phase advance. Attenuated re-entrainment for phase delay. Unable to entrain to skeleton photoperiod* | [99] [115] |
| | $VPAC_2R$-OE | Faster re-entrainment for phase advance and delay | [116] |

In summary, decades of fastidious work have gone into our pursuit for understanding how we interact with the natural world around us. From the evolutionarily ancient, yet distinct, pathways that drive melanopsin phototransduction to the intricate behavioral phenotypes of transgenic animals, the hunt for better understanding of the processes that underlie our basic biology on this Earth continues to push the boundaries of the imagination and scientific creativity. The knowledge gained from these endeavors sheds much needed light on physiological processes that are essential to health and well-being and illuminate a path toward understanding the chronobiological bases for human disease.

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# Chapter 18
# Mechanisms of Non-photic Entrainment

**Emma J. Wams, Sjaak J. Riede, Ivor van der Laan, Tim ten Bulte, and Roelof A. Hut**

**Abstract** This chapter reviews how non-photic cues like temperature, food, anxiety and induced activity lead to circadian rhythm entrainment in mammals. Generally speaking, when dealing with entrainment by different photic and non-photic zeitgebers, we are only able to show that a certain type of stimulus can or cannot entrain the circadian system, therefore, the comparison is difficult to be quantified for the purpose of interpretation. For example, the effect of temperature as a zeitgeber could be different when used alone or when used in anti-phase with the light-dark cycle. Timed feeding and anxiety can also cause non-photic entrainment of circadian rhythms, with a potentially strong selective benefits for prey species like mice and rats.

Although significant work has been done on non-photic entrainment, there are still many open questions to date. Here we will describe different aspects of non-photic entrainment of the circadian system in mammals, with focus on certain open questions especially when nocturnal and diurnal animals are compared.

## 18.1 Introduction

### *18.1.1 Overview of Non-photic Zeitgebers*

In Chap. 3 we dealt extensively with circadian entrainment by light or photic entrainment. Apart from light, other cues have been described that can also lead to mammalian circadian entrainment. Among such cues are temperature, food, anxiety and induced activity. In comparing the strength of different types of photic and non-photic zeitgebers, we face the problem of interpretation. We are only able to show that a certain type of stimulus can or cannot entrain the circadian system,

Dedicated to Nicholas Mrosovsky (1934–2015) and Maroli Krishnayya Chandrashekaran (1937–2009)

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but the comparative quantification of different stimuli is difficult to interpret [1]. In mammals, constant temperature can have a strong and functional influence on activity profiles over the day, but under a light-dark cycle, anti-phased temperature cycles cannot invert the activity patterns, and therefore, temperature cycles in mammals are usually considered to be a weak zeitgeber in comparison to light [2]. Although cells and tissues of the mammalian body are easily entrained by temperature cycles, the SCN seems more resilient [3]. Timed feeding can entrain circadian rhythms and can even overrule light entrainment. Irrespective of the presence of an intact SCN, nocturnal rodents will become increasingly more active in anticipation of the feeding time, even when feeding time is in the middle of the light phase [4]. Anxiety inducing stimuli have recently been proposed as an environmental zeitgeber with a potential role for the amygdala and the SCN [5]. - Anxiety-related zeitgebers could certainly bare a strong selective benefit for a prey species like a mouse or a rat when predators use daily routines for finding prey [6–8]. Apart from the ecologically relevant non-photic zeitgebers mentioned above, the term non-photic entrainment is historically mentioned to indicate the circadian effects of induced activity or arousal on circadian the circadian system. Nicholas Mrosovsky [9, 10] was among the first to describe and study such non-photic effects of induced activity systematically. His lab published numerous studies on this topic, and reviewing all of them would be beyond the scope of this chapter. Although his work identified induced activity as a potent zeitgeber, there are still many open questions to date. The present paper will review key findings and provide new data in a first attempt to answer some of the open questions especially when nocturnal and diurnal animals are compared.

## 18.2 Induced Activity or Arousal as a Potent Non-photic Zeitgeber in Mammals

The first description and comparison of non-photic stimuli came from M. K. Chandrashekaran, who described non-photic entrainment in bats kept in a cage inside a dark cave where the rest of the colony members were able to fly in and out [11]. These freely moving bats were entrained to the environmental light-dark cycle, but apparently their behavioural rhythms in activity and calling were sufficient to entrain the captive bats that were unable to receive light information. Other people showed non-photic phase shifting and entrainment in hamsters, mice and degus [12–17] and coined it ‘activity feedback’ to the circadian system [14, 18]. The repeatability of the experiments in different labs, the consistency of the results and the size of the effects led to the conclusion that induced activity might be a potent zeitgeber for the circadian system in mammals.

## 18.3 The Mechanism of Non-photic Effects on the Circadian System

A non-photic phase response curve (PRC) in the nocturnal hamster was established by Mrosovsky in 1988, for social interactions and for cage cleaning-induced arousal and later for novel wheel-induced activity [13]. All these curves were very similar in shape and produced high resemblance with the dark pulse PRC for hamsters under LL (PRC atlas) [19]. This led to the early insight that the non-photic effects, found by induced activity, seem to be opposite of the effects of light. Indeed, this seems to be in line with suppression of electrical activity in the SCN by behavioural activity in nocturnal mice [20]. Reduced firing rates in SCN neurons might be produced by inhibiting neuropeptide Y (NPY) projections from the intergeniculate leaflet [9] and by serotonergic projections from the raphe nuclei. The involvement of the IGL in non-photic phase shifting was shown by a lesion study where IGL-lesioned hamsters failed to respond to novel wheel- and cold-induced activity. The median raphe was shown to be a likely candidate that mediates behavioural activity input to the SCN through inhibitory serotonergic signalling, whilst the dorsal raphe may mediate activity input to the SCN through excitatory serotonergic signalling to the IGL (Fig. 18.1) [21].

## 18.4 Circadian Activity Feedback and Social Entrainment: A Functional Interpretation Problem

While evaluating the function of non-photic activity-induced phase shifting, Edgar and co-workers [14, 18] coined the term 'activity feedback'. Nocturnal animals respond to induced activity in such a way that activity would advance when the

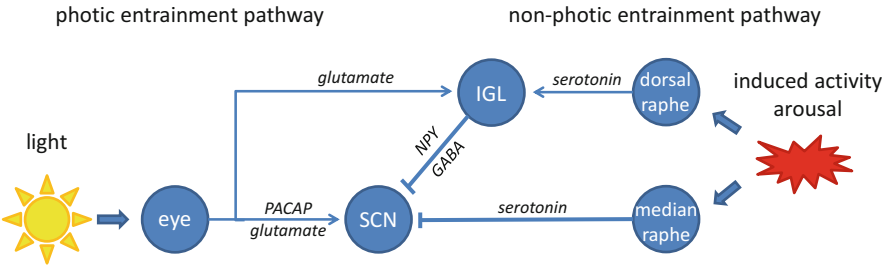


**Fig. 18.1** Photic and non-photic entrainment pathways to the SCN. Photic input received directly and indirectly by melanopsin containing intrinsically photosensitive retinal ganglion cells (ipRGCs) projecting to the SCN through excitatory PACAP and glutamate projections. ipRGCs have also dense glutamatergic projections to the IGL, where they interact with the non-photic pathway. In the non-photic pathway, serotonin projections from the dorsal raphe nuclei elicit excitatory responses in IGL neurons, which project with inhibitory NPY and GABA signalling on SCN neurons. Median raphe neurons also use serotonin projections, which directly inhibit SCN neuronal activity. Neuronal activity in the dorsal and medial raphe is enhanced by induced locomotor activity or arousal

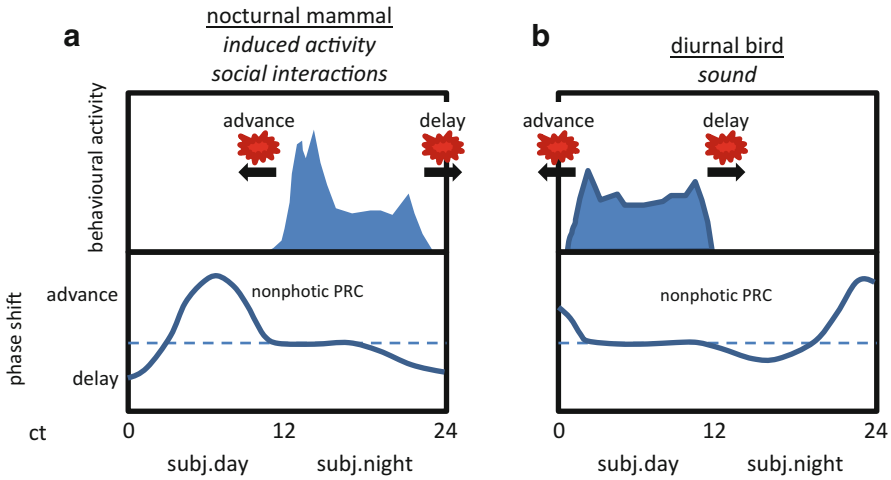


**Fig. 18.2** Activity feedback in a nocturnal rodent (**a**) versus social synchronisation in a diurnal bird (**b**). Non-photic phase shifting would be functional in a nocturnal rodent, where induced activity in the subjective day would elicit phase advances, and induced activity during the late subjective night would cause a phase delay. The activity peak during the early subjective night would thus be drawn towards the timing of induced activity in subsequent days of entrainment in a nocturnal rodent (**a**). In a diurnal bird, the social interactions, activity induction and sound need to elicit a similar phase response as a normal light pulse PRC would do, because social interactions, sound and activity induction should normally coincide with the light phase in entrained diurnal animals. This result was indeed found by Reebs (1988) in house sparrows

stimulus preceded the normal activity peak, while it seemed to delay when the stimulus would follow the normal activity peak (Fig. 18.2). The non-photic PRC for induced activity (Fig. 18.2a) shows that the timing of the stimulus would 'draw' the activity rhythm towards it, such that the next day the animal would be active around the time where activity was induced. This matched very well with the finding that social interactions in nocturnal animals produced a very similar PRC as induced activity does [9].

The validity of this argument of 'activity feedback' as coined by Edgar [14, 18] was tested by comparing non-photic responses in diurnal and nocturnal animals. A clear prediction can be formulated here: a diurnal animal would face social interactions and conspecific vocalisations during daytime, and the phase shifting responses to such stimuli thus need to be similar to the effect of light to cause stabilisation of entrainment. In other words, the non-photic PRC for arousing stimuli in diurnal animals needs to be in anti-phase with the non-photic PRC for nocturnal animals. Indeed, Stéphan Reebs, in 1988, showed that the diurnal house sparrow entrained to a sound stimulus at the end of the subjective night, with the stimulus directly preceding their activity bout [22]. The experiments were carried out under continuous darkness to avoid confounding effects of light, which previously obscured similar effects found in earlier studies [23]. As shown in Fig. 18.2 (comparing A vs. B), the non-photic PRC for diurnal animals seemed to be almost in anti-phase with the non-photic PRC for nocturnal animals.

## 18.5 The Non-photic Paradox of Activity Feedback: Social Synchronisation in Diurnal and Nocturnal Mammals

However accurately performed, the Reebs 1988 experiment does not resolve the non-photic problem. The phase difference between the non-photic PRCs from nocturnal mammals (Fig. 18.2a) and diurnal birds (Fig. 18.2b) can be either attributed to (1) differences between nocturnal and diurnal animals, (2) the type of social stimulus (sexual stimulus for hamsters, sound in sparrows) or (3) a general difference between birds and mammals. Data from Erkert and Schardt in 1991 might resolve this issue in part. They showed social entrainment by conspecific sound in the diurnal primate, the common marmoset [24]. Monkey pairs housed in continuous conditions were able to entrain to the vocalisations of another pair housed under light entrainment. The social entrainment experiment showed that the activity patterns of the entrained pair coincided with that of the pair which provided the social vocalisations, which in turn coincided with the light phase of the light-dark cycle. These results thus indicate that the underlying circadian PRC for social vocalisations in diurnal mammals should have a similar shape as the light pulse PRC, since vocalisations occur at daytime during the active phase and because both signals yielded a similar phase of entrainment (Fig. 18.3a). Likewise, social synchronisation of the bats in Chandrashekaran's study would need a dark-pulse like PRC to yield nocturnal activity patterns and synchronisation with their conspecifics. The non-photic PRC for social synchronisation thus seems to be in anti-phase between nocturnal (dark pulse-shaped PRC, Fig. 18.2a) and diurnal mammals (light pulse-shaped PRC; Fig. 18.3a).

## 18.6 The Non-photic Paradox of Activity Feedback: Induced Activity in Diurnal and Nocturnal Mammals

Erkert and Schardt's results beautifully showed social entrainment in primates, but how activity feedback in diurnal mammals would precisely work was not addressed [24]. Would the induced activity PRC in diurnal mammals also be in anti-phase with that of nocturnal mammals, as is the case with social interactions? The best method to study the validity of the 'activity feedback' argument would thus be to induce activity in a diurnal mammal in a similar way as it was done in nocturnal mammals. This approach was first tested by Hut, Daan and Mrosovsky in 1999, when Nicholas Mrosovsky was a visiting scientist at the laboratory of Serge Daan in Groningen, the Netherlands, at the time when the last author of this chapter was a graduate student in Serge Daan's lab. He performed circadian experiments with the most extreme diurnal mammal known to date, the European ground squirrel (*Spermophilus citellus*), and thus was well equipped to test the activity feedback hypothesis in a diurnal mammal [25]. Entrainment experiments where the animals were handled and locked up in a running wheel for 3 h with a periodicity of 23.5 h forced the ground squirrels to entrain with advances. The phase angle of

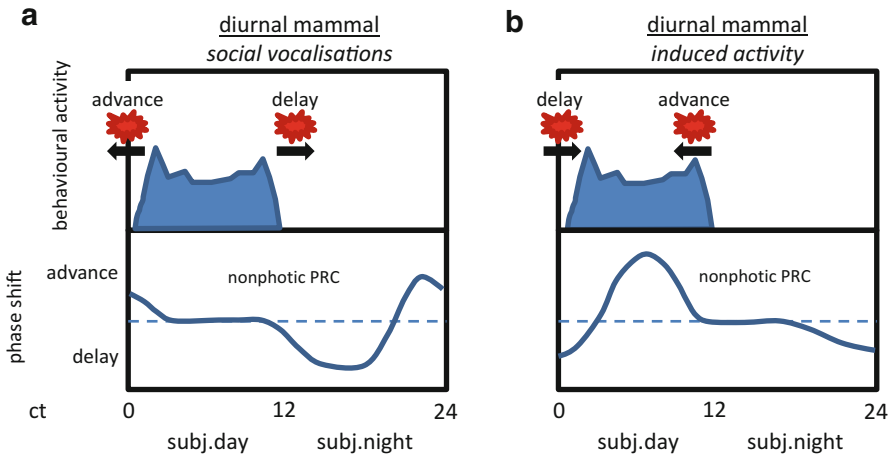


**Fig. 18.3** Non-photic PRCs for social vocalisations and induced activity in diurnal mammals. Social vocalisations in marmosets (primates) cause entrainment through a PRC (**a**) which is in anti-phase with the non-photic PRC in nocturnal mammals (Fig. 18.2a) and also in anti-phase with the non-photic PRC of induced activity in diurnal mammals (**b**)

entrainment would tell whether the underlying non-photic PRC would be in phase with that of nocturnal mammals or in anti-phase. The results clearly indicated that the advances occurred at the end of the active phase, which for a diurnal mammal would be the end of the subjective day (Fig. 18.3b) [25]. The surprising conclusion needs to be that the underlying non-photic PRC for *induced activity* would thus be *in phase* with that of nocturnal mammals (compare Fig. 18.3b with Fig. 18.2a), whereas we showed above that the non-photic PRC for *social stimuli* has to be in *anti-phase* between nocturnal and diurnal animals.

## 18.7 The Non-photic Paradox: Induced Activity Versus Social Synchronisation in Diurnal Mammals

The ground squirrel results left us with a non-photic PRC problem: the social synchronisation PRC in marmosets [24] was in anti-phase with the induced activity PRC in ground squirrels [25]. This could either be a species-specific issue or some kind of by-product of the different experimental approaches. In collaboration with Mrosovsky, David Glass (2001) took a similar approach with marmosets as with the ground squirrels. Activity in marmosets was induced by the presence and activity of the experimenter while the animals were kept in continuous dim light [26]. The results showed that entrainment by phase delays occurred at the beginning of the active phase (beginning of the subjective day), where light would normally cause advances (Fig. 18.3b). The underlying non-photic PRC for induced activity in

ground squirrels and marmosets had to be in phase. Together, these experiments show that marmosets, and most likely all diurnal mammals, have to have at least two types of non-photic PRCs: one for social entrainment and one for induced activity (Fig. 18.3a, b, respectively).

## 18.8 Non-photic Entrainment in Humans: Social Cues Versus Induced Activity

The results above indicate that light entrainment may interact with non-photic entrainment, either in synergy (as in nocturnal mammals) or through opposing effects (induced activity in diurnal mammals). In humans, we would predict that (as indicated by Fig. 18.3a) social stimuli would enhance normal light entrainment. Indeed, Klerman and colleagues [27] found that in totally blind people, without a functional retina and without any non-image-forming light responses, about two-thirds were able to maintain entrained to 24 h with relatively normal phase angles of entrainment [27], as the marmosets of Erkert and Schardt did [24]. Although the precise social stimulus was not identified [28], Klerman's result suggests that humans also have a non-photic PRC for social interactions, which works in synergy with light entrainment in humans with intact photic light input (as in Fig. 18.3a).

This leaves the question unanswered whether humans, like marmosets and ground squirrels, would also have a non-photic PRC for induced activity which would be, like in the ground squirrels, in anti-phase with the PRC for social entrainment. To answer this question, we conducted a pilot experiment in humans where we scheduled activity in the early afternoon or in the early evening in four young adults (20–25 years old, BMI<30). Scheduled activity consisted of 1 h of indoor cardio training under artificial lighting for five consecutive days, timed in the early afternoon (13:00–14:00 GMT+1) or in the early evening (18:00–19:00 GMT+1). Early afternoon treatment and early evening treatment were performed in each individual in a random order design. Dim light melatonin onset (DLMO) measured in each individual before the experiment (baseline) and the day after the morning or evening exercise condition was used as a proxy for the phase of the circadian clock. Wrist activity data (MotionWatch, CamNTech, UK) were collected in three participants. The results indicate that induced activity in the early afternoon advances DLMO, whereas induced activity in the late afternoon did not elicit major advances (Fig. 18.4).

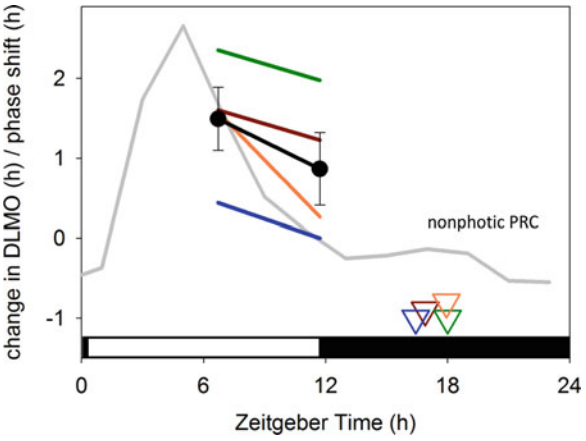


**Fig. 18.4** Induced activity in humans causing advanced phase angle of entrainment in normal day life. Scheduled activity in the early afternoon caused more advanced in entrained phase when compared to scheduled activity in the late afternoon. Grey curve is the non-photic PRC for induced activity in hamsters from Mrosovsky et al. (1992). *Line colours* indicate phase shift of DLMO in different subjects after being exposed to 5-day scheduled exercise at 13–14:00 (ZT6.7) or at 18–19:00 (ZT11.7). Baseline DLMO for each subject is indicated with *triangles*. *Black circles* indicate average change in phase angle of entrainment as measured by DLMO after scheduled exercise. Afternoon exercise, but not evening exercise, elicited significant phase shifts (one sample *t*-test $p < 0.03$ and $p < 0.15$, respectively), and each subject advanced more by afternoon exercise than by evening exercise (paired *t*-test A>E: $p < 0.04$). White bar indicates local light phase using civil twilight times during the experiment (Feb.21st). Local noon (12:47 GMT+1) was set as ZT6. Data indicate similar responses to induced activity in nocturnal mammals (Fig. 18.2a) diurnal mammals (Fig. 18.3b) and humans (Fig. 18.4), with large advances around mid (subjective) day

## 18.9 Conclusion

Data of non-photic effects on the circadian system so far indicate that the PRC for social synchronisation in diurnal animals has a similar shape as the light pulse PRC and seems in anti-phase with that of nocturnal animals. Surprisingly, the non-photic PRC for induced activity in diurnal and nocturnal animals seems to have the same phase with large advances around (subjective) midday. This indicates that the signalling pathway for social synchronisation in nocturnal and diurnal mammals may be different. On the other hand, the circadian signalling pathway for induced activity in nocturnal and diurnal mammals is probably quite similar. This may present another argument for the mammalian nocturnal bottleneck hypothesis, stating that all mammals have a common nocturnal ancestor [29]. The evolutionary benefits and functionality of activity-induced advances during midday remain unclear, especially for diurnal mammals that seem to move away in time from the induced activity event rather than moving towards it (like nocturnal mammals do).

The finding that humans advance their circadian phase of entrainment through repeated induced activity during midday may have practical applications in modern

societies. Regular scheduled activity during midday could reduce the number of late chronotypes in our society [30], treat delayed sleep phase syndrome and social jet lag [31], reduce late chronotype-related obesity [32] and increase school performance in late chronotypes [33, 34].

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# Chapter 19
# Temperature Input for Rhythmic Behaviours in Flies: The Role of Temperature-Sensitive Ion Channels

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**Abstract** The present chapter revisits the circadian clock in the fruit fly and the mechanisms of temperature entrainment of the circadian clock. Temperature is a crucial abiotic factor influencing the property of clocks and regulates development, growth and reproduction. Therefore, apart from light, thermal cycles also synchronise physiological and behavioural rhythms as evident in insects. Thermosensors in flies and the molecular mechanism of thermal entrainment have been extensively studied and they have revealed the neuronal circuits mediating the effect of temperature on clock properties. The temperature-sensitive ion channels, TRPA1, have functional importance, and their role has been discussed in detail along with the behavioural output of flies when multiple cues are present in nature.

## 19.1 Introduction

Among the various abiotic environmental factors that organisms encounter on a daily basis, temperature is crucial as it can affect vital processes such as the rate of development, growth and reproduction of organisms and also regulate many physiological, biochemical and metabolic processes. It is therefore understandable that extreme high or low temperatures can be detrimental to survival. The temperature at a given geographical location depends upon a number of factors such as latitude, altitude, distance from sea or other water bodies, vegetation, wind conditions, etc.

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Climatic specifications of a particular region have a large influence on the habitat and geographic distribution of species. Besides these factors, the rotation of the earth around its own axis changes the temperature in a daily fashion (being hot during the day and cold during the night). Furthermore, the fact that the earth's axis has an ~23° tilt in relation to its equatorial or rotational axis gives rise to seasonality accompanied by annual changes in temperature.

Possibly, due to the remarkable regularity in the daily changes of many environmental factors in the real world, living organisms have evolved internal time-keeping mechanisms (circadian clocks) that help them anticipate daily changes in the environment and synchronise behaviours and physiological processes to those changes and help organisms to adapt to seasonal changes throughout the year [1]. Since many animals use shelters such as dens, nests or burrows possibly to escape from predators and avoid extreme weather conditions such as hot afternoons and chilly nights in a daily manner, it is quite likely that, along with changes in light levels, cyclic temperature cues are dominant external time cues or 'zeitgebers'. In this chapter, we review the various behaviours exhibited by insects that are influenced by thermal time cues. We discuss our current understanding of how insects, especially *Drosophila melanogaster*, perceive temperatures that are reliable indicators of time of day. We focus on several thermosensors that have been identified in flies and their possible roles in modulating activity/rest rhythm.

## 19.2 Rhythmic Behaviours of Insects That Can Be Synchronised by Thermal Cycles

Insects exhibit a daily rhythm in a wide variety of behaviours such as egg hatching (moth *Antheraea pernyi*, phasmid *Carausius morosus*), pupation (mosquito *Aedes vittatus*), pupal eclosion (*Drosophila melanogaster* and *D. pseudoobscura*), locomotion (grasshopper *Schistocerca gregaria*), sleep (*D. melanogaster*), mating (cricket *Acheta domestica*, *D. melanogaster*) and egg laying (lepidopteran pink bollworm, *Pectinophora gossypiella*). An extensive compendium of rhythmic behaviours exhibited by individuals or populations can be found in Tables 1 and 2 of Saunders (1976) [2]. These behaviours are either examined in constant conditions of light (LL) or darkness (DD) or under cycles of light and dark (LD, typically with 12:12 h light/darkness), and a number of studies document the role of thermal cues in modulating rhythmic behaviours and physiological processes in insects. Perhaps the earliest report is that of the Mediterranean flour moth *Anagasta* (*Ephestia*) *kuehniella*, whose adult emergence rhythm could be synchronised by thermal cycles, i.e. thermophase (warm): cryophase (cold) cycles, henceforth TC cycles [3]. Similar phenomena were subsequently reported in other insects, such as the Queensland fruit fly *Dacus tryoni* [4] and the tobacco budworm *Heliothis virescens* [5] including other behaviours involved in reproduction such as courtship,

mating and egg laying [6]. A recent study on the alfalfa leafcutting bee *Megachile rotundata*, which makes nests in microhabitats where access to natural light cycles are limited, showed that very low-amplitude TC cycles (as small as 2 °C) were sufficient to elicit synchronous emergence of adults as opposed to constant temperatures [7]. The adult emergence rhythm of the onion fly *Delia antiqua* was also seen to be strongly influenced by TC cycles with amplitudes as small as 1 °C, while the phase of the eclosion peak was susceptible to changes in amplitude of the thermal cycles [8]. In several insect vectors of disease and agricultural pests, TC cycles may synchronise rhythms in behaviour and physiology that have yet to be examined but could potentially be exploited for the effective management of these insect species that have biomedical and economic relevance.

## 19.3 Evidence for Temperature as a Time Cue for Circadian Clocks of Flies

For a cyclic environmental factor to be considered as a true zeitgeber, it must fulfil the following criteria: (i) it should synchronise the overt rhythm with a stable and reproducible phase relationship; (ii) upon removal of the zeitgeber, the rhythm must continue to free-run with the phase set by the zeitgeber. In addition, in the quest to understand the cellular and molecular bases of entrainment to a zeitgeber, it is common for circadian biologists to perturb candidate pathways or genes and determine how many transient cycles are required to re-entrain to phase-advanced or phase-delayed cycles. Many studies starting from the work of Colin S. Pittendrigh [9] have established that TC cycles can act as entraining agents of the circadian clock in *Drosophila*. To the best of our knowledge, this was the first conclusive demonstration of circadian entrainment of a rhythm by temperature cycles. The authors showed that emergence rhythm of *D. pseudoobscura* could be phase-shifted by temperature pulses or step up or step down in temperature. Moreover, they observed that the size of the phase shift was dependent on the phase of the circadian clock and that cycles of warm and cold temperatures could stably synchronise this behaviour [9]. More recently, a study evaluated the effects of light and temperature cues on emergence rhythm in *D. melanogaster* stocks that have been artificially selected for morning or evening emergence (early or late chronotype, respectively). The 'early' and 'late' populations have an early or late phase of emergence compared to controls. The authors report that the divergence in phase of emergence peak between early and late populations was enhanced dramatically under TC cycles as compared to LD cycles, whereas subjecting flies to in-phase light and TC cycles suppresses these differences in phase [10] suggesting that temperature cues play an important role in adult eclosion. The authors hypothesise that thermal cycles promote divergence between chronotypes, while light cycles inhibit these differences.

The vast majority of studies on rhythmic behaviours in flies have been on the more easily assayed activity/rest rhythm due to the extent of automation and minimally invasive methodology now available to assay rhythms of individual flies in contrast to the populational-level emergence rhythm. One of the earliest studies of thermal cycles influencing this behaviour came from the laboratory of Jeff Hall who showed that TC cycles with a temperature difference as small as 1–2 °C can entrain both activity/rest rhythms and the underlying molecular clocks in pacemaker cells of flies [11]. The study also showed that flies lacking a functional circadian clock (such as $per^{01}$) could not entrain to the above TC cycles which suggested that those molecular components of the circadian clock that were known until then were also important for entrainment to thermal cycles. A further study showed that TC cycles with a 5 °C difference are able to entrain activity/rest rhythm of flies even under LL [12], a condition which is thought to disrupt core clock functions [13]. Interestingly, the laboratory of Kenji Tomioka went on to show that, while under DD the activity/rest rhythm of flies could only entrain to 12:12h TC cycles, under LL, they were able to entrain to a range of TC cycles (with period length from 8 to 32h, e.g. 4:4 TC up to 16:16 TC) [14]. Moreover, the phase of activity peaks was found to be a function of the period of the thermal cycle, suggesting 'true' entrainment under LL. The authors hypothesised that perhaps the LL regime, previously considered to be deleterious to the overall functioning of circadian clocks, is in fact more conducive for entrainment to thermal cycles [14]. Subsequently, several groups showed that activity/rest rhythm of *Drosophila* could also be entrained by TC cycles in DD [15–17]. One study which applied a modified TC where the temperature changed in a gradual manner to mimic naturally changing TC cycles in the presence of rectangular LD cycles of varying day length [18] showed that wild-type flies entrain to shifted natural-like TC and that the morning and evening peaks of activity are closely associated with the rising and falling phases of temperature, respectively. Moreover, the study showed that even flies with null mutation in *period* and *timeless* genes could entrain to such TC cycles [18] once again showing that other components of the molecular clock may drive these rhythms. In addition, various studies have exposed flies to warm temperature pulses (~29 °C) and found that the resulting phase shifts vary as a function of the phase of the circadian clocks, providing additional evidence for TC cycles being able to entrain the clock [15].

In *D. melanogaster*, in addition to emergence and activity/rest rhythms, other rhythmic behaviours have been demonstrated to be entrained by TC cycles. For instance, TC cycles can also entrain egg-laying rhythms [19], although they appear to be a weaker zeitgeber compared to LD cycles. Flies also exhibit temperature preference rhythm (TPR), which could be considered as analogous to body temperature rhythm (BTR) exhibited by homeothermic mammals [20]. When adult wild-type flies are exposed to a thermal gradient, the preferred temperature range lies between 24 and 26 °C [21, 22], whereas larvae prefer temperatures of around 18 °C [23–25]. It is reasonable to hypothesise that the thermosensors that allow flies to orient themselves towards their preferred temperature range may send inputs to circadian clocks and that they function in tandem [26]. It was recently demonstrated

that indeed flies do exhibit a circadian clock-controlled daily rhythm in their preference for a given temperature when offered a gradient [27, 28]. TPR in flies is almost bimodal with preference for low temperatures in the morning and preference for high temperatures just before lights off (ZT10–12); another attenuated peak occurs during late night (ZT19–21). This rhythm persists for several days in the absence of cyclic light cues, but clock mutants such as $per^{01}$ and $tim^{01}$ fail to exhibit robust TPRs, suggesting that this rhythm is under circadian clock control [28]. While the study rules out the role of canonical 'M cells' and 'E cells' in TPR (subsets of circadian pacemaker neurons that are critical for anticipation of lights-ON or 'morning' and amplitude of the morning activity peak and phasing of the evening activity peak, respectively), it posits that DN2 neurons (a pair of cells implicated in temperature entrainment, see Fig. 6.5, Chap. 6 by Helfrich-Förster) control TPR in flies during the day (ZT1–ZT2). The authors suggest that TPR persists even in LL conditions as it is primarily driven by DN2 neurons [28] which may belong to the temperature-responsive neurons (see section below) which do not express the blue-light sensitive circadian photoreceptor CRYPTOCHROME (CRY).

## 19.4 Thermosensors in Flies

To understand how TC cycles may influence circadian clocks, it would be useful to know how flies sense temperature and if any of the known fly thermosensors are involved in entrainment of circadian rhythms to TC cycles. A robust thermosensory system is a prerequisite for ectotherms to detect changes in the external environment. Unlike endotherms that maintain a 'constant' core body temperature irrespective of the outside temperature, the core body temperature of ectotherms follows the changes of their external environment. Under very warm or cold external temperature conditions, small insects with a large surface area/volume ratio can suffer severe consequences. Thus, small insects such as *D. melanogaster* must be able to sensitively detect external temperatures and move in order to avoid harmful temperature zones; this active avoidance of temperature extremes is referred to as negative thermotactic movement. Most insects are known to have peripheral thermosensors, and the antenna is an important organ known to house thermoreceptors across several insect models such as cockroaches [29], dragonflies [30], locusts [31], etc. In the cockroach, the optic lobe is known to be the site of central clocks that governs activity/rest rhythm [32]. However, even in animals where optic lobes were removed, TC cycles of various lengths ranging from 12 to 48h cycles could elicit rhythmicity [33] suggesting the presence of another perhaps weaker oscillator outside of the central clock that regulates this rhythm. Both locomotion and flight activity of the mosquito *Culex pipiens pallens* were found to entrain to 24h TC cycles, and the authors proposed that two separate oscillators exist that 'move in different directions in response to the TC cycles [34]. Flies, like many other organisms, have two broad classes of thermoreceptors – innocuous

receptors that function in the ambient temperature range and noxious thermoreceptors, which function in higher temperature range and are associated with pain sensation. Noxious cold receptors have not yet been identified in *Drosophila*.

Distinct thermosensory organs have been identified in flies and chordotonal organs, or stretch receptors have been implicated in expressing thermosensors in both larvae and adults. They primarily function as mechanoreceptors in exoskeletal joints of antenna, limb joints, wings, etc. and are composed of a group of sensilla connected to skeletal tissues (for review see [35]). At the molecular level, most of the known thermosensors belong to TRP (transient receptor potential) family of ion channels. Thermo-TRPs are selectively active in a defined range of temperatures and are involved in mediating avoidance of extremely hot, innocuous warm or cold temperatures both in larvae and in adult flies [36, 37]. Previously, terminal organs at the anterior end of larvae were believed to house neurons' cold sensors [38], whereas the multidendritic body wall (md class IV) neurons of the abdomen were thought to express a hot sensor [39]; for review see [40]. Molecular identity of cold larval sensor is not yet known but Painless TRP channels in md neurons mediate nocifensive cork-screw rolling movement [39]. A recent study suggest that the dorsal organ, another structure, also located at the anterior end, houses three thermosensory cells which are responsive to both warming and cooling [41]. Larval thermotactic movement away from noxious warm temperatures and towards optimal temperature of 18 °C is mediated by dTRPA1 channels [23, 24, 42]. Also, TRPL and TRP ion channels, belonging to the TRPC family, are involved in the perception of cool temperatures [25]. Further, larval chordotonal organs also express the thermosensitive TRPV ion channel, Inactive (Iav), which enables *Drosophila* larvae to distinguish minor temperature changes and allows them to choose their preferred optimal temperature of 18 °C among slightly lower temperatures [23]. Thus, larvae possess a wide range of thermosensors that allow them to respond to thermal cycles although there are no reports of larval behaviours that are rhythmic under thermal cycles. This could be due to the larval microhabitat and physiology being adaptive to be arrhythmic or our inability to detect a rhythm in larvae. Having said that, it must be pointed out that, in larvae, only one behavioural output has been found to exhibit a circadian rhythm thus far, i.e. negative phototaxis, which peaks at dawn [43].

In the mid 1990s, Sayeed and Benzer were the first to demonstrate that the third antennal segments of adult flies are involved in thermosensation; however, the identity of these receptors was unknown at that time [22]. In addition, the study suggested the presence of other thermosensors outside the antennae functioning at relatively warmer temperatures (above 31 °C). After almost two decades, a study from the laboratory of Charles Zuker reported the identity of thermoreceptors in the aristae and sacculus of the fly antenna [44]. The cold sensors in the aristae express Brivido (*brv1*, *2*, *3*), ion channels belonging to TRPP family [44]. The base of the aristae also houses three neurons that express hot thermosensors which were later identified to be a gustatory receptor, Gr28d [45]. Thus, all six aristal neurons of the fly antenna are involved in thermosensation, and distinct neurons respond to hot and cold temperatures. These thermosensory aristal neurons are very sensitive,

responding to temperature changes as minute as 0.5 °C change as seen by quantifying neuronal activity via two-photon calcium imaging [44], implying that the antennal thermosensors are crucial in mediating thermosensation in flies. Notwithstanding the discovery of temperature-sensitive mutants such as *bizarre* (biz) and *spineless* ($ss^a$) [22] and TRP mutants, the neural circuitry-encoding thermosensation in flies remains poorly understood. The central complex and mushroom body (MB) in flies make up the higher processing centres in the brain, and signal inputs from sensory modalities are believed to be integrated in these brain areas. The protocerebral bridge in central complex has been implicated in nociception in adult flies [46], but the neural circuit from the thermoreceptors to their corresponding projection neurons and to the higher brain centres is yet to be elucidated. Some studies propose a labelled-line coding for thermosensation in adult brains [44] implying that warm and cold signals are conveyed to higher centres by distinct pathways. Aristal neurons expressing cold and hot sensors project to spatially distinct glomeruli in the lateral margin of antennal lobes, and these glomeruli are called cold and hot glomeruli, respectively. The 'hot glomerulus' receives warm thermal signals from sensory neurons both from the periphery (aristal neurons) and from the central brain (dTRPA1-expressing neurons), thus, suggesting that this is an important centre for integration of warm thermal signals for higher-order processing [44]. Further, dTRPA1-positive neurons themselves receive thermal input from pyrexia neurons from second antennal segment in the periphery [47]. Two recent reports further dissect thermosensory circuits in the central brain by identifying distinct thermosensory projection neurons (tPNs) through which hot and cold sensations are encoded in the brain thermosensory network [48,49]. Using a number of genetic-labelling techniques, these studies identified second-order tPNs. They found that tPNs predominantly send projections to three centres: the calyx of the mushroom body (MB), the lateral horn (LH) and the lateral protocerebrum (PLP). Most tPNs are found to be narrowly tuned either responding to hot or cold stimuli, but some are broadly tuned neurons. In summary, thermoreceptors are housed in the antennal organ in the periphery – at the base of the aristae and the sacculus or the third antennal segment. Thermosensory neurons expressing distinct hot or cold thermoreceptors send their projections to distinct antennal glomeruli. Second-order projection neurons convey hot and cold signals to the triad of higher processing centres in the brain of thermal signals – MB, LH and PLP. How these hot and cold signals are integrated in these higher-order brain regions and the pathways through which signals are sent to motor centres to modify temperature-dependent behavioural responses remains to be understood. However, none of the thermosensors located in the antennae are necessary for circadian entrainment to thermal cues since even antenna-less flies can efficiently entrain to TC cycles [26]. Instead, peripheral structures such as chordotonal organs that primarily function as mechanoreceptors in exoskeletal joints of antenna, limb joints, wings, etc. and are composed of a group of sensilla connected to skeletal tissues [35] have been implicated in sending temperature cues for circadian entrainment into the brain under the control of the aptly named *no circadian temperature entrainment* gene *nocte* [50].

## 19.5 Molecular Basis of Circadian Entrainment to Thermal Cycles

In fruit flies, the molecular mechanisms for entrainment to TC cycles are not as well understood as compared to entrainment to LD cycles (reviewed by Helfrich-Förster in Chap. 6 of this book). In brief, photoentrainment of circadian clocks is mediated through the activation of the blue-light sensitive circadian photoreceptor CRY which binds to TIMELESS (TIM) and marks it for ubiquitin-dependent proteosomal degradation [1, 51]. In the absence of TIM, its partner PERIOD (PER) is hyperphosphorylated via the doubletime (DBT) kinase, which destabilises PER and leads to proteosomal degradation of PER. Due to decreased PER and TIM levels in the cytoplasm, stable heterodimers of PER and TIM cannot be formed; PER-TIM heterodimers do not enter the nucleus and are thus unable to bind to the CLK-CYC complex to inhibit their own transcription, an essential step of completing the transcription-translation loop that defines the molecular circadian clock. Therefore, the transcriptional activator CLK-CYC heterodimer complex binds to the regulatory regions of *per* and *tim* genes beginning another cycle of transcription. Thus, light activates CRY, leading to TIM degradation which resets the circadian clock [51].

Until recently, the striking difference in thermal versus photic entrainment was the lack of evidence for a role for CRY (or an equivalent thermoreceptor) at least under physiologically and ecologically relevant conditions [15]. A new study proposes that photoentrainment and thermal entrainment converge at the degradation of TIM mediated by light or temperature cues, respectively [52]. While in photoentrainement, light activates the circadian photoreceptor CRY, which in turn tags TIM for ubiquitin-mediated proteosomal degradation, in thermal entrainment, temperature cues facilitate $Ca^{2+}$ influx within circadian neurons (most likely through cationic TRP channels such as dTRPA1 and Pyx). Calcium-dependent calmodulin (CaM) binds to its target sites on TIM, thus marking it for calcium-dependent degradation which is mediated by calpain proteases. Out of four known calpain classes, A, B, C and small optic lobes (SOL), the latter was found to mediate degradation of CaM-bound TIM [52]. Temperature-triggered SOL-dependent degradation of clock proteins is also conserved in mammals where the mammalian homolog SOLH preferentially targets mPER2 when activated by temperature pulses within the physiologically relevant range of 36–38.5 °C and resets the clock [52]. Thus, temperature-dependent degradation of clock proteins via a non-canonical SOL calpain protease that mediates temperature-induced resetting of circadian clock circuit is conserved between vertebrate and invertebrate model systems [52]. The degradation of TIM via SOL calpain protease also defines a cell-autonomous mode for temperature entrainment. This challenges the previous model which proposed that brain clock cells depend on signals from peripheral thermosensors to entrain to TC cycles [50]. However, in the cell-autonomous model of thermal entrainment, a few key players remain unknown, e.g. the identity of ion channels that lead to $Ca^{+2}$ influx within circadian neurons in response to

temperature. Whether dTRPA1 or any other TRP member was to be involved remains an open question, and future studies unravelling the identity of these channels are required to shed light on our understanding of temperature entrainment of circadian clocks.

## 19.6 Neuronal Circuits Mediating Temperature Entrainment

In adult *Drosophila*, approximately 150 neurons in the brain are known to comprise the circadian clock neuronal circuit. It is believed that the transcription-translation feedback loops described above ultimately result in rhythmic outputs of firing frequency and neurotransmitter release from these pacemaker neurons, thus, modulating rhythmic behaviour and physiology (see Chap. 6 of Helfrich-Förster). Several research groups took different approaches to examine the neuronal circuitry involved in thermal entrainment. In this endeavour, a few thermoreceptor molecules (norpA, nocte, Ir25a, TRPA1, described in detail in the next section) and subsets of circadian neurons (especially the dorsal neurons (DNs)) have been implicated both in larvae [53] and adults [54]. Nevertheless, our understanding of how temperature inputs impinge on circadian clocks to entrain behavioural rhythms continues to lag behind our understanding of pathways' underlying photic entrainment.

In the larval circadian clock circuitry, two pairs of CRY-negative DN2 neurons are temperature sensitive [53]. Under TC cycles, phasing of molecular rhythms of circadian clock proteins PER and TIM in different circadian neuronal subgroups appears to be synchronised by DN2 cells. In the absence of a functional clock in DN2, the PER oscillations entrained to LD cycles but became disrupted under TC cycles, suggesting that a functional molecular clock in the DN2 cells is a prerequisite for circadian neurons to entrain to TC cycles [53]. In LD cycles, phase of molecular oscillations of clock proteins, PER and TIM, in lateral neurons (LNs) and other dorsal neurons (DN1) was 'almost antiphasic' to that of molecular oscillations in DN2 neurons. Importantly, the phase of molecular oscillations of clock proteins in DN2 neurons under both LD and TC cycles remains similar [53]. In contrast, under TC cycles, the phase of molecular oscillation in all LNs and DN1 neurons shifts by almost 12h to match the phase of PER in DN2 neurons [53]. This suggests that under TC cycles, DN2 neurons act as circadian pacemakers in the larval brains and that they synchronise the phase of clock protein oscillations in other clock neuronal subsets. Furthermore, in adults, the DNs and the lateral posterior neurons (LPNs) are thought to be more sensitive to thermal cycles as their molecular oscillations appear to shift more readily in response to the shift in TC cycles as compared to the more light-sensitive LNv and LNd cells [55].

## 19.7 Temperature-Sensitive Ion Channels Modulating Daily Rhythms

At the molecular level, only a few thermosensors known to be playing a role in temperature entrainment of circadian clocks have been isolated so far. These include nocte, norpA, two TRP channels, dTRPA1 and pyrexia and ionotropic channel, IR25a. Two studies from the laboratory of Ralf Stanewsky advanced our understanding of temperature entrainment of circadian clocks in flies. The first study [26] showed that flies with ablated antennae or those carrying the *spineless* mutation (antennae transformed into legs) could entrain to TC cycles, thus, ruling out the notion that antennal thermosensors might play a role in circadian entrainment. A mutant for the gene *nocte* was isolated via a chemical mutagenesis screen under TC cycles in LL and exhibited defects in entraining to TC cycles. In *nocte* mutants, *per* oscillations were dampened, as quantified through bioluminescence of the *per-luc* construct (luciferase reporter fused downstream of *per* promoter), and their activity/rest rhythm took longer to entrain to TC cycles than controls [26]. The same study also reported that *norpA* mutants exhibited moderate defects in temperature entrainment as these mutants took more transient cycles than wild-type control flies did to re-entrain to phase-shifted TC cycles under LL [26]. In a follow-up study from the same group, they reported that nocte is expressed in the chordotonal organs and rectified their previous observation that isolated brain tissue can entrain to TC cycles in LL [50]. The same study also demonstrated that while peripheral tissues can autonomously entrain to TC cycles, brain tissues need signals from nocte-expressing chordotonal organs for thermal entrainment [50]. Notwithstanding the implication of nocte for thermal entrainment of circadian clocks, not much is known about how this protein functions and how it enables circadian entrainment. The current understanding is that non-clock neurons from chordotonal organs send temperature inputs either to peripheral clock neurons or directly to temperature-sensitive clock neurons of the brain. This communication pathway is reminiscent of the one observed for sex-peptide signalling in *Drosophila* [56] where peripheral neurons from reproductive organs send signals to the brain. However, as mentioned above, a recent study [52] proposed that a cell-autonomous mechanism for temperature entrainment exists in flies. The new study also hypothesises that the entrainment of circadian clocks to TC cycles may involve dTRPA1 ion channels that gate $Ca^{2+}$ entry into the cell upon receiving thermal stimuli, thus triggering a SOL-dependent degradation pathway [52]. However, a report from our laboratory [16] and a study from the Stanewsky laboratory [17] demonstrate that dTRPA1 is not required for circadian thermal entrainment, at least in the range of temperatures between 20 and 29 °C. However, it is possible that dTRPA1 ion channels, among others, mediate influx of intracellular $Ca^{2+}$ levels in response to warm temperature cues, thus, indirectly aiding in temperature entrainment. Moreover, it has been shown that Pyx neurons in the second antennal segments make synaptic connections with AC neurons and contribute to thermal sensitivity of AC neurons at warm (27–29 °C) temperatures [47], further indicating that AC neurons may function as a

crucial component in integrating warm signals from periphery and central brain neurons.

The first member of the TRP family to be implicated in temperature entrainment was pyrexia [57]. Pyrexia channels were previously reported to be activated only at noxious hot conditions of 40 °C [48]. However, a more recent study demonstrated that Pyx-TRP, which are expressed in peripheral chordotonal organs, are involved in entrainment of circadian clocks to TC cycles in the cool range (20:16 °C in LL) but are not required for entrainment to TC cycles in warm conditions (29:25 °C in LL) [57]. Moreover, restoring pyrexia expression in a few neurons in *pyx* null mutants only leads to a partial rescue of thermal entrainment [57]. This suggests that other thermoreceptors besides the Pyx-TRP channels send thermal cues to circadian clocks. Future studies that unravel the molecular mechanisms of Pyx-TRP activation and neural circuits that modify Pyx-dependent temperature entrainment would possibly explain how the Pyx-TRP channel functions at both noxious (40 °C) and cool (16–20 °C) ambient temperature ranges.

Another report claimed that dTRPA1 is involved in influencing behaviour under TC cycles [59]. The authors claimed that dTRPA1 null flies are unable to show stable entrainment under asymmetric 18:6°h TC cycles (29:18 °C) [59]. However, the role of dTRPA1 in circadian entrainment to TC cycles has been questioned ever since because even the wild-type flies do not seem to entrain to the asymmetric TC cycles [59] (for further details see footnote in [60]). Moreover, even a non-thermosensitive isoform, dTRPA1-B, is able to rescue the defects of dTRPA1 null mutant flies under imposed TC cycles, raising questions on the influence of temperature in determining the mutant phenotype [59]. Studies have shown that dTRPA1 is expressed in circadian clock neurons including the three CRY-positive LNd neurons [16, 61, 62] and that dTRPA1 null mutants have dampened PER oscillations in LNd subsets [59] based on which Montell and colleagues propose that dTRPA1 is involved in synchronising circadian behaviours to TC cycles.

Taking together all these lines of evidence, we posit that dTRPA1 ion channels may not be the dedicated circadian thermosensors in mediating entrainment but could be instead key players in the entrainment cascade where its predominant function is to enhance intracellular $Ca^{2+}$ levels in response to thermal cues in order to facilitate SOL-dependent TIM degradation. Recently, a non-TRP receptor, namely, the ionotropic receptor, IR25a, has been reported to be involved in mediating entrainment to low-amplitude (a difference of 2 °C between thermophase and cryophase) TC cycles [63]. Previously known for its role in chemosensation and, importantly, as a co-receptor for IRs in olfactory system [64–66], the new study shows that IR25a, expressed in chordotonal organs in legs, is important for flies to exhibit stable entrainment to 25:27 °C TC cycles imposed under LL or DD. Furthermore, IR25a is also required for molecular oscillations of clock proteins in circadian clock neurons [63]. It is also believed that IR25a may function in other peripheral chordotonal organs and that inputs from these sensory neurons, which pick up small changes in temperature, may help circadian clocks in flies to remain immune to phase changes in response to short temperature pulses.

## 19.8 TRPA1 Ion Channels Exert Opposite Behavioural Responses Under Rectangular Versus Ramped Thermal Cycles

Among the known thermo-TRPs in flies, dTRPA1 is the only member to be expressed in neurons in the brain. The dTRPA1-expressing anterior cells (AC neurons) have been referred to as the 'internal thermosensors' in flies because knocking down dTRPA1 expression in AC neurons disrupts the ability of flies to choose their preferred temperatures within a thermal gradient [21]. Out of the four known isoforms of dTRPA1, the two temperature-sensitive isoforms function in the range of 24–29 °C (dTRPA1-A isoform) and 34 °C and above (dTRPA1-D isoform) [67], which overlaps with the temperature range that flies are prone to encounter in their natural habitat, especially in tropical climates (see Table 2 in [68]). These features make dTRPA1 ion channels an attractive candidate to convey thermal inputs to brain centres and to modulate rhythmic, behavioural responses. We employed behavioural assays and neural imaging to elucidate the neural correlates of temperature-dependent behaviours in flies. We find that under LD cycles with constant warm ambient temperatures ~30 °C, dTRPA1 is necessary for flies to advance the phase of morning peak and delay the onset of evening activity [16]. Further, dTRPA1 null mutants show an advanced phase of evening peak compared to controls when subjected to TC cycles in LL [16], reiterating the role of dTRPA1 signalling in modifying phase of circadian clock-controlled activity/rest rhythms. We also studied activity/rest behaviour of flies lacking dTRPA1 ion channels or with modified expression in a subset of neurons in the brains; flies were subjected to conventional laboratory incubator-generated rectangular (12:12h) 21:29 °C TC cycles [16] or to gradually changing ramped TC cycles as observed in nature [69]. Our studies unravelled that dTRPA1 receptor-mediated signalling is involved in modifying different aspects of rhythmic, circadian clock-controlled activity/rest behaviour in flies and, most importantly, that dTRPA1 signalling has opposite effects on behavioural output depending on whether the temperature cue was acute or chronic. This is likely the first report of a temperature-dependent behavioural modification in opposite ways mediated by a single thermosensor.

Our results show that under rectangular (12:12h) TC cycles, dTRPA1 plays a very crucial role in regulating 'siesta' during the middle of the day (mid-thermophase under TC cycles). dTRPA1 null mutant flies or flies with RNAi knockdown of dTRPA1 only in a subset of dTRPA1-expressing neurons in the brain do not suppress activity during the mid-thermophase [16]. A lack of siesta in dTRPA1 null mutants during mid-thermophase has been proposed to be due to the lack of sleep during afternoon [17], and preliminary studies suggest that it can reduce the lifespan of these flies (*Antara Das and Vasu Sheeba*, *unpublished data*). The expression pattern of dTRPA1 neurons has been defined by promoter-driven reporter expression as the anti-TRPA1 antibody labels only three pairs of cells in the brain [21] in contrast to ~75–80 neurons targeted by $TrpA1^{KI\text{-}GAL4}$ driver [61]. A previously characterised driver, *dTRPA1-GAL4* [42], targets an even larger

number of neurons; however, we are able to narrow down the function of dTRPA1 in neurons (henceforth, dTRPA1$^{SH+}$ neurons) targeted by a narrow driver, *dTRPA1$^{SH}$-GAL4* [21]. Our study demonstrates that dTRPA1$^{SH+}$ neurons are both necessary and sufficient to mediate suppression of mid-thermophase siesta under TC cycles [16]. Anatomically, GFP-driven expression under *dTRPA1$^{SH}$-GAL4*, as reported by different research groups, revealed that a subset of GFP-tagged dTRPA1$^{SH+}$ neurons overlap with circadian neurons, viz. one s-LNv, fifth s-LNv, three CRY$^{+ve}$ LNd and one DN1a [16, 61, 62]. We, however, show that knocking down dTRPA1 in circadian neurons overlapping with dTRPA1 promoter-driven expression of GFP has no effect on mid-thermophase activity under TC cycles unlike dTRPA1 null mutants. This suggests that dTRPA1 expression in non-circadian neurons mediates suppression of activity during mid-thermophase [16]. Taken together, our results suggest a crucial role for dTRPA1 receptor-mediated signalling primarily in dTRPA1$^{SH+}$ neurons in modifying phasing of morning and evening activity peaks and activity levels during siesta in a temperature-dependent manner. We summarise the role of dTRPA1 in influencing activity/rest rhythms under different light and temperature regimes in Fig. 19.1.

## 19.9 Behaviour of Flies Under Multiple Time Cues in Nature

In the past few years, chronobiologists have embraced the idea of studying rhythms under natural conditions [68-82], although a few older studies under seminatural (SN) conditions have been reported earlier [83–85]. This offers two major advantages over laboratory-based studies in that rhythmic behaviours can be studied in an enriched and complex environment with multiple zeitgebers present and external zeitgebers such as light, temperature and humidity levels change in a more gradual manner, in contrast to laboratory conditions that impose abrupt rectangular cycles. Since it was not possible to track all possible environmental factors in nature that may influence daily rhythms, most studies have mainly focused on how light, temperature and humidity levels may influence behaviours. Since these experiments will invariably impose several levels of artificial interventions, despite being conducted outdoors, we will, henceforth, refer to such regimes as seminatural (SN) conditions, although a previous study refers to such assays as being performed under natural conditions [81].

Studies to examine the impact of natural sensory cues have sparked much interest, and many different behaviours such as activity/rest rhythm [68, 71, 76, 81], emergence rhythm [72, 78, 79] and rhythms in courtship [71] have been assayed. Feeding behaviours and foraging-based learning are also being studied in 'free-flying' greenhouse arenas that mimic natural conditions [86]. In addition to *D. melanogaster*, a comparative study examining four other *Drosophila* species in parallel with *D. melanogaster* across a span of 1.5 years has also revealed

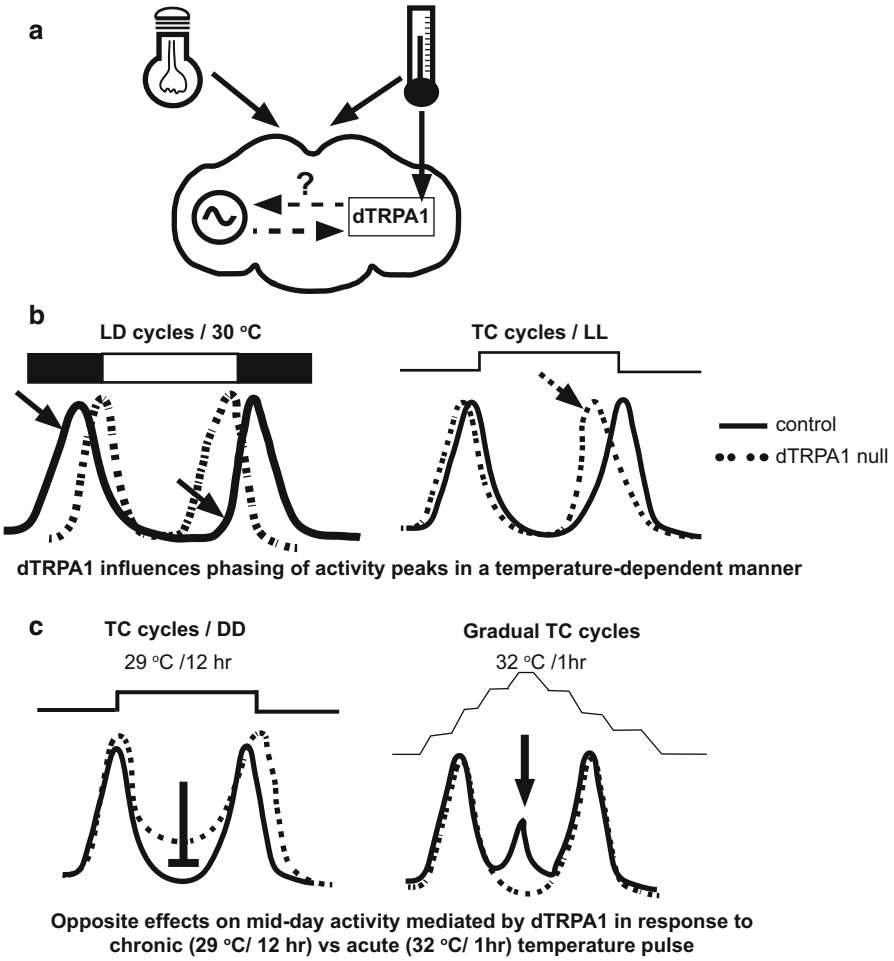


**Fig. 19.1** dTRPA1 influences activity/rest rhythms in a temperature-dependent manner. (**a**) Light and temperature cues synchronise circadian clocks in the brain giving rise to activity/rest rhythms. Thermosensory dTRPA1 channels are activated by temperature inputs, but how signals from circadian clocks and dTRPA1 neurons interact to modify behaviour remains unclear. (**b**, *left*) dTRPA1 signals enable advance of morning peak and delay of evening onset (*arrows*) under constant warm LD/30 °C conditions. (**b**, *right*) Under TC cycles in LL, the lack of dTRPA1 signals result in an advance in the evening peak phase (*dotted arrow*), suggesting that dTRPA1 influences phase of evening peak. (**c**) dTRPA1 mediates opposite effects on midday activity in a temperature-dependent manner. (**c**, *left*) Under TC cycles in DD, when thermophase temperature is 29 °C for 12 h, dTRPA1 signalling is required to inhibit midday activity (control – *solid line*), whereas dTRPA1 null mutants do not efficiently suppress midday activity (*dotted line*). (**c**, *right*) In contrast, under gradual TC cycles wherein peak temperature is 32 °C for 1 h, dTRPA1 mediates the occurrence of afternoon peak (or **A-peak**) in wild-type flies, whereas dTRPA1 null mutants do not display A-peak (*dotted line*)

a variety of subtle differences in temporal patterns of activity/rest among these sympatric species [68, 87]. In addition, the nature of the oscillations of the molecular circadian clock in terms of mRNA levels and protein levels under SN has also revealed unique features [75, 81, 88] that remain to be fully explained.

In contrast to the report of other researchers who have focused on the behaviour of *D. melanogaster* alone [81], our studies on four Drosophilid species under SN suggest that the bimodal activity peaks that are seen under laboratory rectangular time cues of light or TC cycles are in fact the most prominent and persistent feature of locomotor activity rhythms across seasons [79]. Our studies consisted of at least 12 independent assays (each assay spread across at least 6 days) and showed that *D. melanogaster* males exhibit clear morning and evening peaks (>93 % flies exhibit both peaks) in all except one instance (1 out of 12 assays) for *D. melanogaster*. In the case of the one assay, where the M-peak was exhibited by fewer (~68 %) flies, the only environmental variable that could explain such a behaviour was the relatively low-temperature minimum ($T_{min}$) which coincided with the M-peak ($12.7 \pm 0.7$ °C) [79]. On the other hand, the afternoon (A) activity peak, which has been touted as an 'unexpected feature of circadian rhythms' revealed by studies under natural conditions [81], was seen in 100 % of the flies only in one assay (1 out of 12). In one assay it was exhibited by 68 % flies, in three others by less than half of the (35–47 %) flies. In all other assays, as few as 10 % or none at all exhibited the A-peak; see Table 3 in [68]. Another important point to note is that in our assays in no instance do the A-peak increase in amplitude to levels above the M- and E-peaks. Once again this is in sharp contrast to the A-peak reported by some others [81, 89] where the A-peak of wild-type *D. melanogaster* flies appears to be the most prominent feature with its amplitude at par or even higher than M and E-peaks. The above studies as well as others have suggested that high daytime temperature is most likely to influence A-peak [68, 69, 71, 76, 87]. However, we have also seen that, even at much lower temperatures, if flies are exposed to very high light intensity during midday, this can also trigger an A-peak [68, 69, 71, 76, 87]. In fact, our studies showed that with a sufficient separation between environmental light intensity and temperature peaks in SN, we could detect two peaks: one coinciding $L_{max}$ and the other coinciding with $T_{max}$. We also showed that the later peak is dependent on the temperature-sensitive ion channel dTRPA1 [69]. A similar dependence of the A-peak on dTRPA1 was also demonstrated by another study [89]. In our previous study, we showed multiple lines of evidence for shade seeking contributing to A-activity under high light intensity and relatively high temperature (but less than $T_{avg}$ 29 °C) [71]. Our results depict that flies exhibit a greater preference for the middle region of the tube (which appeared to be relatively shaded compared to the rest of the tube) during the afternoon which corresponded to low visually observed activity but high activity recorded from the DAM system (Figure 2A, B, [71]). Therefore, we posited that afternoon peak observed in our study to be mainly the result of shade-seeking behaviour.

Additional evidence came from results showing that flies tend to prefer the shaded regions of the tubes in the afternoon when regions near the food or in the

middle were artificially covered with tape and that such a preference was not observed when the tube was left uncovered (Figure S2B, [71]). We also showed activity from a DAM5 monitor which was not shaded in the middle region. In the absence of additional shading afternoon activity occurred, however, it was significantly increased when the middle region was artificially covered with tape (Figure 2A, [71]). We also showed that there are differences in the amplitude of activity when activity was recorded from different parts of the tube (near food, in the middle or near cotton; Figure S2A, [71]) which suggests that the activity recorded in the DAM system may be influenced by preferences of flies towards different regions despite locomotor activity counts being similar.

In light of the fact that these A-peaks appear to be acute responses to uncomfortably warm temperatures and/or high light intensity, we asked whether such behaviours might be truly circadian clock controlled. Multiple lines of evidence suggest that they are not: (1) the dTRPA1-dependent A-peak can be induced at multiple phases of the day or night as long as temperatures rise above 29 °C ([69] and unpublished data); (2) the fact that the A-peak can be induced even under constant light [69], when it is thought that the canonical circadian clock is disrupted [13]; and (3) the A-peak phase is not altered in a predictable manner upon introducing *per* gene mutations, including a *per* null mutation [71, 76, 81]. Therefore, studies using TriKinetics DAM2 monitors need not concern themselves about a circadian clock-driven A-peak, particularly if the TriKinetics monitors are always kept at temperatures below 30 °C since temperatures above this are too stressful for flies especially when they are placed in constricted spaces [22]. However, it has been proposed that circadian clocks confer an adaptive advantage with respect to the afternoon activity: flies which do not possess an intact clock due to the absence of *per* or severe mutations in other circadian genes such as *Clock* ($Clk^{Jrk}$) and *timeless* ($tim^{01}$) all exhibit 'unproductive' and possibly harmful activity at midday [76].

In contrast to *D. melanogaster*, the species *D. ananassae*, which we have found to be predominantly diurnal with unimodal patt of activity (skewed towards forenoon) when tested under laboratory rectangular cycles of light or temperature [87, 90], continue to exhibit predominantly diurnal activity under all the seasons tested [68]. On the other hand, another less studied yet highly invasive species *D. malerkotliana* is almost identical in its behavioural rhythm to *D. melanogaster* both under laboratory rectangular time cues and under SN conditions [68, 87]. Thus, we find that even in this closely related species, a clear temporal separation of activity is seen under SN similar to our observations under laboratory conditions. Thus, the major features of circadian activity rhythm seen under LD or TC cycles under rectangular time cues in the laboratory are reproduced even under SN across at least four species that we tested. However, many more interesting and useful features of rhythmic behaviours remain to be investigated, and future efforts in this direction which will incorporate more realistic experimental arenas may reveal behaviours that have a more direct bearing on the fitness of flies such as their feeding, mating and egg-laying behaviours. In addition, using net sweeps or fly trap assays across habitats (human dwellings and roofed markets versus gardens,

orchards or open markets) and across seasons, latitudes and altitudes would reveal a more accurate estimate of activity rhythms of fruit flies in nature.

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# Part V
# Circadian Clocks, Metabolism, and Immune Functions

# Chapter 20
# Circadian Clocks, Metabolism, and Food-Entrained Rhythms

**Rohit Chavan, Urs Albrecht, and Takashi Okabe**

**Abstract** The circadian clock is one of the most conserved systems in mammals. It is an important regulator of many biological processes, such as the sleep-wake cycle, hormone secretion, and body temperature, which can influence both cellular and organ-level metabolic functioning. At the molecular level, the circadian system consists of autoregulatory feedback loop that dictates the timing of behavioral and physiological processes. This molecular clock is persistent in all of the central and peripheral tissues. Metabolism can also affect the circadian clock via feeding, or by metabolites which expression is controlled by food intake. Therefore, the current chapter emphasizes the cross-talk between the circadian system and metabolism at the molecular level, and its physiological outcome.

## 20.1 Introduction

The circadian clock is one of the most conserved systems in mammals. It is an important regulator of many biological processes, such as the sleep-wake cycle, hormone secretion, and body temperature, which can influence both cellular and organ-level metabolic functioning. At the molecular level, the circadian system consists of autoregulatory feedback loop that dictates the timing of behavioral and physiological processes. This molecular clock is persistent in all of the central and peripheral tissues. Metabolism can also affect the circadian clock via feeding or by metabolites of which expression is controlled by food intake. Therefore, the current chapter emphasizes the cross-talk between the circadian system and metabolism at the molecular level and its physiological outcome.

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## 20.2 Organization of the Circadian Clock

The central clock is located in the hypothalamus, within a paired structure above the optic chiasm called the suprachiasmatic nuclei (SCN). The SCN play an important role for rhythmic secretion of hormones and the regulation of locomotor activity [1–3], and they hierarchically dictate the circadian timing system. They appear to be the central conductor that is orchestrating the other clocks and entrain the circadian system to the 24-h light/dark cycle. The SCN coordinate the synchronization of peripheral clocks, which is essential to ensure temporal organization of physiological processes [4].

The molecular links responsible for the circadian system is the core complex established by the transcription factors CLOCK and BMAL1 [5]. They heterodimerize and drive the transcription of many *clock-controlled genes* (*CCGs*) by binding to E-box sites (CACGTG) within their own promoters. CLOCK and BMAL1 also regulate the transcription of their own repressors, the members of *period* (*per1/2/3*) and *cryptochrome* (*cry 1/2*) families. The increased transcriptional level of *per* and *cry* genes causes the accumulation of the circadian repressors PER and CRY, which inhibit the transcription driven by CLOCK/BMAL1. Additional elements such as the orphan nuclear receptors ROR and REV-ERB are involved in the global circadian regulation as well.

## 20.3 Connection Between Clocks and Metabolism

### *20.3.1 Evidence from Studies in Mice*

Studies on mice deficient for clock genes have shown that circadian rhythms play a key role in metabolism. Some evidence was found in clock mutant mouse studies. They revealed that mice having arrhythmic feeding and locomotor activity showed hyperphagia, hyperlipidemia, hyperglycemia, and hypoinsulinemia (6). Studies on mice lacking *Bmal1* showed disrupted adipogenesis and carbohydrate metabolism in the liver [7–9]. Furthermore, asynchronous dietary cues may modify glucose homeostasis via their interactions with peripheral molecular clocks [8]. Mice with a liver-specific deletion of *Bmal1* exhibited hypoglycemia restricted to the fasting phase of the daily feeding cycle, exaggerated glucose clearance, and loss of rhythmic expression of hepatic glucose regulatory genes [7]. *Clock* and *Bmal1* mutants show impaired glucose tolerance, reduced insulin secretion, and defects in size and proliferation of pancreatic islets that worsen with age [10].

Metabolic alterations have also been found in deficient mice for target genes of BMAL1/CLOCK. For example, PER2 interacts with PPARγ, which leads to a time-dependent modulation of lipid metabolism. Accordingly, *per2*-deficient mice display altered lipid metabolism with drastic reduction of total triacylglycerol and nonesterified fatty acids [11]. Glucocorticoids and glucocorticoid receptor (GR) are

involved in regulation of glucose homeostasis. It is reported that the *cry1/2* physically interacts with GR [12]. Through this interaction, the expression of the *phosphoenolpyruvate carboxykinase 1* (*Pck1*), which is directly regulated by GR, is reduced, and subsequently *cry*-deficient mice show the increased *Pck1* expression. The *Rev-Erbα*, which is the major suppressor of *Bmal1* transcription [13], is expressed in a variety of tissue types, including brown fat, skeletal muscle, and liver [14,15]. In addition to its action in the circadian clock mechanism, the biological function of *Rev-Erbα* has been implicated in metabolism [16,17]. In the case of *Rev-Erbα* knockout (KO) mice, although these animals showed altered expression of clock components, they did not show disrupted rhythms of behavior [13]. The study on *Rev-Erbα* and *β* double KO mice also revealed the link with lipid metabolism [16,17]. These animals displayed altered locomotor activity and disrupted lipid metabolism. Feeding-induced obese mice treated with Rev-Erb agonists showed decreased fat mass, weight gain, triglyceride, and cholesterol levels [18]. Interestingly, overexpression of *Rev-Erbα* in the liver caused disrupted lipid metabolism [19].

### *20.3.2 Evidence from Studies in Humans*

In humans, several genetic studies showed a connection between circadian clock and metabolism. Genetic variants of *Clock* and *Bmal1* have been related with metabolic syndrome [20–24]. *Per2* variants are also involved in metabolic syndrome such as high blood glucose levels and abdominal obesity [25,26]. *Cry2* variants are also related to glucose homeostasis [27,28]. Thus, several genetic studies in humans revealed a close connection between clock disruption and metabolic syndrome.

### *20.3.3 Evidence from Genome-Wide Profiling Studies*

Studies using genome-wide profiling have provided evidence for a close link between circadian clock and metabolism. A recent study showed that around 15 % of the liver transcriptome is expressed in a circadian manner and manipulation of feeding schedule can regulate the expression of these genes [29]. In the liver, these include DBP, TEF, and HLF [30], E4BP4 [31], the Krüppel-like factors KLF10 [32] and KLF15 [33], and nuclear receptors [34]. These transcription factors are reported to regulate genes that are involved in the metabolism.

## 20.4 Metabolic Feedback onto Clock Genes

SIRT1 plays an important role in nutrient availability, liver gluconeogenesis, lipolysis, and insulin secretion [35]. SIRT1 deacetylates histones and several transcription factors [36]. SIRT1 physically interacts with CLOCK/BMAL1 heterodimers, resulting in a rhythmic deacetylation of BMAL1 [37] and degradation of PER2 [38]. Since activity of SIRT1 depends on the nicotinamide adenine dinucleotide ($NAD^+$), it seems that SIRT1 can connect metabolism to the circadian network. Furthermore, SIRT1 also influence the activity of PPARα [39] and PGC-1α [40]. PGC-1α seems to be connected to the circadian clock because its expression is rhythmic and it can be a co-activator of ROR [41], which is known as an activator of transcription of *Bmal1* (Fig. 20.1). Since SIRT1 activity is regulated by availability of the $NAD^+$, studies were performed for understanding if this cofactor could be a circadian target as well. The rate-limiting enzyme in $NAD^+$ biosynthesis is nicotinamide phosphoribosyltransferase (NAMPT). Increased NAMPT results in increased synthesis of $NAD^+$, which increases the activity of SIRT1, also known as $NAD^+$-dependent deacetylase. The ratio of $NAD^+$/NADH modulates CLOCK/BMAL1 DNA-binding activity [42]. It is also reported that in the liver the activity of poly(ADP-ribose) polymerase 1 (PARP-1), a $NAD^+$-dependent ADP-ribosyltransferase, oscillates in a daily manner and is regulated by feeding [43]. PARP-1 is known to bind and poly-ADP-ribosylate CLOCK at the light phase. Thus, loss of PARP-1 increases the activity of CLOCK/BMAL1 and influences the circadian rhythmicity of *Per* and *Cry*. As a consequence, *parp-1*-deficient mice show altered expression of genes regulated by CLOCK/BMAL1.

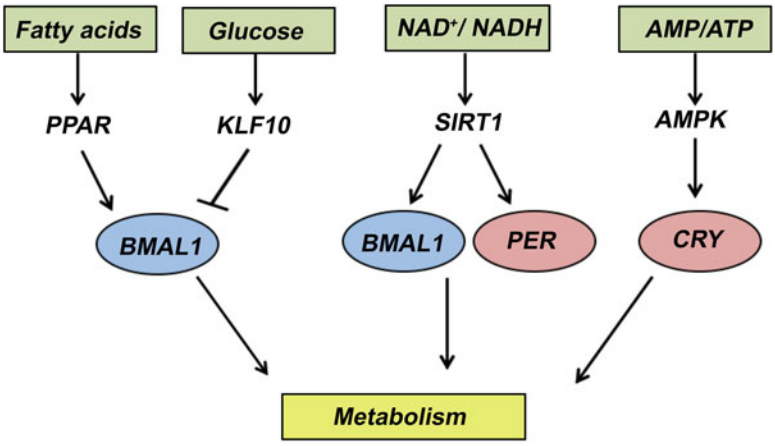


**Fig. 20.1** Metabolic regulators and circadian clock components. A schematic representation of the interactions between metabolic regulators with clock components in the liver is shown. Krüppel-like factor 10 (*KLF10*) and PPARs regulate *Bmal1* expression by binding to the corresponding recognition sequences in the *Bmal1* promoter. Levels of NAD+ influence SIRT1 activity, which deacetylates BMAL1 and PER2 proteins. AMPK, which is a major sensor for the AMP/ATP ratio, can directly phosphorylate CRY1, leading to destabilization and degradation of CRY1

Glucose is also involved in regulation of circadian clock. Glucose appears to upregulate KLF10 [44]. It is known to bind to GC-rich elements in the *Bmal1* promoter and subsequently represses *Bmal1* transcription. Another important factor for metabolic state is the ratio between AMP and ATP. A major sensor for the ratio between AMP and ATP is adenosine monophosphate-dependent protein kinase (AMPK). It can directly phosphorylate and destabilize CRY1 [45]. Food components can also affect circadian clocks. When mice are fed with ketogenic diet, circadian rhythmicity of wheel-running activity under constant darkness is altered [46]. High-fat diet also causes a significant reformation of circadian oscillation of transcripts and metabolites in the liver [47]. These can be explained both by interfering with recruitment of CLOCK/BMAL1 to chromatin and by inducing PPARγ-driven oscillation on noncyclic genes.

## 20.5 Entrainment of Peripheral Clocks by Feeding Cues

At the systemic level, the circadian clock mechanism is related to metabolism. One of the most important external inputs to the metabolic systems are feeding signals. Through the temporal feeding restriction, the link between the central clock and the liver clock can be disrupted [48]. A recent study also revealed that time of feeding can regulate circadian rhythmicity in hepatic gene expression [29]. They used distinct feeding and fasting paradigms on wild-type (WT) and circadian clock-deficient mice. Accordingly, both food availability and the temporal pattern of feeding determined the phase and amplitude of the circadian transcriptome in WT liver.

## 20.6 Food-Anticipatory Activity and Food-Entrainable Oscillator

Food availability is a strong external cue that modulates locomotor activity in rodents. Under ad libitum feeding condition, nocturnal animals (such as mice and rats) favor food intake during the active phase (i.e., subjective nighttime). Under ad libitum feeding condition, the mealtime and its associated behavior are coordinated by the SCN [49]. When food access is restricted to daytime, mice display preprandial locomotor activity, preprandial rise in the body temperature, and the release of corticosterone. Such daily arousal and prefeeding activity around 2–3 h prior to the mealtime is designated as "food-anticipatory activity (FAA)" (Fig. 20.2). Under the normal light/dark cycle (LD) and daytime RF condition, mice display FAA in the daytime with subsequent reduction in nighttime activity. In addition, FAA is also observed in animals subjected to constant dark (DD) or constant light (LL) condition. When mice were shifted from restricted feeding to ad libitum

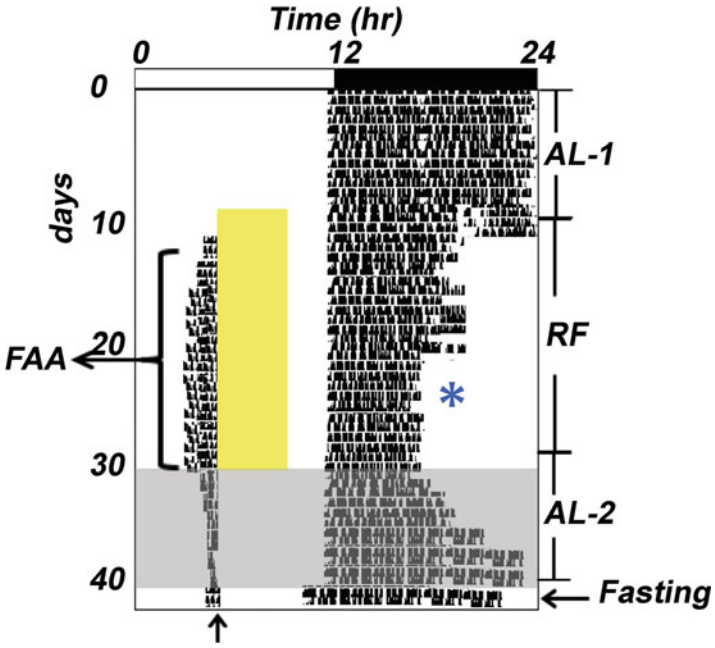


**Fig. 20.2** An exemplary plot of wheel-running actogram of a nocturnal rodent under 12 h:12 h LD cycle. From day 1 to 10, the animal showed activity in dark phase under ad libitum food access (AL-1). When subjected to temporal restricted feeding (*RF*) condition (*yellow area* indicates duration and time of food availability), the animal exhibited increased wheel-running activity 2–3 h preceding to mealtime, defined as food-anticipatory activity (*FAA*). Furthermore, RF condition is marked by decrease in nighttime activity (indicated by *). The animal was kept under constant darkness (*gray shaded area*) with food ad libitum (AL-2) and followed by fasting for the last day. In AL-2 and fasting condition, the animal displayed the food-anticipatory bouts at low magnitude but in phase with previously entrained mealtime; it indicates presence of self-sustained food-entrained circadian system (*arrow*)

feeding condition, FAA appeared at the expected time for several days [50]. This demonstrates the presence of another internal circadian timing system, which allows mice to adapt and memorize their own mealtime. It means that FAA is not a momentary phenomenon generated by acute fasting. This internal clock predicting the feeding time is called the "food-entrainable oscillator (FEO)," and FAA is a behavioral manifestation of the FEO.

### *20.6.1 FEO Is Independent of the SCN*

The SCN has been identified as the prime site of the light-entrainable oscillator [3], and the underlying molecular mechanism depends on the interlocking autoregulatory feedback system of clock genes [5]. On the other hand, the FEO

location and underlying molecular mechanism remain to be elusive. Since the SCN is the circadian pacemaker, it was postulated that this region could be one of the components of the FEO, but specific experiment did not confirm this hypothesis. Indeed SCN ablation in rats led to arrhythmicity in their day-night locomotor activity, but FAA and feeding-induced corticosterone rhythms still persisted. When SCN-damaged rats were shifted from RF to total food deprivation, FAA was still observed for several days. Furthermore, FAA is readily observable under feeding a regimen with a period within circadian range, which highlights the limits of entrainment [49,51]. Considering the strong evidence showing that FAA remains after the SCN lesion, the FEO may be present in locus or loci outside of the SCN.

### *20.6.2 Location of the FEO*

Following the trail of light into the brain led to the discovery of the SCN as a site of a master circadian clock; a similar approach was applied to the FEO search. The strategy consisted of manipulating or destroying the communication pathways, which may transmit food-related cues to the central nervous system. Some studies aimed to detect those properties of food, which can convey mealtime information to drive FAA. These properties include gustatory, olfactory, food digestion-induced stimuli, and nutrients in the bloodstream. However, nasal epithelium disruption [52], olfactory bulb ablation [53], alteration of hepatic function [54], and subdiaphragmatic vagotomy [55] did not affect food-anticipatory behavior in rats.

The SCN lesion study provided an important methodological approach to quest of FEO location. Ablation of specific brain area was performed, and mice were subjected to a temporal restricted feeding condition. Because of the involvement of the hypothalamus in the regulation of the circadian clock, food intake, and satiety signal processing, it was one of the structures likely to harbor the FEO. However, lesions of the paraventricular hypothalamus (PVN) [50], the lateral hypothalamic (LH) [56], the arcuate nucleus (AN) [57], the ventromedial hypothalamus (VMH) [58], the nucleus accumbens (NAc) [59], the hippocampus, and the amygdala [60] showed weak or no influence on FAA expression. The hindbrain possesses various nuclei, which are involved in control of food intake and hunger. The lesions of caudal brainstem nuclei such as the nucleus of the solitary tract (NTS) [61], the area postrema (AP) [62], and the parabrachial nucleus (PBN) [63] did not abolished FAA.

The dorsomedial hypothalamic nucleus (DMH) is one of the important components in various neural and humoral pathways regulating feeding behavior and body weight. It is connected to other brain regions, which are involved in the regulation of the sleep-awake cycle and body temperature [64]. The cell-specific lesion in DMH had abolished the preprandial rise in body temperature, locomotor activity, and wakefulness. In addition, neuronal activity and *period* gene expression in the DMH entrained to mealtime under scheduled feeding conditions [65,66]. In another report, rats with complete and fractional DMH ablation displayed normal FAA

relative to intact ones. Hence, the DMH seems not exclusively and explicitly responsible for FAA [67]. However, earlier studies suggested that the DMH actively inhibits the SCN output and facilitates functioning of the FEO located elsewhere in the brain. Thus, the DMH can play a modulatory role in FAA expression [68]. Taken together, the role of the DMH in FEO is still highly contentious. Further functional rescue experiments will be needed to explain the role of the DMH in FAA.

Numerous contradictory observations from single central substrate lesion studies suggested that FAA expression might be an integrated outcome of a network of brain structures. Under RF conditions, *c-Fos* showed temporal expression profile in the PVN, LH, and DMH [69]. In addition, brain stem nuclei, particularly the NTS and PBN, showed neuronal activation in phase with feeding time [70]. This indicates that brain stem nuclei may deliver necessary neuronal entraining information to hypothalamic nuclei for FAA. Increased c-Fos expression at expected mealtime was observed in some other structures involved in motivational process such as the prefrontal cortex, lateral septum, stria terminalis, paraventricular thalamic nucleus, and NAc, except in the hippocampus. This suggests a relevant role of corticolimbic structure in the FEO [71]. Another strategy involves the assessment of local cerebral metabolic rate for glucose in RF during FAA expression. It was hypothesized that structures showing significant alteration in glucose utilization under RF would be part of a system responsible for FAA appearance. The intergeniculate leaflets and the paraventricular hypothalamic and the arcuate nuclei showed decreased glucose metabolic rate during food anticipation [72].

All these findings indicate and support the concept of a multi-structural organization of the FEO, which appears to be composed of several hypothalamic, extrahypothalamic, and peripheral tissues.

### *20.6.3 Clock Gene Mutation and FAA*

The discovery of clock genes has steered the insights into the molecular and genetic working of the circadian clock. The plausible question was raised, i.e., whether the same clock complex regulates the FEO. In order to address this question, various clock gene mutant mice were subjected to temporal RF conditions. It was hypothesized that the clock gene mutation that is critical for FEO functioning should show impaired FAA. *Clock* mutant mice exhibited FAA, when subjected to temporal restricted food access in LD and DD conditions. This indicated that the *Clock* gene is not necessary for FAA expression [73]. *Npas2*-deficient mice did not show any major changes in their circadian behavior relative to WT [74]. Under RF conditions *Npas2* knockout mice showed delayed FAA expression. It implies that NPAS2 is responsible for adaptability to food restriction [75]. In a report published in 2005, *Cry1*/*Cry2* double mutant mice developed less stable food-anticipatory rhythms with delayed onset compared to their littermate controls. It suggests alteration, but

not complete loss of FAA in *Cry1/Cry2* double mutant mice [76]. It appears that the *cry* genes are not essential, but necessary for the stability of FAA expression.

It has been demonstrated that *Per1* and *Per2* genes play important roles in light-induced synchronization of the SCN [77]. Therefore, *Per* genes may be responsive to other external synchronizer such as food. *Per1* mutant and WT mice did not show significant difference in FAA, when challenged with temporal RF condition. Surprisingly, *Per2* mutant mice failed to anticipate the mealtime [78]. These results highlighted a role of *Per2* in molecular regulation of the FEO. Pendergast et al. (2009) has reported that *Bmal1*-deficient mice exhibited FAA during RF in LD condition and robust FAA in DD condition [79]. Though FAA is unstable and imprecise in a 24-h feeding regimen, *Per1/Per2/Per3 triple mutant* mice were able to anticipate mealtime [80].

Some clock gene mutant mice showed unchanged or reduced FAA amplitude. But, lack of food-anticipatory rhythms in *Per2*-deficient mice suggested the involvement of one circadian clock component in the FEO mechanism. Studying FAA in tissue-specific clock gene knockout mice might provide critical inputs in FEO search.

## 20.7 Food Entrainment and Peripheral Oscillators

Feeding-related cues could entrain clock gene expression in peripheral tissues. The temporal restricted food access modulates clock gene expression in the liver, kidney, heart, and pancreas without affecting the phase of clock gene expression in the SCN [48]. The feeding-induced phase resetting occurred faster in the liver compared to other peripheral organs, shifting rhythms by 10 h in 2 days [81]. In SCN-lesioned mice, temporal feeding can entrain and reorganize the circadian profile of liver gene expression [82]. It indicates that metabolic changes caused by feeding cycles can lead to dissociation of peripheral oscillators from the SCN. In ad libitum food access, feeding behavior is associated with the activity phase, and the metabolic signals cycle in harmony with the SCN. Thus, the SCN can set the phase of clock genes in peripheral tissues. However, under RF conditions, many metabolic and hormonal signals are shifted according to the mealtime. These signals include glucose, free fatty acids, glucocorticoids, ketone bodies, and some hormones [83]. Different blood-borne factors alone or in combination with other signals can reset the clock in the peripheral tissues [84]. These metabolic and hormonal cues may play a role in FEO functioning.

Glucocorticoids can alter the circadian clock gene expression in many peripheral organs [85], implying a role of glucocorticoids in phase resetting of peripheral tissues. Under RF, corticosterone (CORT) level peaked prior to the mealtime. The correlative rise of CORT and increased prefeeding activity have led to the hypothesis that CORT may act as signal for FEO-related behavioral output [86]. However, the adrenalectomized rats showed no noticeable difference in the magnitude of FAA compared to intact ones [87]. This finding indicates that CORT is not able to

express scheduled feeding-induced behavior. Yamamoto et al. (2005) showed that CORT-induced *Per1* expression does not cause a phase shift in clock gene expression [88]. Furthermore, adrenalectomized rats entrained rapidly to RF compared to intact animals [89]. Taken together, the role of glucocorticoids as an entraining signal for peripheral clocks is still controversial.

Acyl ghrelin and des-acyl ghrelin are gastrointestinal peptide hormones synthesized by gastric oxyntic cells and in medial-lateral hypothalamic nuclei. Ghrelin peptides can stimulate feeding in rats and mice. Food-restricted rats and mice revealed preprandial rise in plasma ghrelin. Temporal RF condition can entrain daily rhythms of circadian clock gene expression in oxyntic cells of the stomach [90]. The FAA examination in ghrelin ligand and ghrelin receptor knockout mice indicated that ghrelin is not necessary for FAA [91,92]. In contrast, Davis et al. (2011) showed that ghrelin receptor signaling was necessary for adaptation to the scheduled feeding-induced anticipatory response [93]. In addition to ghrelin, several other metabolic hormones such as leptin, insulin, glucagon, and glucagon-like peptide were assessed for their role in food-entrainable rhythms. However, these metabolic hormones are not necessary for FAA expression, but can act as cues to modulate FAA [94].

The peripheral oscillators have the ability to be entrained rapidly to feeding schedule and metabolic cues. Although they are not necessary for FAA expression, they can play a role in the modulation of the anticipatory activity. Coordination of central and peripheral oscillator systems need to be studied under RF conditions to elucidate the mechanism behind the FEO.

## 20.8 Clinical Relevance

Because circadian clocks and the metabolism are strongly coupled and clock gene mutant mice exhibit altered lipid and glucose homeostasis, the circadian clock mechanism seems to be related with a number of metabolic diseases such as obesity, dyslipidemia, and diabetes. Hepatic overexpression of *Cry1* lowered blood glucose concentrations and improved insulin sensitivity in insulin-resistant db/db mice [95]. In primary hepatocytes, the synthetic CRY1/2 agonists inhibited glucagon-induced gluconeogenesis [96]. These studies suggest that compounds that enhance *cryptochrome* activity may provide therapeutic benefit to individuals with type 2 diabetes. The use of a PPARα agonist causes a phase advance of locomotor activity and feeding rhythm. Given that disrupted circadian rhythms lead to obesity, activation of PPARα can serve as a clinical target for the modulation of both circadian rhythms and metabolism [97]. As highlighted in this chapter, food entrainment can affect the circadian clock mechanism, suggesting that lifestyle can affect human health through the circadian clock mechanism. Given that meal timing plays an important role in synchronizing behavioral and physiological rhythms, it is possible that defects in food entrainment could impair circadian organization of physiology and metabolic function. These studies reveal the

potential of operating on circadian clocks as strategy toward therapeutics for metabolic diseases.

## 20.9 Summary

- Studies on clock gene-deficient animal models have revealed that circadian rhythms have a key regulatory function in metabolism.
- The clock machinery controls the expression of genes essential for numerous metabolic pathways at the molecular level.
- Circadian oscillators exist not only in the SCN but also in most peripheral tissues, and alterations in feeding rhythm can affect the circadian system.
- Scheduled feeding can organize and entrain peripheral circadian clocks.
- Numerous studies highlighted distributed location and integrated mechanism among central and peripheral clocks behind FEO.

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# Chapter 21
# Circadian Regulation of Metabolism in Health and Diseases

**Breanna Sarkisian, Neelu Jain Gupta, and Satchidananda Panda**

**Abstract** The importance of circadian rhythm has been established through its evolutionary conservation and its connection to many health conditions. Circadian deregulation has emerged as an important risk factor for metabolic disruption and related chronic diseases. Chronic diseases are on the rise worldwide, and emerging evidence points toward restoration of circadian rhythms as a propitious approach to preventing and improving prognosis of many chronic disorders. This review will outline the evidence supporting the importance of the circadian system in metabolism by debriefing the molecular basis for the interaction between circadian timing and metabolic health and behavioral, genetic, and human epidemiological studies indicating the health implications of chrono-disruption.

## 21.1 Introduction

Metabolic homeostasis is central to a healthy lifespan. Traditionally, homeostasis is described as the property of a living system to establish and maintain a condition of equilibrium in its internal environment, even when faced with acute changes. As most plants, animals, and microbes in this planet have evolved to adopt to the predictable changes in light, temperature, humidity, and food availability, mechanisms maintaining metabolic homeostasis have also evolved to adapt to this diurnal changes. Accordingly, a major function of the circadian clock is to anticipate the daily cycle of nutrient intake and nutrient expenditure and maintain an internal equilibrium within a defined range of parameters. As a large number of metabolites and cellular contents are to be maintained at equilibrium in different organs, the circadian oscillator makes intricate connection with homeostatic mechanisms in a tissue-specific manner. Disruption to this homeostasis of a given metabolite can

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occur due to chronic changes in the availability, storage, or use of the metabolite or due to disruption to the circadian modulation of homeostatic regulation.

Metabolic disruption leads to many chronic diseases, including cardiovascular diseases, obesity, diabetes, and related comorbidities. As of 2012, it is estimated that half of adult population in the USA has one or more chronic health conditions [1], and 35 % suffer from metabolic syndrome [2]. To be diagnosed with metabolic syndrome, one should have at least three of the five metabolic risk factors: large waistline, high triglyceride level, low HDL cholesterol, high blood pressure, and high fasting glucose [3]. As of now, 60 % of deaths worldwide are due to noninfectious chronic diseases, and it is estimated that as much as 80 % of premature stroke, heart disease, and diabetes could be prevented [4]. An important approach to preventing these consequences involves restoring healthy lifestyle. As the daily rhythm in activity-sleep and feeding-fasting and the dependent rhythms in energy metabolism are integral part of lifestyle, it is becoming increasingly apparent that circadian regulation of metabolism has a central role in both prevention and prognosis of chronic metabolic diseases.

From an evolutionary perspective, for most part of human history, humans have lived with a robust daily rhythm in activity-rest and the associated rhythm in feeding-fasting. Early hunter-gatherer populations often ate sparingly mostly during the daytime, and, even today, these populations rarely suffer from cardiovascular diseases and other metabolic complications [5]. Accordingly humans and animals have developed adaptations for an intermittent food supply. This adaptive mechanism includes organs for *the uptake* and storage of rapidly mobilizable glucose and longer-lasting energy substrates, such as fatty acids in adipose tissue. Biochemical programs have also evolved to adapt to intermittent food intake: anabolic metabolism during feeding and catabolic metabolism during fasting. With the predictable nature of feeding-fasting cycle tied to the daily cycle of activity-rest, circadian oscillators have evolved to integrate ~24 h rhythms to anabolic and catabolic metabolism from single cell to the whole organism. Hence, organisms have evolved to go through the cycles of feeding, energy storage, use of stored energy during fasting, and trigger of hunger to reinitiate the cycle with a bout of feeding. As modern humans are untethered from the natural light-dark cycle and food is plentiful, chronic disruption of this genomic program of going through intermittent daily cycle of feeding and fasting is emerging as an important disruptor of metabolic homeostasis.

## 21.2 Organization of Circadian System in Mammals

### *21.2.1 Core Clock Players*

The circadian clock circuitry acts to produce rhythms in behavior and physiology in anticipation of environmental changes. For metabolic functions, this can be

accomplished via rhythmic expression of genes encoding regulators and enzymes of important metabolic pathways. The core components of the circadian clock network include transcription factors CLOCK and BMAL1, which heterodimerize and bind to the E-box motifs on clock-controlled genes (CCGs) to drive their transcription. Two such CCGs include PER and CRY genes which, when activated throughout the day, cause accumulation of PER and CRY proteins by the night and form heterotypic complexes with additional proteins [6]. These complexes associate with CLOCK-BMAL1 heterodimers and repress their transcription activation potential. The autorepression of Per1/2 and Cry1/2 genes by their own PER/CRY proteins leads to a decrease in accumulation of these proteins, eventually allowing a new cycle of Per1/2 and Cry1/2 transcription to occur [6].

Similarly, another feedback loop of the clock system involves proteins REV-ERB, whose levels increase during the day and bind ROR-REVERB-response elements (RREs) within Bmal1 promoter, thus inhibiting Bmal1 transcription. At night, REV-ERBα protein amounts are low, allowing Bmal1 transcription to rise. The repressive effects of REV-ERB are in constant competition with the retinoic acid related orphan receptors (RORs) (a, b, g), which also bind to RRE on Bmal1 promoter to activate transcription [7, 8]. These transcriptional-translational regulatory loops operate in most tissues and control a notable fraction of the mammalian genome, with many of them also being implicated in metabolic pathways [9–11].

This modulation of transcription and subsequent feedback based on protein accumulation allows the circadian system to fine-tune its regulation based on internal and external factors. In addition to self-regulation via product accumulation, the clock system can be affected by feeding timing and other stimuli.

### *21.2.2 Local vs. Systemic Control*

Every cell in an animal must adapt to the daily rhythm of food availability. At the same time, metabolic homeostasis involves tight coordination among several metabolic organs and neuroendocrine tissues. Furthermore, this complex coordination for energy homeostasis needs to be coupled to the organism's activity-rest cycle. The circadian regulatory system functions to temporally coordinate these complex mechanisms. The circadian system in mammals has a hierarchical architecture, with a central oscillator in the hypothalamic suprachiasmatic nucleus and peripheral clocks in almost all organs in the body. The SCN oscillator is entrained to the ambient light to be in sync with the 24 h light/dark cycle [12]. The cell-autonomous molecular circadian oscillator has nearly the same makeup in the SCN as well as in the periphery, but cells in the SCN are coupled via synaptic and paracrine signals. Coupling among the individual SCN neurons constitute a robust oscillator and makes it less susceptible to mild perturbations [13]. Peripheral oscillators do not appear to communicate with each other through tight local communications. Rather, they rely on systemic signals for phase coherence. Indeed, in SCN-lesioned mice, peripheral tissues gradually become out of phase with the

external light-dark cycle and with circadian oscillations in other tissues [14]. Similarly, synchronization is gradually lost in tissue explants and transiently synchronized fibroblasts [15, 16].

The SCN imparts temporal information to the peripheral clocks via systemic cues [14]. The peripheral oscillators are also sensitive to nutritional cues. In this way, circadian physiology gets modulated systemically from the central clock or locally via tissue-specific clocks in response to local metabolites. The discrimination between these two routes has been seen in mouse liver, in a circadian transcriptome analysis in mice with or without functional hepatocyte clocks [17, 18]. These studies have shown the liver circadian transcriptional rhythms are partly mediated by the cell-autonomous clock and "driven" by systemic rhythmic signals generated elsewhere in the body. Multiple systemic candidate signals have been suggested including steroid hormones and fluctuation in core body temperature [19]. In experiments relevant to circadian coordination of metabolism, daytime feeding of naturally nocturnal rodent can shift the phase of circadian gene expression in the liver, regardless of lighting conditions, leaving the phase of cyclic gene expression in the SCN unaffected. Thus, changes in feeding timing can uncouple peripheral oscillators from the central pacemaker [20–22].

Feeding modulation of peripheral clock suggests that time of eating is a strong zeitgeber (entraining cue) for peripheral clocks and might even supersede the synchronization cues received from the SCN clock. Under optimal conditions, the periphery and SCN are synchronized: the SCN imposes feeding rhythms by driving rest-activity cycles using food-dependent zeitgebers in addition to other cyclic cues (like body temperature or blood-borne signals) to set the phase in the periphery. However, alteration of metabolism, whether through food content or feeding timing, can lead to desynchronization of circadian clocks and a reciprocal disruption of metabolic pathways that seems to underlie the resulting adverse health effects to be termed as chrono-disruption. The mechanisms underlying this cross talk between food consumption and clock-driven metabolic control are complex [23] and require further elucidation; essential components of this system are discussed in the sections to follow.

## 21.3 Cross Talk: Molecular Basis of Circadian Oscillator-Metabolism Interface

There is extensive interplay between the clock system and metabolic inputs and outputs. Many clock target genes are directly involved in metabolic pathways, including lipid metabolism and the regulation of metabolic timing in peripheral tissues. One general example is the action of CLOCK/BMAL on the set PAR bZIP proteins DBP, HLF, and TEF, which causes these proteins to be transcribed with robust circadian rhythms in the SCN and peripheral tissues such as the liver. PARbZIP proteins have been found to target various enzymes involved in major

xenobiotic metabolic pathways [24]. Additionally, the metabolic states can be communicated to and modulated by glucose-sensing pathways, NAD+ sensing pathways, and nuclear receptor pathways.

### 21.3.1 Glucose Homeostasis

CLOCK/BMAL1 plays an integral role in glucose homeostasis. The clock circuitry can both act on and respond to glucose levels. BMAL1 itself promotes hepatic gluconeogenesis [25, 26], and there are many additional indirect pathways by which clock is involved in glucose regulation. As previously stated, CLOCK/BMAL1 activates CRY and PER genes, which are heavily implicated in glucose sensing and regulation, via interactions with AMPK, CREB, KLF10, and insulin pathways.

AMP-activated protein kinase (AMPK) serves as a sensor for the ratio of AMP/ATP within cells, putting it at the center of cellular energy homeostasis [27]. Increase in this ratio will lead to phosphorylation and activation of AMPK. AMPK activation leads to increased glucose uptake and decreased gluconeogenesis. It is also implicated in hepatic fatty acid oxidation, mitochondrial biogenesis, and insulin sensitivity. There is a consensus motif for phosphorylation of AMPK within CRY, so that phosphorylation of AMPK leads to phosphorylation and destabilization of CRY/PER complexes, disrupting their accumulation and thus derepression of the CLOCK complex [28]. This provides one pathway that communicates metabolic state to the CLOCK circuitry.

CREB also acts in glucose regulation via its action on gluconeogenesis. Fasting state leads to glucagon binding to its membrane receptor in hepatocytes, which activates a downstream G protein *Gs* and increases intracellular cAMP levels, leading to the phosphorylation of CREB [29]. pCREB shows robust rhythmic expression in the liver and acts to increase gluconeogenesis. CRY can also interact with Gs or adenylate cyclase to regulate temporal expression of pCREB and thus temporal regulation of gluconeogenesis [30]. In this way, clock genes provide important regulation in glucose homeostasis.

Conversely, there are also pathways by which glucose levels can affect clock action. The mRNA accumulation of KLF10, a negatively acting $Zn^{2+}$ finger transcription factor, follows a diurnal rhythm in mouse liver that may be driven by intracellular glucose concentrations. Increased glucose upregulates KLF10, which binds to BMAL1 promoter and dampens BMAL transcription [31].

### 21.3.2 $NAD^+$ Sensing Pathways

$NAD^+$ levels fluctuate with metabolic state. Glycolysis oxidizes NADH to NAD+ during the reduction of pyruvate to lactate. Fatty acid synthesis also produces

higher NAD+ levels. NAD+ level shows daily oscillations in the liver, likely due to oscillations in Nampt transcription [32, 33]. NAD+ levels also vary in response to DNA damage, via damage-induced poly(ADP-ribose) polymerase-1 (PARP1), which acts to reduce NAD+ levels [34]. PARP normally has rhythmic accumulation in the liver. Additionally, in day-fed rodents, PARP oscillation is inverted, suggesting circadian patterns of PARP are food dependent. In the absence of PARP activity, the hepatic clock takes a long time to readjust to a shifted feeding window, suggesting PARP plays an important role in the phase entrainment of liver oscillators [35]. PARP activity directly impacts NAD+ levels, which can subsequently affect sirtuins, a family of NAD+-dependent deacetylases that impact a multitude of metabolic pathways and the circadian clock.

SIRT1 participates in cross talk with FoxO genes, providing a mechanism by which SIRT impacts metabolic pathways. During long-term fasting, NAD+ levels increase, which upregulates SIRT activity. This leads to deacetylation and activation of FoxO, which in turn impacts an array of metabolic pathways [36–38].

Additionally, NAD+-activated SIRT1 deacetylates and destabilizes sterol regulatory element-binding protein (SREBP), a transcription factor that upregulates synthesis of enzymes involved in sterol biosynthesis. The destabilization causes SREBP to build up and increase fatty acid and cholesterol synthesis [39]. The increase in sterols and fatty acids means an increase in ligands for nuclear receptors like PPAR and ROR (discussed in more detail in the next section). These can act on the CLOCK/BMAL system directly or via effects on PER/CRY accumulation, modulating the feedback pathway.

Worth noting, there are many other pathways by which SIRT can influence metabolism. Sirt1 can act on STAT3 in gluconeogenesis, NFKB in insulin secretion and sensitivity, LXR in lipid metabolism, CRTC2 in gluconeogenesis, and PGC1a in gluconeogenesis and fatty acid oxidation (reviewed in [40]). SIRT1 acts both as a sensor and regulator of metabolic pathways. The fact that it is regulated by circadian expression of NAD+, combined with its ability to influence pathways that regulate the core clock system, puts SIRT1 at an important node of the cross talk between metabolism and circadian circuitry [41].

### *21.3.3 Nuclear Receptor Pathways: PPAR, Reverb, ROR*

In the mouse genome, 20 of the 49 nuclear receptor genes are expressed in a circadian manner, and nearly all of these nuclear hormone receptors implicated in metabolic processes [42]. Several of these are also implicated in clock machinery, either directly or indirectly. These include, among others, PPAR, REVERB and ROR.

As mentioned earlier, ROR proteins are activators of Bmal1 transcription. REVERB inhibits ROR function by competing for the same DNA binding site and thus inhibits Bmal1 transcription, thereby producing rhythmic levels of Bmal1 mRNA. Functionally active ROR-REVERB-response elements (RREs) are also

found to regulate the accurate temporal expression of additional circadian oscillator components [43–45].

Nuclear receptor corepressor (NCoR) and HDAC3 complex is recruited to enhance resident REVERB where it is tethered to DNA by tissue-specific transcription factors. Given the Reverb is strongly circadianly expressed, rhythmic repressive action of NCoR/HDAC3-REVERB exerts circadian regulation of tissue-specific outputs that are independent of ROR regulation [46].

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptors that function as transcription factors for numerous target genes, especially those encoding enzymes important for metabolic pathways. All three PPAR isoforms are expressed in a circadian manner in mouse tissue, and PPARα and PPARγ are direct regulators of core clock components, Bmal1 and Reverbα. Conversely, PPARα is also a direct Bmal1 target gene. PPAR lipid regulators continue to express with daily rhythms even when clock-disrupted mice are subject to daily eating-fasting rhythms [47, 48]. This data implicates rhythmic PPAR expression is supported by both the circadian clock and the feeding-fasting cycle.

## 21.4 Health Implications of Circadian Disruption

In the last century, the leading causes of morbidity and mortality in modern societies have shifted from infectious diseases to noninfectious chronic diseases including hypertension, hyperlipidemia, coronary artery disease, diabetes, cancer, and diseases of chronic inflammation [49]. Chronic disruption of circadian rhythm in experimental animals or as commonly experienced among shift workers raises the risk for many of these diseases. These correlative observations have catalyzed a rapidly expanding research into understanding the mechanism underlying the connection between circadian disruption and chronic diseases. We will discuss a few examples here and encourage the readers to explore these connections in several recently published excellent reviews [23, 41, 47, 50, 51].

### *21.4.1 Genetic Perturbations*

Genetic perturbation of circadian oscillator in mice predisposes to metabolic pathologies. Such genetic perturbation models have been invaluable in understanding the mechanistic link between the circadian clock proteins and metabolic homeostasis. A comprehensive summary of metabolic diseases in circadian perturbation mouse models has been published recently [51]. Here we will cite a few examples. The simultaneous inactivation of Cry1 and Cry2 results in hyperglycemia [52], while liver-specific BMAL1 knockout mice show hypoglycemia [25]. Altered glucose homeostasis is a hallmark of insulin resistance and type 2 diabetes. $Clock^{\Delta 19}$ mutant mice exhibit greatly attenuated daily feeding rhythm,

with increased caloric intake during the day. These mice are obese and develop a metabolic syndrome [53]. Likewise, adipocyte-specific Bmal1-null mice exhibit increased food intake during the light phase and increased body weight [54]. These experiments provide a link between disruption of clock components and metabolic pathologies.

### *21.4.2 Inflammation*

A common feature of a vast majority of chronic diseases is an increase in local or systemic inflammation marked by increased sensitivity of immune system to antigens and elevated levels of cytokines, lymphocytes, or macrophages in circulation or in specific tissue types. Circadian regulation in immune function is supported by observations in both animals and humans. Experimentally induced circadian disruption alters innate immune responses, with heightened release of pro-inflammatory cytokines in response to endotoxic shock in shifted mice vs. -non-shifted controls [55, 56]. In humans, dysregulation of the sleep/wake cycle affects the number of circulating lymphocytes [57]. These immune changes are likely mediators in many of the adverse health effects seen from circadian disruption, as inflammatory responses have been implicated as risk factors in multiple diseases including obesity, diabetes, IR, cancers, neurodegeneration, and cardiovascular complications.

Both immune cell proliferation (maturation, activation, trafficking) and immune cell function may be affected by circadian clock components. Proliferation and release of mature lymphocytes from bone marrow have been shown to be circadian [58]. Several trafficking factors including CXCL1 and SDF1 are also regulated in a circadian manner, and this regulation is likely mediated by JARID1a [59], which also modulates CLOCK/BMAL1 function [60]. Additionally, transcriptional regulators DEC1 and ROR-gamma have been implicated in both circadian regulation and lymphocyte maturation [61]. In differentiated cells, the function of the major immune regulator NF-kappaB is inhibited by CRY proteins, so that Cry-deficient mice show elevated levels of IL6 and succumb to LPS challenge [62]. Circadian regulation of immune function is not limited to a handful of genes. Genome-wide transcriptome studies have found hundreds of genes that are expressed in a circadian manner in mature macrophages [63].

### *21.4.3 Cardiovascular Complications*

Similar to other systems, the cardiovascular system exhibits daily and seasonal rhythms. Heart rate, cardiac output, and blood pressure show daily rhythms [64]. Genes encoding core clock components and CCGs relevant to heart function show oscillations in heart tissues and in the aorta [65–69]. The SCN also stimulates

the pineal gland to produce melatonin at night. A relationship has been reported between melatonin and light/dark variations in inflammatory systemic markers [70]. Melatonin may act as a potent antioxidant, reducing myocardial damage induced by ischemia reperfusion. Patients with lower nocturnal concentrations of melatonin appear to have a higher chance of developing heart failure or cardiac death [71]. Circadian regulation of a key transcription factor KLF15 in mammalian heart sustains cardiac electrical properties. Disruption (either deficiency or overexpression) of KLF15 causes loss of rhythmic QT variation, abnormal repolarization, and enhanced susceptibility of arrhythmia [72].

### *21.4.4 Cancer*

Epidemiological observations have linked circadian rhythms with cancer risk. Women with more hours per week and years working at night have a moderate increase in breast cancer. Shift work is also increasingly associated with elevated risk for cancer to a point that WHO has recognized shift work as a "potential carcinogen" [73]. After cancer diagnosis, prognosis is poorer in patients with disrupted circadian rhythms, while chronotherapy, where timing of drug administration is considered during treatment, has been shown to increase efficacy of some chemotherapeutic agents [74, 75]. These observations in humans support an intimate connection between circadian rhythm and cell cycle regulation; however, the mechanism appears to be more complex.

At least three different connections between circadian clock and cancer have been described: circadian regulation of cell cycle regulators, shared components between DNA damage response and circadian rhythm, and circadian regulation of metabolism. Circadian regulation of genes related to the cell cycle creates a compelling case for the involvement of circadian timing in cancer development. Circadian expression of WEE1 (G2/M transition) and MYC (G0/G1 transition) and cyclin D1 (G1/S transition) in mammals [76] can partly explain circadian gating of cell cycle regulation. Accordingly, partial hepatectomy-induced liver regeneration is slowed down in $Cry1^{-/-}$;$Cry2^{-/-}$ mice, indicating CRY is an important link between circadian physiology and cell proliferation [76]. PER1 and PER2 have been shown to be tumor suppressors in mice, and expression of all three PER genes is deregulated in breast cancer [77]. Some of the effects of PER proteins in cancer may relate to DNA damage response. PER1 protein can interact with DNA-damage response kinases ATM and CHK2 [78], which offer a direct link between clock component and cancer. Another immediate responder to DNA damage is PARP protein that rapidly synthesizes poly(ADP-ribose) polymers and thereby depletes cellular NAD store. The intimate connection between NAD, SIRT, and circadian clock and the demonstration that genetic perturbation of PARP or SIRT pathway affects both circadian clock and DNA damage response offer another link between cancer and clock. Recently, DNA damage response has been shown to alter the circadian clock via Hausp-dependent stabilization of CRY1 protein [79].

As cancer cells have an anabolic demand and are known to depend on glycolysis and autophagy to meet their energy requirement, circadian regulation of metabolism and autophagy underlies one connection. Cancer cells show an altered energy metabolism, using glycolysis rather than oxidative phosphorylation for energy, known as the Warburg effect [80]. In multiple tissue types, components of both mitochondrial oxidative phosphorylation and of glycolysis show daily rhythms [11]. Furthermore, diurnal rhythm in nutrient availability also modulates glycolytic pathway and mitochondria [22]. Therefore, for cancer cells to adapt a different energy metabolism strategy, the circadian rhythm regulation of metabolism must be disrupted. Similarly, some cancers become increasingly dependent on autophagy to recycle their cellular constituents, and many circadian clock components directly or indirectly regulate autophagy. Circadian expression of C/EBP beta [81], a potent activator of autophagy, and Rev-erb regulation of autophagy gene expression in the liver offer a temporal regulatory mechanism for autophagy [44]. Therefore, similar to energy metabolism, the cancer cells addiction to autophagy must override the circadian regulation of autophagy.

### *21.4.5 Obesity, Diabetes, Insulin Resistance*

Association between disruption of circadian timekeeping and risk of metabolic syndrome, obesity, and type 2 diabetes has been extensively established [82]. Obesity, diabetes, and insulin resistance maintain close cause and effect relationships. BMAL knockout mice show reduced insulin and lack rhythmicity in insulin activity. When rhythmicity is rescued via expression of BMAL2, insulin action and activity is restored. Mice whose circadian function has been compromised – either via BMAL knockout or WT exposure to constant light – are more obese-prone when on a high-fat diet than normal controls [82]. In humans, circadian disruption may increase diabetes risk via inflammatory mechanisms independent of sleep loss, leading to decreased insulin sensitivity without compensatory increase in insulin secretion [83].

## 21.5 Relevance of Circadian-Eating Pattern Interactions

Given the extensive reciprocal interaction between circadian oscillator and metabolic regulators, simple change in daily eating pattern will likely perturb the temporal regulation of metabolic homeostasis and result in altered body composition, weight gain, and metabolic diseases. Experimental results in both animals and humans support this notion. Laboratory male mice typically consume a major portion of their daily diet during the dark phase of the light-dark cycle. However, mice housed in constant bright/dim light eat more than usual during the light phase [84]. Overall caloric intake and level of motor activity remain similar to that of

mice under standard conditions, but the shift of consumption to the light phase leads to weight gain and glucose intolerance [84]. In wild-type mice under nighttime-restricted feeding of regular chow, total caloric intake is unaffected, but hepatic triglyceride content decreases by 50 % [85].

Daily rhythm of feeding-fasting alone can drive rhythmic expression of hepatic mRNAs and metabolites in mice. Feeding rhythms could phase-entrain the accumulation of over 600 mRNAs and several metabolites in the liver of oscillator-deficient Cry1/Cry2 double-knockout mice [22]. These results indicate the powerful influence of feeding timing on circadian regulation of metabolism [86, 87]. The imposed daily eating-fasting rhythm may counteract some of the adverse metabolic effects of genetically perturbed circadian clock. In a Per1 phosphorylation mutant mouse, increased daytime eating predisposes to weight gain. However, this genetic predisposition to weight gain can be countered without reducing caloric intake by imposing a daily rhythm of feeding-fasting [88]. These results are highlighting that the circadian regulation of metabolic homeostasis is a synergistic product of direct cell-autonomous regulation and indirect feeding-fasting regulated processes.

Moreover, few human studies have indicated managing the daily pattern of eating-fasting might be a new approach to controlling obesity and metabolic disease. In a weight loss study in Spain, subjects who ate earlier lunch showed higher weight loss than those who ate later – even when both groups were controlled for total caloric intake and physical activity [89]. In an evidence-based eating time survey, majority of adult non-shift workers were found to spread their daily nutrient intake $>$14 h. A small subset of volunteers, after adopting a 10–11 h eating schedule, reported sustained weight loss of ~4 % over a year with associated improvement in subjective measure of energy level during the day and sleep quality at night [90]. The impact of daily eating-fasting rhythm on health may be pleiotropic. In a retrospective analyses of self-reported 24 h food recall, a correlation between overnight fasting of $\geq$13 h and reduction in blood biomarkers of cancer risk has been found [91]. This raises the possibility that daily pattern of feeding-fasting even under the modern lifestyle of extended illumination can provide protection against several chronic diseases.

## 21.6 Conclusions and Future Directions

The importance of circadian timing has been demonstrated both through evolutionary relevance and observed health implications of disrupted clock systems. While the pathways that lie in the clock-metabolism interface are extensive and require further elucidation, molecular connections between the two are well established and provide an important gateway by which circadian disruption contributes to metabolic syndrome and related pathologies.

Promising studies have shown alteration in circadian rhythm to have therapeutic effects. The timing of feeding and fasting, irrespective of total caloric intake, can reduce weight gain and improve metabolic parameters. It has been shown that time-

restricted feeding can have long-term effects against preexisting obesity even when applied only 5 days per week [86]. These results underlie the importance of further study of the function and regulation of internal clocks and the integral role these mechanisms play in human health and behavior.

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# Chapter 22
# Circadian Clocks and Immune Functions

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**Abstract** In mammals, circadian rhythms modulate many physiological processes, including the immune system. This system is divided into two interconnected arms, the innate and the adaptive immune systems. Immunocompetent cells, such as macrophages, natural killer cells, and lymphocytes, have a functional circadian clock. Indeed, daily variations are observed in numbers of circulating cells, as well as in their capacity to secrete cytolytic factors and cytokines. The daily variation is also observed, for example, in lymphocyte expansion after immunization with an antigen across the day and in effector functions against the antigen. In this chapter, we review the current knowledge of circadian rhythms in the immune system, from the first line of immune defense (the innate immune response) to the pathogen-specific control of infections (the adaptive immune response). We end with some examples of immune pathologies influenced by the circadian system.

## 22.1 Introduction

Circadian rhythms are generated by clocks throughout the body, with a master clock located in the suprachiasmatic nucleus of the anterior hypothalamus that is synchronized by external light-dark cycles. Most organs and cell types have their own functional clocks [1]. This includes cells of the immune system [2–6]. Therefore, it is not a surprise that circadian clocks control different aspects of the immune system,

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both in its innate and adaptive arms. Expression of functional clock in immune cells and therefore in their functions confers an evolutionary advantage to organisms, by maximizing the immune functions at the most relevant time (Table 22.1).

The innate immune system includes a broad range of responses that are not specific to a particular pathogen. Instead, they are usually designed as a first line of defense that is often sufficient to get rid of the invader. Various cells compose the innate system and allow the detection of infectious agents and recruitment of immune cells to the infection site. However, in some cases, pathogens are able to evade or overcome innate immune defenses. In this case, the adaptive immune response (i.e., T lymphocytes and B lymphocytes) is recruited to fight off the infection. After clearance of the pathogen, a fraction of the activated T and B cells become memory cells. When a second infection occurs with the same pathogen, the immune system will eliminate it faster and more efficiently. In this chapter, we will provide an overview of the circadian control of the different steps of the immune responses, from the innate to the adaptive steps.

## 22.2 Circadian Control of the Innate Immunity

Physical barriers (i.e., epithelium surfaces, such as skin) combined with the innate immune system represent the first line of immune defense. When the epithelial barrier is broken, leading to the invasion of infectious agents, the innate immune system is set up. The innate immune system is a set of nonspecific (or broadly specific) defense mechanisms designed to clear the infection. It involves the action of various cell types, including cells derived from myeloid lineage, monocyte-derived cells (macrophages and dendritic cells) and granulocytes (e.g., eosinophils, basophils, neutrophils, and mast cells), and from lymphoid lineage: natural killer (NK) cells (Fig. 22.1). These cells use different mechanisms, such as phagocytosis (i.e., major mechanism of elimination of pathogens and cellular debris by vacuolar internalization) and production of effector mediators (e.g., cytokines, chemokines, cytolytic factors) to recruit immune cells and eliminate infected cells. Among these mechanisms, several cell types, including macrophages and dendritic cells, use pattern recognition receptors (PRRs) expressed either at the cell surface (e.g., Toll-like receptor 4 (TLR4)) or at the endosomal membrane (e.g., TLR9) to control the infection. These receptors recognize specific motif of pathogens, leading to the activation of various pathways, such as the NF-κB pathway, important for the expression of cytokines and then the elimination of the infectious agent. The specific location of PRR is important for their functions; for example, TLR4 is expressed at the cell surface to recognize molecules of bacterial wall, whereas TLR9 recognizes bacterial DNA within the endosomes. Here, we review the current knowledge about the circadian control of innate responses mediated by macrophages, natural killer cells, dendritic cells, and neutrophils.

**Table 22.1** Summary of the circadian control of the immune response

| Parameter studied (general) | Parameter studied (detailed) | Acrophase | Species | Cell/subset | References |
|---|---|---|---|---|---|
| Clock gene expression | *Bmal1*, *Clock*, *Cry1*, *Cry2*, *Dbp*, *Dec-1*, *E4bp4*, *Npas2*, *Per1*, *Per2*, *Per3*, *Rev-Erbα*, *Rev-Erbβ*, *Rorα*, *Rorγ* | – | Mouse | Monocyte | [7] |
| | | | | Peritoneal macrophage | [3, 7–10, 13, 15] |
| | | | | Splenic macrophage | [4, 7] |
| | | | | Dendritic cell | [4] |
| | | | | Thymocyte | [34] |
| | | | | T lymphocyte | [6, 34] |
| | | | | B lymphocyte | [4] |
| | | | | Splenic natural killer cell | [17] |
| | | | | Spleen | [3, 49] |
| | | | | Thymus | [32, 49] |
| | | | | Lymph nodes | [3, 40] |
| | *Bmal1*, *Clock*, *Dbp*, *Per1*, *Per2* | – | Rat | Splenic natural killer cell | [5, 20, 29] |
| | *BMAL1*, *CLOCK*, *CRY1*, *CRY2*, *DBP*, *E4BP4*, *PER1*, *PER2*, *PER3*, *REV-ERB*, *REV-ERBα*, *RORα* | – | Human | Peripheral blood mononuclear cell | [2] |
| | | | | Monocyte | [30] |
| | | | | Macrophage | [8] |
| | | | | Neutrophil | [30] |
| | | | | Leukocyte | [30] |
| | | | | CD4 T lymphocyte | [6] |
| Trafficking/ cell numbers in the blood | – | ZT8 | Mouse | Monocyte | [7] |
| | | – | | Peritoneal macrophage | [8, 9] |
| | | CT0 | | Neutrophil | [31] |
| | | Morning | Human | Natural killer cell | [16] |
| | | Night | | T helper lymphocyte | [16, 35–37] |
| | | Night | | T regulatory lymphocyte | [16] |
| | | Night | | B lymphocyte | [16] |
| Cell proliferation | LPS stimulation | ZT5 | Rat | T lymphocyte | [41] |
| | Concanavalin A stimulation | ZT5 | | B lymphocyte | |
| | Anti-CD3 stimulation | CT10/ CT14 | Mouse | T lymphocyte | [40] |
| | PMA/ionomycin | CT14/ CT18 | | | |
| | CpG-OVA-immunized mice, restimulated with OVA peptide | ZT19 | | Lymph node T lymphocyte | [13] |

(continued)

**Table 22.1** (continued)

| Parameter studied (general) | Parameter studied (detailed) | Acrophase | Species | Cell/subset | References |
|---|---|---|---|---|---|
| Immune response | Innate functions | | | | |
| | Phagocytosis | ZT6 | Mouse | Peritoneal macrophage | [8] |
| | Cytolytic activity | ZT19 | Rat | Splenic natural killer cell | [19] |
| | | Morning | Human | Natural killer cell | [18] |
| | NF-κB after TLR5 stimulation | ZT22-ZT2 | Mouse | – | [12] |
| | *Tlr9* expression | ZT19 – CT2 | | Spleen | [13] |
| | | ZT12 – CT12 | | Macrophage | |
| | | ZT16 | | B lymphocyte | |
| | | CT16/CT24 | | Dendritic cell | |
| | Vaccination response after TLR9 stimulation | ZT19 | | – | |
| | Cytokine/chemokine expression | | | | |
| | *Ccl2* | ZT20 | Mouse | Blood monocyte | [7] |
| | *Tnfα* | ZT14 | | Peritoneal macrophage | [8] |
| | *Il6*, *Il1β* | ZT6/ZT8 | | | |
| | *Ccl2* | ZT10 | | | |
| | *Granzyme B*, *Perforin*, *Ifnγ* | ZT19/ZT4 | Rat | Spleen | [19] |
| | *Granzyme B*, *Perforin*, *Tnfα, Ifnγ* | ZT19 | | Splenic natural killer cell | [20] |
| | *Ifnγ* | ZT18 | Human | T lymphocyte | [6] |
| | Cytokine/chemokine secretion | | | | |
| | IL-1β, IL-6, IFNγ, CCL2 | ZT8 | Mouse | Monocyte | [7] |
| | TNFα, IL-6 | CT8-CT12 | | Peritoneal macrophage | [3] |
| | IL-6, G-CSF, TNFα | CT6 | | BAL: bronchoalveolar tissue | [31] |
| | CXCL5 | CT18-CT0 | | | |
| | Granzyme B, perforin, TNFα, IFNγ | ZT19 | Rat | Splenic natural killer cell | [20] |
| | IFNγ, IL-2, IL-4 | ZT18 | Human | T lymphocyte | [6] |
| | Antigen-specific response | | | | |
| | T cell expansion (in vivo) against antigen | ZT6 | Mouse | Spleen | [40] |
| | $IFN\gamma^+$ T cell expansion (in vivo) against antigen | ZT6 | | CD8 T lymphocyte | |

(continued)

**Table 22.1** (continued)

| Parameter studied (general) | Parameter studied (detailed) | Acrophase | Species | Cell/subset | References |
|---|---|---|---|---|---|
| Lymphocyte development/ differentiation | T cell subsets | | | | |
| | Th1/Th2 balance | 1 AM | Human | Th1/Th2 lymphocyte | [42] |
| | Th17 development | – | Mouse | Th17 lymphocyte | [44, 45] |
| | | – | | | |
| | Treg lymphocyte development | – | | Treg lymphocyte | [45] |
| | Treg: suppressive activity | 2 AM | Human | Treg lymphocyte | [47] |
| | B lymphocytes | | | | |
| | B lymphocyte development | – | Mouse | B lymphocyte | [49] |
| Immune-related pathologies and circadian clocks | Rheumatoid arthritis | | | | |
| | Joint stiffness | Morning | Human | – | [50] |
| | Prolactin and melatonin secretion | Night | | Synovial membrane cell | [53, 54] |
| | Allergic reactions | | | | |
| | Allergic asthma attacks | Late night/ early morning | Human | – | [56] |
| | Cutaneous allergic reaction | ZT10 | Mouse | – | [57] |
| | Mast cell degranulation | ZT12 | | Mast cell | |
| | FcεR1β expression | ZT8 | | | |
| | IL1, CCL2, histamine secretions | ZT4 | | Serum | [59] |

Zeitgeber time (ZT) 0 corresponds to the beginning of the day (lights on), and ZT12 is the beginning of the night (lights off), in a 12 h light/12 h dark cycle.
Circadian time (CT) 0 corresponds to the beginning of the subjective day and CT12 as the beginning of the subjective night, in constant darkness conditions.
The acrophase is the time of peak of the daily or circadian variation.

### *22.2.1 Rhythmic Expression of Clock Genes in Monocytes and Macrophages*

Monocytes are derived from precursors located in the bone marrow. These cells circulate in the bloodstream and differentiate into macrophages or dendritic cells that migrate to inflamed tissues. Resident macrophages also exist, located in strategic places where microbial infections are more subject to develop; this is,

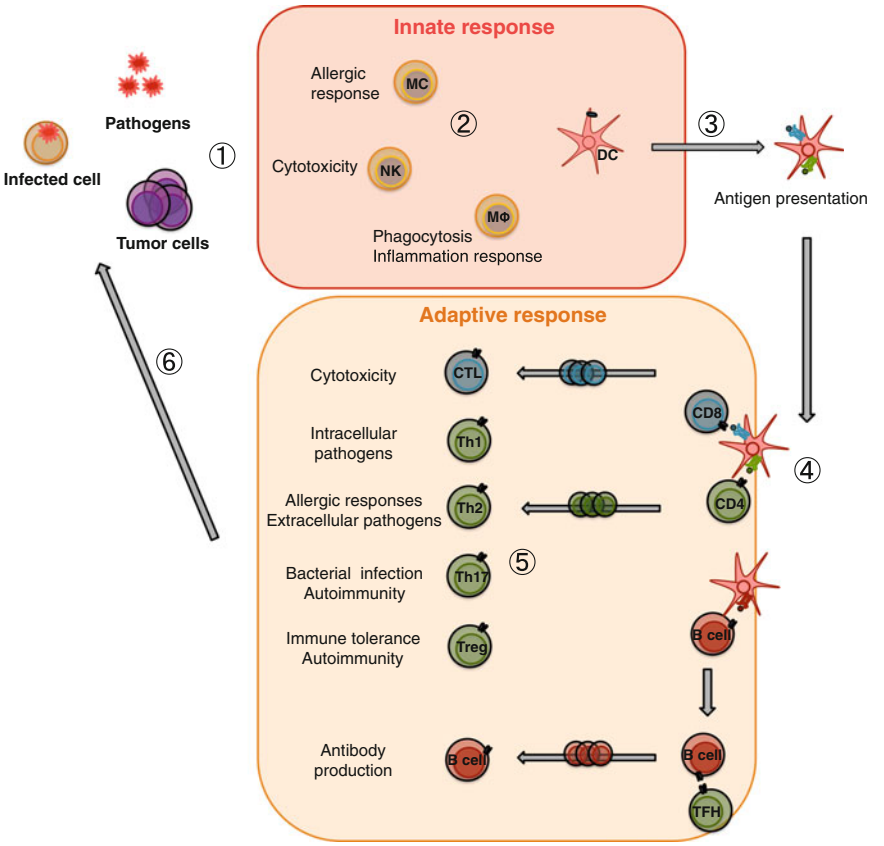


**Fig. 22.1** The innate and adaptive immune responses. The innate immune responses (*top*) involve macrophages (MΦ), dendritic cells (DC), natural killer (NK) cells, and mast cells (MC) (and other granulocytes, not shown). It is the first line of defense against invaders, which occurs during the first hours of the infection (*1*, *2*). When the infection potentially cannot be controlled, the adaptive immune response is set up to eliminate the danger (*bottom*). Antigen-presenting cells, such as dendritic cells (*3*), migrate to the lymph nodes to activate T lymphocyte in a pathogen-specific manner (*4*). T lymphocytes then become activated and proliferate. CD8 T lymphocytes differentiate into cytotoxic T lymphocytes (CTL), while CD4 T lymphocytes differentiate into Th1 cells to control intracellular pathogens, Th2 cells for extracellular pathogens and allergic responses, Th17 to control bacterial infection, or regulatory T cells (Treg) for the control of the other T cell subsets (*5*). Dendritic cells also activate B cells, which then migrate to interact with T follicular helper (TFH) cells to produce specific antibodies against pathogens to eliminate it afterward. Various mechanisms of the innate/adaptive immune cells lead to the elimination of the pathogen, infected cells, or tumor cells (*6*)

for example, the case of peritoneal macrophages, involved in the immune surveillance of the intestines.

Like other cellular clocks in the body, monocytes and macrophages have a clock mechanism involving circadian clock genes. Studies have shown a rhythmic expression of clock genes in monocytes, peritoneal macrophages, and splenic

macrophages in mice, and in a human macrophage cell line, suggesting that these cells have a functional clock [3, 4, 7, 8]. Indeed, peritoneal macrophages from PER2::LUC mice and cultured ex vivo showed persistent rhythms during 7 days (PER2::LUC mice have the PER2 protein fused to the luciferase enzyme, such that bioluminescence allows to track clock rhythms in the cells) [3].

### 22.2.2 *Peritoneal Macrophages Express Many Genes with a Circadian Rhythm*

A microarray study on mouse peritoneal macrophages revealed that nearly 10 % of their transcripts follow a circadian rhythm [3]. Some of these genes are important for immune responses (e.g., components of NF-κB pathway). Given that the expression of many genes in monocytes and macrophages present a circadian rhythm, it can be expected that the functions of these cells are also rhythmic. Accordingly, diurnal variations have been found for various mechanisms in these cells, which we will describe below (Fig. 22.2).

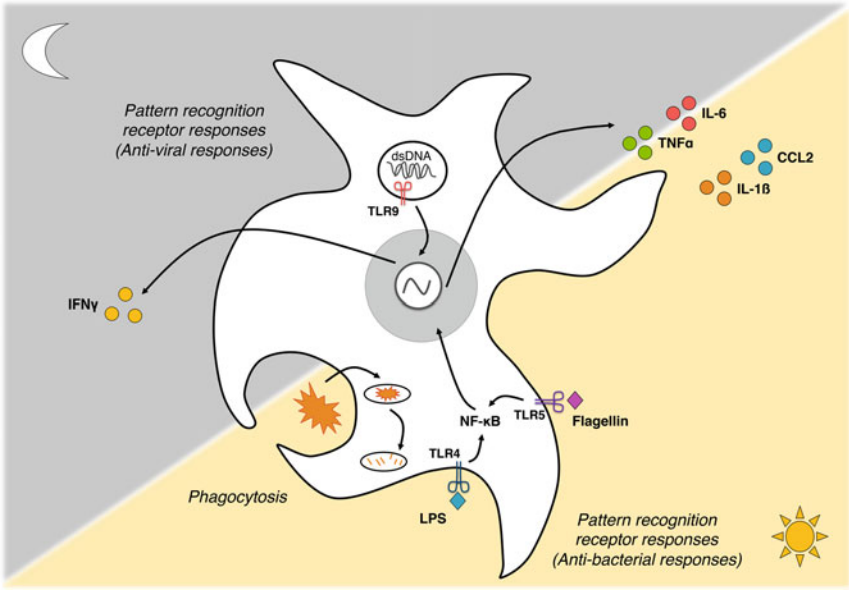


**Fig. 22.2** Circadian rhythms in monocytes and macrophages. Various aspects of monocyte and macrophage functions vary with a circadian rhythm. Here, they are shown according to whether they show highest levels in the daytime or nighttime. See text for details. *LPS* lipopolysaccharide, *dsDNA* double-stranded DNA, *TLR* Toll-like receptor

### 22.2.3 *Various Functions of Monocytes and Macrophages Follow a Circadian Rhythm*

#### 22.2.3.1 Phagocytosis

Macrophages are phagocytes that can engulf dying cells and cellular debris. In vitro experiments with mouse peritoneal macrophages revealed that phagocytosis presents a diurnal variation [8]. Indeed, phagocytosis of fluorescent particles was higher during the middle of the day than the middle of the night.

#### 22.2.3.2 Macrophage Trafficking

Macrophages differentiated from monocytes migrate into the tissues to act against pathogens. This migration occurs because of chemokines, which are chemoattractant molecules. Studies have shown a direct control of the gene encoding such a chemokine, CCL2, by clock proteins. In the human macrophage cell line RAW264 where *Bmal1* expression was suppressed by RNA interference, *Ccl2* transcription was decreased [8]. Overexpression of the transcriptional repressor REV-ERBα in the same cell line repressed the *Ccl2* expression [9]. Conversely, overexpression of *Rorα*, a transcriptional activator that binds the same DNA elements as REV-ERBα, increased *Ccl2* expression. Also, overexpression of *Rev-Erbα* in human macrophages alters the recruitment and the migration of macrophages, whereas the overexpression of *Rorα* has the reverse effect [9]. However, these two mechanisms were studied in in vitro experiments. It will be important to determine if these mechanisms occur in vivo. In this regard, an interesting study has shown a rhythm in the circulating and tissue levels of the Ly6C$^{hi}$ inflammatory monocytes [7]. The underlying mechanism seems to involve a negative regulation of the *Ccl2* gene by BMAL1 (via the recruitment of the polycomb repressor EZH2 onto the *Ccl2* promoter by BMAL1, inhibiting gene expression). This occurs in a cell-autonomous manner, as monocyte/macrophage-specific *Bmal1* KO mice lose the *Ccl2* rhythmic regulation as well as the inflammatory monocyte fluctuations [7]. Moreover, monocyte trafficking rhythms seem to underlie a time-dependent variation in susceptibility to *Listeria monocytogenes* infection, a rhythm that is also lost in these monocyte-specific KO mice [7].

#### 22.2.3.3 Production of Cytokines and Chemokines

Various cytokines produced by peritoneal macrophages, stimulated with LPS (i.e., lipopolysaccharide, a molecule of the cell wall of gram-negative bacteria), are rhythmic [3, 8]. Thereby, *Tnfα* expression peaks in the beginning of the night, whereas *Il1β* and *Il6* expression (i.e., pro-inflammatory cytokines) is higher during the middle of the day without any stimulation [8]. Concerning protein secretion

after LPS stimulation, TNFα, IL-6, and IL-1β secretion peaks at the end of the day during the transition to activity in mice [3, 7, 8, 10].

Glucocorticoids such as cortisol (in humans) and corticosterone (in rodents) are known to be inhibitors of the transcription of pro-inflammatory cytokines. Given that their levels vary in a circadian manner, with peak levels during the late night and early morning (in humans; the reverse in nocturnal rodents), it seems possible that these rhythms are the source of rhythmic cytokine secretion. However, circadian rhythms of pro-inflammatory cytokine expression in spleen-derived macrophages were conserved in cells harvested from mice after removal of the adrenal glands, the tissue that secretes corticosterone [3]. Therefore, there must be other mechanisms than glucocorticoid regulation to explain these rhythms.

#### 22.2.3.4 Pattern Recognition Receptor Response

A mechanism by which the circadian rhythm could regulate cytokine response could be the involvement of PRRs. These receptors are expressed in different immune cell types (including macrophages, dendritic cells, B lymphocytes) and allow them to recognize and respond to conserved microbial molecules. The TLR4 is the PRR for LPS. This pathway is involved in the secretion of pro-inflammatory cytokines such as TNFα and IL-6. However, the rhythmicity of the expression of these cytokines cannot be explained by TLR4, which are not rhythmically expressed [11]. Therefore, upstream and/or downstream mechanisms must be at play to explain the rhythmicity of cytokine response to LPS. Mouse macrophage transcriptome study showed that among the 8.1 % of genes under circadian regulation, some are involved at every level of the TLR4 signaling pathway (e.g., molecules involved in LPS binding to TLR4, transcription factors involved in cytokine expression, molecules involved in cytokine mRNA stability and location) [3].

Mice with a monocyte/macrophage-specific *Bmal1* deletion showed a loss of the morning-evening difference of the cytokine response to LPS [10]. Although this showed a cell-autonomous rhythmic control, a similar phenotype was seen in *Rev-Erbα* KO mice, despite a generally functional clock in those cells [10]. Therefore, it appears that REV-ERBα, which has rhythmic abundance levels, conveys the circadian clock-dependent rhythmic regulation to a subset of rhythmic genes important for the events downstream of TLR4.

Another PRR presents a daily rhythm in its response: TLR5, which is the receptor for bacterial flagellin. The binding induces the secretion of pro-inflammatory cytokines by activating the NF-κB pathway. The induction of this pathway was stronger in the middle of the day when mice were injected with a TLR5 ligand [12]. Additionally the rhythm of TLR5 response seems to be due to a regulation of NF-κB activity by the CLOCK protein. TLR9, another PRR that is involved in the recognition of bacterial DNA, shows a daily rhythm of expression in peritoneal macrophages [13]. This rhythmic expression has a functional consequence as immunization of mice in the presence of a TLR9 ligand was more

effective when done around the middle of the night (when TLR9 levels are high) than around the middle of the day (when TLR9 levels are low) [13].

Molecular circadian control downstream of PRR activation could also involve NF-κB. Several studies have highlighted a modulation of NF-κB activity by clock proteins: CLOCK can act as a histone acetyltransferase leading to the acetylation and activation of the NF-κB subunit p65 [12]; CRY proteins indirectly repress NF-κB activity via a downregulation of cAMP levels [14]; and a recent study showed an interplay between BMAL1, NF-κB, and miR-155, a microRNA which is itself activated by LPS and regulates the magnitude of inflammation [15]. Indeed, in the context of an inflammatory response, the pro-inflammatory miRNA miR-155 inhibits BMAL1 expression.

### *22.2.4 Natural Killer Cells Have Rhythmic Cytolytic Functions*

Natural killer (NK) cell numbers in the blood of human subjects follow a diurnal rhythm, with a peak during the waking phase [16]. NK cells are involved in the immune surveillance against fungal, bacterial, and viral infection and are also involved in the cellular response against tumor cells and metastasis formation. NK cells accomplish their cytolytic function (i.e., killing target cells) via the secretion of granules that contain perforin and granzyme B (Fig. 22.3). Perforin causes transmembrane pores in the target cells, allowing granzyme B to enter and activate the apoptotic pathways, leading to the elimination of the infected or malignant cells. The NK cells are the only immune cells that express constitutively the cytolytic machinery and do not need to be preactivated to eliminate target cells. NK cells also release cytokines, such as IFNγ and TNFα.

Clock genes are expressed in spleen NK cells of rodents [5, 17]. This suggests that NK cells harbor a circadian clock and that NK cell functions might be regulated according to the time of day. Indeed, NK cells from the human blood have a peak of their cytolytic activity during the morning [18]: lymphoma K562 cells used as a target were loaded with $^{51}$Cr, and the cytolytic activity of the NK cells was assessed by measuring $^{51}$Cr release. Similar experiments were done using splenic NK cells of rats: the NK cells showed high activity during the dark phase and low activity during the light phase [19] (Fig. 22.3).

What is the underlying mechanism? A simple possibility would be a rhythm in the expression of cytolytic factors and cytokines. Interestingly, the expression of *Perforin*, *Granzyme B*, and *Ifnγ* mRNAs showed a daily rhythm in rat splenocytes and splenic NK cells, with a peak in the beginning of the day and low levels during the night [19]. Interestingly, the protein amounts showed a different phase, as they were higher during the night (i.e., when the mRNAs are weakly expressed). This suggests that there is posttranscriptional mechanism involved in the circadian regulation of these factors in NK cells. Moreover, it is interesting to note that

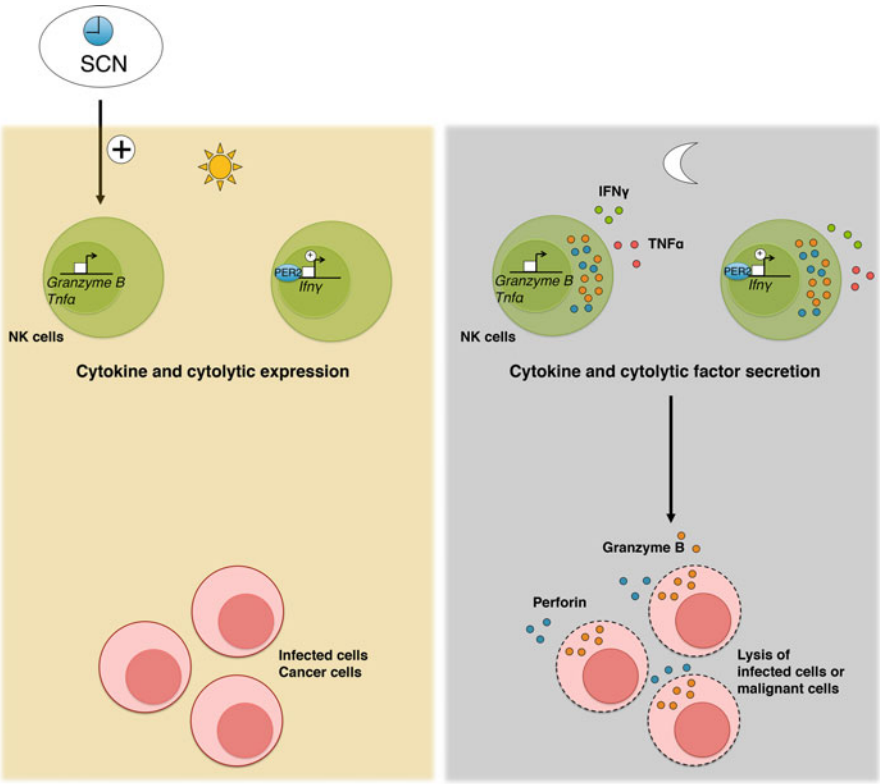


**Fig. 22.3** Circadian control of the cytolytic activity of natural killer cells. Natural killer (NK) cells are involved in the immune surveillance against microorganisms and are involved in a cellular response against malignant cells. The master clock of the suprachiasmatic nucleus (SCN) controls the *Granzyme B* and *Tnfα* expression, whereas a peripheral clock controls the *Ifnγ* expression in NK cells. Cytolytic factors (perforin and granzyme B) and cytokines (IFNγ and TNFα) are more expressed during the nighttime, leading to the elimination of infected cells or malignant cells

both the expression of cytolytic factors and the cytolytic activity of NK cells present highest levels in the night in rodents.

These rhythms could be driven by a local clock or by the central clock of the suprachiasmatic nucleus (SCN). To address the latter possibility, chemical sympathetic denervation of the spleen was performed in rats by guanethidine treatment. This led to the alteration of clock gene expression in NK cells [20]. The norepinephrine (NE) neural input to spleen was essential to maintain normal rhythms of the molecular clock components in this organ. Moreover, NK cells from denervated spleens failed to show a diurnal expression of *Granzyme B* and *Tnfα*, suggesting that the sympathetic nervous system is essential for the diurnal rhythms of these cytolytic factors. Interestingly, guanethidine treatment did not alter the *Ifnγ* and *Perforin* expression in NK cells [20] (Fig. 22.3). Thus, NE neural input to the spleen

is not necessary for the expression of these genes, and *Ifnγ* and *Perforin* expression might rather be controlled by the NK cell-intrinsic clock or others peripheral clocks.

In the context of LPS-induced endotoxic shock, susceptibility of bacterial endotoxin (LPS) depended on the time of day in mice [21]. In contrast, in *Per2* mutant mice, the resistance to LPS injections was high in all times [22]. Thus a circadian clock influences the resistance to LPS-induced septic shock. After the septic shock in *Per2*-deficient mice, *Ifnγ* expression was reduced. This reduction was not due to lower number of NK cells, but to a production defect of *Ifnγ* in NK cells. Indeed, the cellularity of most of the immune cell subsets in the spleen of LPS-challenged mice was normal. These results suggest that the *Per2* gene controls the expression of *Ifnγ* in NK cells during sepsis.

One of the important roles of NK cells is to eliminate tumor cells. Various epidemiological studies have highlighted an increased incidence of some types of cancer in long-term shift workers, compared to individuals working on a day-oriented schedule [23–26]. Circadian disruption in rodents was also shown to lead to a higher incidence of tumors or faster tumor growth [27, 28]. In order to test the possible involvement of a disruption of cytolytic cell function, such as that in NK cells, rats were subjected to repeated shifts of the light-dark cycle, which suppressed perforin levels [29]. Accordingly, NK cells from shifted rats showed reduced cytolytic activity in vitro compared to cells from control rats. This was paralleled in vivo, with a higher frequency of lung tumors in shifted rats injected with MADB106 tumor cells, compared to controls [29]. This suggests that NK cells might be involved in the increased risk of cancer in shift workers.

### 22.2.5 *Other Clock-Controlled Innate Immune Cells (Dendritic Cells and Neutrophils)*

Dendritic cells (DCs) are the most potent cells for the presentation of antigens to naive T cells (T cells are major components of the adaptive immune response; see below). Like most of the immune cells, mouse DCs express clock genes, suggesting that they may have a functional intrinsic clock [4]. There are various subsets of DCs, such as the inflammatory DCs (TNFα and iNOS-producing dendritic cells, or Tip-DCs), which are not involved in the antigen presentation process. During *L. monocytogenes* infection, the clearance of the bacteria is higher 2 days postinfection when mice were infected at in the late light period than in the beginning of the light period, associated with a higher amount of Tip-DCs [7]. However, little is known about the clock in dendritic cells and the cellular functions that it controls.

Neutrophils are recruited to inflamed tissues very early to eliminate the infection. As macrophages, they are phagocytes and they also can secrete antimicrobial agents to clear the infection. Clock genes are expressed in human neutrophils, suggesting that they have a circadian clock [30]. In the context of pulmonary

inflammation in mice, neutrophil recruitment in the lungs after a challenge with aerosolized LPS presents a circadian rhythm [31]. This rhythm of recruitment might be due to a 24-h variation of expression of the chemokine CXCL5 in bronchiole epithelial cells.

## 22.3 Circadian Control of the Adaptive Immunity

Adaptive immune responses occur mainly when innate immunity is not sufficient to defend the organism against the pathogens. The adaptive responses are specific for particular pathogens. Two major components of the system are T lymphocytes (or T cells), involved in inducing both cell-mediated and humoral responses, and B lymphocytes (or B cells), involved in humoral immunity via the secretion of immunoglobulins (antibodies). The adaptive immune response is very specific because it is initiated by the presentation of a specific antigenic peptide by DCs to T cells, which initiates activation, proliferation, and acquisition of effector functions by these cells. In parallel, B cells specifically recognize an antigenic peptide complex expressed by DCs and become activated in lymph nodes. B cells then interact with specific CD4 T cells and T follicular helper cells, which initiate proliferation and secretion of specific antibodies against pathogens (humoral response). After clearance of the pathogen, while most T and B cells die, a subset survives for a much longer time, and these cells become memory cells that will respond quickly after a second infection with the same pathogen. Here, we will address the circadian control of T cell and B cell responses.

### *22.3.1 T Cell Frequency, Activation, and Differentiation Are Controlled by the Circadian System*

#### 22.3.1.1 Rhythms of T Cell Numbers and Response to Antigen

T cells develop in the thymus, a primary lymphoid organ. Their development is characterized by different stages of recombination of the T cell receptor and by various selection steps based on the recognition of self-antigens presented by antigen-presenting cells (e.g., thymic epithelial cells and dendritic cells). This differentiation process generates a repertoire of T cells that will be useful to fight infection while ensuring self-tolerance. The thymus is therefore mainly composed of immature, undifferentiated cells. After the differentiation has completed, T cells can either be CD4-positive T cells (helper T cells) or CD8-positive T cells (cytotoxic T cells). Although thymocytes (immature T cells) were shown to express clock genes, this expression is not rhythmic, and it is not affected by mutation of the gene *Clock*, which led to the suggestion that this organ seems not to bear a clock [32]. However, it remains possible that thymocytes are rhythmic at the single cell

level. Similar to results obtained from testis (another organ with an active differentiation program), circadian clock function only occurs after complete differentiation of these cells (in line with [32, 33]). In contrast to arrhythmic thymocytes, bioluminescence rhythms observed in mature CD4 T cells of PER2::LUC mice as well as the expression of clock genes in splenic CD4 T cells (i.e., mature CD4 T cells) were found to be rhythmic [6, 34].

After T cell development has been completed, the T cells leave the thymus and circulate between the different secondary lymphoid organs (e.g., spleen, lymph nodes) via the bloodstream until the recognition of an antigen presented by an antigen-presenting cell. In the human blood, lymphocyte numbers (B cells, CD4 T cells, and CD8 T cells) show a daily rhythm, with highest levels during the night that decline in the morning and remain stable during the daytime [16, 35–37]. When T cell subsets are measured, only naive and memory T cell numbers are highest at nighttime, whereas effector CD8 T cell numbers peak in the day. No rhythms were found for the numbers of CD4 effector T cells [36]. The same study highlighted the role of cortisol and epinephrine, two hormones with a circadian rhythm of secretion, in regulating T cell circulation rhythms. After an infusion of cortisol, circulating T cells decreased, whereas epinephrine infusion increased effectors CD8 T cells in the blood of the subjects. Similarly, perfusion of hydrocortisone in mice resulted in decreasing number of T cells in bloodstream, due to their sequestration in the bone marrow [38]. The T cell homing in the bone marrow results in the expression of CXCR4 on T cells (a chemokine receptor that binds CXCL12 ligand expressed in bone marrow). In humans, after hydrocortisone infusion during the sleep period, when the endogenous release of cortisol is low, T cell numbers decrease in the bloodstream, and CXCR4 expression increases on T cells [39]. These results suggest that cortisol regulates the diurnal rhythm of T cell circulation in bloodstream, by modulating CXCR4 expression.

T cells recognize antigenic peptides presented by antigen-presenting cells in lymphoid organs. This antigen recognition occurs via a specific receptor, the T cell receptor. Because of the diversity of the T cell receptor allowing the recognition of various antigenic peptides, T cells need to proliferate via a clonal expansion after their activation to control the infection. Following this, T cells differentiate in different subsets. In an in vitro experiment conducted in mice, the proliferation of CD4 and CD8 T cells in response to T cell receptor activation was shown to vary in a circadian manner [40]. Both CD4 and CD8 T cells were more responsive during the early night than during the early to midday. This day-night difference was lost in *Clock* mutant mice. These results are consistent with previous research conducted in rats, where B and T cell proliferation after LPS stimulation of B cells and concanavalin A stimulation of T cells followed a diurnal variation [41]. Moreover, rhythmic cytokine (IFNγ, IL-2, IL-4) secretion by human T cells stimulated with PMA and ionomycin was observed (PMA activates protein kinase C and ionomycin triggers calcium release in the cell, important for the NFAT pathway involved in cytokine expression) [6]. The analysis of rhythmic transcripts in T cells suggested that this variation might be explained by the rhythmic expression of *IκBα* and *SGMS2*, involved in the NF-κB pathway. Moreover, in an ex vivo experiment

conducted with blood lymphocytes from human subjects, stimulation with either adenovirus antigen lysate (that stimulates CD4 T cells) or *Staphylococcus aureus* enterotoxin B (that stimulates both CD4 and CD8 T cells) led to a higher number of IFNγ- and IL-2-producing cells during the late night [37].

Overall, T cell proliferation and effector functions show diurnal variations in vitro. To test whether this is also occurring in vivo, experiments were conducted in mice and revealed a higher CD8 T cell response after an intravenous immunization with dendritic cells loaded with an antigen (the OVA peptide SIINFEKL) [40]. The expansion of these cells was higher when the immunization was done during the middle of the day than in the middle of the night [40]. This day-night difference was also recapitulated by the frequency of IFNγ-producing cells. Therefore, T cell responses also show daily variations after immunization in vivo.

#### 22.3.1.2 Circadian Rhythms in CD4 T Cell Subsets

Following antigen recognition, CD4 T cells must differentiate into the proper effector subsets that will be adapted to the type of infection that occurs. This is very important as different effector mechanisms are regulated to control the growth of extracellular and intracellular bacteria, intracellular parasite, and helminthes. The innate response provides the key signal, cytokines that will orient T cell differentiation. This leads to the expression of master transcription factors that promote CD4 T cell differentiation and acquisition of proper effector functions.

Th1 cells are generated in response to intracellular viral and bacterial infections. They release IFNγ, IL-2, and TNFα, involved in cellular responses such as macrophage activation. On the other hand, the Th2 cells are involved in humoral responses against extracellular pathogens by releasing IL-4, IL-5, IL-13, leading to stimulation of mast cells, eosinophils, and B cells. Thereby, immune responses that are adapted to the type of pathogen, and thus more efficient, are established by balancing the Th1 and Th2 responses. Human studies have shown that sleep leads to a shift in the Th1/Th2 balance [42]. Indeed, the IFNγ/IL-4 ratio (indicative of the Th1/Th2 ratio) was increased during the early sleep and decreased during the late night.

More recently, two new members were added to the Th cell subsets: the Th17 cells and the regulatory T (Treg) cells. Recent studies have shown a circadian regulation of both of these cell types. The Th17 cells, which express IL-17 as a signature cytokine, are necessary to control bacterial and fungal infections at mucosal surfaces and are associated with inflammatory bowel disease (IBD). IBD was associated with polymorphisms in the *Nfil3* gene, known to encode a clock-regulated transcription factor [43]. *Nfil3*-deficient mice exhibited a higher IL-17 and RORγt-positive Th17 cell (RORγt is the master transcription factor of Th17 cells) frequency in the small intestine and colon than wild-type (WT) mice, suggesting an important role for *Nfil3* [44]. Indeed, in activated *Rev-erbα* KO CD4 T cells, *Nfil3* expression is higher, and the polarization of naive CD4 T cells to Th17 is reduced. The BMAL1/

CLOCK complex is essential for *Rev-Erbα* expression; therefore, in *Clock* mutant mice, *Rev-Erbα* expression is decreased, and *Nfil3* expression in CD4 T cells is higher, leading to defective Th17 cell development. These results strongly suggest that a circadian clock controls Th17 cell development and potentially immune homeostasis, because an increased number of Th17 cells are associated to autoimmune disease development.

Recent work on patients with multiple sclerosis (MS) has shown a negative correlation between the level of melatonin and MS relapses [45]. Melatonin is produced in a circadian manner and is known to control several immune mechanisms [46]. Thereby, it is not surprising that this hormone affects the differentiation of Th cells and their effector functions [45]. Experiments in mice highlighted the inhibition of Th17 cell differentiation and the activation of Treg cell differentiation by two distinct mechanisms involving melatonin. The first mechanism leads to the increase of NFIL3 and consequently the inhibition of RORγt expression, thus inhibiting Th17 cell differentiation, and the second one leads to the activation of RORα, allowing the expression of IL-10 (an immunosuppressive cytokine expressed by Treg). These two combined mechanisms lead to a less severe disease in a mouse model of MS [45].

Autoimmunity is characterized by a self-recognition by immune cells and antibodies, leading to the destruction of specific tissues. The risk of autoimmunity is reduced by the action of Treg cells, which suppress the activity of Th1, Th2, Th17, and CD8 T cells. To address whether Treg-mediated suppression of other Th subsets is daytime dependent, human effector T cells were cultured in the presence or absence of Treg cells from the same individuals at different times of day, to measure the suppression of Th1, Th2, and Th17 cells by analyzing IFNγ/TNFα, IL-4/IL-10, and IL-6/IL-17 secretion, respectively [47]. Only IL-2, IFNγ, and TNFα production was inhibited by Tregs at 2:00 h, but not IL-4, IL-6, IL-10, and IL-17. Another human study showed a circadian rhythm of Treg counts in the blood, independent of sleep [48]. However, T cell proliferation and activity (secretion of IL-2) were less inhibited when Tregs came from donors with sleep deprivation than others with sufficient sleep. Thus, Treg numbers in the blood follow a circadian rhythm independent of sleep, whereas their functions are dependent on sleep. This might be an aggravating factor of chronic inflammatory diseases such as rheumatoid arthritis or allergic responses.

### *22.3.2 Circadian Rhythms in B Cell Development and Numbers in the Blood*

B cells are involved in humoral immune responses via the secretion of antibodies specific to the pathogen. B cell development occurs in the bone marrow. The clock component BMAL1 seems to be important for B cell development because *Bmal1* KO mice have fewer B cells in the bone marrow, spleen, and bloodstream than WT

mice [49]. The alteration of B cell development is not due to a clock in B cell precursors themselves, as shown using the bone marrow chimeras. Host mice (either *Bmal1* KO or WT) were irradiated and then implanted by bone marrow from a WT mouse, implying that *Bmal1* expression in cells of the bone marrow microenvironment could be essential for the development of B cells. However, it could also be due to other clocks in the organism, which was not tested in this study.

Like T cells, B cell numbers in the bloodstream of healthy human subjects follow a diurnal rhythm [37]. In contrast to T cells though, B cell numbers present a bimodal rhythm, with a first peak during the midday and a second and more prominent peak during the late night. In mice, clock genes are expressed in B cells [4], but their possible involvement in B cell functions has yet to be examined.

## 22.4 Immune-Related Pathologies and Circadian Clocks

The extensive control of circadian clocks over various immune mechanisms has implications for diseases that involve immune dysfunction. This part of the chapter will describe a few examples of pathologies that might be influenced by the circadian system.

### *22.4.1 Rheumatoid Arthritis*

Rheumatoid arthritis (RA) disease is an autoimmune disease (i.e., where the immune system attacks self-antigens), and it is characterized by cartilage and bone erosion. Symptoms vary over the day, with morning stiffness of the joints that decreases during the rest of the day [50]. In a mouse model of RA, the rhythmic expression of clock genes is altered in nuclei of synovial cells and in splenocytes [51]. Moreover, clock genes appear to control the disease. Indeed, in arrhythmic *Cry1*/*Cry2* KO mice, the proportion of activated TNFα-producing T cells in the spleen after RA induction was increased compared to WT mice, leading to a more pro-inflammatory environment and aggravation of the disease. Interestingly, the increased arthritis score in *Cry* KO mice can be attenuated by blocking TNFα action. Furthermore, in cultured human rheumatoid synovial cells, TNFα modulates *Per2* gene expression through the action of D-box-binding transcription factors to *Per2* promoter [52].

In human studies, two hormones were shown to be higher in the night in RA patients' sera compared to control patients: prolactin and melatonin, establishing a pro-inflammatory environment in synovial membrane cells [53, 54]. In another study, RA patients with high disease activity exhibited increased morning levels of plasma cortisol, an anti-inflammatory hormone [55]. Thereby, the pathogenesis of RA might be influenced by circadian cues including circadian hormones.

### 22.4.2 Allergic Reactions

The allergic response is a hypersensitive immune reaction caused by a normally harmless substance termed allergen in this context. The exposure to the allergen leads to the secretion of immunoglobulin type E (IgE) that binds to basophils (in the blood) and to mast cells (in tissues). The first exposure to the allergen is not responsible for the allergic reaction. This is the sensitization phase. A second exposure to the same allergen causes the release of substances (histamine, prostaglandins, and leukotrienes) by the basophils and mast cells, leading to the manifestations of allergic symptoms (i.e., swelling and inflammation of the tissue). Among allergic diseases, allergic asthma presents a diurnal pattern [56]. Indeed, attacks occur mainly during the night and early morning in human, suggesting a circadian control of the disease.

In a mouse model of cutaneous allergic reaction, the allergic response is exacerbated during the day and is lower at night [57]. This variation is absent in *Per2* mutant mice and in mice after ablation of the adrenal glands (which normally secrete rhythmic corticosterone). The authors suggested that the local clock in mast cells might gate the response to corticosterone regulation. Thus, to address the role of a mast cell clock, they generated mice with a dysfunctional clock only in these cells: the daily variation of cutaneous allergic reaction was also lost in these mice [57, 58].

A major mechanism of the disease is the interaction of IgE with an Fcε receptor at the cell surface of the mast cells, leading to the degranulation of these cells. This degranulation leads to manifestation of the different symptoms of the disease. Mast cell degranulation displayed daytime dependence in mice [57]. Furthermore, the transcription of the Fcε receptor gene was also shown to be under control of the mast cell clock. Finally, the rhythmic secretion of CCL2, IL-6, and histamine during a systemic allergic reaction was abolished in mice with an SCN lesion [59] Taken together, these results suggest a strong implication of the circadian clock in allergic reactions.

### 22.4.3 Acquired Immune Deficiency Syndrome

Acquired immune deficiency syndrome (AIDS) is a pathology caused by the human immunodeficiency virus (HIV). This disease is associated with sleep difficulties such as insomnia [60]. The HIV has a CD4 T cell and macrophage tropism (i.e., HIV preferentially targets CD4 T cells by binding to the co-receptor CD4 and macrophages by binding the receptor CCR5 and the co-receptor CD4). After cell infection, the viral genome is reverse-transcribed to DNA and integrated in the cellular DNA. The viral DNA can remain silent for an extended period of time, and at some point can be expressed, to generate new virus that might infect other cells.

A mouse study showed that Tat, a specific viral transcription factor important for the replication of the HIV virus, can reset the circadian clock [61]. This action is due to amino acids 1–72 of the Tat protein, also involved in cell surface binding leading to the endocytosis of Tat. Another mouse study examined the effect of chronic exposure to Tat in GFAP immunoreactive cells (astrocytes), which are abundant in the SCN, on circadian rhythms, and their entrainment to a light-dark cycle. The wheel-running activity levels were significantly decreased in these mice [62]. Given that HIV infection is impossible in rodents, the relevance of such studies for the knowledge of possible effects of HIV infection in humans is unclear. However, similar results were observed in monkeys infected with simian immunodeficiency virus (SIV), a virus similar to HIV: these monkeys displayed low levels of motor activity [63]. These studies suggest that the decreased motor activity and circadian rhythm amplitude in infected individuals with HIV or SIV might be due to altered Tat expression.

## 22.5 Conclusion

Various pathologies present a circadian pattern in terms of symptoms and pathogenesis development. The impact of circadian clocks on immunocompetent cell function has been demonstrated in various studies both in murine models and in human subjects. In some immune cells, the cell-intrinsic clock was shown to be essential for the circadian regulation. However, it remains important to analyze more deeply the role and the consequence of each of these immune clocks in immune responses and in the fight against pathogens and cancer. Understanding these mechanisms might improve treatment based on chronobiology in immune diseases as well as vaccination.

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# Chapter 23
# Clock Genes and Cancer

**Silke Kiessling and Nicolas Cermakian**

**Abstract** Mismatch between the external time and the internal circadian time causes loss of circadian organization and is frequently linked to cancer. This chapter describes the role of the molecular circadian clock in the incidence and progression of cancer. The first section will present the strong association between disrupted clock gene expression in either the host or the tumor tissue with cancer progression. Furthermore, it will be evaluated whether timed clock gene expression is a relevant factor for tumor development. Possible processes that are regulated by the circadian clock and may trigger tumor growth during circadian disruption will be summarized in the second section. The last section will highlight the importance of circadian timing for the development of effective cancer therapies.

## 23.1 Circadian Disruption Is Associated with Cancer

Changes in environmental conditions can disrupt the molecular circadian clock. For example, abrupt shifts in the day/night cycle, as experienced during jetlag or shift work, result in desynchronization within and between circadian clocks in the suprachiasmatic nucleus (SCN) and in peripheral tissues [1]. Furthermore, increasing evidence links dysfunction of the clockwork with tumor progression [2]. Thus, circadian disruption caused by mismatch of the external time with the internal time is believed to be an underlying factor for the risk of cancer. Indeed, in 2007, an agency of the World Health Organization classified shift work with circadian disruption as "probably carcinogenic" to humans [3] based on results from various experimental and epidemiological studies (reviewed by [4]). For example, a higher incidence of endometrial and colorectal cancer was found in nurses exposed to night shift work compared to their colleagues working on day shifts [5]. Another study indicated an increased risk of people working under night shift conditions to develop non-Hodgkin lymphoma [6].

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Studies in humans were supported by experiments in rodents, e.g., chronic jetlag condition promotes the incidence of lung cancer in rats following injection of tumor cells [7] and enhances the progression of Glasgow osteosarcoma in mice [8]. These mice show circadian disruption on multiple levels, such as disturbed rhythms of clock gene expression in the SCN, body temperature and hormone levels.

Whether circadian disruption is directly linked to cancer occurrence and enhances tumor progression was further investigated by genetic approaches. Results obtained from studies on mice with genetic defects in clock genes, which lead to circadian dysfunction, should therefore match the results obtained from studies on shift workers. Indeed, numerous studies discovered a close association between single nucleotide polymorphisms (SNPs), deletion, deregulation, or epigenetic silencing of circadian genes in humans or targeted gene ablation in animal models; and increased cancer risk (recently reviewed in [9]).

### *23.1.1 Disturbance of Clock Genes in the Host Is Associated with Cancer*

#### 23.1.1.1 Clock Gene Polymorphism in Humans

Recent studies report significant associations between polymorphisms in clock genes and cancer risk, in particular in *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *TIM*, *BMAL1* (or *ARNTL*), *CLOCK*, *NPAS2*, *REV-ERBα* (or *NR1D1*), *DBP*, *DEC1*, *DEC2*, and *CK1ε* [10–12]. For instance, a polymorphism in the *PER3* gene has been associated with prostate and breast cancer risk [13, 14]. However, other studies did not find such an association [10, 15, 16]. The different outcomes of these studies did not verify a clear association between *PER3* and cancer risk; thus, the data have recently been reanalyzed and support an association of *PER3* variants with the incidence of breast cancer, but not with glioma, prostate and colorectal cancer [17].

Breast cancer risk has additionally been associated with variants in either *BMAL1*, *CRY1*, and *NPAS2* [15, 16] or *BMAL1*, *CLOCK*, *CK1ε*, and *NPAS2* [10]. Variants of *NPAS2* have also been associated with prostate cancer risk [14]. Moreover, prostate cancer risk and lymphomagenesis were linked to variants in *CRY2* [18].

Taken together, genetic associations between clock gene variants and cancer risk indicate that variation in clock functions can act as a risk factor for cancer. This is consistent with the possibility that disturbed clock function may enhance the incidence to develop cancer.

#### 23.1.1.2 Clock Gene Mutations in Mice

Apart from spontaneous genetic variation in human clock genes, cancer cell lines were established and various circadian mutants have been identified and designed in rodents. These mutants show circadian phenotypes of different magnitude, from arrhythmicity to minor changes in their behavior. Here we summarize the implication of mutations in specific clock genes in the development and progression of cancer.

*Per1* and *Per2* double-knockout (KO) mice lack a functional circadian clock, whereas *Per2* mutants ($Per2^{m/m}$) have a functional but disturbed circadian clock. However, abnormal cell growth was reported in both of these mouse lines and is further reflected in increased radiation-induced tumor occurrence and progression [2, 19]. Accordingly, *Per1* or *Per2* overexpression results in slower tumor growth [20, 21]. Thus, *Per* genes have been characterized as tumor suppressor genes.

Similar to *Per1*/*Per2* double-KO mice, animals lacking both *Cry1* and *Cry2* have a disrupted circadian clock and show increased tumor development following γ-irradiation [2]. However, a different study by Gauger and Sancar reported that animals with targeted disruption of both *Cry* genes did not differ from their wild-type (WT) littermates regarding the frequency of tumor development [22].

*Bmal1* gene ablation in mice abolishes circadian rhythmicity [23]. A computational model predicted that perturbation of BMAL1-mediated transcription can generate circadian phenotypes similar to those observed in metastatic cell lines [24]. Indeed, *Bmal1* KO mice show increased radiation-induced tumor development similar to *Per* or *Cry* double-KO mice [2]. Accordingly, knockdown of *Bmal1* promoted tumor growth in mice [25], whereas its overexpression inhibited colorectal cancer cell proliferation [26].

Comparable to *Bmal1* KO mice, mice with a *Clock* gene mutation ($Clock^{\Delta 19}$) were unable to maintain circadian rhythms in constant darkness [27]. However, these mice only show a moderate cancer-related phenotype [28]. In particular, $Clock^{\Delta 19}$ mice do only exhibit increased tumor formation when exposed to long-term γ-irradiation.

Abolished circadian rhythmicity is not necessarily needed to promote cancer. Indeed, deregulation of a single clock gene may be sufficient to change the overall level of other clock genes and account for the cancer-related phenotype. Mice lacking the clock kinases CK1δ, which have an abnormal period, were reported to have an increased incidence to develop mammary carcinogenesis and a shorter life span [29]. Also, as mentioned above, *Per2* mutant mice exhibit a cancer phenotype comparable to arrhythmic *Per1*/*Per2* double-KO mice [19].

Taken together, it is necessary to address the role of each clock component within the molecular clockwork at the systems level, especially in relation to an increased risk to develop cancer. In this regard, the expression levels of clock genes have been correlated with the magnitude and prognosis of cancer. In the next section, results are summarized showing a correlation between the gene expression level for specific clock genes and the incidence of cancer.

### *23.1.2 Clock Gene Expression Within Tumor Cells Correlates with Cancer Progression*

#### 23.1.2.1 Tumor-Intrinsic Clock Gene Expression Is Associated with Cancer

In humans or WT animals, multiple recurring changes in the light-dark cycle disrupt the molecular clock in the SCN and in peripheral tissues [1]. Aside of circadian disorganization, animals undergoing repeated jetlag exhibit increased tumor frequency and faster tumor growth [7, 8]. Interestingly, circadian disruption has been reported, not only in the host's tissues, but also in various tumor cell lines and tumor tissues. However, the direction and magnitude of the changes in clock gene expression can be in opposite directions for different cancer types. For example, *CRY1* levels are decreased in pancreatic cancer [30], but increased in ovarian cancer [31]. Furthermore, changes in the same clock gene can have opposite effects on tumor growth and prognosis, e.g., *PER2* suppression in human pancreatic cancer cells results in reduced proliferation [32], whereas the overall survival in patients with low *PER2* levels in pancreatic tumors was found to be reduced [30]. In conclusion, the relationship between clock gene expression and cancer incidence seems to be gene and cancer type specific and does not always match between cancer cell lines and their related tumor tissues. Table 23.1 lists the studies in humans and rodents reporting significant correlations of clock gene expression with the incidence of cancer and overall prognosis.

#### 23.1.2.2 Circadian Rhythm Disruption in the Tumor Is Associated with Cancer

Various associations of clock gene expression with cancer have been reported (see Table 23.1). However, only an analysis of the time-dependent – circadian – effect of clock genes can allow addressing the role of the circadian rhythms in tumor development and progression. In this regard, disturbed circadian rhythms of clock genes have been documented in various cancer cells and tumor tissue (Table 23.2). Interestingly, circadian rhythmicity of *Per1*, *Per2*, *Rev-Erbα*, and *Dbp* was significantly reduced in colon tumor tissue, but not in healthy colon tissue surrounding the tumor [33], while the rhythmicity of *Bmal1* was abolished on both sides, indicating that tumor-intrinsic circadian rhythms may play a more pronounced role in cancer progression and development than rhythms in the host. Indeed, timed manipulation of circadian rhythms in the tumor was shown to accelerate tumor growth and strongly influence the magnitude of symptoms and prognosis. For example, lack of *Per1* or *Per2* increases tumor growth only at times when their intrinsic expression levels were high [34]. Thus, tumor-intrinsic circadian rhythms may represent a new target for cancer-related therapies. Indeed, since various tumor

**Table 23.1** Clock gene expression levels associated with cancer

| Clock gene | Species | Expression/ manipulation | Tissue | Outcome | References |
|---|---|---|---|---|---|
| *Per1* | Human | Decreased | Pancreatic cancer | Negative | [30, 64] |
| | Human | Increased | Pancreatic cancer cells | Negative | [65] |
| | Human | Knockdown | Pancreatic cancer cells | Positive | [32, 65] |
| | Human | Decreased | Colorectal cancer | | [66] |
| | Human | Decreased | Colorectal cancer | Negative | [21, 67–70] |
| | Human | Overexpression | Colorectal cancer cells | Positive | [21] |
| | Human | Decreased | Gastric cancer | Negative | [71] |
| | Human | Decreased | Breast cancer | | [72, 73] |
| | Mouse | Decreased | Breast cancer | | [74] |
| | Human | Enhanced | Breast cancer | Positive | [72] |
| | Human | Decreased | Liver cancer | | [75] |
| | Human | Knockdown | Liver cancer cells | Positive | [65] |
| | Mouse | Increased | Lung cancer | | [76] |
| | Human | Decreased | Lung cancer/cells | | [77] |
| | Human | Overexpression | Lung cancer cells | Positive | [77] |
| | Human | Decreased | Glioma cells | | [78] |
| | Rat | Overexpression | Mammary adenocarcinoma | Positive | [79] |
| | Mouse | Mutated | Mammary cancer | Negative | [34] |
| | Human | Decreased | Prostate cancer cells | | [80] |
| | Human | Decreased | Ovarian cancer | | [31] |
| | Human | Decreased | Endometrial carcinoma | | [81, 82] |
| | Human | Decreased | Skin melanoma | | [83] |
| | Human | Decreased | Squamous cell carcinoma | | [84] |
| | Human | Decreased | Pleural mesothelioma | | [85] |
| *Per2* | Human | Decreased | Pancreatic cancer | Negative | [30] |
| | Human | Suppression | Pancreatic cancer cells | Positive | [32] |
| | Human | Overexpression | Pancreatic cancer cells | Positive | [86] |
| | Human | Decreased | Colorectal cancer | | [70] |
| | Human | High | Colorectal cancer | Positive | [68] |
| | Mouse | Mutation | Colon cancer | Negative | [87] |
| | Mouse | Suppression | Colon cancer cells | Negative | [87] |
| | Human | Decreased | Gastric cancer | Negative | [71] |
| | Human | Decreased | Breast cancer | | [73] |
| | Mouse | Decreased | Breast cancer | | [74] |
| | Mouse | Suppression | Breast cancer | Negative | [88] |
| | Human | Increased | Breast cancer | Positive | [72] |
| | Human | Restored | Lung cancer | Positive | [89] |
| | Human | Decreased | Liver cancer | | [75] |
| | Human | Decreased | Kidney cancer | | [90] |
| | Human | Decreased | Glioma cells | | [78, 91] |

(continued)

**Table 23.1** (continued)

| Clock gene | Species | Expression/ manipulation | Tissue | Outcome | References |
|---|---|---|---|---|---|
| | Mouse | Mutated | Mammary cancer | Negative | [34] |
| | Human | Decreased | Skin melanoma | | [83] |
| | Human | Decreased | Squamous cell carcinoma | | [84] |
| | Mice | Overexpression | Human sarcoma cancer | Positive | [92] |
| | Human | Decreased | Ovarian cancer | | [31] |
| | Human | Overexpression | Osteosarcoma cells | Positive | [93] |
| *Per3* | Human | Decreased | Pancreatic cancer | Negative | [30] |
| | Human | Suppression | Pancreatic cancer cells | Positive | [32] |
| | Human | Decreased | Colorectal cancer | | [66] |
| | Human | Decreased | Colorectal cancer | Negative | [68, 70] |
| | Human | Enhanced | Breast cancer | Positive | [72] |
| | Human | Decreased | Liver cancer | | [75] |
| | Human | Increased | Ovarian cancer | | [31] |
| | Human | Decreased | Squamous cell carcinoma | | [84] |
| | Human | Decreased | Pleural mesothelioma | | [85] |
| *Cry1* | Human | Decreased | Pancreatic cancer | | [30] |
| | Human | Overexpression | Colorectal cancer | Negative | [94] |
| | Human | Increased | Ovarian cancer | | [31] |
| | Human | Decreased | Mucinous cancer | | [31] |
| | Human | Decreased | Skin melanoma | | [83] |
| | Human | Decreased | Squamous cell carcinoma | | [84] |
| *Cry2* | Human | Decreased | Pancreatic cancer | | [30] |
| | Human | Decreased | Colorectal cancer | | [70] |
| | Human | Enhanced | Breast cancer | Positive | [72] |
| | Human | Decreased | Liver cancer | | [75] |
| | Human | Decreased | Ovarian cancer | | [31] |
| | Human | Decreased | Squamous cell carcinoma | | [84] |
| | Human | Decreased | Pleural mesothelioma | | [85] |
| *Bmal1* | Human | Decreased | Pancreatic cancer | Negative | [30] |
| | Human | Decreased | Colorectal cancer | | [70] |
| | Human | Increased | Colorectal cancer | | [66] |
| | Human | Increased | Colorectal cancer | Positive | [26] |
| | Mouse | Knockdown | Colon cancer cells | Negative | [25] |
| | Human | Increased | Ovarian cancer | | [31] |
| | Human | Suppressed | Ovarian cancer | | [43] |

(continued)

**Table 23.1** (continued)

| Clock gene | Species | Expression/ manipulation | Tissue | Outcome | References |
|---|---|---|---|---|---|
| | Human | Overexpression | Ovarian cancer | Positive | [43] |
| | Human | Decreased | Mucinous adenocarcinomas | | [31] |
| | Human | Decreased | Squamous cell carcinoma | | [84] |
| | Human | Increased | Pleural mesothelioma | | [85] |
| | Human | Increased | Mesothelioma cells | | [41] |
| | Human | Knockdown | Mesothelioma cells | Positive | [41] |
| | Human | Overexpression | Glioma cancer cells | Positive | [95] |
| *Clock* | Human | Reduced | Pancreatic cancer | Negative | [30] |
| | Human | Increased | Colorectal cancer | | [66] |
| | Human | Increased | Colorectal cancer | | [67, 68] |
| | Human | Mutated | Colorectal cancer | | [45] |
| | Human | Restored | Colorectal carcinoma cells | Positive | [45] |
| | Human | Enhanced | Breast cancer | Positive | [72] |
| | Human | Knockdown | Glioma cells | Positive | [49] |
| | Human | Decreased | Ovarian cancer | | [31] |
| | Human | Decreased | Skin melanoma | | [83] |
| *Npas2* | Human | Decreased | Colorectal cancer | | [96] |
| | Human | Knockdown | Colorectal cancer | Negative | [96] |
| | Human | Enhanced | Breast cancer | Positive | [72, 97] |
| | Human | Increased | Glioma | Negative | [98] |
| | Human | Increased | Pleural mesothelioma | | [85] |
| *Dec1* | Human | Decreased | Pancreatic cancer | | [64] |
| | Human | Increased | Pancreatic cancer cells | | [99] |
| | Human | Increased | Breast cancer | | [100] |
| | Human | Decreased | Lung cancer | | [47] |
| | Human | Knockdown | Lung cancer cells | Negative | [47] |
| | Human | Overexpression | Lung cancer cells | Positive | [47] |
| | Human | Increased | Liver cancer | | [101] |
| | Human | Reduced | Liver cancer | Positive | [101] |
| | Human | Variant | Kidney cancer | Negative | [102] |
| | Human | Decreased | Esophageal cancer cells | | [103] |
| | Human | Restoration | Esophageal cancer cells | Positive | [103] |
| | Human | Decreased | Lymph node metastasis | | [104] |
| | Human | Increased | Gastric cancer | | [105] |
| *Dec2* | Human | Increased | Endometrial carcinogenesis | | [106] |

(continued)

**Table 23.1** (continued)

| Clock gene | Species | Expression/ manipulation | Tissue | Outcome | References |
|---|---|---|---|---|---|
| *CK1ε* | Human | Decreased | Pancreatic cancer | | [30] |
| | Human | Reduced | Pancreatic cancer | Negative | [30] |
| | Human | Increased | Colorectal cancer | | [68] |
| | Human | Decreased | Ovarian cancer | | [31] |
| | Human | Decreased | Endometrial carcinoma | | [82] |
| | Human | Decreased | Squamous cell carcinoma | | [84] |
| *Rev-Erbα* | Human | Decreased | Breast cancer | | [107] |
| | Human | Enhanced | Breast cancer | Negative | [108, 109] |
| | Human | Increased | Breast cancer cells | | [110] |
| | Human | Knockdown | Breast cancer cells | Positive | [110] |
| | Human | Agonist | Breast cancer cells | Positive | [48] |
| | Human | Increased | Papillary carcinoma | | [111] |
| | Human | Decreased | Pleural mesothelioma | | [85] |
| *Rorα* | Human | Decreased | Colorectal cancer | | [112] |
| | Human | Decreased | Breast cancer cells | | [113] |
| | Human | Enhanced | Breast cancer | Negative | [114] |
| | Human | Increased | Prostate cancer cells | Positive | [115] |
| | Human | Knockdown | Breast cancer cells | Positive | [114] |
| | Human | Decreased | Breast cancer/cells | Negative | [116] |
| | Human | Restored | Breast cancer cells | Positive | [116] |
| | Rat | Agonist | Pituitary cancer | Positive | [117] |
| | Rat | Antagonist | Pituitary cancer | Negative | [117] |
| *Rorγ* | Human | Enhanced | Breast cancer | Positive | [72] |
| | Human | Decreased | Thyroid cancer | | [111] |
| *Tim* | Human | Decreased | Pancreatic cancer | | [30] |
| | Human | Increased | Colorectal cancer | | [70] |
| | Human | Decreased | Liver cancer | | [75, 82] |
| | Human | Decreased | Kidney cancer | | [90] |

tissues and human cancer cell lines harbor a dysfunctional circadian clock, the strategy to improve circadian rhythms in those cells becomes obvious.

#### 23.1.2.3 Restoring Circadian Rhythm in the Tumor Inhibits Tumor Growth

Only a limited number of studies have addressed whether tumor-intrinsic clock manipulations may become important for cancer prevention or therapy. For example, the inhibitory effect of the drug seliciclib on tumor growth was enhanced when administration was done at times of the day when it stimulates a high amplitude of

**Table 23.2** Disturbed circadian rhythms in cancer cells and tumor tissue

| Clock gene | Species | Tissue | Circadian expression | References |
|---|---|---|---|---|
| *Per1* | Human | Breast cancer | Disturbed | [118] |
| | Human | Breast cancer | Dampened | [73] |
| | Human | Neuroblastoma | Dampened | [119] |
| | Human | Astrocytoma | Arrhythmic | [119] |
| | Human | Hepatoma cells | Arrhythmic | [119] |
| | Human | Myeloid leukemia | Disrupted | [120] |
| | Hamster | Buccal mucosa cancer | Decreased | [121] |
| | Mouse | Colorectal cancer | Decreased | [33] |
| *Per2* | Human | Breast cancer | Disturbed | [118] |
| | Human | Breast tumors | Dampened | [73] |
| | Rat | Human breast cancer cells | Arrhythmic | [122] |
| | Rat | Human breast cancer | Repressed/disrupted | [122] |
| | Human | Myeloid leukemia | Disrupted | [120] |
| | Mouse | Colorectal cancer | Decreased | [33] |
| *Per3* | Human | Breast cancer | Disturbed | [118] |
| | Human | Myeloid leukemia | Disrupted | [120] |
| *Cry1* | Human | Myeloid leukemia | Disrupted | [120] |
| *Cry2* | Human | Myeloid leukemia | Disrupted | [120] |
| *Rev-Erbα* | Mouse | Colorectal cancer | Decreased | [33] |
| *Dbp* | Mouse | Colorectal cancer | Decreased | [33] |
| *Bmal1* | Rat | Human breast cancer | Repressed/disrupted | [122] |
| | Rat | Human breast cancer cells | Arrhythmic | [122] |
| | Human | Myeloid leukemia | Disrupted | [120] |
| | Mouse | Colorectal cancer | Abolished | [33] |
| *CK1ε* | Human | Myeloid leukemia | Disrupted | [120] |

clock gene expression in the tumor [35]. These results match observations by Li et al. indicating reduced tumor growth when circadian rhythms were restored by time-restricted food access [36]. Both studies are limited by the possible side effects of the drug and the feeding schedule on other peripheral circadian clocks, such as the liver. Whether restoration of circadian oscillations specifically in the tumor is sufficient to inhibit tumor growth needs to be validated in future studies.

Taken together, changes in overall clock gene expression as well as disruption of their rhythmic expression have been documented in the host and in cancer cells. However, a correlation between clock gene alterations, circadian disruption, and their role in delaying cancer development is an indication but not evidence for a causal relationship in either one or both directions. Thus, two distinct hypotheses can be made. A circadian clock gene may be influencing cancer incidence or tumor development (1) due to their gene-specific activities on target genes involved in cancer-related pathways or (2) through their involvement in circadian clock functions regulating cancer-related pathways. In line with the first hypothesis, clock gene alterations or mutations do not necessarily lead to disruption of the circadian system, either centrally or peripherally. Nevertheless, changes in clock gene

expression have been correlated to the incidence to develop cancer. In contrast, the best indication for the second hypothesis is that WT mice with an environmentally disrupted circadian system show enhanced tumor progression. Moreover, deregulated clock genes are frequent in human cancer cells and tumor tissue.

## 23.2 Possible Mechanisms Linking the Circadian Clock with Cancer

Cancer development may be pronounced when tumor suppressor functions of either the circadian clock or specific clock genes are lacking. Transcriptome analysis has revealed that many important genes involved in cancer-related pathways, such as the cell cycle, cell proliferation, apoptosis, and DNA damage, are targets of the circadian clock [37]. This section will describe the current knowledge regarding the involvement of the circadian clock in processes which are dysregulated during tumorigenesis. Another possible mechanism involves the circadian control of immune responses, in particular NK cell cytotoxicity. The reader is referred to Chap. 22 for more details.

### *23.2.1 The Circadian Clock Controls the Cell Cycle*

The cell cycle is the process by which a cell prepares for and accomplishes its division into two daughter cells. This occurs through different phases: G1, where the cell prepares for DNA replication; S, where DNA replication occurs; G2, where the cell prepares for mitosis; and M (mitosis), where the cell divides to give rise to the two daughter cells. The cell cycle involves a network of cyclin-dependent kinases (CDKs) forming complexes with cyclins, which regulate each phase of the cell cycle by controlling major checkpoints. This gene network is coupled to the circadian clock through circadian variations in the levels of major players regulating cell cycle checkpoints, such as WEE1, c-MYC, p21, and cyclin E [19, 38] (see Fig. 23.1).

The important checkpoint kinase Wee1 shows rhythmic expression, established through gene activation by CLOCK/BMAL1 and repression by PERs/CRYs [21, 38]. WEE1 inhibits the entry into the M phase by suppression of CDK1 and CDK2 activity [38, 39]. Consequently, reduced synthesis of WEE1 favors the entry into mitosis and may even shorten its duration. Dysregulated circadian expression of WEE1 in turn could induce disruption of the cell cycle, which may result in uncontrolled fast proliferation. Indeed, low and arrhythmic levels of *Wee1* have been reported in *Clock* mutant mice [40] and after *Bmal1* deletion in cancer cells [41]. In contrast, loss of *Cry* genes increased *Wee1* expression, which inhibited the G2/M transition and may account for slower liver regeneration [38].

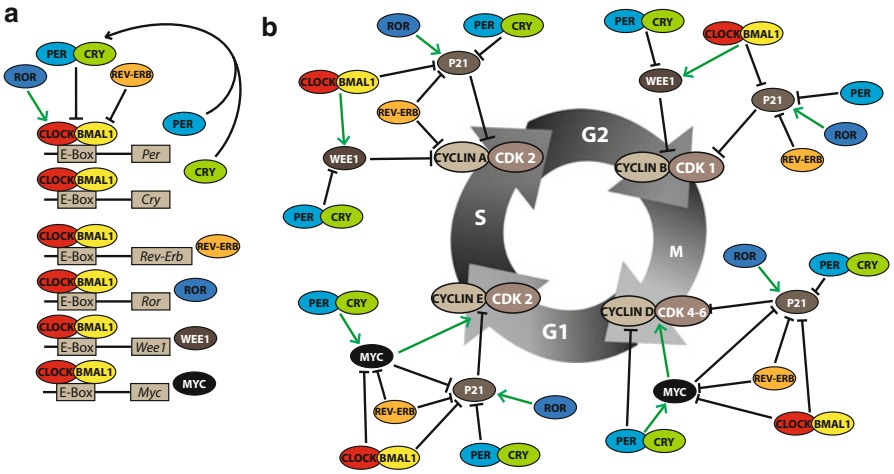


**Fig. 23.1** The circadian clock controls the cell cycle on multiple levels. The circadian clock (**a**) interacts with all cell cycle phases by controlling major cell cycle factors, such as WEE1, MYC, p21, cyclin A, and cyclin D (**b**). The cell cycle network consists of cyclin-dependent kinases (CDKs) forming complexes with cyclins and regulating each phase of the cell cycle by controlling major checkpoints: G1 and G1/S phase transition are regulated by cyclin D/CDK4–6 and cyclin E/CDK2, respectively; entry into S phase and thereby DNA replication as well as S-G2 transition are controlled by cyclin A/CDK1; cyclin B/CDK1 finally elicits G2/M phase transition [21, 38, 39]

Another important cell cycle regulator, the oncogene *c-Myc*, is rhythmically regulated via promoter elements for CLOCK/BMAL1 [19]. In contrast to *Wee1*, *c-Myc* expression is repressed by CLOCK/BMAL1 and REV-ERBα but elevated by PER1 [21]. Cell proliferation is regulated by c-MYC via activation of cyclin E/CDK2 and cyclin D/CDK4–6 in parallel, to inhibition of cell cycle inhibitors p21 and p27 [39]. Importantly, *c-Myc* expression was found to be highly elevated in many human tumors [42] and mouse mutants [19]. For example, enhanced expression of *c-Myc* correlates with increased γ-irradiation-induced cell proliferation and tumor development in *Per2* mutant mice, which exhibit suppressed *Bmal1* expression. Accordingly, overexpression of *BMAL1* can suppress elevated *c-MYC* levels and restore its rhythmic activity in ovarian cancer cells [43]. c-MYC is required for G0/G1 transition and elicits S-phase entry, and thus overexpression in cancer cells may be a crucial link for enhanced tumor development [42].

Circadian rhythmicity of the CDK inhibitor *p21* is achieved via inhibition by REV-ERB factors and activation by ROR factors [44]. Additionally, *p21* harbors CLOCK-binding elements [45], and *Per1* overexpression was reported to repress *p21* [21]. P21 inactivates various cyclin/CDK complexes, which induce cell cycle phase entries, such as S-phase transition by cyclin E/CDK2. Other than *Wee1*, *p21* is upregulated and arrhythmic in *Clock* mutant and *Bmal1* KO mice [40, 44]. Consequently, decreased proliferation rate was observed in *Bmall* KO hepatocytes, which can be rescued by *p21* knockdown. In contrast and despite enhanced *p21* levels, increased cell proliferation was found in the epidermis of *Bmal1* KO mice [46], and

enhanced tumor development after tissue-specific ablation of *Bmal1* has been documented [25]. An explanation might be that the regulatory function of p21 in clock gene KO mice may be masked by other cell cycle regulators also regulated by CLOCK/BMAL1, such as c-MYC or WEE1. This is seen, for example, in pleural mesothelioma cells, where *P21* and *WEE1* and *CYCLIN E* levels were altered upon knockdown of *BMAL1* [41].

Adding to this picture, other cell cycle genes were identified as targets of the circadian clock, such as cyclin D1 and A [19, 47, 48].

Taken together, the clock-controlled cell cycle proteins have different sometimes even antagonistic effects on cell cycle progression. This may explain why different clock gene mutants exhibit different cell cycle- and cancer-related phenotypes. Nevertheless, this complex interaction between the circadian clock and cell cycle events probably underlies at least in part the key role for circadian rhythm alterations in carcinogenesis.

### *23.2.2 DNA Damage Response*

Errors during repair of damaged DNA can cause mutations [49]. Genomic instability or accumulation of mutations within the genome is a hallmark of cancer. When damaged DNA is detected by sensor kinases, such as ATM or ATR, cells activate the cell cycle checkpoint kinases CHK1 and CHK2, which stabilize the oncogene p53. P53 in turn activates a series of genes that restrict cell cycle progression and stimulates DNA repair or, in the case of irreparable damage, triggers apoptosis [50]. Important p53 targets are *Mdm2*, *Gadd45α*, and the CDK inhibitor *p21*, a major cell cycle player which mediates G1 arrest and is controlled by the circadian clock (see above). Additionally, p53 and its targets *Mdm2* and *Gadd45α* are regulated by the circadian clock [19]. MDM2 activates cell cycle arrest, but it also feeds back on p53 and inhibits its functions [37, 38, 51]. GADD45 inhibits the G2/M transition and triggers apoptosis [37].

Daily oscillation of DNA excision repair was documented in mouse skin [52]. At the clock gene level, BMAL1 was found to suppress p53 functions in human fibroblast cells and thus to induce the release from cell cycle arrest [51]. Accordingly, overexpression of *BMAL1* inhibited DNA damage sensitivity [26], whereas tissue-specific ablation of *Bmal1* increased the risk of genomic instability and cell cycle arrest in the epidermis [46].

In *Per2* mutant mice, *p53* induction and *Gadd45α* and *Mdm2* rhythmicity were deregulated [19]. Accordingly, daytime-dependent DNA damage-induced apoptosis was perturbed in thymocytes of *Per2* mutant mice [53]. Recent studies demonstrated that PER proteins modulate apoptosis and cell cycle arrest by controlling ATM and CHK2 [21, 54]. In this regard, overexpression of *PER1* triggered DNA damage-induced apoptosis, whereas inhibition of *PER1* blocked apoptosis in human cancer cells [21]. In contrast, *CLOCK* knockdown increased γ-irradiation-induced cell cycle arrest and apoptosis in human glioma cells [49].

Interestingly, *p53*, *Gadd45α*, and *Mdm2* are additionally controlled by *c-Myc*, which is in turn regulated by BMAL1 [37]. Thus, clock-controlled *c-Myc* expression may be another important factor for tumorigenesis by integrating the cell cycle and DNA damage response with the circadian clock. Indeed, jetlag in mice was sufficient to uncouple p53 and c-MYC signaling in the thymus and induce tumor development [2].

Taken together, the circadian clock has been implicated in both DNA damage-induced apoptosis and DNA repair. Consequently, in cases of circadian deregulation, the occurrence of DNA mutations may increase. Moreover, mutated cells may bypass their apoptosis, which would result in accumulation of mutated cells and thereby inducing cancer. Consequently, a reasonable explanation for how clock gene manipulation leads to enhanced cancer incidence or growth rate could be their interaction with transcriptional regulators controlling cell proliferation, DNA repair, and apoptosis.

## 23.3 Does the Tumor Disrupt Circadian Rhythms?

The interconnection between the circadian clock and the cell cycle does not allow conclusions about the cause of cell cycle deregulation and circadian perturbation in cases of cancer. An intriguing hypothesis is that disturbance of one of the cycles during cancerogenesis, either the circadian clock or the cell cycle, can in turn disrupt the function of the other one. In this section, we address the possible mechanisms for the disruption of clock function in tumors.

### *23.3.1 Cancer-Related Genes*

Interestingly, recent evidence supports the idea that cancer-related signals may interfere with the circadian clock machinery. The clock-controlled major cell cycle regulator MYC can induce circadian malfunction by indirectly repressing *Bmal1* through upregulation of REV-ERBα and PER2 [55]. Importantly, previous studies mentioned an upregulation of *Myc* in human tumor tissues [42] and circadian mouse mutants [19]. Consequently, MYC is an even more important candidate for cancerogenesis by integrating disturbed circadian rhythms and cell cycle dysfunctions and thus is considered as an important target for the development of cancer therapies.

Perturbation of another oncogene, RAS, has been reported to cause circadian clock disruption. RAS transformation induced major phase shifts of *Bmal1* promoter-driven luminescence in human keratinocytes, mouse fibroblasts, and human colorectal cancer cells [24]. Moreover, decreased *PER2* levels and upregulated *CRY1* expression were observed after RAS transformation, supporting the possibility that the activity of RAS might modulate the circadian disruption in cancer cells by influencing CLOCK/BMAL1.

### 23.3.2 Epigenetic and Posttranscriptional Modifications

DNA methylation plays an important role in modifying gene expression posttranscriptionally, and promoter hypermethylation is a hallmark of cancer. Most core clock genes are predominantly downregulated in various cancer cell lines and tumor tissues (see Table 23.1). Thus, improper DNA methylation may suppress clock gene expression, contributing to the development and progression of cancer. Interestingly, methylated DNA immunoprecipitation microarray identified BMAL1 among the genes that are differentially methylated in ovarian cancer cells [43]. Cancer cell growth could be restored by rescuing *BMAL1* expression, indicating that DNA methylation may be an important mechanism to suppress the circadian clock in cancer cells and induce cancer proliferation. Indeed, hypermethylation has been found on the promoters of core clock genes, such as *PER1*, *PER2*, *CRY1*, and *BMAL1* in breast cancer tissue [56].

Another tumor-intrinsic mechanism which may disrupt circadian rhythms is ubiquitination. For example, transfection with the oncogenes E6/E7 in mouse fibroblasts led to BMAL1 ubiquitination and degradation after the action of the UBE3A ubiquitin ligase on this clock protein and suppression of circadian rhythms in these cells [57].

Histone modifications such as acetylation/deacetylation can also control gene expression and in turn underlie circadian clock control [58]. Interestingly, the histone deacetylase sirtuin 1 (SIRT1) was identified as a circadian clock component, as it deacetylates BMAL1 and PER2 [59]. Low levels of *SIRT1* were documented in various colorectal cancer cell lines and tumor tissues [60], and a correlation was found between expression levels of *SIRT1*, altered clock gene expression and the outcome of pancreatic adenocarcinoma in patients [61]. Collectively, these data indicate that tumor components may direct epigenetic modifications, leading to disruption of circadian rhythms.

## 23.4 Conclusions and Perspectives

In conclusion, circadian disruption within tumor tissues and in the host enhances cancer progression, and a poorer prognosis was documented in cancer patients with altered circadian rhythms. Thus, improving circadian rhythms in the host and in the tumor may be an important strategy to address cancer therapy.

Also important is cancer chronotherapy – the timed administration of anticancer drugs. The circadian regulation of physiological processes, such as metabolism or detoxification, has severe consequences on the outcome of anticancer therapies [62]. For example, the treatment efficacy and patient survival were improved by rhythmic delivery of the therapeutic into colorectal cancer [63]. Studying the timing of anticancer therapies will allow maximal therapeutic effect with minimal cytotoxic side effects, which may dramatically enhance the life quality of cancer patients.

However, the molecular mechanism linking circadian disruption and cancer should be examined based on specific cancer subtypes. The alteration of clock gene expression differs between cancer subtypes and thus does not allow generalizations about the function of circadian clock genes or the overall circadian system on the development of cancer. Precise characterization of specific cancer subtypes could be used to develop therapeutic approaches involving circadian control, tailored for each cancer type.

## Key Questions of Interest and Suggested Readings

- Is circadian clock disruption the cause of cancerogenesis or does cancer induce circadian clock disruption? Hints exist to support both hypotheses, but further studies are required to address this key question.
- How could circadian clock disruption enhance tumor growth? Dysregulation of the cell cycle by altered expression of cell cycle regulators such as WEE1 or c-MYC in circadian clock mutant mice affects the speed of the cell cycle and thus may regulate cancer progression [19, 38].
- What tumor-intrinsic mechanism could downregulate clock genes? Possible factors are DNA methylation [43], ubiquitination [57], or histone modifications [59].
- How can we take advantage of the link between the circadian clock and cancer? Improving circadian rhythms in the host and tumor tissue may reduce cancer progression [35, 36]. Cancer chronotherapy [63] uses the circadian time to treat cancer most effectively.

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# Part VI
# Pineal, Melatonin, and Biological Timekeeping

# Chapter 24
# The Timezyme and Melatonin: Essential Elements of Vertebrate Timekeeping

**Surajit Ganguly and David C. Klein**

**Abstract** One of the most highly conserved features of vertebrate physiology is a robust daily rhythm in melatonin synthesis by the pineal gland. This in turn controls the daily rhythm in circulating melatonin, characterized by high values occurring at night. This high-at-night pattern is seen in all animals, independent of lifestyles. This signal has broad effects on biological processes, as documented in over 10,000 publications since the discovery of melatonin in the late 1950s by Aaron Lerner, the father of the melatonin field.

One interesting aspect of melatonin is that it does not have a specific, well-conserved effect on a single-target process, as is the case for other hormones, e.g., growth hormone and insulin. Rather, melatonin is a circulating time signal that is interpreted and used differently by different species to optimally entrain their physiology to the environment. This has been reviewed by many experts in the field and is beyond the scope of this chapter. Rather, this chapter will focus on a less well-studied facet of melatonin, which is how the daily rhythm in melatonin is regulated.

## 24.1 Daily Rhythm in Melatonin Production and the Unique Role of the "Timezyme"

The last four decades of research on this topic have revealed that the large changes in melatonin synthesis are driven by large changes in a one enzyme, arylalkylamine *N*-acetyltransferase (AANAT; EC 2.1.3.87), which acetylates serotonin (5-HT) to form *N*-acetylserotonin (NAS; Fig. 24.1) [4]. The activity of AANAT increases tenfold to 100-fold at night, causing a dramatic increase in melatonin synthesis

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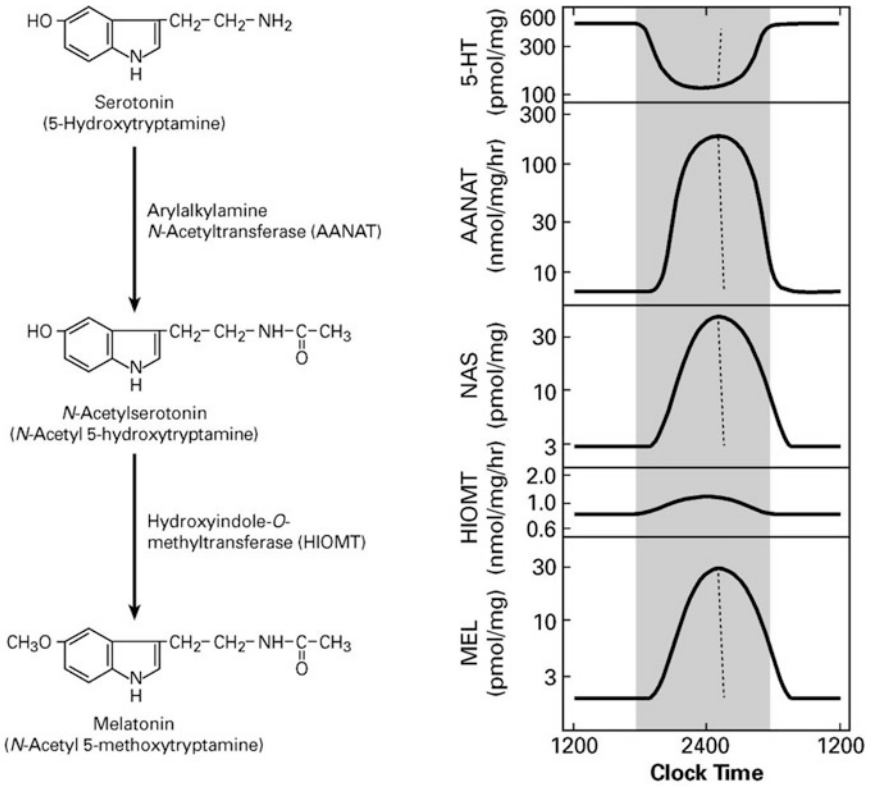


**Fig. 24.1** Melatonin synthesis steps and daily rhythm in pineal indoleamines. Serotonin (5-hydroxytryptamine, 5-HT) levels decrease at night (*shaded*) due to a large increase in arylalkylamine *N*-acetyltransferase (AANAT) activity. AANAT catalyzes the transfer of an acetyl group from AcCoA to 5-HT. The increase in AANAT activity results in an increase in the intracellular concentration of N-acetylserotonin (5-hydroxy-N-acetyltryptamine, NAS), which is converted to melatonin (N-acetyl-5-methoxytryptamine, MEL) by hydroxyindole-O-methyltransferase (HIOMT or ASMT). Exposure to light at night causes a rapid return to the daytime levels (dotted line). Shaded area represents nighttime values. It is to be noted that the rhythmic pattern continues in constant darkness as AANAT is stimulated by the SCN-driven endogenous circadian clock [1, 2] (This figure is used from the review by Klein et al. [3])

leading to immediate release of melatonin and its elevation in circulating levels. Circulating melatonin levels are a direct reflection of its synthesis. The close relationship between the cellular synthesis and the circulatory levels is made possible by the rapid catabolism of circulating melatonin by the liver [4, 5]. The precisely regulated changes in AANAT activity are remarkably rapid; the doubling time in rodents is close to 15 min. Moreover, exposure of animals to light at night causes AANAT activity to rapidly disappear with a halving time ($T_{1/2}$) of ~3.5 [6, 7]. Although this rhythmic pattern is conserved, the amplitude and duration of the nocturnal increase in melatonin vary from species to species and are used by each species to optimally control seasonal and daily changes in physiology [4].

AANAT acquired the nickname "the timezyme" on the basis of the unique role this enzyme plays in biological timekeeping in vertebrates [1]. It is unequivocally the most important single enzyme in interfacing the environment with the internal milieu and serves as the target of multiple mechanisms which have evolved to control melatonin synthesis through control of the activity of this one enzyme. Whereas other enzymes are required for melatonin synthesis, no other enzyme contributes significantly to the daily dynamics of circulating melatonin.

## 24.2 Distribution of the Timezyme

AANAT is consistently found at high levels in the pineal gland of all vertebrates [3]. It is also expressed in the retina at levels that vary from species to species; in some cases expression in the retina and pineal gland is similar [8–10]. AANAT expression has also been detected in the pituitary gland [10–12]. The production of melatonin in extrapineal sites is low and does not appear to contribute to circulating melatonin, which is totally dependent upon pineal-derived melatonin [5, 13, 14]. AANAT activity in the retina and pituitary gland has been implicated in local signaling. This could involve melatonin, N-acetylserotonin, N-acetyltryptamine, or another acetylated arylalkylamine.

## 24.3 Evolution of the Timezyme

The name AANAT refers to a family of enzymes within the large Gcn5-related superfamily of acetyltrasnferases (GNAT) composed of over 100 members [15, 16]. Members N-acetylate a broad range of substrates, ranging from proteins to small molecules. Whereas each family member exhibits high specificity for a specific substrate, all share structural features, referred to as the GNAT domain, involved in acetyl-CoA (AcCoA) binding.

The AANAT family is divided into vertebrate and non-vertebrate AANATs [17, 18]. A single AANAT gene is found in all vertebrates except for fish, which have two or more [19]. The preferred substrates are aromatic amines in which a carbon side chain separates the amine and the aromatic ring, hence the name arylalkylamine; these include tryptamine, serotonin, phenylethylamine, and similar compounds. Non-vertebrate AANATs are present in a broad range of organisms, including plants, yeast, and invertebrates. The vertebrate form of AANAT evolved rapidly ~500 million years ago, coincident with the evolution of the eye and pineal gland. This evolution is characterized by two distinct changes. One is an increase in catalytic capacity, resulting from acquisition of a short peptide that converts a relative immobile loop of protein into a highly floppy structure and acquisition of histidines, which facilitate elimination of protons, as part of a proton chain during catalysis. The second is the addition of regulatory sequences that flank the catalytic

core, which mediate changes in the stability and kinetics of the enzyme and are essential for rapid changes in activity.

A dominant evolutionary pressure to acquire the features seen in the vertebrate AANAT is likely to have been detoxification [20, 21]. In general, amines are potentially toxic because they can interact with macromolecules nonspecifically and as a result compromise cellular function. In the case of AANAT, a more specific pressure came from the potential of arylalkylamines to react with and destroy retinal (retinaldehyde) through Schiff base conjugation; this compromises light detection in photodetectors. Photodection in vertebrates begins with a photon-induced photoisomerization of 11-*cis*-retinal to all-*trans*-retinal. A complex process has evolved to recycle and reuse each molecule of retinal; the evolution of AANAT enhanced this by protecting against loss of retinal through conjugation with arylalkylamines. Moreover, this daily rhythm in AANAT activity likely evolved to optimize light sensitivity at night.

By-products of the evolution of the nocturnal increase in AANAT were N-acetyltryptamine and melatonin. Ultimately, melatonin was recognized as a signal of nighttime – a valuable bit of information that organisms could use to tell time. With this came the evolution of melatonin receptors and ultimately encouraged the evolution of a robust melatonergic signaling system through which the environment could communicate with cells through circulating melatonin and melatonin receptors. This advantage of having such a signaling system increased pressure to develop a stronger melatonergic signaling; this and the pressure to enhance photosensitivity created a conflict fueled by competition for ATP by melatonin synthesis versus photodetection and increased destruction of retinal through interaction with the higher levels of intracellular serotonin required for melatonin synthesis. This resulted in the divergence of the primitive photodetector/melatonin factory into the evolution of the pineal gland primarily dedicated to melatonin production and the retina dedicated to photodetection. In this light, AANAT is seen playing a critical role in the independent evolution of the retina and pineal gland from a common ancestral photodetector [20, 21].

## 24.4 Melatonin Synthesis Pathway

As a melatonin factory, the pineal gland has high levels of expression of all the enzymes involved in the conversion of tryptophan to melatonin; similarly, the enzymes required to generate cofactors for these enzymes are also expressed at notably high levels. Melatonin is synthesized from serotonin (5-HT) via sequential actions of two enzymes – AANAT and ASMT (acetylserotonin O-methyltransferase; alternative nomenclature HIOMT, hydroxyindole-O-methyltransferase; Fig. 24.1). Pineal 5-HT levels are highest during the day, increasing tenfold in some cases. This is a reflection of very high levels of production of hydroxytryptophan, due to exceptionally high levels of expression of tryptophan hydroxylase-1(Tph-1). It is not predominantly expressed in other

brain areas; tryptophan hydroxylase in other brain regions is mostly encoded by the related gene, Tph-2 gene. Hydroxytryptophan is decarboxylated to 5-HT by dopamine decarboxylase (DDC), which is present in many tissues.

During the day, pineal AANAT activity is low; and as a result, *N*-acetylserotonin (NAS) and melatonin levels are low in the pineal gland. The situation is reversed at night, when AANAT activity increases tenfold to 100-fold, thereby elevating both NAS production and accumulation. The increased accumulation drives melatonin production by mass action [2, 3, 22]. This change in activity of the timezyme in all species reflects posttranslational phosphorylation; in addition, in some species transcriptional mechanisms are involved. These are addressed below in detail. The increase in AANAT activity has reciprocal effects on the oxidation and acetylation pathways of serotonin metabolism. That is, not only does the increase in AANAT activity decrease the 5-HT levels, but this also decreases oxidation products including hydroxyindole acetic acid. Being the last enzyme in melatonin synthesis pathway, ASMT exhibits relatively small or no day/night variation [23–25]. It is very selectively expressed at high levels in the pineal gland, with expression in most other tissues undetectable or at very low levels.

In the absence of daily changes in ASMT activity, the cellular rate of *O*-methylation is believed to be largely a function of substrate availability, whereas the *N*-acetylation step is regulated by the availability of active AANAT protein. The major features of the daily (24 h) pattern of pineal indole metabolism outlined in Fig. 24.1 are conserved among vertebrates. Although it is clear that large changes in AANAT activity are responsible for the daily rhythm in pineal indoles, question still remains what limits the minimum daytime and maximal nighttime rates of melatonin production. The determining factor could be the availability of cofactors, the uptake of tryptophan, or the activity of other enzymes required for the conversion of tryptophan to melatonin [26–30]. Accordingly, the use of the term “rate limiting” may not be appropriate for AANAT, because it ignores the complex nature of melatonin synthesis and what elements limit production during the day and during the night. However, there is little question that the single most important factor controlling the dynamic changes in melatonin synthesis is AANAT, the timezyme.

## 24.5 Endogenous Clock Regulation of AANAT

The daily changes in AANAT in essentially all animals are driven by endogenous clock mechanisms. However, the links between clocks and AANAT differ. In the mammalian pineal gland, as described in Fig. 24.2, there is a neural circuit from the master oscillator located in the suprachiasmatic nucleus (SCN) that carries signals that elevate neural stimulation of the pineal gland by the release of norepinephrine at night [31–35]. Light acts on the system through projections from the retina to the SCN [31, 36–38]. In birds and fish, the pineal cells contain melatonin synthesis machinery, photodetectors, and an endogenous clock that regulate the rhythm in

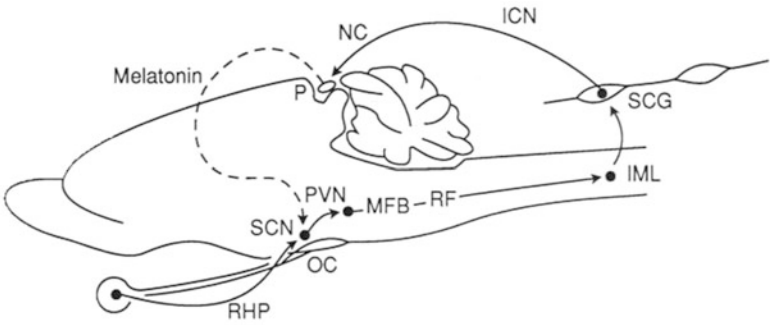


**Fig. 24.2** Neural network from retina to pineal gland controlling melatonin rhythm. The clock regulating AANAT in the pineal gland, located in the suprachiasmatic nucleus (*SCN*), is entrained by light which acts via a retinal hypothalamic projection (*RHP*) to the SCN. Monosynaptic connections exist between the SCN, paraventricular nucleus (*PVN*), and preganglionic cells in the intermediolateral cell column (*IML*) which in turn innervate a subpopulation of cells in the superior cervical ganglia (*SCG*). The SCG is connected to the pineal gland (*P*) through the inferior carotid nerve (*IC*) and nervi conarii (*NC*). Thus, SCN seemed to be hardwired to the pineal gland enabling the light-entrained clock to control the stimulation period of the pineal gland by adjusting the release of norepinephrine (*NE*) from the sympathetic cells of SCG. In addition, light acts downstream of the master oscillator to gate transmission of signals from the SCN to the pineal gland. Stimulation of pineal gland at night by NE leads to the increase in AANAT activity resulting in an increase in melatonin production and secretion. Melatonin is also believed to act on SCN directly. *OC* optic chiasm, *MFB* medial forebrain bundle, *RF* reticular formation (The image is taken from the review by Ganguly et al. [2])

AANAT activity [39–41]. A similar system functions in the retina [42, 43]. An important link between the intracellular endogenous circadian clock and AANAT activity in the retina and pineal cells of fish and birds is cyclic AMP.

## 24.6 Regulation of AANAT by Second Messengers

Cyclic AMP plays a highly conserved role in regulating vertebrate AANAT. The importance of this has been revealed in experiments with several mammalian systems, as well as with pineal glands from fish and chicken [31–33, 44]. Cyclic AMP controls AANAT activity through protein kinase A (PKA). In all vertebrates, cyclic AMP has posttranslational effects mediated by two protein kinase A (PKA)-mediated phosphorylation sites (PKA sites) in the regulatory flanking regions of the protein, as detailed below [1]. Cyclic AMP also regulates AANAT transcription in some mammals; specifically, cyclic AMP response elements (CREs) in the rodent AANAT gene mediate cyclic AMP regulation of AANAT gene expression via PKA-mediated phosphorylation of CRE-binding (CREB) protein [2, 3, 22]. It should also be noted that $Ca^{++}$ has a second, less well-understood, combinatorial influence on pineal signal transduction (Fig. 24.2), mediating $\alpha_1$-adrenergic potentiation of $\beta_1$-adrenergic stimulation of AANAT [45]. $Ca^{++}$ potentiates the effects of

cyclic AMP downstream of cyclic AMP generation [46]. It is not clear whether this effect of $Ca^{++}$ reflects an action on transcription or posttranslational regulation or on both.

## 24.7 Regulation of AANAT at the Transcriptional Level

AANAT mRNA levels are continually elevated with little or no daily rhythm (day versus night) in ungulate and primate pineal glands. Accordingly, changes in transcription play no role in the regulation of AANAT activity in these species. In rodents, however, the expression of AANAT transcript dramatically increases more than 100-fold at night; smaller increases are seen in zebrafish, chicken, and other species [3, 22]. The functional importance of the species-specific variation in transcription profile may be linked to the patterns of melatonin production generated in these species; certainly, transcriptional mechanisms provide an additional control over melatonin production.

In ungulates and primates, melatonin increases shortly after lights off at night. The immediate increase in melatonin may reflect in part the instant availability of AANAT mRNA, which makes it possible for AANAT protein to be synthesized immediately promoting increased protein levels. This increase, as discussed below in details, is a reflection of the accumulation of AANAT protein by virtue of the protection of the AANAT protein against proteasomal degradation system. In contrast, the daytime level of AANAT mRNA in rodents is virtually undetectable. This necessitates the new synthesis of AANAT mRNA at the onset of the dar period (nighttime) resulting in a lag period on the increase in AANAT protein and hence melatonin synthesis. This tailored pattern of melatonin production may be of special significance as it prevents the increase in AANAT protein and melatonin synthesis in the early-night period, which is known to be an important period for effects of melatonin in hamsters [47]. As noted above, the increase in AANAT mRNA in rodents is due to cyclic AMP-dependent PKA phosphorylation of CREB, which in turn binds to the AANAT promoter via multiple CRE sites. The CREB phosphorylation (pCREB) activates transcription. The extent of this activation is claimed to be controlled by a transcription repressor designated as inducible cyclic AMP early repressor (ICER), which is believed to compete with CREB for CRE sites on the AANAT promoter region so that AANAT transcription reflects the CREB/ICER occupancy of CRE [48, 49]. In rodents, ICER transcription is elevated approximately 100-fold at night by an adrenergic-cyclic AMP-mediated mechanism. However, the day/night difference at the protein level is relatively small (about fourfold). Further, it is to be noted that ICER does not control minute-to-minute daily dynamics of AANAT mRNA [50]. Rather, changes in ICER protein occur gradually with a week-by-week time course to control the peak amplitude in AANAT mRNA by gradually shifting the relative CREB/ICER occupancy of CRE sites in the AANAT promoter.

The cellular mechanisms that induce the decrease or disappearance of AANAT mRNA at the end of the night period lack extensive investigation compared to the events that lead to the augmentation of the AANAT transcript. However, recent discoveries of the role of miRNAs (micro-RNAs), namely, miR-483 [51], in the pineal gland along with the possible interplay with the pineal-enriched phosphodiesterase isoform (PDE4B2) [52] appear to contribute to the disappearance of cyclic AMP and effects of cyclic AMP on AANAT mRNA levels. It seems reasonable to suspect that the decrease in cyclic AMP levels at the end of the night period is via the action of PDE4B2 that destroys cyclic AMP, leading to a decrease in phosphorylation of CREB, resulting in suppression of AANAT transcription. On the other hand, parallel actions of miR-483- and RNA-binding proteins like hnRNP Q, hnRNP L, and hnRNP R binding at the 3′UTR region of AANAT mRNA are believed to destabilize the AANAT transcript leading to the disappearance of the mRNA [53].

Additionally, other regulatory promoter elements in the AANAT gene exist and play a role in modulating expression of the AANAT. Specifically, the rat AANAT gene transcription is regulated by an E-box sequence that directly interacts with clock proteins [54]. This E-box element appears to control the daily rhythm in AANAT transcript in the chicken pineal gland as well. Daily rhythms of other clock genes including mPer1 (in mouse) and rPer1 (in rat) and transcription factors including Fra-2 have been demonstrated in the pineal gland [2, 3]. However, the relationship of these proteins to the regulation of AANAT gene expression has not been fully established.

Interestingly, more AANAT gene-expression regulatory elements have been discovered at the turn of the century, particularly from the studies using zebrafish AANAT-2 (zfaanat-2) gene as a model system [55]. These elements are not restricted only within the 5′-untranslated region but also located at about 12 kb away at the 3′-downstream region. The 3′-sequence (257 bp in length), designated as pineal-restrictive downstream module (PRDM), contains three photoreceptor conserved elements (PCE) and a single perfect E-box sequence. These elements interact to play multiple functional roles in zfaanat-2 expression: (a) enhancement of pineal expression, an intrinsic property of the PRDM; (b) inhibition of extrapineal expression, mediated by an interaction between PRDM and the upstream regulatory region of zfaanat-2 promoter; and (c) driving rhythmic expression. These functions are guided by a synergistic action of nuclear factors like BMAL/CLOCK and orthodenticle homeobox 5 (OTX5) [56]. It is envisaged that the mechanism via OTX5-BMAL/CLOCK “interactome” controlling the tissue specificity and rhythmic expression of genes could be a conserved feature in the pineal gland across most species (for suggested reading see below).

## 24.8 Regulation of AANAT at the Translational Level

Until recently, the mechanisms involved at the translational level of AANAT expression were perhaps the most ignored area in understanding the regulatory control of melatonin synthesis. Emerging evidence indicates that temporal regulation of AANAT mRNA during translation might contribute another layer of mechanistic feature that fine-tunes the upregulation of AANAT protein exclusively at night regardless of its species-specific mRNA profiles [53]. This rhythmic control of AANAT mRNA translation is believed to be mediated via an interaction between a highly conserved internal ribosome entry site (IRES) element within the AANAT 5′-untranslated region and its binding partner hnRNP Q (heterogeneous nuclear ribonucleoprotein Q) [57]. The expression hnRNP Q protein is highest in the middle of the night. The binding of hnRNP Q to IRES element results in fine-tuning of the peak amplitude in the AANAT protein profile that parallels melatonin production. However, hnRNP Q might not be the only factor contributing to the translational control of AANAT protein synthesis. Recent works indicate the involvement of a translational complex that involves the participation of another RNA-binding protein, heterogeneous ribonucleoprotein R (hnRNP R) in the translation of AANAT. The norepinephrine-inducible hnRNP R protein also interacts with the 5′ untranslated region (UTR) of AANAT mRNA [58]. These discoveries suggest that "cap-independent" translation of AANAT transcript involves crucial temporal regulatory features that facilitate the recruitment of translational machinery to AANAT mRNA ultimately contributing to the rhythmic synthesis of melatonin.

## 24.9 Regulation of AANAT at the Posttranslational Level

The robust nocturnal increase of melatonin was initially thought to be primarily regulated via transcriptional activation of the AANAT gene by critical transcription factors as described above. This mechanistic paradigm explaining the rapid dynamics of melatonin rhythm evolved at an accelerated pace after the cloning of the AANAT gene [10] followed by several significant studies using the rodent model system [3]. However, mechanisms based solely on transcriptional control could not justify the remarkably robust profiles of the melatonin rhythm in the ungulates and primates where changes at the mRNA levels of AANAT were modest at best in comparison to the enormous amplitudes of AANAT transcript levels in the rat pineal gland [3, 9].

This gap in understanding was partly fulfilled with the recognition of a critical posttranslational mechanism that involves the cyclic AMP-dependent stabilization of the AANAT protein against rapid disappearance by the proteasome-based protein degradation machinery in the pineal gland [59]. Further investigation has revealed that the posttranslational mechanism controlling AANAT protein stability and the rapid surge in nighttime catalytic activity reflect the binding of

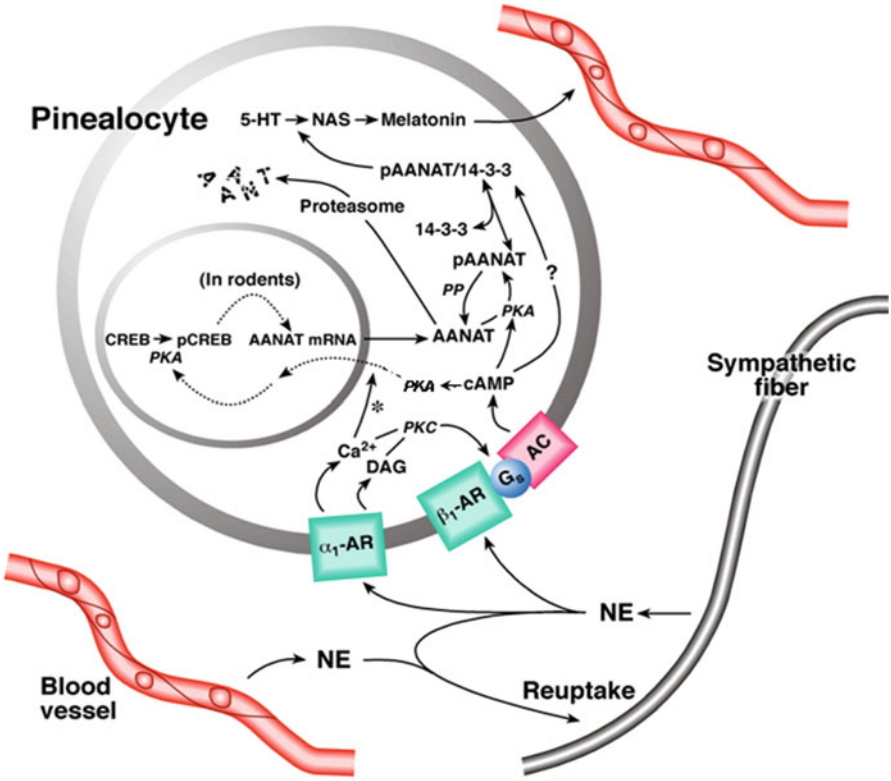


**Fig. 24.3** Overview of the multifaceted regulation of AANAT and melatonin synthesis in the pineal gland. Norepinephrine (*NE*) from sympathetic processes is released into the perivascular space and diffuses to the pinealocytes where it binds to and activates β-1- and α1-adrenergic receptors (*AR*). β-1-AR activation results in a G-protein (*Gs*)-mediated stimulation of adenylate cyclase (*AC*) which in turn activates protein kinase A (*PKA*); α1-AR activation elevates Ca++ and diacylglycerol (*DAG*), which leads to activation of protein kinase C (PKC). The increase in PKC potentiates the β-1-AR-mediated stimulation of AC through a post-receptor mechanism causing a rapid increase in the production of intracellular cyclic AMP [2, 46]. Cyclic AMP acts through multiple mechanisms to increase AANAT activity and melatonin production. As described in the text, in rodents, cyclic AMP causes AANAT mRNA to increase >100-fold at night. This is induced by phosphorylation of the transcription factor CREB by PKA. However, in many mammals, including the rhesus monkey and ungulates, AANAT mRNA is maintained at high levels throughout the day [3, 9], and the cAMP-mediated regulation seems to be mostly occurring at the AANAT protein level. In all vertebrates, cyclic AMP-dependent phosphorylation of AANAT (*pAANAT*) allows it to form a reversible complex with 14-3-3 protein causing AANAT to be activated and protected from proteolysis [2]. On dephosphorylation of pAANAT by protein phosphatases (*PP*), AANAT is rapidly degraded by proteasome [59]. The (*?*) symbol in the figure is to indicate the possibility of a role PKC might play at the protein level via PKC phosphorylation sites on AANAT [8]. The (*) symbol denotes Ca++ potentiation of cyclic AMP induced increases in AANAT activity [46]; the mechanism of which is still not clear. The (*?*) symbol in the figure is to indicate a direct role cyclic AMP might play on the activation of pAANAT/14-3-3 complex whose mechanism is unknown so far [1]. In addition, the AANAT mRNA is subjected to translational regulation as described in the text in details. Taken together, a series of regulatory cellular events converge to insure the increase in AANAT activity, thereby accelerating the conversion of 5-HT to NAS, which in turn is rapidly converted to melatonin at night. Hence, melatonin being highly lipophilic appears to be immediately released, raising the circulating

phosphorylated AANAT to 14-3-3 protein [60, 61]. This mechanism is perhaps the principle regulatory paradigm of rhythmic melatonin production across vertebrate species investigated so far; it is particularly important for species like ungulates and primates, where transcriptional regulation may not be correlated to drastic nocturnal activation of melatonin synthesis. It provides a rapid and efficient mechanism through which cyclic AMP controls AANAT activity and protein levels leading to melatonin production by facilitating the catalytic efficiency and promoting the protection of AANAT protein. In short, cyclic AMP switches the fate of AANAT from destruction to protection and activation (Fig. 24.3) [60].

In the absence of cyclic AMP, AANAT is destroyed by proteasomal proteolysis [59]. Cyclic AMP prevents this through PKA-dependent phosphorylation on the threonine (T) residue of the N-terminal PKA site, which leads to binding of AANAT to 14-3-3 proteins (Fig. 24.3). When cyclic AMP is elevated, it appears that most, if not all, of AANAT is phosphorylated and bound to 14-3-3 protein in a reversible complex. On binding to 14-3-3 protein, AANAT appears to be protected against proteasomal proteolysis and dephosphorylation by protein phosphatase(s). In addition, binding increases the affinity of AANAT for low concentrations of 5-HT that is reflected by lowering of Km (Michaelis-Menten constant) by about sevenfold as compared to the unbound full-length AANAT [62]. The increased affinity appears to be aided by the interaction with 14-3-3 protein that restricts the movement of a floppy loop of AANAT (Loop 1) in a configuration favoring binding of AcCoA and hence 5-HT binding (Fig. 24.4a, b) [61, 63].

X-ray crystallographic analysis indicates that the region containing the N-terminal PKA site of AANAT is bound to an amphipathic binding groove in 14-3-3 proteins [63]; multiple contacts exist between the phosphorylated threonine-31 (pT31, the 31st residue of ovine AANAT sequence; [1]) and 14-3-3 (Figs. 24.4c and 24.5). Although there are numerous contacts involving amino acid residues in the vicinity, the unphosphorylated protein binds very poorly to 14-3-3, and this modest "nonspecific" interaction seems to have no influence on the enzyme catalysis. This indicates that cyclic AMP-dependent phosphorylation of T31 functions as a binding switch as that interaction triggers other potential binding contacts, including the C-terminal phosphorylated S205 (ovine sequence), enabling AANAT to snugly fit into the amphipathic binding groove of the 14-3-3 protein dimer. Though cyclic AMP-PKA-mediated phosphorylation favors formation of the complex, the AANAT-14-3-3 complex is reversible. Free phospho-AANAT (pAANAT) can be dephosphorylated, which markedly reduces association with 14-3-3; free AANAT is then destroyed by proteasomal proteolysis. However, in the presence of high levels of cyclic AMP, formation of phosphorylated form of

**Fig. 24.3** (continued) melatonin levels. When neural stimulation of the gland ceases (decrease in NE), there is a rapid decrease in cyclic AMP levels, leading to the dissociation of pAANAT/14-3-3 complex, with a concomitant dephosphorylation and destruction of AANAT. This eventually leads to a decrease in melatonin production and an increase in 5-HT (The figure is updated from the one published in Ganguly et al. [2])

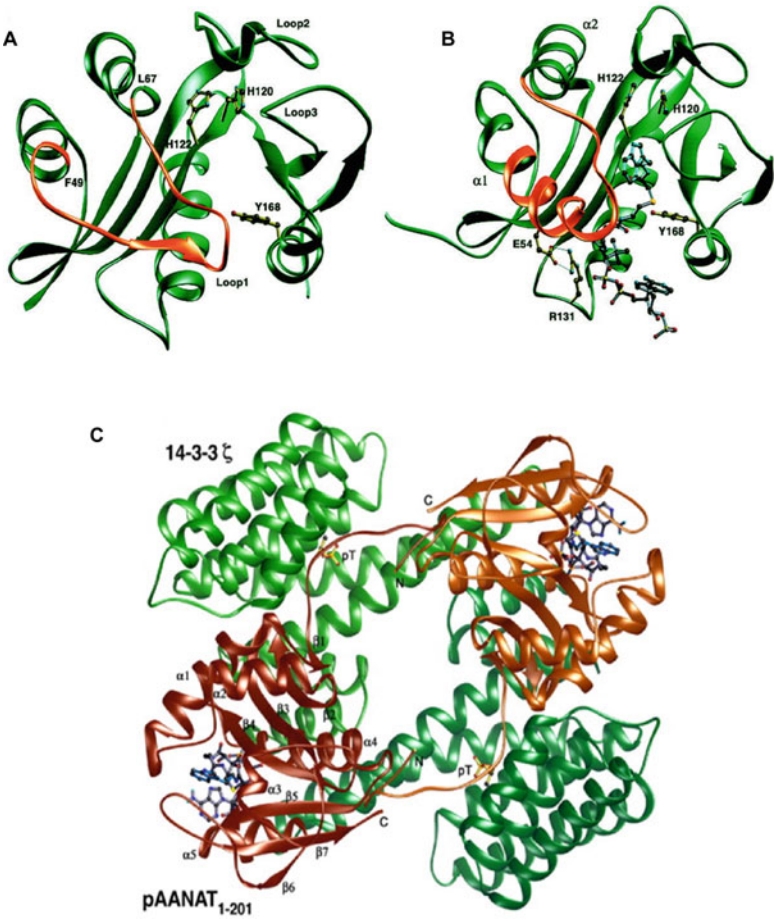


**Fig. 24.4** Importance of AANAT Loop 1 structure in catalysis and the mechanistic significance of the complex formation with 14-3-3 protein. (**a**) Structure of ovine AANAT showing the extended segment in the polypeptide portion (*RED*) between phenylalanine 49 (*F-49*) and Leu-67 (*L-67*) called Loop 1 [63, 64]. This loop is "floppy" and converges over substrate-binding site when compared with (**b**) ovine AANAT protein bound with the bisubstrate analog, coenzyme A-S-acetyltryptamine. This structure demonstrates that AcCoA binding induces a large conformational change by displacement of Loop 1 leading to the formation of the serotonin-binding site in a bi-bi catalysis mechanism. Loops 2 and 3 are relatively rigid compared to Loop 1. It is important to note that the substrate bound form in (**b**) has helix α1 extended to pull out Loop 1 away from the pantetheine-binding site, accompanied by the loss of strand β2. In the absence of the bisubstrate analog in (**a**), a portion of Loop 1 can extend to interfere with the pantetheine-binding space [61, 63]. (**c**) Phosphorylated AANAT/14-3-3 complex. This is a view from the top (half-pipe section) of the complex between 14-3-3 dimer (*in green*) and phosphorylated ovine AANAT (1-201 amino acid sequence; $pAANAT_{1\text{-}201}$) (in *red*), bound with the bisubstrate molecule (*blue*). The phosphorylated threonine-31 (pT31) is shown to be in contact with the 14-3-3 protein. This interaction has resulted in pulling the AANAT Loop 1 (extension of helix α1) away from the AcCoA-binding pocket of AANAT [61] increasing the affinity of AcCoA (reducing Km). Further,

AANAT (pAANAT) is favored, resulting in formation of the 14-3-3 complex and maintenance of high levels of AANAT activity and melatonin synthesis. Taking together the regulatory features described so far, it seems likely that multiple layers of mechanisms are in place to preclude the possibility of an inappropriate increase in melatonin production during the day or during periods of the night when melatonin synthesis is physiologically not desired.

## 24.10 Mechanism of Acetyl Transfer

AANAT is a globular 23-kDa cytosolic protein with a catalytic core encompassing the arylalkylamine and AcCoA-binding pockets. The catalysis of acetyl transfer by AANAT involves an ordered bisubstrate reaction between AcCoA as acetyl donor and arylalkylamine as acetyl group acceptor. AcCoA binds AANAT through major contacts via the pantetheine moiety aligning the thioacetyl group into the center of the enzyme molecule and thereby pushing the adenosine moiety toward the surface [15, 63]. However, the binding site seems to be sterically hindered by the movement of a "floppy" loop (Loop 1) formed by the residues between F49 and L67 (Fig. 24.4a, b). 14-3-3 protein binding via pT31 stabilizes Loop 1 favoring the substrate binding and enhancing the substrate affinity of pAANAT/14-3-3 complex (Fig. 24.4c). Arylalkylamines including 5-HT bind in a funnel-shaped pocket formed by three protein loops (Loop 1, Loop 2, and Loop 3) which contact the aromatic ring of substrates via aromatic residues (Fig. 24.4). By analyzing the crystal structure of ovine AANAT, it became apparent that the catalysis is initiated by deprotonation of the protonated amine of the arylalkylamine substrate. This is indirectly facilitated by neighboring histidines (H120 and H122 of ovine AANAT as in Fig. 24.4a, b) that are part of a "proton wire" conducting protons to the surface through a water-filled channel. Deprotonation induces attack on the thioester bond of AcCoA and formation of a transient ternary complex. This intermediate complex transforms into more stable products: N-acetylated arylalkylamine and coenzyme A (CoA). The acetylation of the amine leads to the increase in hydrophobicity resulting in its ejection from the catalytic pocket. Release of CoA from the binding pocket requires tyrosine (Y168)-dependent protonation of CoA (Fig. 24.4a, b) [63].

**Fig. 24.4** (continued) multiple interactions of helices α1 and α2 of AANAT with the 14-3-3 enhance the stability of the "floppy" Loop 1, perhaps making AANAT more efficient for substrate binding. Thus, 14-3-3 protein not only protects AANAT from degradation by shielding the disordered N-terminal region but also enhances the affinity of AANAT for the substrates leading to its activation [61] (Original images were previously published [61, 63])

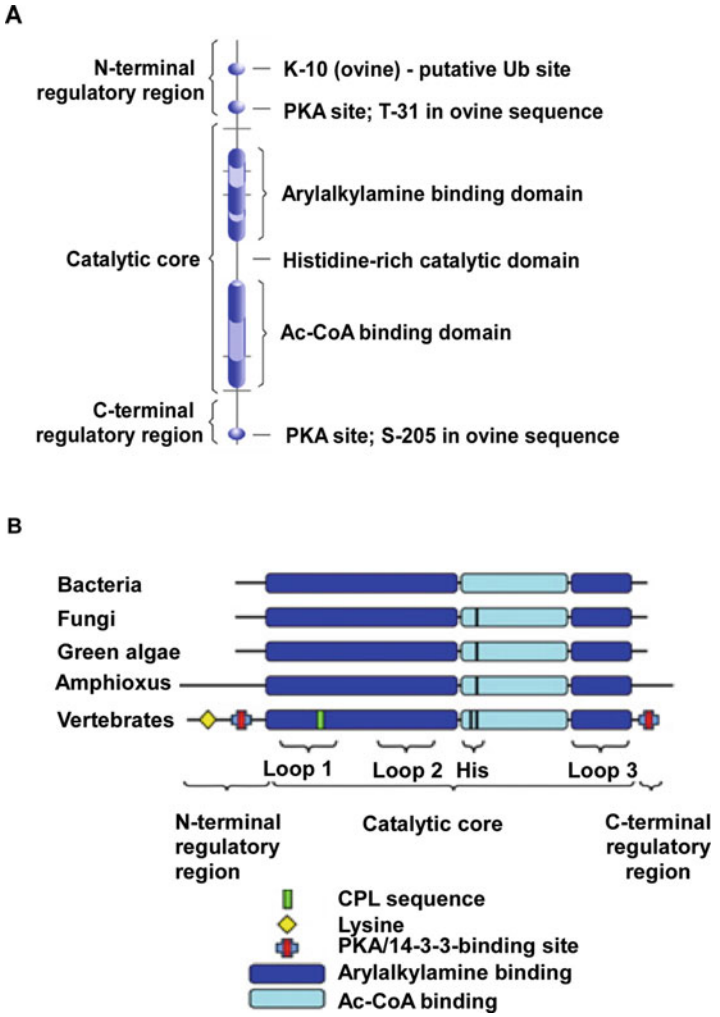


**Fig. 24.5** Critical structural domains of AANAT protein. (**a**) Conserved structural features of vertebrate AANAT. Vertebrate AANATs (MW ~23 kDa) are composed of a central catalytic core and flanking regulatory sequences at the N-terminus and C-terminus region, each of which has a PKA site encompassed by a 14-3-3 protein-binding site. Phosphorylation of the threonine-31 (T-31) residue converts the 8-residue sequence containing the PKA site (underlined) into a 14-3-3-binding motif: RRHT$^{31}$LPAN → RRHpT$^{31}$LPAN (ovine AANAT amino acid sequence numbering used). The phosphorylation on T-31 triggers binding of AANAT to 14-3-3 as indicated by functional studies and X-ray crystallographic analysis of the complex [60, 61]. The conserved lysine-10 (*K-10*) is predicted to be a putative ubiquitination (Ub) site [59]. The arylalkylamine-binding domain characterizes the AANAT family. The AcCoA-binding domain is similar to that found in the Gcn5 acetyltransferase superfamily [15]. (**b**) Comparison of the organization of the structural domains of vertebrate AANAT with AANAT homologs. Major differences are the presence of N-terminal and C-terminal regulatory features, a conserved tripeptide (64CPL66; residue numbering is based on ovine AANAT) sequence within Loop 1 of the catalytic core, and the number of histidines (His) in the AcCoA-binding pocket of the vertebrate AANAT [1]. However, core catalytic domains are conserved across all species shown (Images are from published review [1])

## 24.11 AANAT Terminal Regulatory Regions

The major molecular features that differentiate between the vertebrate and other species, where AANAT has been identified, are the N-terminal and C-terminal regulatory regions [1, 2]. These flanking regulatory sequences (Fig. 24.5) are acquired through evolution as one of the key regulatory components of vertebrate timekeeping. The "signature" flanking region of the vertebrate AANAT allows cAMP/PKA-mediated signaling to rapidly regulate the activity and stability of the enzyme, as described in above sections. The N-terminal regulatory region (about 35 amino acid residues) comprises a PKA-dependent phosphorylation site at T-31 (in ovine sequence) encompassed by a 14-3-3-binding motif (Fig. 24.5a). The primary structure of the PKA/14-3-3 motif with sequence $^{28}$RRHpTLP$^{33}$ (ovine AANAT sequence) resembles that of a canonical "mode I" 14-3-3-binding motif [RSX(pS/pT)XP]; however, the three-dimensional configuration of this sequence in the AANAT/14-3-3 protein complex resembles that of a 14-3-3-bound peptide structure with a "mode II" sequence [RX(Y/F)X(pS/pT)XP] [61]. The protein sequence toward the N-terminus of this site is disordered; the only conserved feature is a lysine (Lys10), which is thought to be critical for proteasomal proteolysis (Fig. 24.5) [59]. The C-terminal regulatory region contains the PKA/14-3-3 motif (sequence): $^{200}$RRNpSG(C/R)$^{205}$ (ovine AANAT sequence). This represents a "mode III" 14-3-3-binding motif [(pS/pT)X1–2-COOH]. AANAT can interact with 14-3-3 proteins via either PKA/14-3-3 motif with varying affinity; however, binding via both the N-terminal and C-terminal phosphorylated sites is required for maximal activation of AANAT [62]. As described in Fig. 24.5b, these N-terminal and C-terminal flanking regulatory regions are conserved in vertebrates only and are not present in AANAT homologs of other lower eukaryotes and bacteria. Thus, it appears that the vertebrate AANAT evolved from the ancestral homologs with broad substrate specificity and multitasking ability, as a precisely regulated and tightly controlled enzyme with high substrate specificity by acquiring the flanking regulatory regions along with a stringent substrate-binding pocket.

## 24.12 Future Perspectives

What does the future hold for AANAT? Perhaps the most important advances will be made in revealing the role AANAT plays in local signaling throughout the brain. It is likely that sites will be identified where the enzyme is expressed in small populations of cells in close association with cells that express melatonin receptors, thereby identifying sites at which AANAT controls biological functions through melatonin and other products via paracrine and autocrine mechanisms. Similarly, it will be important to search for regulatory mechanisms which link AANAT to non-melatoninergic signaling, as has been found to be the case in the retina and hippocampus, where N-acetylserotonin has been found to act by binding to

tropomyosin receptor kinase B (TrkB) reminiscent of brain-derived neurotrophic factor (BDNF) [65, 66]. Another area of discovery is regulation. It is known that circadian clocks are found in many cells outside of the SCN. These cellular clocks might be directly linked to AANAT as is the case in the retina [43]. Accordingly, such clocks might regulate local production of AANAT signaling products independently of the master clock, as regards phase relationships. Likewise, cyclic AMP regulation of AANAT under control of transmitters linked to adenylate cyclase might also be involved in the regulation of the enzyme in extrapineal and extraretinal sites.

AANAT is a unique enzyme, and further investigations will surely reveal new and surprising features of this enzyme and the impact it has on biology. Investigators dedicated to this enzyme will be on the edge of an exciting new frontier in AANAT-related neurobiology.

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# Chapter 25
# Pineal Gland, Melatonin, and Timekeeping in Nonmammalian Vertebrates: Avian Perspective

**Amit Kumar Trivedi, Devraj Singh, Anand Shankar Dixit, and Vinod Kumar**

**Abstract** The pineal gland, shaped somewhat like a pinecone, is a small organ that lies deep in the brain of mammals, but is superficially placed in the brain of nonmammal vertebrates. In birds, its anatomical location is very distinct, lying superficially in the triangular space formed behind the junction of cerebral hemispheres and the anterior aspects of cerebellum. The known product of the pineal gland is melatonin, which is produced at night regardless of the nature of the species, diurnal, nocturnal, or crepuscular. Melatonin, an indoleamine hormone, is almost universally present, albeit with varied functions, and is generally called as evolutionary connecting molecule. In birds as in other vertebrates, melatonin is involved in the circadian organization behavioral and physiological functions as well as in photoperiodic measurement. Unlike in mammals in which the duration of melatonin is a critical determinant of the seasonal reproductive response, daily changes in melatonin profile seem not critical for photoperiodic induction of gonadal growth and development. Melatonin also plays significant roles in the other daily and seasonal functions, like the immune responses. As part of the multioscillatory circadian timekeeping in nonmammal species, melatonin perhaps is involved both as the pacemaking and coupling agent. As latter, it contributes to the flexibility of the clock system in concurrence with changes in the external environment (e.g., photoperiod). The degree to which these roles are assigned could be linked to the interdependence of the self-sustainment of participating oscillators of the circadian oscillations, the photoperiod environment, as well as the annual life-history state of the species. Finally, whether results from laboratory studies would hold true for free-living populations remains largely unknown at this time.

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## 25.1 Introduction

The pineal gland, shaped somewhat like a pinecone, is a small organ that lies deep in the brain of mammals. The inaccessible anatomical location of the pineal has been perhaps acted as deterrent for early experiments from investigations, compared to other glands like the pituitary, thyroid, and adrenal. In 1898 Heubner [1] presented an interesting case of a boy of 4.5 years old who showed less pronounced somatic but marked precocious sexual development as puberty. In the following year, the case was found at autopsy with a teratoma of the pineal [2]. Almost at the same time, another similar case with precocious sexual development was found in a boy of 6 years of age [3]. Within the next 10 years, more than 40 such cases were reported [4]. During the early twentieth century, most observations on pineal were aligned with the concept of hypopinealism (hypertrophy of gonads), hyperpinealism (adiposity), or apinealism (cachexia, also described in cases of apituitarism), which were purely based on clinicopathological observations [5].

During later years, much attention has been directed toward understanding physiological roles of pineal gland in animals. Also, there is now vast literature on both the structure and function of the pineal gland across vertebrate taxa. In contrast to mammals, the location of the pineal gland in the brain is superficial perhaps due to its photoinducible properties in nonmammalian vertebrates. In these species, the pineal gland is a non-image forming light-receptive vesicle-like structure located just below the skull in the area where the bone is either thin or absent or the cranium region lacks melanophores, forming a de-pigmented "pineal window." The pineal vesicle is connected to the roof of the diencephalon by a stalk. In most species, the pineal lumen opens in the third ventricle and is filled with the cerebrospinal fluid (CSF). In this chapter, we propose to very briefly review the role of pineal gland and melatonin in the timekeeping of nonmammalian vertebrates, with particular emphasis because of our own bias and expertise based on birds.

## 25.2 Functional Organization of the Pineal Gland

### *25.2.1 Species Variations*

The pineal gland develops from a circumscribed area of the diencephalic roof between the habenular and posterior commissures. The cellular components of the pineal complex are derivatives of the primitive neuroepithelium. The structure of the pineal gland has changed dramatically from lower vertebrates (fish) to highest grade of body organization, i.e., mammals (Fig. 25.1). This appears due to the unique evolutionary trend that led to the replacement of the photosensitivity of nonmammalian pineal by photo-insensitivity of the pineal gland in mammals

[7]. In lampreys and teleost fishes, the pineal complex consists of a pineal organ proper and a parapineal organ located inside the skull (Fig. 25.1). The parapineal organ of lampreys comprises a wide lumen that is reduced to a capillary space in the teleost parapineal. In all fish species, the parapineal is neuronally connected to the left habenular nucleus. The pineal organ proper of lampreys and teleosts displays a wide lumen that is in open communication with the third ventricle. The pineal vesicle epithelium consists of mostly photoreceptor and supporting cells, which are non-photoreceptive in nature. Inner and outer segments of photoreceptor cells remain protruded into the lumen of pineal vesicle. Anatomy of the amphibian pineal complex shows variation. In anurans, a frontal organ located in an extracranial position in the skin can be distinguished from the intracranial pineal organ proper (Fig. 25.1); urodeles and caecilians lack the frontal organ [8]. The pineal organ proper of all amphibian species is a hollow structure whose lumen communicates with the third ventricle. Among reptiles, the pineal complex is highly variable. In crocodiles, e.g., *Alligator mississippiensis* and *Crocodylus niloticus*, the complex is absent. In other reptiles, the pineal organ proper superficially lies in between the habenular and posterior commissures. The distal component of the reptilian pineal complex, the parietal eye, is located in the parietal foramen of the skull (Fig. 25.1). In the adult stage, it persists in some lizards, and if present, the parietal eye is highly differentiated, possessing a cornea and a lens and thus resembling the lateral eyes. The parietal eye is connected to the left habenular nucleus via the parietal nerve [9]. The pineal organ proper comprises a conspicuous

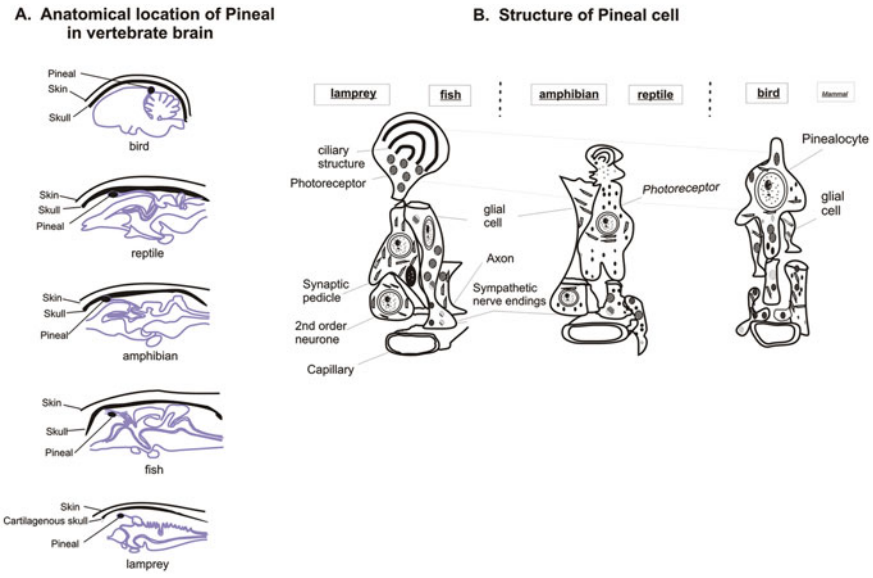


**Fig. 25.1** Schematic diagram showing (*i*) location of the pineal gland in the brain (**a**) and (*ii*) evolution pineal cell types (**b**) in nonmammalian vertebrates, fish to birds (The figure has been drawn based on Falcón et al. [6])

lumen in lacertilian species; it is solid and gland-like in ophidians. Avian pineal represents intermediate evolutionary position and is characterized by a very complex internal structure and functional organization [6]. In contrast to mammals where pineal gland is only endocrine, in birds it is both photoreceptive and endocrine [6, 10, 11]. It lies superficially in the triangular space formed behind the junction of cerebral hemispheres and the anterior aspects of cerebellum [12] and connected to the roof of the third ventricle via the choroid plexus with the help of a long stalk [12, 13]. There is scanty information for pineal gland in nocturnal birds. In nocturnal temperate owl (*Strix uralensis*), a very small pineal gland with a poor system of tubules and follicles without a vascular network has been reported [14]. However, spotted owlet, *Athene brama*, has been reported with a well-developed pineal gland [15].

### *25.2.2 Pineal Photoreceptors*

The pineal gland consists of three types of cells, the pinealocyte, glial cell, and neurons of the pineal parenchyma. Pinealocytes exhibit conspicuous variations among different classes of vertebrates. On the basis of their ultrastructure, pinealocytes have been divided into pineal photoreceptor cells, modified pineal photoreceptors, and pinealocytes sensu stricto ([16, 17]; Fig. 25.1). The pineal photoreceptors resemble that of the retinal cones. The outer segment protrudes into the pineal lumen and consists of numerous disks (10–300 in numbers) produced by successive invaginations of the plasma membrane. This outer segment is connected to inner segment via connecting piece having a cilium. Immunocytochemical investigations have shown that pineal photoreceptors contain molecules, which are very closely related to those expressed by the retinal photoreceptors. A number of opsin molecules have been reported in the pineal gland of different nonmammalian vertebrates. Rhodopsin is localized in the outer segment of pinealocytes of various species of fish [18, 19], anurans [20], reptiles [21], and birds [22]. Similarly, the pinopsin is reported in the pineal from chicken and pigeon [23] and reptiles [24]. The teleost pineal does not express pinopsin but instead has another novel rhodopsin-like molecule, the exo-rhodopsin [18].

The modified pineal photoreceptors have rudimentary outer segment which is less regular than the true pineal photoreceptor cells; some modified photoreceptors lack membrane disks and consist of only a bulbous cilium. The basal part of the cell contains synaptic ribbons intermingled with clear vesicles and dense granules. The number of basal granules varies from species to species. The dense core granules are considered as an indication of a high secretary activity of the modified photoreceptor cells. Immunohistochemically, these photoreceptors in the avian pineal contain photoreceptor-specific proteins [21, 25]; few rhodopsin-positive elements

have been reported in the pineal of Japanese quail, *Coturnix c. japonica* [26], and chicken [17, 27].

Pinealocytes sensu stricto are the pineal photoreceptors and intra-pineal second-order neurons, which have been gradually reduced in the course of evolution. In mammals the parenchyma of the pineal gland consists of pinealocytes sensu stricto. In some species like hamster (*Mesocricetus auratus*) and opossum (*Didelphis virginiana*), certain pinealocytes are directly exposed to the cerebrospinal fluid and are called CSF contacting pinealocytes [28].

## 25.3 Melatonin: An Evolutionary Connecting Molecule

In 1958, Lerner et al. [29] discovered melatonin (N-acetyl-5-methoxytryptamine) as the main secretory product of the pineal gland. The presence of melatonin has now been reported in a number of invertebrates and vertebrates as well as the plants, suggesting that it exists in nearly all kind of life forms (Fig. 25.2). Hence, the presence of pineal gland is not a prerequisite for the melatonin production, and the molecule of melatonin has been evolutionarily conserved. Melatonin is involved in varied functions ranging from an antioxidant and agent of cellular protection against free radicals to the regulation of sleep and seasonal behaviors. Many invertebrates exhibit diurnal vertical migration (rising to the surface of water bodies during the night and moving to a depth during the day), which is akin to the sleep in higher vertebrates. The role of melatonin in diurnal vertical migration or melatonin-dependent signaling in annelid worm, *Platynereis dumerilii*, along with the role of vertebrate sleep is a good example of melatonin as the evolutionary connecting molecule of the animal kingdom [31].

### *25.3.1 Main Sources and Biosynthesis of Melatonin*

The pineal gland is a major source of melatonin production in vertebrates. The other extrapineal sources of melatonin include retinal cells, cells in Harderian gland, and some somatic cell groups of the gastrointestinal tract. It is uncertain whether all these sources produce melatonin in all the vertebrate groups. Even if they do, their relative contributions may vary from species to species. For example, most, if not all, circulating melatonin in house sparrows (*Passer domesticus*) comes from the pineal gland; pinealectomized sparrows do not have a detectable melatonin profile [32]. On the other hand, only half of the total circulating melatonin comes from pineal gland in the Japanese quail and pigeon, *Columba livia* [33, 34]. Also, in the absence of pineal, blood melatonin loses day-night differences in the desert iguanas, *Dipsosaurus dorsalis*, and ruin lizards, *Podarcis sicula* [35, 36].

Most studies on the regulation of synthesis and secretion of melatonin, an indoleamine hormone, have focused on pineal gland, particularly the pinealocytes.

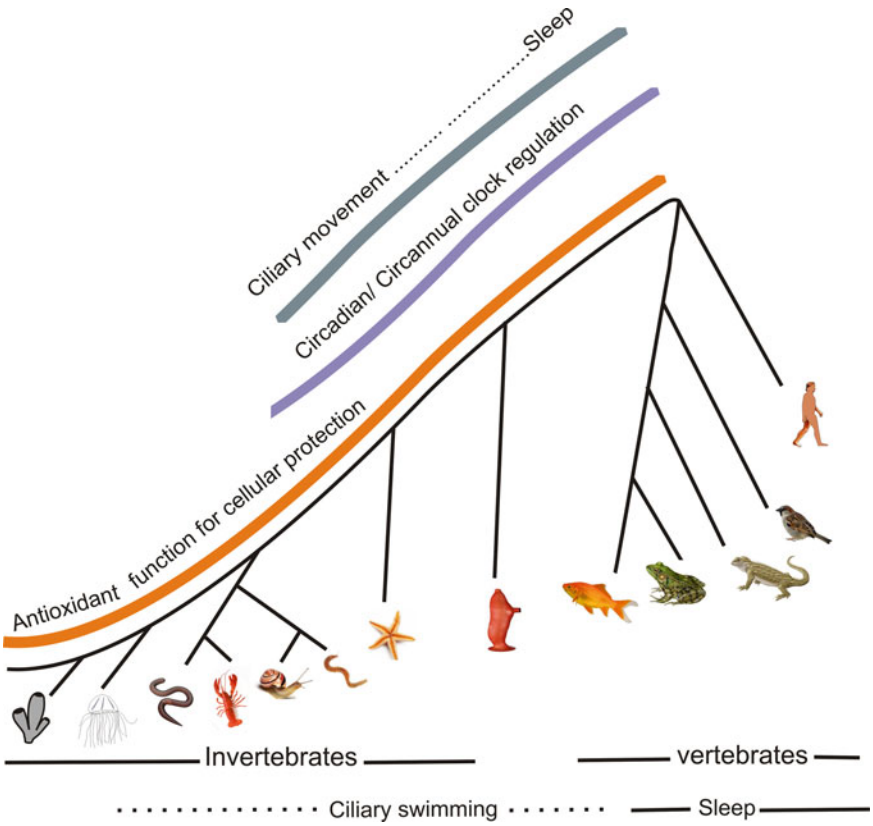


**Fig. 25.2** A sketch of the proposed scheme for melatonin as evolutionary connecting molecules between different animal taxa, based on Schippers and Nichols [30]. Basically, the antioxidant functions of melatonin have been conserved across the animal groups, both invertebrates and vertebrates. The role of melatonin in circadian and circannual processes has been evolved during the course of evolution. Melatonin performs diverse roles, acting as signaling molecule for ciliary movements in invertebrates to regulatory molecule for more complex behavior, such as sleep, in vertebrates

Regardless of the habitat of organism, melatonin is secreted at night. Tryptophan, a dietary amino acid and substrate for melatonin synthesis, is taken up from the bloodstream during the daytime and hydrolyzed to 5-hydroxytryptophan (5-HTRP). This is further converted into 5-hydroxytryptamine (5-HT, serotonin) mediated by enzyme aromatic amino decarboxylase (AADC). Serotonin is accumulated during the day, and then at night, N-acetyltransferase (arylalkylamine N-acetyltransferase, AANAT) produced causes N-acetylation of serotonin, which is again methylated by addition of a methyl group at the 5-hydroxy position via hydroxyindole-O-methyltransferase (HIOMT) enzyme [37]. The rate-limiting step during melatonin biosynthesis is catalyzed by AANAT, and rhythm in the activity of this enzyme underlies the rhythmic production of melatonin [38]. The AANAT activity is

remarkably regulated both at the levels of transcription and translation by cAMP [39]. Although melatonin is supposed to be quickly released into circulation, the pineal gland concentration of melatonin is always manyfold higher than the circulating melatonin levels, perhaps implicating storage in the pineal gland.

## *25.3.2 Regulation of Melatonin Synthesis and Secretion by the Pinealocytes*

### 25.3.2.1 Neural Regulation

The role of adrenergic control of pineal melatonin synthesis has been established. In particular, norepinephrine (NE) pathway runs via superior cervical ganglion and plays a significant role in the diurnal control of melatonin production, although with group differences. In mammals, for example, NE by acting mainly on beta-adrenergic receptors potentiates the activity of cAMP and enhances melatonin production by the pinealocytes at night. In birds, NE inhibits cAMP accumulation via alpha-2 adrenergic receptors and, in turn, inhibits AANAT activity and melatonin production during the daytime. Hence, the role of NE in control of melatonin biosynthesis is not the same in birds and mammals, probably due to differences in the nature of melatonin-producing tissues. Whereas avian pineal is photoreceptive, pineal in mammals gets light information exclusively from the retina, as decoded by the activity of the suprachiasmatic nucleus (SCN).

### 25.3.2.2 Circadian Clock Regulation

In contrast to mammals, in which the disconnection of pineal from the SCN causes loss of rhythmic pattern in melatonin production, nonmammalian vertebrate pineal glands are independent from the SCN. They possess components that are light sensitive, show self-sustained circadian melatonin rhythms both in vivo and in vitro entrained to light-dark (LD) cycles [40, 41], and are equipped with circadian genetic machinery that generates the circadian oscillations [42]. Light by its entraining effects programs melatonin secretion at night and by its suppressive effects restricts high melatonin as per duration of the dark phase [43, 44]. Results from T-cycle experiments (in which the period of LD cycle is changed from 24 h to shorter or longer durations, e.g., 22 or 26 h) also conform to the circadian regulation of melatonin production [45, 46]. The circadian clock control operates both at the transcriptional and translational levels in the melatonin biosynthetic pathway, as suggested earlier in Chap. 11. The pinealocytes possess all canonical clock genes, which are implicated in the generation of circadian rhythmicity in the hypothalamic clock and other peripheral tissues [47–49]. Notably, genes coding for arylalkylamine N-acetyl-transferase (AANAT) are expressed at night (or during subjective night under constant darkness, DD) [49, 50].

### 25.3.3 *Melatonin: Input to or Output of the Circadian Clock System*

#### 25.3.3.1 Melatonin as an Input

Several lines of evidence show that melatonin provides an input to the clock system and helps organize (if not drive) the circadian clock functions (Figs. 25.3 and 25.4). A summary of few findings among nonmammals is as follows: (i) House sparrows loose circadian rhythmicity when pineal was removed, but not when it was neurally disconnected [51]. (ii) Transplant of pineal into anterior chamber of eye provides phase, not period, to circadian rhythms of activity in house sparrows [52]. (iii) Periodic application of exogenous melatonin by infusion or via drinking water restores rhythmicity in arrhythmic pigeons, house sparrows, and Indian weaverbirds [42, 53, 54]. (iv) The absence of pineal (pinealectomy) causes arrhythmicity in 2-DG uptake and melatonin administration restores rhythmicity [55, 56]. (v) Melatonin is involved in circadian regulation of body temperature in

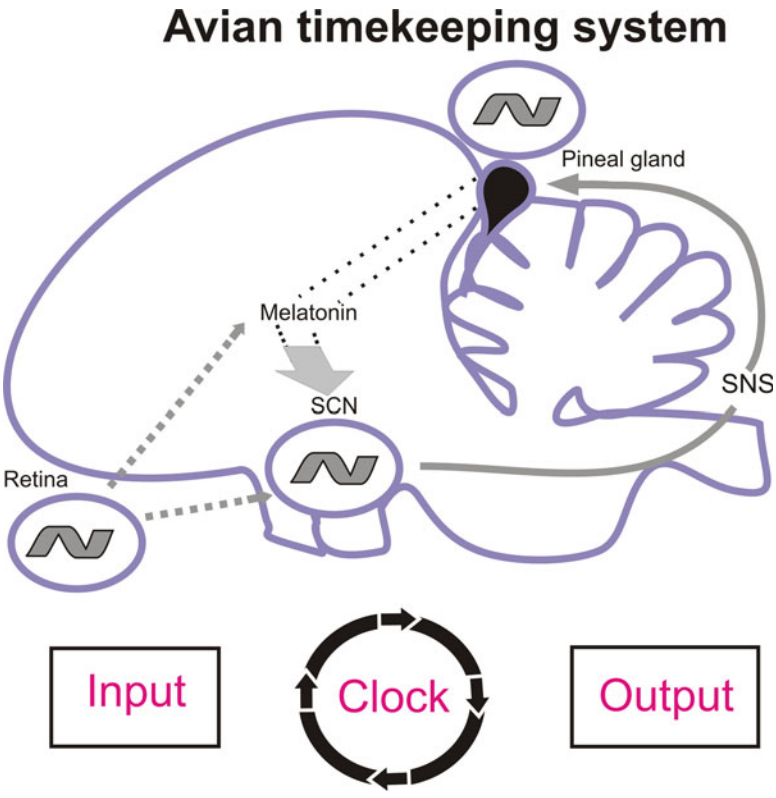


**Fig. 25.3** A schematic generalized view of the interacting retina, pineal, and SCN as component oscillators of the multioscillatory avian timekeeping system. All the three components are self-sustained and contain the input pathway(s), clock or pacemaker, and the output pathway(s)

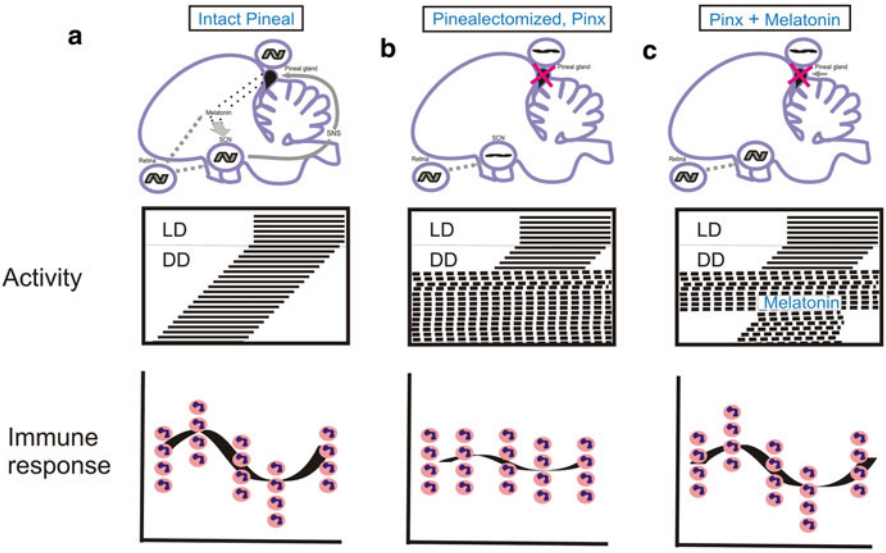


**Fig. 25.4** Cartoon diagrams showing scheme on how melatonin regulates rhythmic locomotor activity and immune responses, the latter by changes in immune cells profiles. The three *vertical panels* show possible results from animals with intact pineal (**a**), the pinealectomized animals (**b**), and pinealectomized animals administered with exogenous melatonin (**c**). Note disruption of rhythms in activity and immune response in (**b**) and restoration of these in (**c**)

Japanese quails [57]. A very recent study on migratory buntings (*Emberiza bruniceps*) showed the involvement of pineal in generation of the circadian oscillations of clock genes in central and peripheral clock tissues. Pinx and sham-operated buntings entrained to short days (8 h light: 16 h darkness, 8L:16D) were sequentially exposed for 10 days each to stimulatory long days (13L: 11D) and constant dim light (LLdim; a condition that tests the circadian rhythm persistence). Whereas activity-rest pattern was monitored continuously, the mRNA expressions of clock genes (*bmal1*, *clock*, *npas2*, *per2*, *cry1*, *ror*α, *rever*α) were measured in the retina, hypothalamus, telencephalon, optic tectum, and liver tissues at the circadian times (CTs) 1, 6, 13, 17, and 21 (CT 0, activity onset) on day 11 of the LLdim. The absence of the pineal gland did not affect the development of long-day-induced *Zugunruhe* but caused the decay of circadian rhythm in Zugunruhe (the nighttime migratory restlessness in caged birds as well as the clock gene oscillations in the hypothalamus, but not in the retina. Further, there were variable effects of pinealectomy on circadian gene oscillations in the peripheral brain and liver tissues [58].

### 25.3.3.2 Melatonin as a Circadian Output

Melatonin is one of the most stable efferent endocrine outputs of the circadian clock. It is readily measurable in the plasma and other body fluids like saliva, and

circulating blood levels provide a robust and reliable estimate of the clock functions. Duration of night melatonin secretion reflects the length of night, and hence the day length, in all species [43]. In birds and other vertebrates, the duration of night melatonin also decodes daily and seasonal photoperiod information, and thus daily and seasonal melatonin profiles convey the internal information used for daily and seasonal timing [40, 59, 60]. Apart from birds, seasonal variations in plasma melatonin levels have been reported in ruin lizards [61], common dentex (*Dentex dentex*) [62], and box turtle (*Terrapene c. triunguis*) [63]. However, inconsistent with these, photosensitive and photorefractory black-headed buntings were not dramatically different in the night melatonin profiles [64]. Also, the influence of season was not found on the amplitude of melatonin rhythm in Hyline chickens, and both winter and summer melatonin profiles were similar in 16-day-old chickens [65].

## 25.4 Role of Melatonin in Daily and Seasonal Timekeeping

A vertebrate circadian system comprises three components: input pathway(s), a time generator mechanism(s), and outputs in the form of behavioral, physiological, or endocrinological functions (Fig. 25.3). In nonmammalian vertebrates, pineal gland acts as a photoreceptor (input) and time generator (produces circadian gene oscillations in isolation and produces output in melatonin secretion. A number of studies from diverse groups of organism suggest that daily melatonin rhythm participates in internal synchronization and acts both as a potential clock component and the coupling agent. Melatonin influences either frequency of oscillators or the strength of coupling among the oscillators or both [66–68]. Any change in melatonin profile may alter the circadian system. Tables 25.1 and 25.2 give a summary of important studies that has been done on the role of pineal and melatonin in daily timekeeping in birds.

### *25.4.1 Daily Timekeeping*

Predominantly, the effect of pineal and melatonin has been studied in circadian system-regulated locomotor and feeding rhythms (Tables 25.1 and 25.2). Pinealectomy results in disruption, and periodic application of melatonin by infusion or drinking water restores the circadian rhythmicity [67, 68]. However, the effect of pinealectomy is not universal. In European starlings (*Sturnus vulgaris*), the pineal removal impairs locomotor but not the feeding rhythms [74]. In chicken (*Gallus gallus*) [79] and Japanese quail, the enucleation, not pinealectomy, causes arrhythmicity [81]. In house sparrows, circadian rhythms of locomotor activity [69], body temperature [95], and feeding [96] are disrupted by pinealectomy, but not by neural disconnections of the pineal gland or chemical sympathectomy

**Table 25.1** Effects of pinealectomy on free-running circadian rhythms

| Species | Melatonin rhythm | Circadian function | Effects | References |
|---|---|---|---|---|
| House sparrow (*Passer domesticus*) | – | Locomotor rhythm | Arrhythmicity | Gaston and Menaker [69] |
| White-crowned sparrow (*Zonotrichia albicollis*) | ? | Locomotor rhythm | Arrhythmicity | McMillan [70] |
| White-throated sparrow (*Z. leucophrys gambelii*) | ? | Locomotor rhythm | Arrhythmicity | Gaston [71] |
| House finch (*Carpodacus mexicanus*) | ? | Locomotor rhythm | Arrhythmicity | Fuchs [72] |
| Java sparrow (*Padda oryzivora*) | ? | Locomotor and body temperature rhythm | Arrhythmicity | Ebihara and Kawamura [73] |
| European starling (*Sturnus vulgaris*) | – | Locomotor rhythm | Period decrease or arrhythmicity | Gwinner [74] |
| | | Locomotor and feeding rhythms | No effect | Gwinner and Benzinger [75] |
| | | | | Gwinner et al. [76] |
| Pigeon (*Columba livia*) | + | Locomotor rhythm | Pinx: period change | Ebihara et al. [77] |
| | – | Locomotor and feeding rhythms | Pinx+EX: arrhythmic | Chabot and Menaker [78] |
| Chicken (*Gallus domesticus*) | – | Locomotor rhythm | Slight effect | McBride [79] |
| | | Locomotor rhythm | Period change or arrhythmicity | Nyce and Binkley [80] |
| Japanese quail (*Coturnix coturnix japonica*) | + | Locomotor and melatonin rhythms | Pinx: no effect | Simpson and Follett [81] |
| | – | | Pinx+EX: arrhythmic | Underwood and Siopes [33] |
| | | | | Underwood et al. [82] |
| Spotted munia (*Lonchura punctulata*) | ? | Locomotor rhythm | Arrhythmicity | Pant and Chandola-Saklani [83] |
| Indian weaver bird (*Ploceus philippinus*) | – | Locomotor rhythm | Arrhythmicity | Rani et al. [84, 85] |
| Zebra finch (*Taeniopygia guttata*) | – | Locomotor rhythm | Arrhythmicity | Wang et al. [86] |
| | | Song and call rhythm | | |

(continued)

**Table 25.1** (continued)

| Species | Melatonin rhythm | Circadian function | Effects | References |
|---|---|---|---|---|
| Redheaded bunting (*Emberiza bruniceps*) | ? | Locomotor rhythm | Arrhythmicity | Trivedi et al. [58] |
| | | mRNA rhythm of clock genes | | |

– absent, + present, ? unknown, *Pinx* pinealectomy, *EX* enucleation

[51, 97]. The pineal gland is involved in the organization of metabolic rhythm of 2-deoxy [14C]-glucose uptake, which is abolished in visual system of pinealectomized house sparrows [55] and restored in melatonin-supplemented individuals [56]. These findings implicate that pineal gland confers temporal message to the circadian system humorally through the melatonin signaling.

## *25.4.2 Seasonal Timekeeping: Photoperiodism*

### 25.4.2.1 Photoperiodic Induction of Gonadal Growth

Because of an important component of the avian circadian system, melatonin appears to be involved in the regulation of the photoperiodic effects at one or the other level. However, melatonin seems redundant for photoperiodic control of gonadal growth and development in many birds, including the European starling (*Sturnus vulgaris* [98]), spotted munia (*Lonchura punctulata* [99]), American tree sparrow (*Spizella arborea* [100]), Japanese quail [43], black-headed bunting (*Emberiza melanocephala* [101]), and redheaded bunting (*Emberiza bruniceps* [102]). The absence of an apparent role of melatonin in photoperiodic induction of gonadal growth and development in birds could be explained by a study on ring doves (*Streptopelia roseogrisea*) showing direct innervation of GnRH neurons by deep-brain photoreceptors, DBPs [103]. This study suggested that DBPs, which are necessary and sufficient for sensing the changes in day length, are not linked through circadian system to the reproductive axis in birds and perhaps in other nonmammal vertebrates. A study by Nakane et al. [104] further supports this in showing direct contact of neuropsin containing neurons to the pars tuberalis, which is the site of initiation of photoperiod-induced mechanism. The proposition that DBPs may also contain circadian clock needs investigations. However, some effects of pinealectomy or melatonin injections have been shown in few birds, including the domestic duck (*Anas platyrhynchos* [105]), Indian weaver bird (*Ploceus philippinus* [106]; but see also the findings from Kumar et al. [102], which show the absence of melatonin effects during certain gonadal phases), Indian jungle bush quail (*Perdicula asiatica* [107]), rose-ringed parakeets (*Psittacula krameri* [108]) and lal munia (*Estrilda amandava* [109]). In one of our experiments, we showed the role of melatonin as modulator of testicular growth

**Table 25.2** Effects of melatonin on free-running and synchronized circadian rhythms

| Species | Melatonin treatment | Circadian function | Effects | References |
|---|---|---|---|---|
| House sparrow (*Passer domesticus*) | Pineal transplant | Locomotor rhythm | Entrainment | Zimmerman and Menaker [52] |
| | Oral (drinking water; rhythmic) | Locomotor, feeding | Entrainment | Heigl and Gwinner [87] |
| | | Melatonin + metabolic rhythm | | Lu and Cassone [56] |
| | Continuous | Locomotor rhythm | Arrhythmicity, period shortened | Turek et al. [88] |
| | | Locomotor, feeding + melatonin rhythms | | Chabot and Menaker [89] |
| | Continuous | Locomotor + feeding | Resynchronization period reduced | Abraham et al. [90] |
| | | | | Hau and Gwinner [91] |
| | | | | Kumar and Gwinner [92] |
| House finch (*Carpodacus mexicanus*) | Continuous | Locomotor rhythm | Pinx: birds remained arrhythmic | Fuchs [72] |
| Java sparrow (*Padda oryzivora*) | Continuous | Locomotor + body temperature rhythm | Arrhythmicity | Ebihara and Kawamura [73] |
| European starling (*Sturnus vulgaris*) | Daily injection | Locomotor rhythm | Entrainment | Gwinner and Benzinger [75] |
| Japanese quail (*Coturnix c. japonica*) | Continuous | Locomotor and body temperature rhythms | Period increase, arrhythmicity | Underwood and Edmonds [93] |
| | Oral (rhythmic) | Locomotor and body temperature rhythms | Entrainment | Underwood and Edmonds [93] |
| | Continuous | Locomotor rhythm | No effect | Simpson and Follett [81] |
| Pigeon (*Columba livia*) | Daily injection (infusion) | Locomotor and feeding rhythms | Entrainment | Chabot and Menaker [53, 78] |
| | Continuous (daily infusion) | Locomotor, feeding and melatonin rhythms | Arrhythmicity | Chabot and Menaker [89] |
| | | | | Ebihara et al. [77] |

(continued)

**Table 25.2** (continued)

| Species | Melatonin treatment | Circadian function | Effects | References |
|---|---|---|---|---|
| Domestic canary (*Serinus canaria*) | Continuous | Oxygen uptake and feeding rhythm | Resynchronization period reduced | Pohl [94] |
| Indian weaver bird (*Ploceus philippinus*) | Timed (melatonin and drinking water) | Locomotor rhythm | Entrainment | Surbhi et al. [54] |
| Zebra finch (*Taeniopygia guttata*) | Timed (melatonin and drinking water) | Locomotor rhythm | Entrainment | Wang et al. [86] |
| | | Song and call rhythm | | |

in black-headed buntings. Buntings implanted with melatonin-filled (controls with empty) Silastic capsules showed significantly larger testes under 11.75 h light per day, which is just below the threshold photoperiod as determined in a 4-week treatment duration. These results are consistent with the hypothesis that elimination of melatonin rhythm probably altered the perception of the day length. Trivedi et al. [110] further investigated the modulatory role of melatonin in photoperiodic induction in migratory redheaded buntings, in which the pretreatment with melatonin was found to block the suppressive effects of prolactin on testicular growth and development (cf. [111]).

#### 25.4.2.2 Seasonal Changes in Immune Responses

The relationship between the circadian and circannual rhythms, melatonin, and immune responses seems complex in nature. A schematic figure describes it in the circadian clock context (Fig. 25.4). In chicken, season of hatch (winter vs. summer) determined the development of immune responses: peritoneal inflammation developed quicker and lasted longer in chickens hatched in summer, than those hatched in winter [112]. The mitogen-stimulated proliferation of splenocytes in vitro was also season dependent, with significantly increased proliferation in summer than the winter. These variations in immune measures were correlated with the nighttime increase in the pineal AA-NAT activity, which varies as per the local seasonal environment [112]. Further, a season-dependent effect of melatonin was found in immune responses of chicken reared under photoperiods corresponding to the summer (16 h light per day, 16L:8D), winter (8L:16D), and equinox (12L:12D) photoperiods [113].

In wild living birds, the circulating melatonin levels are elevated during the short photoperiod in concurrence with the enhanced immune response and reduced testicular volume. Opposite to this happens under long photoperiods with shorter nights in summers, when with increased gonadal development the immune

parameters are compromised [114]. The inverse correlation between the immune and reproductive responses supports the hypothesis of physiological trade-off between the two systems, involved in the regulation of reproduction and immune functions [115]. In temperate house sparrows, the constitutive innate immunity was reduced during the energetically demanding mating and breeding stages [116]. Sparrows breed during the summer long photoperiods when nocturnal rise in melatonin has a shorter peak and when there were decreased levels of the innate response [117]. However, seasonal dependence of immuno-enhancement may not be found in migratory birds like the red knots (*Calidris canutus*), in which immune functions seem to be rather linked to the severity of environmental conditions [118]. Possibly, there are changes in the distribution of immune cells between the blood and lymphoid tissues (spleen and thymus) corresponding to the changes in the environment conditions in migrants [119].

## 25.5 Conclusions and Perspectives

Avian timekeeping system is highly complex with at least three participating significant clock components in the retina, the pineal, and the hypothalamus. The system exhibits significant plasticity with different light-history states during the year. The extent of complexity and plasticity can be very diverse and may vary from species to species. Species-specific characteristics of the clock system could provide significant advantages and have tremendous ecological implications. For example, a host of species living in the same environment and hence possibly sharing at least some, if not all, of the resources may adopt strategies for their optimal performance and ultimately survival in the long run. The pineal gland and its product melatonin seem to be the key component of whole scheme of evolution of the successful temporal strategy. In the absence of melatonin, even the hypothalamic pacemakers seem loosing the capacity of the self-sustained circadian oscillations. In spite of significant advancement in the last few decades of research, we are far away as yet from understanding how melatonin meets the demands of the complex and diverse multioscillatory avian circadian system in the temporal environment. One possibility is that melatonin provides flexibility to the timekeeping system by serving both as the pacemaking and the coupling agent. The degree to which these roles are assigned could be linked to the interdependence of the self-sustainment of participating oscillators of the circadian oscillations, the photoperiod environment, as well as the annual life-history state of the species. Finally, whether results from laboratory studies would hold true for free-living populations remains largely unknown at this time.

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# Part VII
# Circannual Rhythms, Photoperiodism, and Seasonal Behavior

# Chapter 26
# Circannual Rhythms Anticipate the Earth's Annual Periodicity

**Barbara Helm and Gerald A. Lincoln**

**Abstract** Life on Earth has evolved in a periodic world, which in most environments cycles predictably between conditions that are favourable or unfavourable for an organism. Organisms use favourable seasons to reproduce and grow, and withdraw during unfavourable seasons. To deal with the predictable changes in their environments, all taxonomic groups have evolved genetically programmed timing mechanisms. These govern seasonal cycles in physiology and behaviour that optimise survival and reproductive success, and have been formally described as circannual rhythms. Experimentally, under conditions of constant day length and temperature, circannual rhythms of many species continue to be expressed, even throughout the life cycle, illustrating the fundamental endogenous control. Under natural conditions, they are usually entrained by seasonal time cues, notably photoperiod, to synchronise the biology to environmental periodicity. Here we review long-term timekeeping strategies from classical vertebrate models to invertebrate and unicell life histories. These circannual rhythms enable organisms to anticipate Earth's periodicity.

## 26.1 Evolution in a Periodic World

On an annual timescale, environmental conditions in which living organisms have evolved are just as fundamentally periodic as described in the preceding sections for daily rhythmicity [1]. All but the equatorial regions undergo annual cycles of exposure to the sun because of the tilt of Earth's axis (23.5°) relative to its orbit around the sun (Fig. 26.1). The daylight fraction of the 24 h day (i.e., photoperiod) is longer in summer than in winter, and the amplitude of the annual photoperiodic cycle increases with increasing latitude. The annual change in exposure to solar radiation has a whole suite of effects on organisms' environment, beyond

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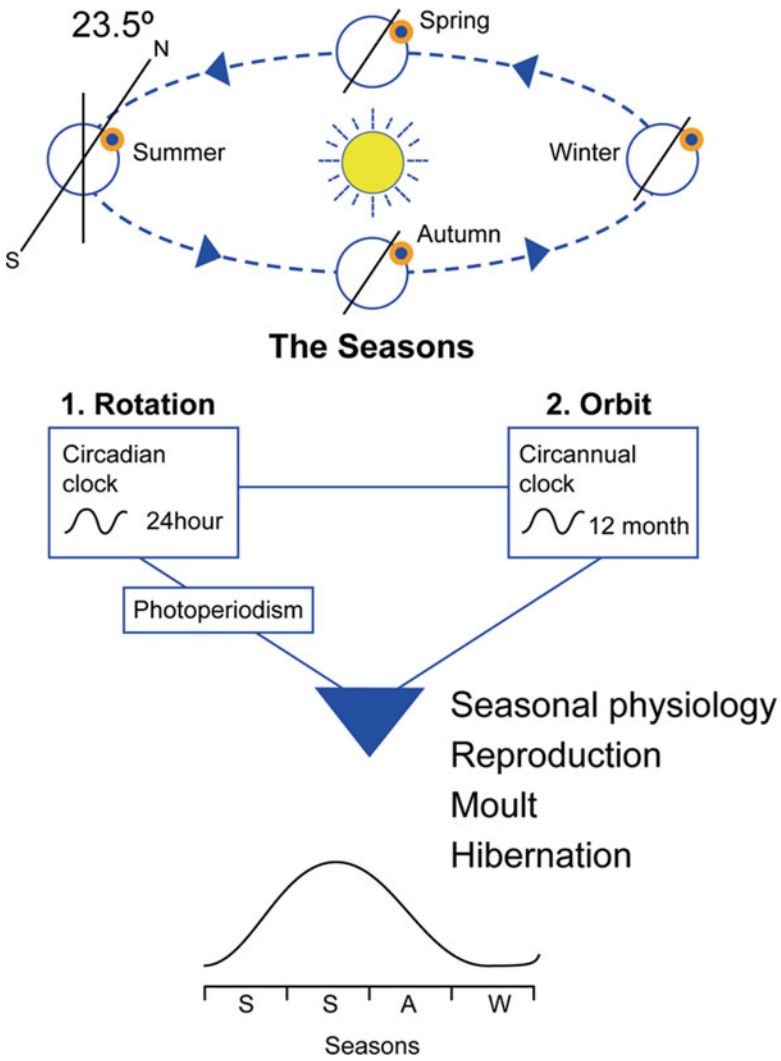


**Fig. 26.1** Endogenous clocks anticipate the Earth's periodicities. The rotation of the Earth on its axis every 24 h (*line* S/N indicates the rotational axis and the *filled circle* represents a point of reference) and orbit around the Sun every 12 months has favoured the evolution of endogenous circadian and circannual clocks. The ability to respond to the annual cycle in day length (photoperiodism) depends on the circadian clock system. Organisms utilise both types of innate timing mechanism to regulate long-term cycles in reproduction, moult, hibernation and other seasonal characteristics (Adapted from [63])

differences in day length. For example, solar exposure affects ocean and land temperatures and, as a consequence, humidity, cloud cover, precipitation, snow cover and air and ocean currents. These knock-on effects lead to annual fluctuations in climatic conditions on a global scale, even at regions that are not directly exposed

to changing photoperiod (such as equatorial or deep sea locations). However, both the amplitude of seasonal changes and the precision in timing differ between locations on Earth.

Living conditions of organisms across the globe are shaped by annual cycles in climate. For example, while organisms at higher latitudes have to cope with alternation between summer and winter seasons, changes between dry and rainy seasons can be similarly challenging for organisms living near the equator. Both plants and animals respond to these annual changes by aligning their biology, using favourable conditions for reproduction and growth, while investing into survival of unfavourable conditions. Consequently, in most parts of the globe, vegetation cover spreads and retracts rhythmically, followed by pulses of animal abundance. This phenomenon has been described as a 'green wave' for habitats as diverse as Serengeti and the Russian Arctic [2, 3]. By undergoing pervasive seasonal changes, the biotic environment effectively potentiates geophysical cycles. Its rhythmicity fundamentally affects the quantity and quality of available food, as well as risks such as predation and infection. These factors have been termed 'ultimate' causes of seasonal life histories because they exert selection pressure on individuals and thereby drive evolution [4, 5].

The most obvious rhythmic response of organisms is alternations between active or reproductive stages and dormant, or reproductively inactive, stages. Examples of inactivity include the annual dieback of deciduous perennial plants to their root-stock, encystment of protists and bacteria to permanent stages, diapause of insects and hibernation of vertebrates. The annual life cycle of organisms often includes further periodic changes in morphology, physiology and behaviour, including moult and migration (cf. Chap. 30). Alternations may occur within individuals, at different stages of the life history (e.g. eggs, larvae, pupae) and transgenerationally (e.g. multiple generations in insects within a year). The particular strategies vary markedly depending on habitat and species' characteristics [6]. For example, in noctuid macro-moths on the British Isles, the most common strategy for survival through winter is the larval stage (48 % of species), and the least common is imago (7 %; G. Lincoln, pers. observation).

It is crucial for the fitness of organisms that these changes are accurately timed. Transitions between stages often require many weeks or months, for example, the growth of the vegetative body of plants, the recrudescence of the reproductive system in mammals and the return of migratory birds from their winter quarters. In order to reproduce at the optimal time, organisms must be able to anticipate future environmental conditions and initiate preparations far in advance. In mammals, the requirement for a long gestational period means that ovulation must be timed to occur many months in advance of the birth season, when environmental conditions will favour survival of the offspring [1]. This example illustrates that timing often cannot be cued by the ultimate causes and instead responds to 'proximate' causes [4]. Photoperiod is the most reliable, proximate predictor of future conditions and is used by most organisms to anticipate the seasons [7, 8]. Other cues include ambient

temperature and changes in food supply and rainfall [1], but organisms must safeguard against following misleading information. For example, in December of 2015, ambient temperatures in northern polar regions were far above freezing. However, initiating summer-type activities under these conditions would be disastrous for polar species.

Accordingly, organisms have evolved an internal representation of time, which governs transitions in the life cycle, and proficient use of environmental cues to time these transitions. Such an internalisation of time has been a key, ancient innovation for coping with seasonal environments [7]. Therefore, across taxa in biology, organisms show genetically programmed, endogenous timing mechanisms. These are modified according to the particular, local environments of individuals and populations. The annual processes can occur periodically under constant environmental conditions and are formally described as circannual rhythms (Latin derivation: *circa* = about and *annus* = year).

## 26.2 Discovery of Circannual Rhythms

Naturalists and researchers have long speculated that organisms must be utilising internal timekeeping mechanisms on an annual timescale. This was based on observations in the wild, rather than on experimental evidence, because long-term studies in isolation from direct influences of the environment were not feasible. In the twentieth century, when experimental capacities were expanded, the evidence for truly endogenous rhythms was consolidated for species across many taxa [9]. Here we illustrate a small selection of examples (Fig. 26.2 [10]).

Few species have captured human interest more than migratory birds (Fig. 26.2a; great knot, *Calidris tenuirostris*). Throughout recorded history, farmers and naturalists have noted their timely return, which is so accurate that in some societies their behaviour is used as an agricultural calendar [1]. Birds were also observed in captivity, and from the eighteenth century, ornithologists described behavioural changes during the migration seasons. Captive songbirds of nocturnally migrating species showed migratory restlessness (*zugunruhe*), hopping and flying at night in their cages, while their wild conspecifics would engage in actual migration [11–13]. This initial evidence for innate migration clocks was then supported by new evidence on the migration patterns of birds. A striking example is the common cuckoo (*Cuculus canorus*) which winters in Africa. The offspring are raised by foster parents and migrate independently to their species' wintering grounds, illustrating an innate sense of time ([9, 14, 15]).

Another group of animals that had long invited speculation about timekeeping are hibernating mammals [15]. Hibernation often occurs in underground burrows, which may be sealed by the animals with plugs and become covered by thick layers of snow (Fig. 26.2b; alpine marmot, *Marmota marmota*). It is hard to conceive how their timely arousal in spring is achieved, other than by the control of some form of an endogenous clock. Indeed, studies of hibernating ground squirrels (*Citellus*

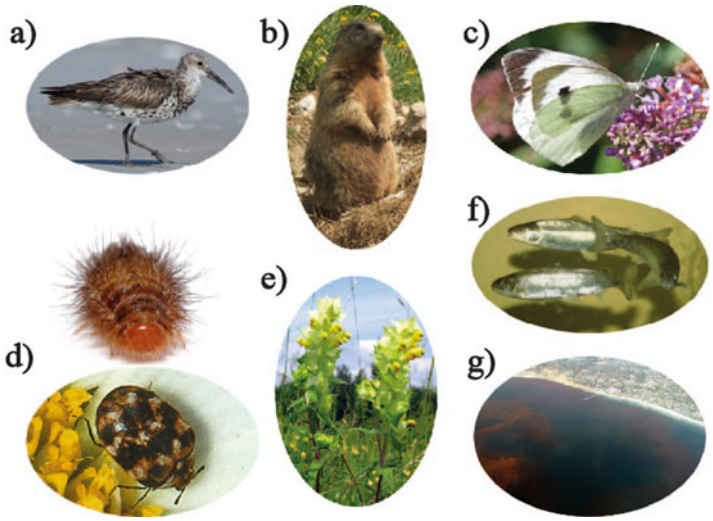


**Fig. 26.2** Model organisms for circannual rhythm research. Many different species have been used experimentally to demonstrate the endogenous nature of long-term cycles. The classic paradigm is to house the test subject in the laboratory under constant conditions of temperature, day length and food supply for more than a year and to repeatedly monitor morphological, physiological and behavioural characteristics. In the species illustrated, the cyclical biology persisted in the absence of environmental cues, thus suggesting innate control. Because the period of the free-running cycles differs from a year, the rhythm is termed circannual. Under natural conditions, outdoors, periodic seasonal environmental cues act to extend or condense the innate cycle to 12 months and to synchronise its phase to the optimal time of year. Time control affects a wide range of characteristics (e.g. body weight, food intake, gonadal activity, moult, migration and hibernation) and is specific for each species. (**a**) Migrating bird, great knot (*Calidris tenuirostris*; by sunphlo); (**b**) hibernating mammal, alpine marmot (*Marmota marmota*; by trldp); (**c**) emerging butterfly, large white butterfly (*Pieris brassicae*; by Gerald Lincoln); (**d**) pupating beetle, varied carpet beetle (*Anthrenus verbasci*; larval form, frontal view by André Karwath, adult form by Hectonichus); (**e**) germinating plant, yellow rattle (*Rhinanthus alectorolophus*; by Bernd Haynold); (**f**) smoltifying Atlantic salmon (*Salmo salar*; by E. Peter Steenstra/USFWS); (**g**) blooming algae ('red tide', mainly *Alexandrium tamarense*; nn). Images except for (**c**) licenced under public domain via wikimedia.org; design: Edda Starck

*lateralis*) provided the first, unequivocal evidence that isolated, individually marked animals show persistent circannual rhythms under constant environmental conditions of light and temperature [16]. These circannual rhythms are not exclusive to the animal kingdom, but also occur in primitive plant species, including marine kelp (brown algae, Phaeophyceae [17]).

While circannual rhythms occur within individuals, some can only be characterised at a population level. These include the timing of transitions between modular stages of development in insects, for example, from egg to larval to pupal to adult. Here, the timing programme gates windows of opportunity for developmental transitions that periodically open and close, but each individual undergoes a given transition only once in its life time. For example, many species of butterfly

show staggered emergence of adults [18]. Of each cohort of pupae, only some produce adults in a given year, while the others wait in pupal stage for another 1 or several years before emerging. One such species is the large white butterfly (Fig. 26.2c; *Pieris brassicae*; Jeanne Robinson, pers. comm.). Circannual rhythms in the frequency of developmental transitions have been characterised in great detail for the varied carpet beetle, *Anthrenus verbasci* (Fig. 26.2d [19]). Pupation of carpet beetles occurs mostly in the first year of larval development, but part of the cohort will suspend this phase and pupate 1 or 2 years later. The population-level circannual rhythm of carpet beetles shares many features with that of circadian rhythms [20]. Another fascinating but still poorly understood example of insect developmental rhythms with extremely long period length is that of periodical cicadas (Hemiptera), whose life cycle lasts up to 17 years. Adults emerge highly synchronously from nymphs, but the periodicities and years of emergence differ between species and regions, indicating innate control [21]. Similar population rhythmicity has also been documented for seasonal events in plants, for example, seed germination of yellow rattle (Fig. 26.2e; *Rhinanthus alectorolophus* [22]), and in vertebrates, for example, the smoltification and migration of salmonid fish (Fig. 26.2f; *Salmo salar* [7]).

Years after the characterisation of endogenous circannual rhythms in multicellular organisms, researchers were surprised to find such rhythms even in unicellular protists [23]. The marine dinoflagellate *Alexandrium tamarense*, which is a major contributor to algal bloom (Fig. 26.2g, 'red tide'), shows circannual rhythmicity in the propensity of a population of cysts to germinate. Because of its tractability and the simplicity of unicellular organisation, this species has recently attracted major interest of researchers who hope to decode the mechanistic basis of circannual rhythmicity (cf. Chap. 29).

## 26.3 The Paradigm of Inherent Rhythms Under Constant Conditions

As detailed above for circadian rhythms, an important stage in the understanding of a biological rhythm requires the documentation of its truly endogenous, self-sustained nature in isolated test subjects in the absence of any environmental time cues. To be considered rigorous evidence for endogenous regulation, biological rhythms must free-run with an individual period length (called '*tau*'), indicated by drift from environmental time [24]. Observed rhythms with a period length that matches those of geophysical cycles cannot be distinguished from direct responses to uncontrolled environmental factors, but may have an equally endogenous basis. To identify period length, two or more sequential cycles must be documented, which for circannual rhythms was a pain-staking process because of the long timescale. However, from the late 1950s onward, such evidence was provided in quick succession for insects, mammals and birds and then subsequently for various

other organisms (Fig. 26.2 [9]). Many circannual studies recorded a whole suite of behavioural and physiological processes within individual subjects, for example, changes in reproductive organs, moult, annual activity patterns and body mass. These processes were shown to recur with period lengths that deviated significantly from the solar year, most typically being short at around 10 months, although in some taxa free-running cycles were consistently longer than 365 days (e.g. great knot; Fig. 26.2a [25]). A remarkable finding of some of these studies was that in many cases, different annual processes desynchronised within the body, for example, moult relative to reproductive cycles [9, 25, 26]. These observations show that circannual rhythms entail independent, presumably tissue-specific processes and do not arise as a mere by-product of sequences of physiological stages [10].

Figure 26.3 shows two examples of extraordinarily long circannual studies. The persistence of the free-running rhythm was recorded for more than 10 years in a tropical songbird, the stonechat (*Saxicola torquata* [5, 27]), and in a hibernating ground squirrel, the chipmunk (*Tamias sibiricus* [28]). In stonechats in particular, the truly innate nature of circannual rhythms has been highlighted by the observations that the rhythms developed spontaneously even in birds that were bred and born under constant conditions and lasted for the entire life cycle (Fig. 26.3a [12]).

In such rigorous studies, subjects were held under conditions of constant food supply, temperature and light. The definition of constant light conditions for circannual studies has been controversial. The most commonly used constant condition was a daily light-dark (LD) cycle of 12L:12D h. This condition occurs at any location on Earth at least twice a year (during the equinoxes) and has been seen as a relatively neutral test condition, much like twilight in circadian rhythm research. However, the 12L:12D h light cycle conveys a specific seasonal signal. Critics have therefore argued that circannual rhythms were physiological artefacts in response to this specific light condition. This criticism was met by documentation from key examples in insects, birds and mammals of free-running cycles in the absence of LD cycles, for example, under continuous dim light, bright light or darkness [20, 28, 29].

While such evidence shows that the expression of circannual rhythms is generally not dependent on a particular light condition, species differ widely in the requirements under which circannual rhythms are expressed. For example, in some species, photoperiodic cues during particular phases of the rhythm are required to advance the rhythm, and in some others, the permissive range entails only a narrow band of constant day lengths (e.g. from above 11.5 to below 12.5 h in the European starling, *Sturnus vulgaris* [30]). Such differences between organisms are intriguing and can give leads to the functional context and mechanistic organisation of circannual rhythms. However, caution needs to be applied when interpreting the presence and absence of circannual patterns under constant conditions. The main function of the constant-condition paradigm is documentation of endogeneity and formal properties of circannual rhythms. This experimental paradigm is highly artificial and contrasts with the real-life environment for which circannual rhythms have evolved. Therefore, hereafter, this chapter will go beyond

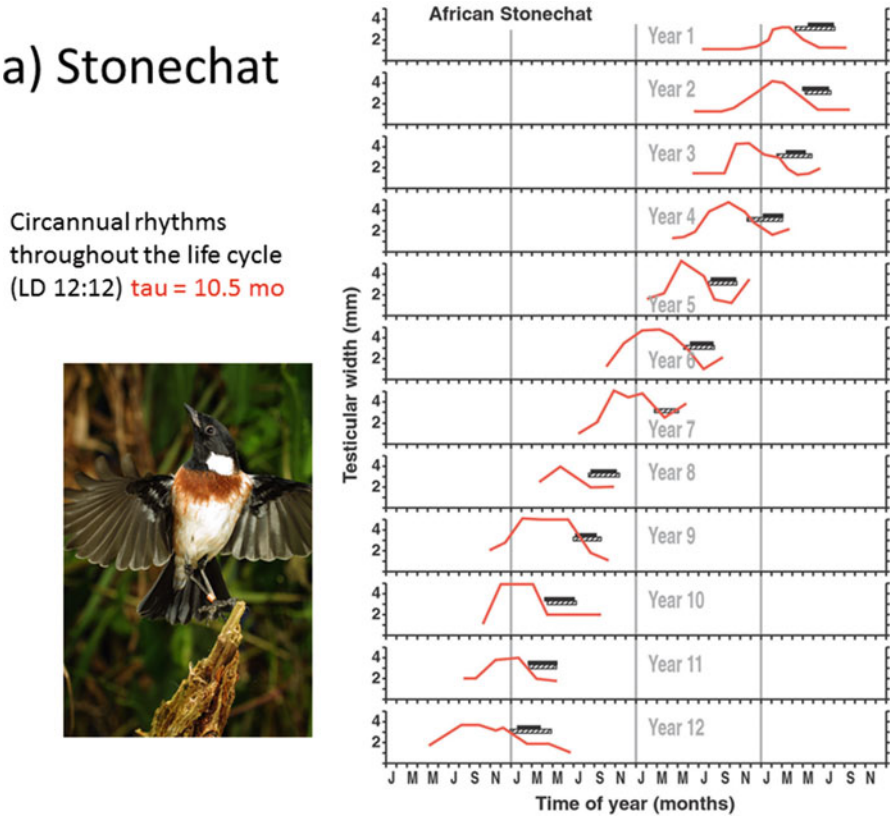


**Fig. 26.3** Circannual rhythms that are sustained across the life history. Two examples where circannual rhythmicity has been shown to persist throughout life in animals maintained under constant conditions. *Above*: African stonechat. Cycles in testis diameter and feather moult in a tame, individual bird caged in the laboratory under constant photoperiod (12L:12D h) throughout its 12-year life span. Reproductive and moult cycles persisted with a free-running period (tau) of 10.5 months: the periodic peaks in gonadal activity became progressively earlier in real time, phase-advancing by a complete cycle. The moult cycle followed a similar pattern and coincided with gonadal regression (Adapted from [64]; image Gerhard Hoffmann). *Below*: chipmunk. (**a**) Circannual body temperature cycles in individual chipmunks housed indoors under constant cool temperature (5 °C) and *dim* light for up to 11 years. The *filled bars* represent the periods of hypothermia associated with hibernation: *HC* hibernation cycle (time from onset to next onset of hibernation, *HD* hibernation duration: values mean +/− SEM). (**b**) Mean period length for the temperature/hibernation rhythm for 16 individual chipmunks under constant conditions (mode, 10.5 months). (**c**) Circannual cycles in the blood concentration of specific liver proteins (called hibernating proteins, HP) in one representative chipmunk related to the temperature/hibernating rhythm (periodic *filled bars*) and western blots of the concentrations of the three different HP proteins that form a complex in the blood. The HP 27 protein is shown to dissociate at the choroid plexus of the brain entering the CSF and affects the cold resistance of brain tissue as part of a seasonal adaptation (Adapted from [28]; image: AndiW, wikimedia.org)

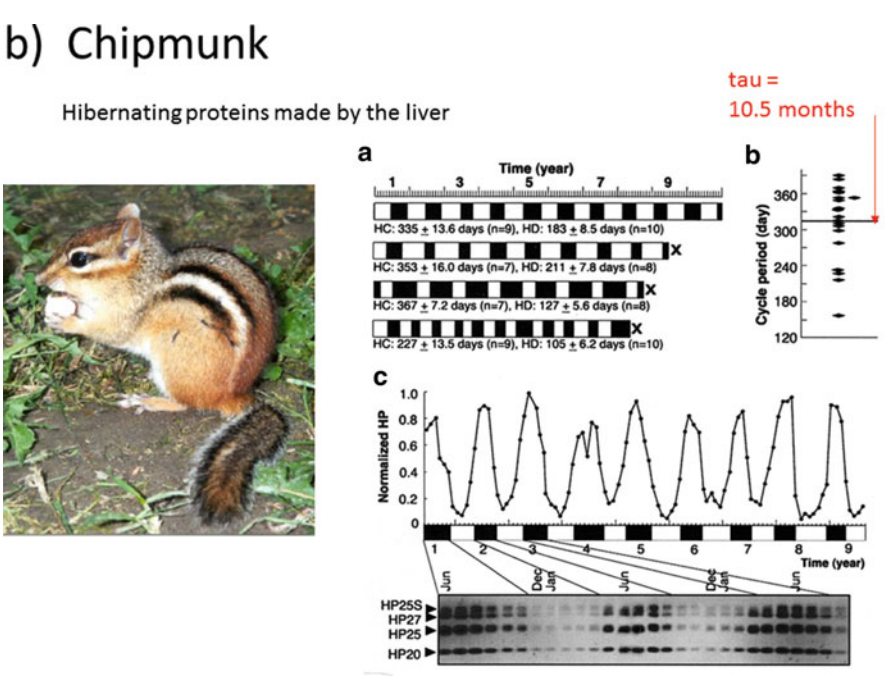


**Fig. 26.3** (continued)

discussing examples that have been formally tested under rigorous constant conditions.

## 26.4 Synchronisation to the Environment

Although circannual rhythms free-run under constant conditions, under natural conditions outdoors, they are usually entrained to the Earth's periodicity by annual time cues (*zeitgebers*). In the vast majority of investigated species, even in the tropics, the periodicity of life cycle stages matches the solar year. Just like circadian rhythms, circannual rhythms have evolved to closely interact with zeitgebers, particularly day length, to adjust the phase and speed (angular velocity [9]) of the cycle (Chap. 29). The only non-photic factor that has been shown to have some zeitgeber effects is ambient temperature [14].

Many species respond very strongly to changes in day length [7, 8]. Photoperiodism occurs throughout global environments, but responses are generally strong at temperate and high latitudes where the timing of seasonal transitions is predictable, and photoperiod is a reliable zeitgeber. The entraining effect of photoperiod can be very dominant driving multiple cycles within 1 year under artificial conditions, e.g. antler cycles in sika deer (*Cervus nippon* [31] Fig. 26.4a). The role of the innate

circannual time mechanisms cannot be revealed in outdoor conditions because it is entrained by the dominant influence of photoperiod, but it still operates. This has been elegantly demonstrated in sheep, where the effect of photoperiod has been silenced by removal of the pineal gland, whose production of the hormone melatonin propagates the photoperiodic signal (Fig. 26.5a). Removal of the pineal reveals the endogenous control of the breeding cycle, which free-runs under natural light conditions until the effect of photoperiod is reinstated using periodic programmed infusions of melatonin [32]. The studies convincingly demonstrate

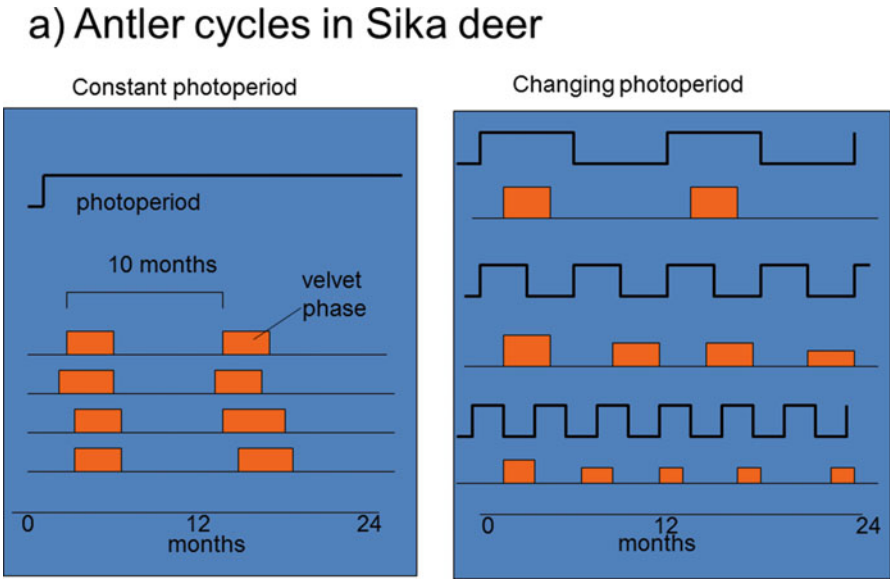


**Fig. 26.4** Circannual cycle timed by changes in day length (photoperiod). (**a**) Antler cycles in sika deer, *Cervus nippon*. *Left panel*, periodic antler cycles (*orange bar* represents the antler growth phase; velvet) in four individual deer housed in a barn under constant long photoperiod (16L:8D h). The antlers are replaced every 10 months due to the endogenous circannual regulation of the seasonal reproductive cycle. *Right panel*, periodic antler cycles in individual sika deer housed under artificial changes in photoperiod (abrupt switches between long days 16L:8D and short days (8L:16D – see *horizontal line*): *top*, once every 12 months; *middle*, twice every 12 months; *bottom*, three-times every 12 months. The accelerated light transitions induced up to three antler cycles within the normal year (driving the circannual cycle in an extreme manner). The treatments altered the phase angle between the light change and timing of the reproductive cycle consistent with an entraining effect on the endogenous circannual timer system (Adapted from [31]). (**b**) Antler cycles (*orange bar*) and sexual cycles (*black bar*; rut) under natural lighting conditions at London Zoo (54° N). *Top*, sika deer show a synchronised autumn rutting season; *middle*, Pere David's deer, *Cervus davidii*, show a synchronised summer rutting season (due to a phase-shifted circannual timer); *bottom*, axis deer (*Axis axis*; data for ten individuals) show annual, asynchronous rutting seasons. This tropical species is non-photoperiodic: having been kept at the zoo at a temperate latitude for over 100 years, the stags still show a seasonality (Adapted from [17, 38]; image: young stags with hard and velvet antlers, respectively; by sumeet.moghe at Wikipedia)

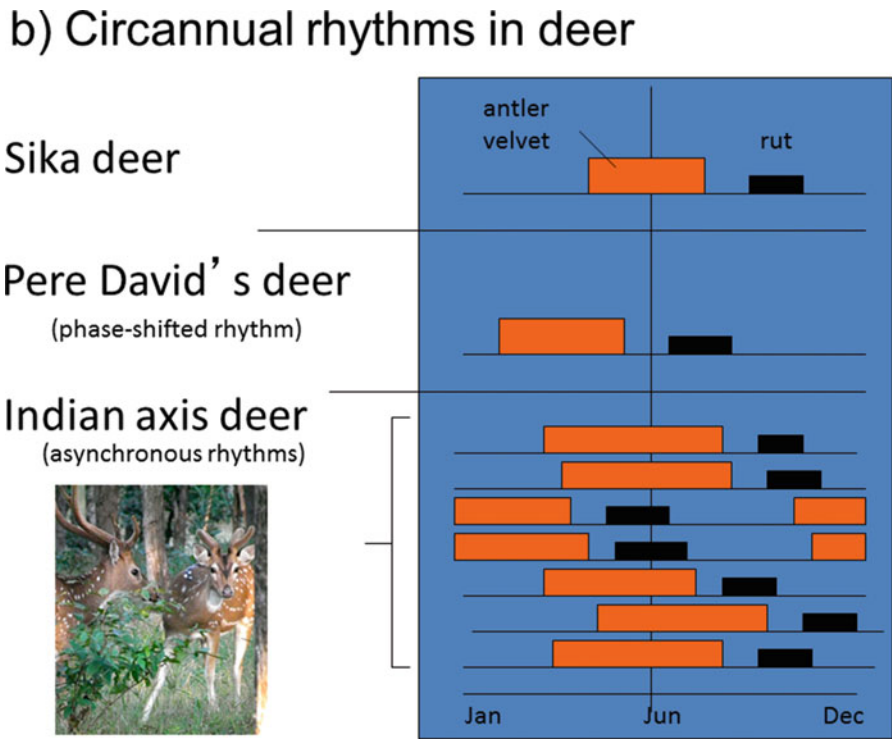


**Fig. 26.4** (continued)

that in sheep, it is the long photoperiod signal of summer that acts to entrain the endogenous circannual system, setting the onset of breeding to the autumn.

Formally, entrainment to zeitgeber*s* has been described in many species and is very similar to that of circadian rhythms. In species with robust circannual rhythms, such as woodchuck (*Marmota monax*), entrainment involves transients (i.e. several intermediate cycles before assuming the final phase of entrainment; P. Concannon cited in [10]). Conversely, species with more responsive circannual rhythms, such as carpet beetles (Fig. 26.2d), show entrainment via fast resetting (type 0 response [20]). These patterns are formally described as circannual phase response curves and have been published for several species [22, 33].

In addition to differences in entrainment properties, species and even local populations may differ in the phase they assume relative to a given photoperiodic cycle (e.g. stonechats, Fig. 26.3a; Helm et al. [34]). In species from different environments, specific seasonal behaviours may occur at various times of year and under a broad range of photoperiods. For example, in temperate regions a given bird species may breed under increasing spring day lengths, but in arid environments, the same species may breed under short days in winter [10]. Similarly, in mammals, the closely related species of sika and Pere David's deer (*Cervus davidii*; Fig. 26.4b) differ considerably in the timing of the rut. When such species are housed under similar conditions (i.e. under 'common garden' conditions), the

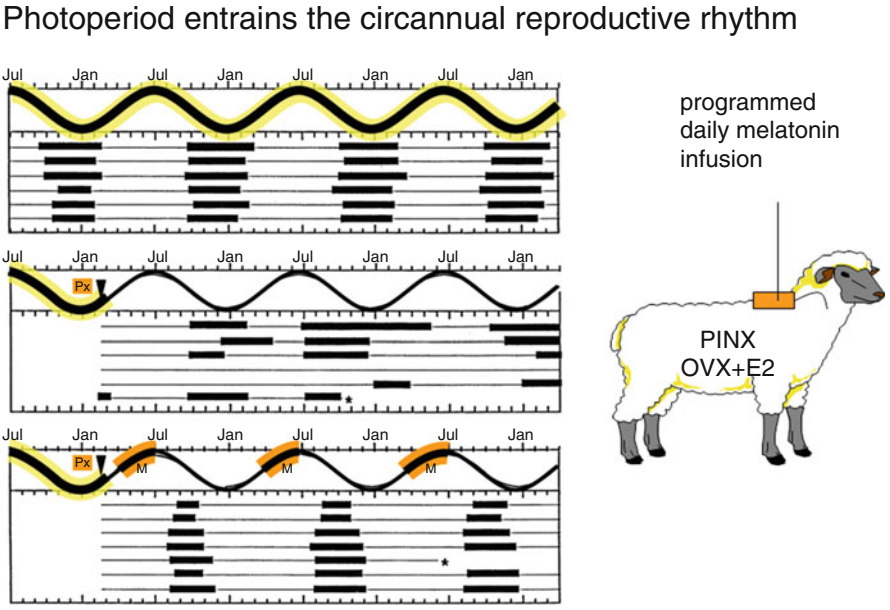


**Fig. 26.5** Entrained circannual rhythms. (**a**) Photoperiodic entrainment of circannual rhythms in sheep. This elegant study investigated the effect of pinealectomy and melatonin replacement on the expression of circannual rhythms in gonadotropin secretion in female Suffolk sheep (*black horizontal bars* represent the sexually active phase for each individual animal). *Top panel*, control group ($n = 6$), untreated, intact animals living outdoors under the natural cycle of day length (represented by the sign wave); note the occurrence of synchronous annual cycles with the sexually active phase in autumn. *Middle panel*, pinealectomised group ($n = 6$), pineal gland removed in spring (Px); note the occurrence of more erratic, asynchronous seasonal reproductive cycles due to the removal of the photoperiod-melatonin relay mechanism. *Bottom panel*, melatonin replacement group ($n = 7$), Px in spring and then treated periodically for 3 months each summer (*orange block*, M) with programmed daily infusions of melatonin (8 h/day to mimic a long day); note the re-synchrony and normal phasing of the seasonal reproductive cycles. Animals treated with programmed infusions of melatonin 16 h/day to mimic a short day failed to show this response (data not shown). Conclusion: long days of summer act to entrain the endogenous circannual clock that regulates seasonal reproduction – this sets the phase of the breeding season to the autumn. All animals were ovariectomised and given an estradiol implant (OVX + E2) to provide a stable sex steroid state (Adapted from [32]). (**b**) Asynchronous annual cycles of free-living male African elephants in Amboseli National Park (Kenya). Fully mature bulls undergo annual cycles of musth (i.e. phases of elevated testosterone with associated physiological and behavioural changes). Each male adopts his preferred annual mating time that is consistent over successive years, but the time varies markedly between males. These specific chronotypes reduce male-male confrontation and allow males to maximise mating opportunities. While it is clear that these animals are closely keeping track of time, the phase of their annual cycles is not driven by photoperiod (Adapted from [39]; image: African elephant bulls in mock fighting; by Profberger at English Wikipedia)

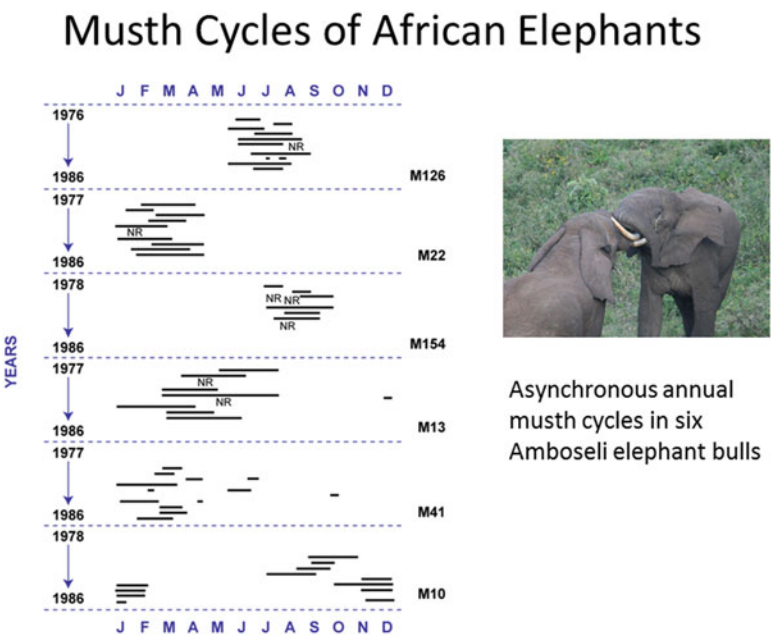


**Fig. 26.5** (continued)

differences in breeding patterns persist, indicating an innate basis. Furthermore, breeding programmes that looked at inheritance in hybrids showed unambiguously that these differences can be based on genetic control (e.g. stonechats, Fig. 26.3a [5]). The genetic differences in the timing may be based on evolutionary modification of the circannual rhythm generation mechanism (e.g. control of period length or of the photoperiodic response mechanism [7, 34]).

Despite the proven strength of photoperiod as a zeitgeber, some species, or individuals within species, are non-photoperiodic. They may be arrhythmic (e.g. American white-footed mice, *Peromyscus leucopus*), or show free-running circannual patterns, or show annual, but asynchronous, cyclicity [35]. Circannual cycles in the wild that clearly deviate from the solar year have been reported for species that inhabit relatively constant habitats, in particular, for seabirds from tropical and subtropical regions [5]. A well-known example are sooty terns (*Onychoprion fuscatus*), whose individual breeding cycles on Ascension Island show average periodicities of approximately 10 months [36].

Annual, but asynchronous, cyclicity has been reported from captivity and the wild. Indian axis deer (*Axis axis*; Fig. 26.4b) translocated to the London Zoo retained individual annual cycles which remained out of phase with each other under the strongly seasonal conditions of Britain. Their asynchronous cycles were confirmed also in captive conditions near the equator [37, 38]. Similarly, free-living male African elephants in Amboseli National Park (Kenya) undergo annual cycles of musth (i.e. phases of elevated testosterone with associated physiological and

behavioural changes) (Fig. 26.5b [39]). Each adult adopts its preferred annual mating time that is consistent over successive years, but the time varies markedly between individuals. While it is clear that these animals are closely keeping track of time, the phase of their annual cycles is not driven by photoperiod.

The ability of species to maintain precise timing near the equator, where photoperiod is relatively constant throughout the year, and the lack of responsiveness of some species to photoperiod, has encouraged the search for alternative zeitgeber*s*. Experimental studies have suggested that tropical birds are able to entrain to subtle, non-photoperiod photic cues, for example, to changes in light intensity between dry and rainy seasons and to small changes in the timing of sunrise and sunset [5]. Based on observations from wild species, effects of rainfall, food quality and social factors are also strong candidates as zeitgebers [35]. Overall, these examples provide very good evidence for endogenous circannual time control that is responsive to a range of subtle environmental cues.

## 26.5 Temporal Organisation Within the Life Histories

With very few exceptions, all organisms have to deal with at least some degree of annual periodicity in their environments. Given the great biodiversity on Earth, there is also a great diversity of mechanisms and strategies by which organisms organise their life histories to align with the change of seasons. Above, we have already referred to some of them, and here we develop a classification of annual cycle organisation, exemplified for the transition from juvenile to reproductively active adult (Fig. 26.6). We first show a generic model of the life cycle of an individual for species that live for less than a year (annual species). We distinguish organisms that progressively mature (Fig. 26.6a; e.g. small rodents) from those that have modular development and undergo a series of transitions between distinct stages (Fig. 26.6b; e.g. annual insects [6, 40]). Continuity of the species' life cycle in the first case depends on staggered, overlapping generations (Fig. 26.6a). In the second case, continuity is achieved by a sequence of resistant developmental stages, which can endure challenging conditions, and short-lived, reproductive adults (temporal compartmentalisation; Fig. 26.6b). While timing programmes are involved in these cases, no apparent circannual rhythm exists within individuals.

Individual rhythmicity can emerge from these generic models in longer-lived species (perennial species) in different ways. Individuals of species whose adult form survives for several years usually undergo repeated annual cycles of reproduction and non-reproduction after first reaching maturity (Fig. 26.6c; e.g. larger mammals, birds, kelp). Various modifications of this pattern have been described, adding to the flexibility of annual life cycle organisation. For example, species such as edible dormice (*Glis glis*), which breed best during irregularly occurring overabundance of food, may pause reproduction for several years. This hibernating species samples its environment annually when first arousing and often prematurely returns to hibernation in poor years [41]. Another modification is heterochrony,

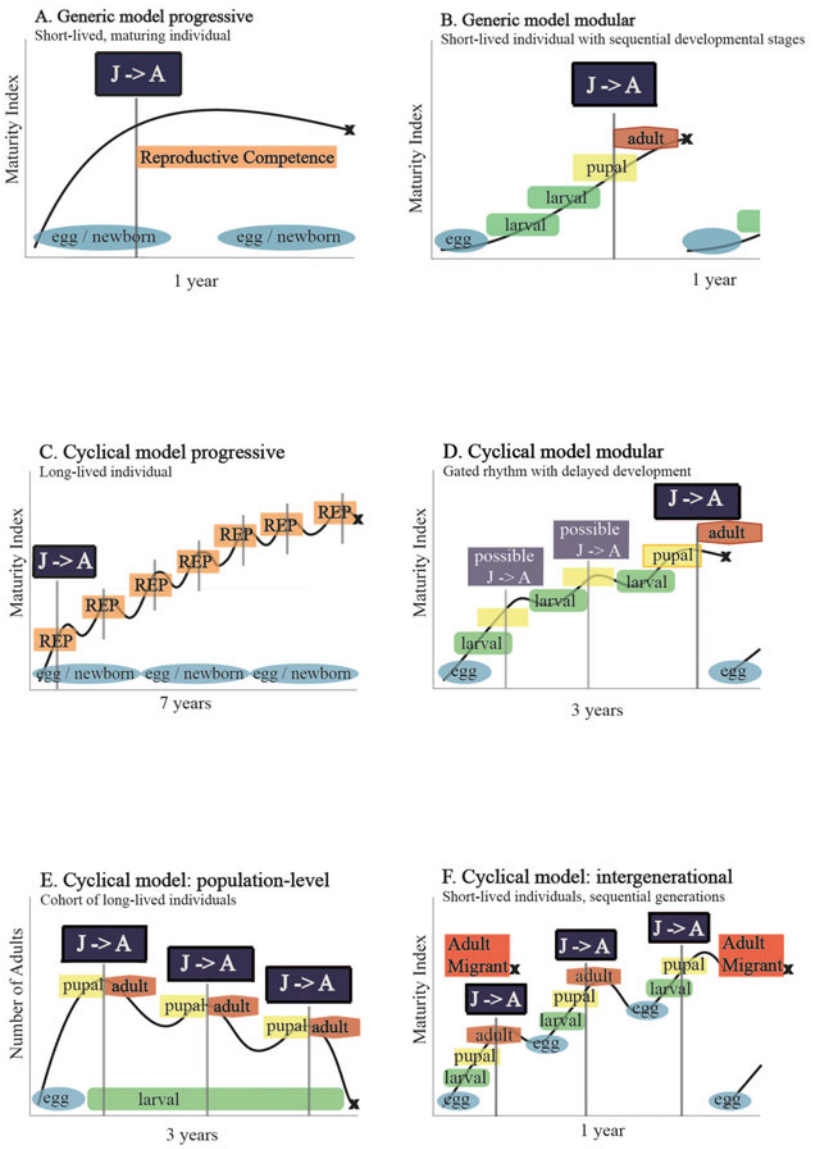


**Fig. 26.6** Circannual rhythms within life histories. We present a schematic classification of annual cycle organisation, exemplified for the transition from juvenile to reproductively active adult (J→R) embedded in the life cycle from egg or newborn to death (indicated by x). We first show generic models of the life cycle of individuals of short-lived species that (**a**) progressively mature and (**b**) undergo a series of modular transitions between distinct stages (temporal compartmentalisation). We then show how circannual rhythmicity occurs for these cases in

where the rate of sexual maturation differs relative to other species or even to conspecifics. In avian and mammalian species that delay maturation, a latent circannual rhythm is likely to be present in individuals that gates the timing of possible developmental transitions to adult maturity [42].

In species whose adult life span is short, individual rhythmicity can emerge during earlier, more long-lived developmental stages (Fig. 26.6d; e.g. carpet beetle, Fig. 26.2d). Although individuals transition to reproductive maturity only a single time, during their immature stages, they may have undergone several cycles of a gating circannual rhythm, as described above for heterochrony. On a population level, this can be observed as annual waves of staggered maturation of a study cohort (Fig. 26.6e). This model is highly flexible, allowing, for example, for a species to endure unfavourable seasons in the most resistant stage, such as the cysts of the dinoflagellate *Alexandrium* (Fig. 26.2g; cf. Chap. 29). In all these cases, individuals possess the genetic programme for sustained circannual rhythmicity.

Finally, we draw attention to a variant in which the completion of the annual life cycle involves several sequential generations (Fig. 26.6f). This situation is found in many invertebrates, for example, in the annual migration cycle of the American monarch butterfly (*Danaus plexippus*). In this species, late-summer adults migrate from the northern USA southward to winter quarters in Mexico. In spring, they fly north for part of the way before laying eggs and dying off. Adults produced from these eggs continue this northward journey, followed by one or more further generations that show this phenotype. In late summer, a final generation emerges that has a larger, more robust phenotype and undertakes the long southern migration back to the winter quarters, thus completing the intergenerational life cycle [43]. The mechanisms underlying the regulation of the timing of this multiple-generation life cycle are only beginning to be understood, but each individual and each generation certainly possesses the entire genomic repertoire that is needed for recurrent, circannual rhythms.

**Fig. 26.6** (continued) longer-lived species. (**c**) If adult forms survive for several years, these adults usually undergo repeated annual cycles of reproduction ("REP") and non-reproduction after first reaching maturity, although cycles can be omitted and the transition to adult maturity can be delayed (heterochrony). (**d**) If adult life span is short, individual rhythmicity can emerge during earlier, more long-lived developmental stages. On a population level, this can be observed as annual waves of staggered maturation of individuals from a study cohort (**e**). Each individual transitions to maturity only once, but in the years leading up to this event, a latent circannual rhythm has likely gated time windows for such transitions. In this way, species can survive in immature endurance stages until a time point is reached for seasonally correct, ephemeral adulthood. (**f**) A variant of this scenario involves sequential generations, rather than sequential stages within an individual. Many invertebrates require several sequential generations to complete their annual life cycle, for example, the annual emergence of sexual or winged propagation stages or the annual emergence of migratory phenotypes in butterflies. Although the mechanistic basis of such multiple-generation life cycle is poorly known, each individual and each generation possess the entire genomic repertoire that is needed for the complete, annual rhythm; image: Edda Starck

## 26.6 Adaptive Significance of Circannual Clocks

The functional significance of entrained biological rhythms under natural conditions is well understood. Demonstrating the functional significance of their endogenous component is proving more difficult because biological rhythms strongly interact with surrounding environmental factors, but recent studies have provided important insights [24, 44]. For circannual rhythms, their wide taxonomic spread, the broad scope of processes that are under their regulation, and the differences between closely related taxa suggest a major functional role under natural conditions. Their adaptive significance is strongly suggested by at least two features of circannual rhythms:

The first critical feature of circannual timekeeping is its predictive power to anticipate and prepare for upcoming seasonal changes in the environment (cf. Sect. 1). In a highly seasonal habitat, where changes in food supply and other selective pressures can be predicted through photoperiod, the timing mechanism allows for very precise regulation of the timing of cycles in physiology and behaviour. The anticipatory mechanism allows timing to be planned much in advance of the ecological events that ultimately determine survival and reproductive success. For example, in red deer (*Cervus elaphus*), the separate events of the seasonal cycle must be integrated to produce births at an optimal time in the spring. This optimal timing is determined by the availability of food to the mothers to lactate the offspring and also by direct effects of environmental conditions on the calves [45, 46]. It has been demonstrated that young being born too early in the spring die of immediate effects of the hostile environment, while those being born too late in the spring die in their first winter, having failed to grow adequately during the summer. For the parents to achieve this timing, all the events across the entire year must be co-ordinated. Regulation of appetite, food intake, accumulation of fat reserves and the development of the appropriate pelage and of antlers and other secondary sexual characteristics all need to be timed by an inherent timing mechanism. In seasonal habitats where photoperiodic changes are small, populations often nonetheless show robust annual cycles, which however may differ between neighbouring populations [5]. For example, rufous-collared sparrows (*Zonotrichia capensis*) on the western and eastern slope of the Andes have robust breeding cycles that are out of phase, and these two neighbouring populations have reduced gene exchange [47]. - Low-amplitude photoperiod conditions also facilitate asynchronous cyclicity of individuals within a population (polymorphisms, Figs. 26.4b and 26.5b; see below). Finally, when seasonal changes are unpredictable, the timing mechanism allows for more flexible regulation of physiology and behaviour. For example, finches on the Galapagos Islands appear to be in a constant state of partial reproductive activation, allowing them to rapidly initiate breeding during vegetation flushes after the scarce rains [48].

Second, circannual timekeeping allows organisms to express robust annual cycles which at specific phases override the effects of proximate cues, including

photoperiod. A classical example is cross-equatorial migratory birds that traverse across hemisphere and thus experience long-day conditions in winter as well as in summer. Similarly, hibernating species may spend many months under constant darkness but arouse on time for vegetation regrowth in spring. Yet another example is high Arctic species, where the organisms are enabled to tolerate the continuous light of summer, and continuous darkness of winter, when regulating their annual rhythms. In such cases, the inertia of the endogenous component continues to drive the rhythm, and its phase-specific response mechanism enables eventual synchronisation to the zeitgebers during highly selective times of year [33].

Species differ in their responsiveness to immediate environmental conditions relative to reliance on internal timekeeping mechanisms. Reliance on endogenous features safeguards against being misled by untimely cues, such as unseasonal weather, but this advantage must be carefully balanced against benefits of direct responses to the environment. Such interspecific differences are highly relevant as environmental conditions are being rapidly altered under climate change [49, 50].

## 26.7 Variation in Circannual Timekeeping

Species and populations vary remarkably in the fine-tuning of seasonal timekeeping, depending on habitat and life history [7]. Species differ in the particular time of year at which a given behaviour, such as breeding, occurs. In some cases these differences can be explained by specific life histories, such as winter breeding of birds that have specialised on conifer mast that becomes available in winter (crossbills [12]). In other cases, social reasons, for example, competition over breeding or feeding opportunities, can lead to staggered life cycle timing. An impressive example of this is the sequential use of island habitat by sympatrically breeding seabirds, which has been associated with allochronic speciation [51]. Based on related observations on a circadian timescale, researchers have introduced the idea that time, like space, may be partitioned as an ecological resource [52]. We propose that the idea of a temporal niche applies likewise on an annual timescale.

Furthermore, within populations, there is variation between individuals in the exact timing of seasonal events, such as the onset of breeding or the onset of migration, with some being early relative to the population average and others being late [53–55]. To the extent that such polymorphisms in annual timing are consistent over several years, individuals can be seen to possess specific, annual ‘chronotypes’. This is analogous to the daily chronotypes described in circadian biology [56, 57]. Such variation is particularly large in environments that are conducive to year-round seasonal activities like reproduction. In contrast, reproductive timing of organisms at high latitudes can often be synchronous to within a few days [4]. Whatever the relative magnitude of such individual differences, they

provide opportunities for partitioning of time as an ecological resource. This is illustrated by the asynchronous musth cycles of Amboseli elephant bulls (Fig. 26.5b), which reduce male-male confrontation and allow males to maximise mating opportunities. Time-dependent reproductive success and mortality have also been demonstrated in birds living at higher latitudes [46, 55]. We propose that an individual's chronotype can function as a temporal equivalent to a spatial territory and suggest the use of 'time territory' to describe this feature of biological timekeeping. Assuming that chronotype is heritable, it can be predicted that this will accelerate evolutionary change in timing programmes, as appears to occur in allochronically breeding seabirds [51].

Finally, even within a single organism, different seasonal processes, such as moult and gonadal development, can vary independently, due to the separate physiological regulation described above [10, 35]. As a consequence, a species can modify the timing of one process without necessarily modifying the timing of another. This promotes the evolutionary potential of circannual phenotypes and determines species adaptability to environmental change [50].

## 26.8 Gwinner's Synthesis: 30 Years Ago

Eberhard Gwinner (Fig. 26.7) was particularly interested in the environmental control and ecological significance of endogenous annual rhythms (migratory warblers, European starling, African stonechat). In 1986 he published a synthesis of the current literature entitled *Circannual Rhythms: Endogenous Annual Clocks*

**Fig. 26.7** Ebo Gwinner's synthesis. Professor Eberhard Gwinner (Ebo) was director of the Max-Planck Institute of Behavioural Physiology (subsequently, Ornithology) at Erling-Andechs in Germany. He had worked there from 1964 until his untimely death in 2004, investigating circannual and circadian rhythms in passerine birds

*in the Organization of Seasonal Processes*. This demonstrated unequivocally the universal importance of circannual timing mechanisms across organisms from all taxa – plants, insects, birds, mammals and even unicells. The formal properties of circannual timing, with parallels to circadian timing, were thereby defined and have since then been confirmed, elaborated and extended:

1. *Robustness*: Circannual rhythms, like circadian rhythms, continue in total isolation from environmental rhythmicity.
2. *Innateness*: Circannual rhythms, like circadian rhythms, are endogenous (self-sustained and innate) and continue throughout the life cycle.
3. *Free-running period*: Compared to circadian rhythms, the range of free-running periods and the extent of inter- and intra-individual variation are larger in circannual rhythms.
4. *Temperature compensation*: Period length of circannual rhythms, like that of circadian rhythms, is largely unaffected by temperature, although evidence is still scarce.
5. *Entrainment*: Circannual rhythms, like circadian rhythms, entrain to zeitgeber*s*, are particularly responsive to photic cues and may involve transients.
6. *Permissive conditions*: The light conditions under which circannual rhythms are expressed are more variable than those of circadian rhythms.
7. *Zeitgeber properties*: The zeitgeber properties under which circannual rhythms entrain are generally more variable than those of circadian rhythms (e.g. range of entrainment, zeitgeber amplitude).
8. *Independence*: Circannual rhythms cannot be explained by frequency demultiplication of circadian rhythms.
9. *Interaction with the circadian system*: The circadian system may contribute to circannual rhythm regulation through annually changing external and internal coincidence or simply through measuring day length. An impaired circadian system does not necessarily disrupt circannual rhythmicity (e.g. SCN-lesioned mammals, pineal-ectomised birds, Arctic damping of clock gene expression).
10. *Broad regulatory scope*: Circannual rhythms, like circadian rhythms, regulate many processes, including alternation between active and inactive phases.
11. *Taxonomic spread*: Circannual rhythms, similar to circadian rhythms, are taxonomically widespread.
12. *Evolutionary lability*: Differences between closely related species suggest that circannual rhythms, like circadian rhythms, are under selection and have high potential for evolutionary change.

## 26.9 Onward Journey: Future of Circannual Studies

Research on circannual rhythms is now at an exciting stage and is beginning to reveal underlying physiological mechanisms (Chaps. 26–31, [10, 58]). This is largely thanks to the revolution in DNA sequencing techniques that can potentially identify all genes in a species' genome that change transcription

across the circannual cycle, and these can then be classified into biological pathways. This may allow the final identification of a cohort of circannual genes, analogous to the canonical circadian clock genes that by interaction generate circannual time. Species-specific molecular tools are also available to measure the chronological changes in gene expression across long-term transitions in physiology. In situ hybridisation and other methodologies are being used extensively to localise the seasonal events within different tissues.

A remarkably wide spectrum of plant and animal models that express annual timing are also now available to be investigated. This includes the detailed genetic characterisation of growth and flowering in the model plant, *Arabidopsis* (Chap. 29). Use of this model is revealing the way epigenetic control of specific ontogenetic genes determines long-term timing processes. There is also a new focus on single-cell organisms, including *Alexandrium* sp., based on the premise that this may reveal the molecular basis of a circannual clock system that is cell autonomous (Chap. 29). At an ecological and evolutionary level, major advances in our understanding of the adaptive significance of circannual timing are predicted. This can involve sampling individuals repeatedly through the annual cycle, at different stages of the compartmentalised life history as in insects and/or across a series of generations [59]. It is probable that information on circannual phase is transferred across generations through the epigenetic modification of gene function (Chap. 29). Plasticity in timekeeping within and between closely related species is also a striking feature in nature which needs to be more fully explored [49]. Such research should reveal the subtle interactions between the internalised clockwork and external environment cues. These may come from climatic features, food quality, the presence of a mate and a plethora of other signals that govern timing on a local ecological scale.

Finally, there is an increasing awareness that circannual biology is of economic and medical relevance to humans. Increasing our understanding of annual cycles in farm animals, family pets and wild species is required to help answer many animal welfare and conservation issues. This is of major current interest because of concern about the unknown impact of rapid climate change, for example, the worrying effects of increasing temperature on the ecology of insects that act as vectors for disease [50]. There are also lessons to be learned from species that show profound seasonal cycles in physiology and behaviour. For example, hibernating mammals utilise a flexible adjustment of insulin resistance to progressively regulate the seasonal change from an obese state at the onset of winter to a life-threatening emaciated state by spring [60]. This may help understand why modern lifestyles that overcome winter deprivation are causing the current human epidemic of obesity and diabetes.

Overall, we emphasise that circannual timing mechanisms are universal. Evolution has operated in a seasonally periodic world throughout Earth's long history, and as a consequence, innate timing processes that predict this periodicity are found in all taxa from unicells to elephants. We now know that humans themselves are no exception – we are a far more seasonal species than previously assumed. In a recent genome-wide screen, using human white blood cells and adipose tissue, a high

proportion of genes were found to change their expression between spring, summer, autumn and winter. This was the case in humans from different ethnic groups and from highly industrialised human societies [50, 61]. Studies of other primates have also revealed that the basic mechanisms of circannual rhythmicity are present in the primate lineage [62]. There can be no doubt ancestral circannual clocks continue to tick in us in still unexplored ways and significantly affect contemporary human life.

**Synthesis**

After several decades of working in the field of seasonal timing, we highlight the following key points:

1. When a seasonal cycle is observed in any organism, it is most likely to be regulated by an *endogenous timing mechanism* rather than by the changing environment. Natural selection for 'optimal timing' operates over countless generations to produce clockwork mechanisms that anticipate the cyclical environment.
2. Responses to photoperiod are *not an essential* requirement for the expression of endogenous circannual rhythms, and moreover, these can obscure insights of the underlying timing process. The past emphasis on characterising the mechanisms of photoperiodic time measurement has distracted from the attempts to resolving the genetic basis of endogenous circannual timing.
3. Circannual rhythms are expressed at the level of the *individual* – due to genetic control. They need to be studied by repeated measurements on known individuals (marked, known ID) across at least two complete circannual cycles. If individuals vary in their cycles (time or amplitude), then population studies are useless at revealing rhythmicity. This applies notably to species that have evolved in equatorial regions, including humans.
4. Within individuals, different circannual processes can become uncoupled from each other, indicating that circannual rhythms consist of independent, *modular components* (e.g. moult, reproductive cycles) and do not result from a sequence of interconnected life cycle stages.
5. In species with a compartmentalised life history, as in insects, the circannual clock *resides permissively* in all stages but may be phenotypically expressed only once in development, e.g. eclosion. The expression of circannual timing can also be *transgenerational*, e.g. where a species undergoes a series of generations across the year.
6. Circannual organisms express *annual chronotypes* (e.g. differences in phase and/or magnitude of their long-term rhythms), analogous to the way circadian organisms express daily chronotypes (e.g. 'larks and owls'). This polymorphism potentially exapts a species to climate change because some seasonal phenotypes will prosper, presumably altering the associated gene frequencies for the circannual timer genes.
7. Just as territorial animals occupy *physical space* to protect food supply and mates (favouring survival and reproductive success), so can an organism occupy *seasonal time space* to be more competitive.
8. Overall, good timing is *adaptive*.

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# Chapter 27
# Seasonal Changes in Brain and Behavior

**Gregory F. Ball, Beau A. Alward, and Jacques Balthazart**

**Abstract** This chapter will focus on how brain and behavior can change with season. Given the breadth of the topic, we will focus on prominent examples discerned from studies of birds though other vertebrate species will be discussed. The chapter will begin with a general discussion of the environmental control of seasonal changes in brain and behavior. We will then consider two types of neural systems that exhibit marked seasonal changes. One consists of neural systems that actually process environmental information and regulate effector systems such as the endocrine system needed to implement seasonal changes in physiology, morphology, and behavior. The other consists of neural circuits that regulate behaviors that exhibit marked changes such as those involved in reproductive behavior.

## 27.1 Introduction: Seasonal Changes in Brain and Behavior and Their Control

Seasonal changes in behavior and physiology have been observed for millennia. Perhaps the most salient event that was observed to change with season was the occurrence of reproduction being associated with a particular time of the year which was noted by humans in writing as early as Aristotle in *On the Generation of Animals*. Humans took a great interest in the timing and control of reproduction of nonhuman animals over the centuries for the obvious reason of desiring to capture wild animals for food and other resources, so it was important to know their habits. The onset of domestication required one to be familiar with the

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environmental regulation of reproduction in order to raise food from captive animals, even out of season, if possible.

A key concept needed to assess the causal basis of seasonal changes is the idea of ultimate vs. proximate causes. Behaviors and related physiological activities change over the course of a year in a systematic way because of their fitness consequences. For example, in the temperate zone, reproduction is more successful in the spring for many species than it would be if conducted in the winter. One can ask two distinct questions about this. One question is "why?", i.e., investigating the ultimate causes, and these questions can be addressed by observing that spring conditions are more favorable for high levels of reproductive success perhaps due to increased temperature, the lack of predators, the availability of food, or some combination of factors. Obviously the exact ultimate cause that is important will be tied to the natural history of the species in question; in birds who directly feed their young, the availability of food is critical, whereas in certain mammalian species where the female is able to feed the young with milk derived from stored fat, the lack of predators or an amelioration in the weather may be more important. The second critical question is "how?" and is concerning the proximate causes of seasonal changes. How does the animal track the time of the year? How are marked changes in brain and behavior implemented to insure that the desired seasonal change occurs? Addressing these questions requires that one implements techniques in the field of environmental neuroendocrinology. It requires that one identifies the critical cues being used by animals to time season, how these cues are processed, and then how the information is transduced to effector systems such as the endocrine system that do indeed modify brain and behavior in significant ways.

A cue that is widely used to time seasonal change is variation in day length or photoperiod. Rowan [1] pioneered studies of photoperiodism in animals based on his attempts to understand in juncos the environmental control of a behavior that changes very prominently with season, migratory behavior. He worked with wild-caught juncos to investigate how changes in photoperiod might alter measures of physiology such as hormones. He hypothesized that these photoperodically induced changes in physiology drive seasonal changes in migratory behavior [1]. Subsequent studies by others of wild species such as European starlings reported that increasing day length rapidly stimulates gonadal growth [2]. Studies of domestic chickens inspired by this work established that the duration of available light also regulates egg laying [3].

From these initial experimental observations, a major field of investigation emerged. Starting in the 1950s, many different vertebrate species were investigated, and the ability of photoperiod to regulate the hypothalamo-pituitary-gonadal (HPG) axis that results in variation in gonadal size became well established (e.g., see [4] for a general review). The establishment of seasonality as a phenomenon regulated by photoperiod inspired systematic investigations as to how light is processed by and transduced into major changes in the regulation of the hypothalamo-pituitary-gonadal (HPG) axis. These sorts of studies lead to many additional questions: Where and how is light detected by the brain? Are biological clocks involved in

the interpretation of long-term variation on photoperiod? Are these clocks circadian or hourglass in nature? How is information about photoperiod conveyed to the HPG axis? Are there other significant environmental cues that are integrated with photoperiod to regulate the HPG axis? What is the role of neural releasing hormone systems in the regulation of endocrine function? How does variation in endocrine physiology code for variation in the environment especially as related to photoperiod (e.g., thyroid hormones and melatonin). These important proximate questions relate to the issue of mechanisms that control how the environment might regulate seasonal changes in physiology. In this review, we focus on aspects of these brain mechanisms that show remarkable seasonal changes for a more general discussion of the neuroendocrine mechanisms regulating seasonality; we direct the reader to an issue in Frontiers in Neuroendocrinology that contains 14 papers on these topics [5].

### *27.1.1 What Sorts of Neural Systems Change with Season?*

The neural circuits that regulate the response of an individual to track the environment represent one set of neural processes that change with season. In particular, cell networks that transduce environmental information perceived by the nervous system to effector systems that mediate adaptive change such as releasing hormones including gonadotropin-releasing hormone are one example of a brain system that can change with season, at least in certain species [6, 7].

An additional question that is also important concerns how these seasonal changes in physiological state relate to seasonal changes in behavior and the underlying neural systems that regulate these behaviors [8]. An important concept that emerges from this work is that the phenomenon of seasonality can result in qualitative changes in one's physiological and behavioral state. Many phenotypic attributes can be used to assess this variation such as brain morphology and physiology and cognition including perception and behavior, but also variation in feeding behavior, immune function, and energy metabolism [9, 10]. Thus the phenomenon of seasonality is an opportunity to link naturally occurring brain variation with different functional outcomes.

### *27.1.2 Seasonal Changes in Behavior: What Changes and What Stays the Same?*

Seasonal cycles are characterized by periodic changes in some behaviors and stability in others. As noted previously, reproduction requires a substantial energetic investment, so by its nature it occurs periodically since individuals cannot manage energy in such a way as to allow them to breed constantly. Most vertebrate species engage in sexual reproduction; this requires that an individual finds a mate,

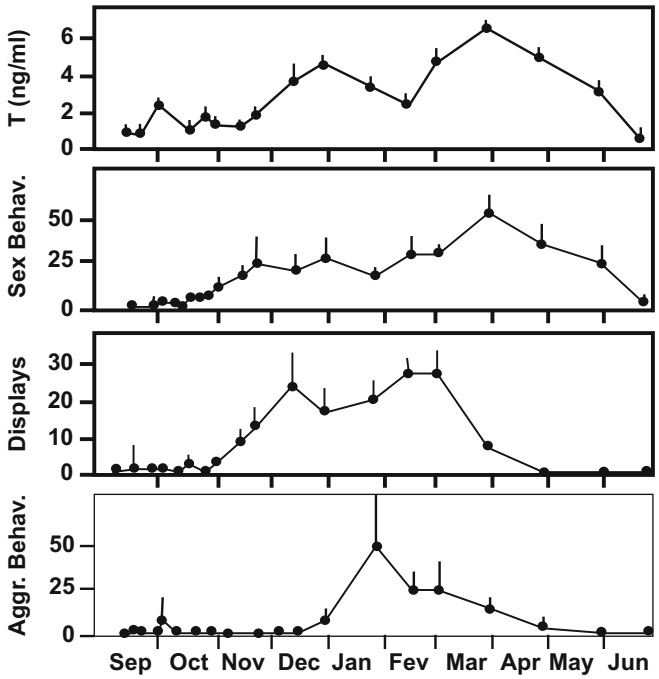


**Fig. 27.1** Monthly changes in behavior as well as in concentrations of testosterone in the blood in mallard ducks. Note that duck aggression peaks just prior to the peak in sex behavior (i.e., copulation). As one would expect, courtship displays (i.e., appetitive sexual behaviors) occurred over a long somewhat variable period before copulation commenced (Taken from [11])

copulates with him/her to exchange gametes, and produces zygotes at a time and place propitious for successful reproduction. In many endothermic vertebrate species especially, high levels of parental care are quite common since the zygotes have to be maintained at a constant temperature to survive. A representative example of a seasonal change in a selection of reproductive and social behaviors in ducks is illustrated in Fig. 27.1 [11]. Other behaviors such preening may change only in frequency but even feeding can change dramatically given that some birds feed in flocks in the winter but individually in the spring and summer.

## 27.2 Seasonal Changes in Neural Systems That Mediate the Environmental Regulation of Brain and Behavior

Reproductive behavior tends to occur with an underlying periodic pattern in relation to season. This timing is regulated by both physical and social cues [12–14]. One well-studied physical cue is the seasonal change in day length or photoperiod. The classic example of the role of photoperiod is revealed in seasonally

breeding species in the temperate zone that breed in the spring when food resources are most available and to support the generation of offspring [15, 16]. Periodic changes in food availability in many environments are accurately predicted by increasing photoperiods in the spring. This has resulted in the evolution of photoperiodic processes such that the HPG axis in a given species can be enhanced by the experience of long days and decreased by the experience of short days. In some species, especially those in harsh environments at high latitude, breeding is terminated well before photoperiod reaches the threshold of the longest day of the year during the summer solstice so that sufficient time is available to raise progeny that needs to migrate to more temperate climes prior to the onset of winter. In these species, a form of photorefractoriness is observed: after experiencing to a certain number of long days, their HPG axis shuts off even though photoperiod is still long (see [13, 17]). The mechanism underlying this change may be directly driven by photoperiod or it could be regulated by an endogenous circannual rhythm entrained by variation in photoperiod [18]. We will not review this topic in this chapter as it is beyond our scope.

Not all species use photoperiod as a cue to time periodic breeding; many species living in the tropics are an example of such species [19]. Avian species inhabiting xeric environments are known in some cases to respond to vegetation growth stimulated by variation in the occurrence of rainfall (e.g., the zebra finch; [20]). Species that inhabit tropical forests may have extended periods of reproduction, but these are often centered on the times of the year when a preferred specific food is most abundant. Tropical species have been shown to be able to respond to slight changes in photoperiod characteristic of the tropics as well as to other cues including the social context and rainfall [21]. This occurrence of long breeding seasons combined with rapid responses to many different cues from the environment seems to be an adaptation to the distribution of food resources that are accessible for long periods of time but never really abundant.

The detections of light required to measure photoperiod involved in the regulation of seasonal cycles do not involve the eyes in avian species but rather depend upon deep brain extraretinal receptors (see [17, 22] for reviews). The search for the photopigment that transduces light in the deep brain and its anatomical location has been an active area of research in recent years [23]. Early studies had suggested that a rhodopsin-like photopigment was involved and that it was present in either the lateral septum or the infundibulum [24–26]. However, subsequent studies were unable to confirm that this was the functional photoreceptor [23]. Three photopigments have emerged as candidates in recent years: melanopsin (OPN4), neuropsin (OPN 5), and vertebrate ancient opsin (VA). OPN4 has been suggested based on anatomical investigations and correlational studies in turkeys as the key extraretinal photoreceptor (see [27]). The mRNA is located in neurons of the premammillary nucleus (PMM; an area just dorsal to the infundibulum) where there is evidence for clock gene expression [27]. Studies in quail localized the OPN5 mRNA to the periventricular organ adjacent to the infundibular region [28]. Disruption of OPN5 expression in canaries with the use of RNA interference did disrupt photoperiodic effects on endocrine physiology, but somewhat

surprisingly it facilitated the expression of thyrotropin-stimulating subunit beta suggesting that OPN5 exerts an inhibitory effect on HPG function [29]. Foster and colleagues have argued that VA rather than OPN5 is the primary photoreceptor mediating the photoperiodic response in birds [23]. They have evidence that VA is expressed in gonadotropin-releasing hormone (GnRH) neurons as well as those expressing arginine vasotocin (AVT). These findings, if they are truly marking a functional photoreceptor in these cell types, would represent a potentially significant change in our thinking about neuroendocrine neuronal systems controlling photoperiod as it has been thought for many years that the photoreceptor is localized in areas of the brain quite distinct from the GnRH system or other systems (e.g., [30]). It is also important to note that there may well be multiple deep brain photoreceptors localized in distinct areas of the mediobasal hypothalamus and having perhaps distinct functional roles.

Engaging in social interactions can profoundly influence various measures of endocrine physiology. In birds the sensory basis of such effects are primarily dependent on the auditory and visual systems [31]. Growth or regrowth of the reproductive endocrine system can be modulated by the appropriate conspecific signals, for example, signals from conspecific females that represent potential mates can enhance male gonadal growth (see [32] for a review). Such effects will in turn obviously influence the expression of male sexual behavior and its seasonal timing. Effects of the presence of a male on female's endocrine state are also known to occur. The presence of a male or a female also has an effect on the timing of sexual responses on a much shorter time scale.

Early studies on the effects of photoperiod on the reproductive endocrine systems focused on males in part because in many avian species, males (even those isolated from other cues) tend to exhibit robust changes in gonadal growth in response to photostimulation in the laboratory, while females do not [33]. This observation highlights an important sex difference in regard to reproduction; females make a much more important (in terms of adaptive significance) physiological decision when committing an egg than males do when committing sperm [34]. Recent work that takes an evolutionary approach to the study of reproductive physiology in birds stresses the importance of understanding processes in females given that many key traits related to success in seasonal reproduction (clutch size, egg size, timing of ovulation, etc.) are regulated by females not by males [34]. This observation along with the fact that females process multiple cues (i.e., photoperiodic and non-photoperiodic) in complex ways in order to ovulate underscores the importance of studying seasonal and endocrine mechanisms in males and females [33]. Some prominent differences include the fact that in some photoperiodic species, input from the eyes is primarily inhibitory in that females that lack visual input lay eggs at a higher rate than those with visual input (reviewed in [33]). Experience with a variety of environmental cues significantly influences how females respond to a given environmental situation, while males will respond strongly to photoperiod alone even in novel situation [33].

### 27.2.1 Neuroendocrine Mechanisms Mediating Seasonal Changes in the Endocrine System Facilitating Reproductive Behaviors in Birds

Signals from the environment that regulate the timing of occurrence of reproduction and reproductive behavior are first processed by the brain which then traduces information to the pituitary that in turn regulates the gonads (e.g., [14, 30, 31]). Initial studies focused on the mechanisms related to how photoperiod regulates the endocrine system (see [7] for a review). The annual change in day length provides a predictive cue in that can be used to predict the time when environmental conditions are suitable for successful reproduction [12, 17]. One of the hallmarks of seasonal breeding, especially in birds, is that they exhibit a remarkable involution (i.e., regression) of their reproductive axis when in a nonbreeding state; however, successful reproduction requires that the recrudescence (i.e., growth) of the reproductive endocrine system occur well before there has been an amelioration of environmental conditions at the breeding site. This process of responding to photoperiod involves a progression through three distinct reproductive states. Day length increases in the spring, and this change stimulates an increase in gonadotropin secretion, gonadal growth, and testosterone concentrations in the blood. Birds in this reproductive state are referred to as being *photostimulated* and are in a breeding condition. Long day lengths do two things: they stimulate gonadal growth, and a long-term exposure also promotes regression of the reproductive system and results in the birds being in a nonbreeding state called *photorefractoriness*. In this condition, the long days that were previously stimulatory are no longer effective in stimulating gonadal growth but seem to be inhibitory. Photorefractory birds are reproductively nonresponsive to long day lengths (even constant light) as well as to other environmental cues that supplement increases in day length to modify reproductive physiology at other seasons including social interactions. Two types of photorefractory responses have been described in birds: absolute and relative photorefractoriness. This review will only consider species that exhibit absolute photorefractoriness. Two criteria have been proposed to define the pattern of absolute photorefractoriness: (1) reproductive regression after prolonged exposure to photoperiods that previously stimulated gonadal growth and (2) the continuation of gonadal regression in the presence of even the longest possible photoperiod, i.e., constant day length [35]. In captive conditions, when birds are transferred from a short day (e.g., 8L:16D) to long day (e.g., 14L:10D), gonadal volumes will increase within 3 weeks, and after approximately 6–7 weeks, there is a clear regression of the gonad associated with the onset of a photorefractory state. Physiological sensitivity (i.e., responsiveness) to long day lengths can be restored, but birds must first experience short days similar to those that occur naturally during the late fall and early winter. Thus, short day lengths result in the resensitization of the neuroendocrine axis; birds in this condition are termed *photosensitive* or pre-breeding.

### 27.2.2 Seasonal Plasticity in the Gonadotropin-Releasing Hormone System

These three physiological states that were first defined based on the pattern of reproductive physiology also are associated with marked brain differences [7, 17, 36]. One of the most surprising findings derived from studies of starlings is that in species that exhibit a pattern of absolute photorefractoriness, there is a marked downregulation of the GnRH-1 protein. The GnRH-I neuronal system is a key part of the network that links the brain processing of environmental information to the endocrine system. This downregulation means that the birds in a nonreproductive state are essentially severing communication between the brain and the reproductive endocrine system. Investigation of changes in the mRNA for GnRH-1 in starlings whose brains were collected during breeding cycle reveals that the onset of photosensitivity involves an upregulation in GnRH-1 mRNA, while the birds are on short days and reproductively quiescent. Expression of this mRNA increases during the development of photosensitivity and the onset of photostimulation it then plummets after photorefractoriness ex attained (see [7] for review). This sort of variation pattern occurs in other species but the degree of plasticity can vary depending on the type of photoperiodic response observed [6]. There is also evidence that there is a functional topography in the organization of the system with some cells responding more to supplementary cues such as social interactions rather than photoperiod [36].

This extreme degree of GnRH-1 plasticity seems to be a feature of seasonal breeding in birds but not in mammals. In the majority of seasonally breeding mammals, GnRH-1 levels appear to remain constant. Some exceptions have been described such as Prairie voles (*Microtus ochrogaster*) where individuals that exhibit photoperiod-induced gonadal regression exhibit not a decrease but an increase in the number of immunoreactive GnRH-1 neurons [37]. In male Siberian hamsters (*Phodopus sungorus*), GnRH-1 expression appears to increase in a stable population of cells in the POA as the animals transition from experiencing short days to long days similar to what has been reported in starlings [38].

Brain plasticity associated with mammalian reproductive cycles was revealed based on studies of another neuropeptide. The discovery of a neuropeptide called kisspeptin (kiss1) turned out to be very significant for the understanding of the cellular basis of the regulation of the neural circuits important in regulating mammalian reproductive cycles. Since the initial discovery of kisspeptin, a number of different isoforms have been isolated, and the primary form kiss1 has been shown based on studies of diverse mammalian species to play an important role in the neuroendocrine regulation of reproduction (e.g., [39]). This is very evident in seasonally breeding mammals, such as the domestic ewe, Syrian hamster (*Mesocricetus auratus*), and Siberian hamsters; variation in reproductive physiology has been linked to extensive kiss1 plasticity in the hypothalamus (see [39] for reviews).

In is interesting to compare these patterns of neuropeptide plasticity that occur in different systems in birds and mammals. In birds, variation in reproductive

physiology is much more apt to correspond directly with variation in the GnRH-1 system than in mammals. For example, several attempts to review in a systematic fashion the phylogenetic distribution of GnRH-1 plasticity in birds have proposed that variation in GnRH-1 plasticity is an adaptive mechanism involved in the timing of reproductive schedules [40]. In mammals it appears that variation in kiss1 plasticity and not GnRH relates systematically to variation in reproductive activity. The degree of change observed in the GnRH system when brains from birds are collected at different times of the year and how this brain plasticity can vary among species is illustrated in Fig. 27.2. As seen in Fig. 27.2, free-living house finches [41], when collected in the spring and in the fall, showed a remarkable decrease in GnRH-1 immunoreactivity in fall birds as compared to spring birds. These birds are highly seasonal and shut down breeding for all of the year except for the spring. In white-winged crossbills, an opportunistic species that breeds in response to food availability, wild-caught birds maintained on a natural photoperiod were sampled at the same months as the house finch study, and the crossbills did not exhibit a change in immunoreactivity; they can breed in a flexible manner during a large portion of the year as long as the appropriate food is available [42].

Another hypothalamic peptide related to reproduction was first identified by Tsutsui and colleagues in Japanese quail who named it gonadotropin-inhibitory hormone (GnIH) (see [43] for a review). This dodecapeptide has been identified in the hypothalamus of several avian and mammalian species [43]. The neuroanatomical pattern seems conserved as cell bodies are consistently present in the paraventricular nucleus (PVN) with fibers projecting to the median eminence [43]

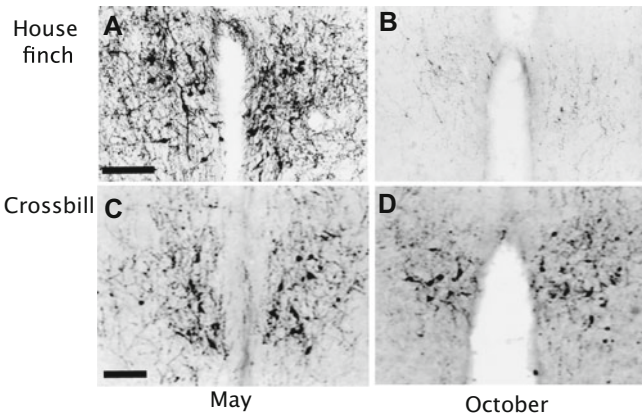


**Fig. 27.2** Seasonal changes in GnRH-I immunoreactivity in two species of wild-caught songbirds. Panels (**a**, **b**) present photomicrographs from the preoptic area of free-living house finches [41] collected in the spring and in the fall. A comparison of the groups reveals a remarkable decrease in GnRH-1 immunoreactivity in the spring birds as compared to fall birds. These birds are highly seasonal and shut down breeding for all of the year except for the spring. In *white*-winged crossbills (see panels **c**, **d**), an opportunistic species that breeds in response to food availability, wild-caught birds maintained on a natural photoperiod were sampled at the same months, and they did not exhibit a change in GnRH-I immunoreactivity. Members of this species can breed in a flexible manner during a large portion of the year as long as the appropriate food is available [42]. Magnification bars = 100 μm in (**a**) (for panels **a**, **b**) and in (**c**) (for panels **c**, **d**)

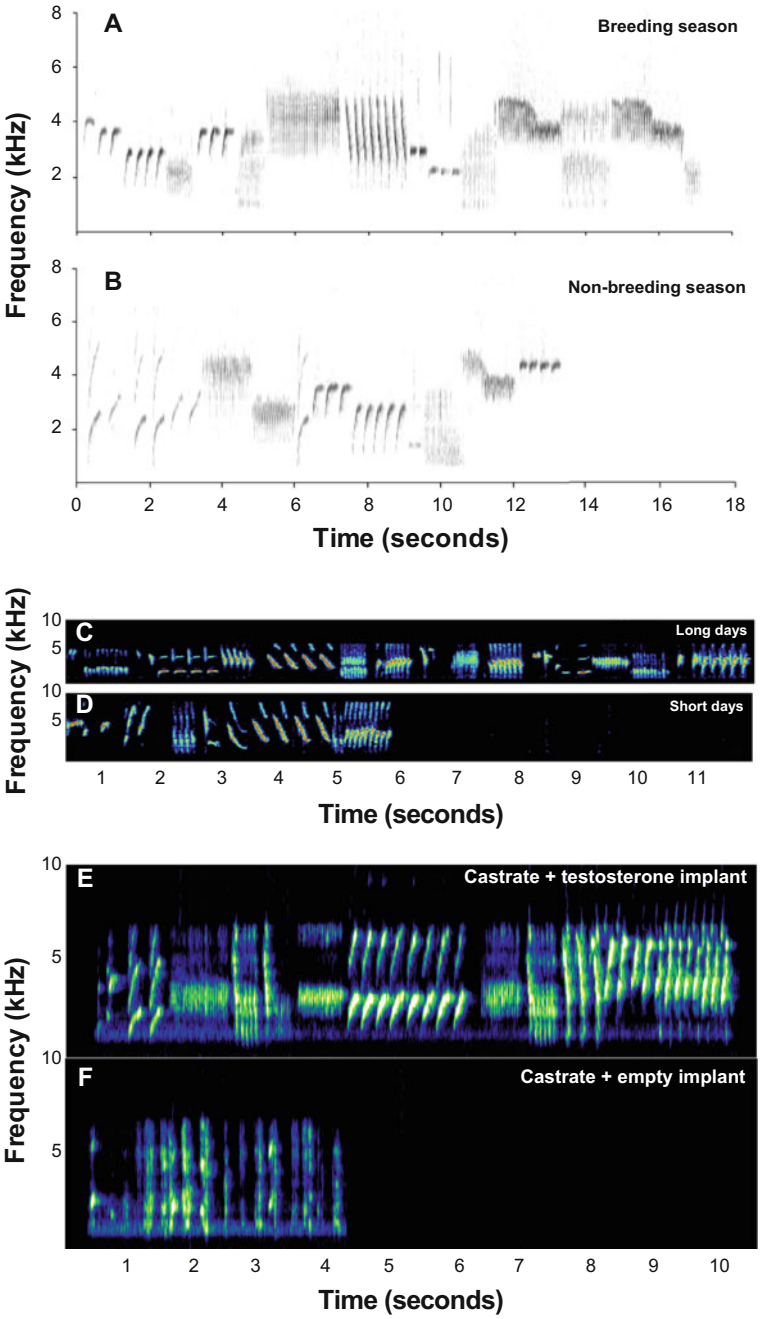


**Fig. 27.3** Role of photoperiod and testosterone in regulating song in male canaries. Male canaries housed in aviaries on naturally changing day lengths show that males (**a**) sing longer songs during the breeding season (i.e., longer day lengths, ~16 h of light and 8 h of dark) compared to (**b**) the nonbreeding season (i.e., shorter day lengths, ~8.4 h of light and 15.6 h of dark) [46]. These results were replicated in a controlled laboratory setting that housed male canaries alone on (**c**) long days

as well as to the preoptic area (POA) and periaqueductal gray (PAG). As one can infer from its name, it can act in many different ways to inhibit reproduction including direct action on the pituitary to inhibit gonadotropin release [43]. This peptide thus appears to act as a modulatory brake on reproduction in contexts where short-term inhibition of reproduction is required. GnIH along with GnRH-I have been studied in relation to the control of seasonality in the Indian weaver, a subtropical avian species that exhibits a more flexible photoperiodic response than birds that go absolutely photorefractory [44]. The data reveal that there are some changes in GnRH-I immunoreactivity but with a pattern characteristic of flexible breeding species rather than highly seasonal ones [44]. Interestingly there are some indications of changes in GnIH so perhaps this peptide has a role in seasonal regulation that still needs to be elucidated.

### *27.2.3 Seasonal Changes in Neural Systems Regulating Reproductive Behaviors*

The avian brain also exhibits a remarkable degree of hormone-regulated neuroplasticity that makes avian species especially attractive for neuroendocrine studies [8]. This phenomenon is perhaps best documented in the avian song control system where Fernando Nottebohm discovered dramatic seasonal changes in the volume of key telencephalic nuclei that control courtship song in male canaries [45]. Figure 27.3 illustrates seasonal variation in song quality of male canaries that were recorded in the field [46] or held in captivity under different photoperiods [47] or in the same photoperiod but castrated and treated with testosterone [48] (Fig. 27.3). These examples illustrate how a seasonal change can be mimicked with seasonally relevant photoperiods and/or by manipulating seasonal changes in testosterone. The seasonal variation in brain area volume was later found to involve changes in cell numbers in some areas of the song system such as nucleus HVC [8].

Is it possible that there can be such variation in neuron number in the adult brain? It was discovered by Nottebohm and colleagues that adult birds have widespread ongoing neurogenesis in the telencephalon at a very high rate [49]. Gonadal steroid hormones clearly play an important role in controlling the brain area volume changes, but they do not seem to regulate the rate of neurogenesis but rather the

**Fig. 27.3** (continued) (LD; 14 h of light, 10 h of dark) and compared them to male canaries alone on (**d**) short days (SD; 8 h of light, 16 h of dark) [47]. Alward et al. [47] also showed SD males sing less frequently compared to LD birds. Panels (**e**, **f**) illustrate the effects of castration and testosterone replacement in male canaries housed on LD [48]. (**e**) Male canaries castrated and treated with a testosterone implant peripherally sing longer and more complex songs compared to (**f**) castrated males treated with an empty implant, which also sing very infrequently. These examples collectively illustrate that the effects of photoperiod on song are likely driven to a large extent by the substantial seasonal changes in circulating testosterone

probability that a new neuron will be incorporated into a functional neural circuit that is a steroid hormone target site [50], although a recent study has identified an effect of testosterone on the rate of cell divisions at the lateral ventricle wall [51]. Plasticity in brain morphology is also observed in other neural systems, for example, the volume of the sexually dimorphic medial preoptic nucleus in quail varies as a function of reproductive state [52]. Plasticity in this case as measured by apparent changes in volume is based on changes in neuron size and cell spacing [52, 53]. Figure 27.4 illustrates changes in a variety of measures of physiology and brain morphology based on studies of wild-caught European starlings [54, 55].

Hormone-mediated plasticity in the adult avian brain can be detected based on other measures besides morphological ones. One reason why avian neuroplasticity is of such interest to neuroendocrinologists is that in comparison to other taxa, proteins involved in steroid hormone action exhibit some useful and unusual features. One example concerns the steroid-metabolizing enzyme aromatase (estrogen synthase) that exhibits a remarkably high degree of enzymatic activity in the avian brain (10–20 times higher in the preoptic area-hypothalamus of various avian species than in rats; see [56, 57]). This means that its detection by enzymatic assays or immunohistochemical methods is quite easy [58–60]. In some species, this high degree of activity is not limited to the diencephalon but can also be detected in the telencephalon, and in some cases the high activity of this enzyme in the brain produces a large amount of estrogens that surprisingly seem to contribute significantly to concentrations in the periphery [61–63]. Based on studies focused on the diencephalon of quail and doves, aromatase activity is clearly regulated by testosterone making investigations of avian species especially useful for studies of the cellular basis of enzymatic regulation [58, 64, 65].

One might ask whether these various examples of neuroplasticity are widespread or whether they are unique to the avian brain. This does not seem to be the case. It is important to remember that the organization and chemical neuroanatomy of the avian brain is far closer to mammalian brain architecture than thought in the past [66]. Also it is important to note that findings first made in birds that appeared suprising have subsequently been discovered to occur in mammalian species, albeit at a lower degree of magnitude, in some cases making them more difficult to detect in this taxa. There are many examples. Adult neurogenesis can be detected in the hippocampus and in some cases even in the cortex in monkeys but the signal is not as prominent as what has been observed in the avian brain [67]. However, studies in white-footed mice have found that photoperiodic condition can modulate the number of new neurons detected in the hippocampus (more new neurons in mice kept on long days) indicating that seasonal changes in the incorporation of new neurons does occur in mammals in a manner similar to what has been described in birds [68]. Another example of such continuity is that the enzyme aromatase is regulated in the mammalian diencephalon in a manner quite similar to that first reported in birds; it was just more difficult to detect, and it therefore remains a more challenging phenomenon to study in these species [56, 69]. Overall, most evidence indicates that data from avian species are of general significance to understanding vertebrate neuroendocrine function.

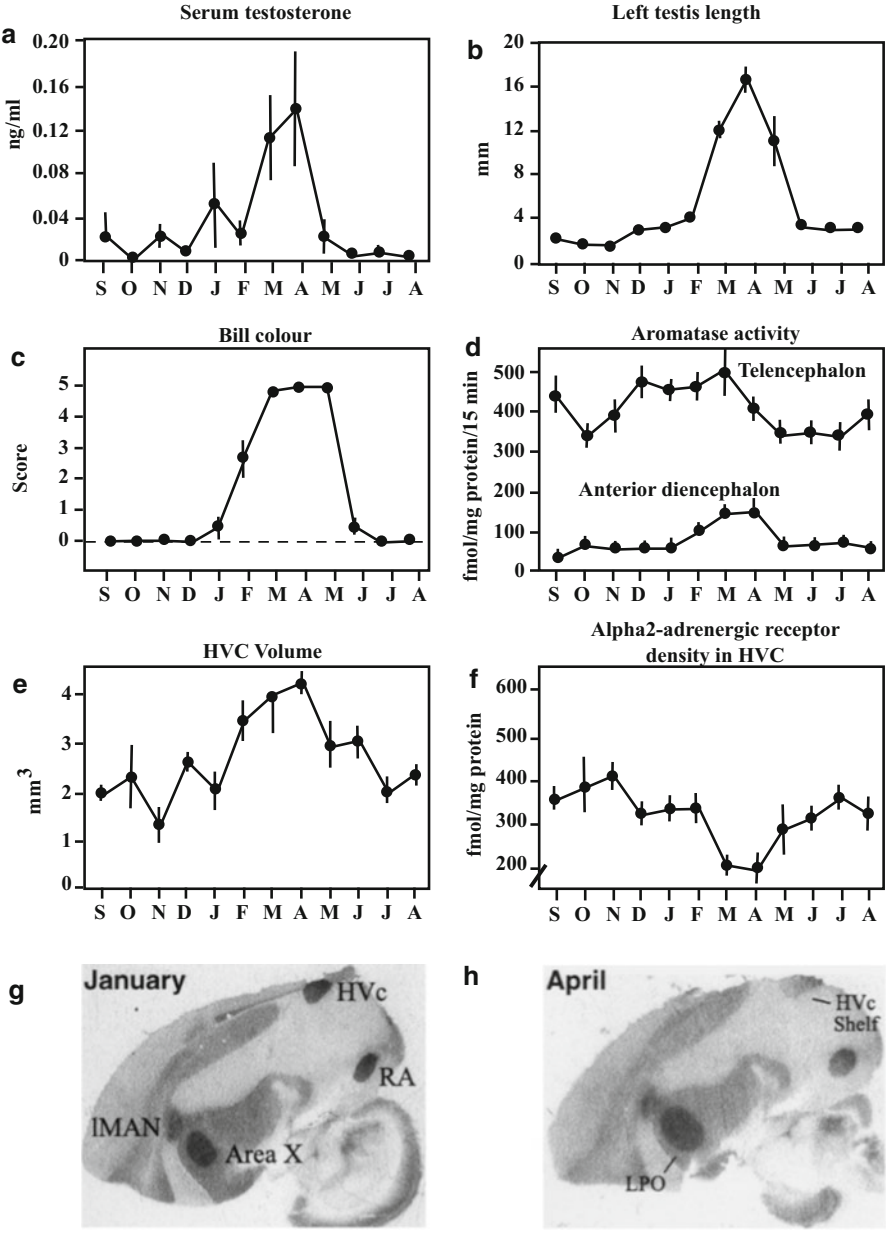


**Fig. 27.4** Seasonal changes in physiology and brain morphology of wild-caught European starlings. The *top* two panels provide data on testosterone concentrations and testis size. Beak color (0=completely black; 5=completely yellow) is illustrated in the next row. The presence of a *yellow* beak in starlings is a testosterone-dependent change. On the same row variation in the volume of the song control nucleus HVC is shown. In the next row densities of alpha-two adrenergic receptors in HVC are shown to change over the course of the year. On the same row, the activity of the steroid-metabolizing enzyme aromatase is illustrated. Note the high levels of activity in the telencephalon that is positively correlated with testis size and testosterone concentration. Finally in the bottom panel, autoradiograms prepared from sections cut in the sagittal plane are presented. Note that HVC volume increases in the spring but the densities of alpha-two adrenergic receptors decreases (Data taken from [54, 55])

## 27.3 Summary and Suggested Readings

This essay highlights some clear examples of seasonal changes in brain and behavior. However, the scope of the chapter is limited so we focused on examples from avian species with many illustrations coming from our own work. This field is in its early days and many important questions remain. For example, there is evidence that sensory systems can change with season; how widespread is this phenomenon, and how are such changes regulated in a way to insure the needed sensory process throughout the year? Cellular changes in the brain can range from those involving the life and death of neurons to subtle changes in morphology. How widespread are these, and what are the causal links between such changes and seasonal variation in physiology and behavior? We focused on gonadal steroids but other hormone systems such as melatonin and thyroid hormones can have substantial effects on behavior. Are there undetected behavioral effects of melatonin and the thyroid hormones? The importance of seasonality in understanding issues related to human health and performance is an important emerging field as is how seasonal cycles in nonhuman animals will function in the age of climate change. Will seasonal breeding be successful in animal populations when a subset of the cues individuals integrate to predict the onset of a season is changing as to how well they correlate with the change of season?

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## *Suggested Readings*

Ball GF, Balthazart J (2015) Seasonal changes in the neuroendocrine system: introduction to the special issue. Front Neuroendocrinol 37:1–2. (This is the introduction to a special issue of Frontiers in Neuroendocrinology that has 14 papers on seasonal changes in brain and behavior)

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# Chapter 28
# Molecular Mechanism Regulating Seasonality

**Keisuke Ikegami and Takashi Yoshimura**

**Abstract** The mechanisms of vertebrate seasonal time measurement were a mystery for a long time, but recent comparative studies have uncovered the photoperiodic signal transduction cascades in birds, mammals, and fish. These studies reveal the universality and diversity of photoperiodic mechanisms. That is, the molecules involved are conserved, while the tissues responsible for these mechanisms are different in different species (Nakane and Yoshimura, Front Neurosci 8:115, 2014).

It is well established that the circadian clock is involved in photoperiodic time measurement. However, the underlying mechanism that defines the photoinducible phase or critical photoperiod (i.e., how organisms measure day length using a circadian clock) is at the heart of photoperiodic time measurement, and this question remains to be answered by future studies.

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## 28.1 Introduction

In non-equatorial zones, marked seasonal changes are observed in temperature, precipitation, and food availability. To adapt to these seasonal changes in the environment, organisms change their physiology and behavior, including their state of reproduction, migration, hibernation, and molting. Among various seasonal environmental cues, the most reliable are the annual changes in day length (photoperiod), because solstices and equinoxes always occur at exactly the same time each year. Although temperature and precipitation also show annual changes, those changes are not as consistent or reliable because cool summers and/or warm winters often occur. Therefore, it is quite plausible that most organisms use changes in the photoperiod as a calendar, and this phenomenon is called photoperiodism [1].

Seasonal reproduction maximizes the survival of offspring. For example, small mammals and birds, whose gestation or incubation periods last for only a few weeks, mate during spring and summer; they are so-called long-day (LD) breeders. In contrast, goats and sheep have gestation periods of ~6 months, and, therefore, they mate during fall; they are so-called short-day (SD) breeders. In both LD and SD breeders, offspring are born and raised during the spring and summer when moderate weather and plenty of food are available.

## 28.2 Models for Photoperiodic Time Measurement

The photoperiodic time measurement system consists of three components: (1) a light input pathway transmitting external light-dark information, (2) a biological clock measuring photoperiod, and (3) an output pathway controlling various aspects of physiology and behavior.

Organisms show photoperiodic responses when day length reaches what is called the "critical day length." Most seasonally breeding organisms have highly accurate mechanisms for photoperiodic time measurement and show dramatic seasonal responses to small changes in photoperiod. For instance, testicular recrudescence could be seen in hamsters that were transferred from SD to photoperiods of 12.5 h or longer [2]. In Japanese quail, day lengths longer than 11.5 h induce testicular growth [3]. These animals have been shown to recognize day length differences as small as 30 min.

### *28.2.1 Bünning's Hypothesis*

In 1936, Erwin Bünning developed a concept of circadian clock-based photoperiodic time measurement [4]. In his hypothesis, a light-requiring phase (photophil) of ~12 h and a dark-requiring phase (scotophil) of ~12 h together make up a 24 h

period. If an animal experiences light only during the photophil, an SD response is induced, while light exposure during the scotophil causes an LD response, and thus light entrains the circadian clock and induces photoperiodic responses. Bünning's hypothesis was groundbreaking in that it predicted the involvement of a circadian clock in photoperiodic time measurement for the first time.

Thereafter, involvement of a circadian clock in photoperiodic responses was experimentally demonstrated using non-24-h light-dark cycles. These experiments are also known as the Bünsow protocol [5] and the Nanda-Hamner resonance experiment [6]. In the Nanda-Hamner experiment, the SD flowering response of soybean plants was investigated with non-24-h light-dark cycles in which 8-h light periods were coupled with dark periods of 8–64 h. Only when the frequency of the light cycle approximated a multiple of 24 h (i.e., 24, 48, or 72 h) did the plants exhibit an appropriate (SD) photoperiodic response. These "resonance" experiments were later repeated in birds (e.g., white-crowned sparrow, house finch, and quail). A 6-h light period coupled with dark periods of varying duration (resonance light cycles) did not cause photoperiodic responses when the duration of each cycle was a multiple of 24 h (e.g., 6 h light, 18 h dark: 6L18D, 6L42D, 6L66D) (Fig. 28.1). However, cycle lengths that were not multiples of 24 h (6L30D, 6L54D) caused a photoperiodic response [7–9]. In these experiments, the 6 h of light would be inserted at the same point in cycles that were multiples of 24 h, while the 6 h of light would be inserted at different points, now called the "photoinducible phase," in non-24-h light-dark cycles, thereby triggering a LD response. Similar observations were also made in mammalian species such as golden hamsters [10]. All of these observations clearly suggest the involvement of the circadian clock in photoperiodic time measurement.

### *28.2.2 External Coincidence and Internal Coincidence Models*

In the 1960s, Colin Pittendrigh and his colleagues proposed two models called the "external coincidence model" [11] and "internal coincidence model" [12] (Fig. 28.2). In the external coincidence model, light has two effects as it does in Bünning's hypothesis (i.e., entrainment of the circadian clock and induction of the photoperiodic response). However, in this model, a photosensitive phase, also known as the photoinducible phase, was hypothesized instead of simple photophil and scotophil periods. When an external light stimulus is coincident with the photoinducible phase, a photoperiodic response is induced (Fig. 28.2). In contrast, the internal coincidence model hypothesized the existence of multiple oscillators. When one oscillator couples with dawn and the other with dusk, the phase relationship between the two oscillators changes with the seasons (Fig. 28.2). In this model, the coincidence of two (or more) internal oscillators causes the photoperiodic response. The external and internal coincidence models are now widely

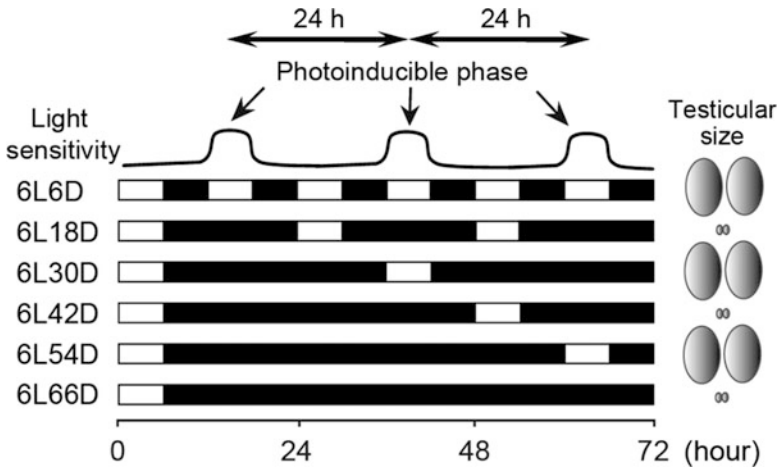


**Fig. 28.1** Nanda-Hamner resonance experiment. Light pulses applied during a specific time interval called the "photoinducible phase" induce testicular growth in long-day (LD) breeders. The 24-h time interval between photoinducible phases suggests that a circadian clock is involved in photoperiodic time measurement

accepted as the basis for photoperiodic time measurement in plants, insects, and vertebrates, and they have helped researchers to understand its underlying molecular mechanisms.

## 28.3 Light Input Pathways in Mammals and Birds

### *28.3.1 Mammals*

In mammals, the eyes are believed to be the only photoreceptor organs, and removal of the eyes abolishes the photoperiodic response [13]. The master circadian pacemaker is located in the suprachiasmatic nucleus (SCN) in mammals. Light stimulation received by the eyes is transmitted to the pineal gland via the SCN [13] (Fig. 28.3). From the pineal gland, melatonin is secreted during the night. Thus, the secretion profile of melatonin encodes the night length. Melatonin plays an important role in the regulation of seasonal reproduction in mammals. Therefore, pinealectomy eliminates photoperiodic responses, while melatonin administration reinstates them in both LD and SD breeders [13]. Since the SCN is required to generate circadian melatonin secretion profiles, SCN lesions also disrupt the photoperiodic response in mammals.

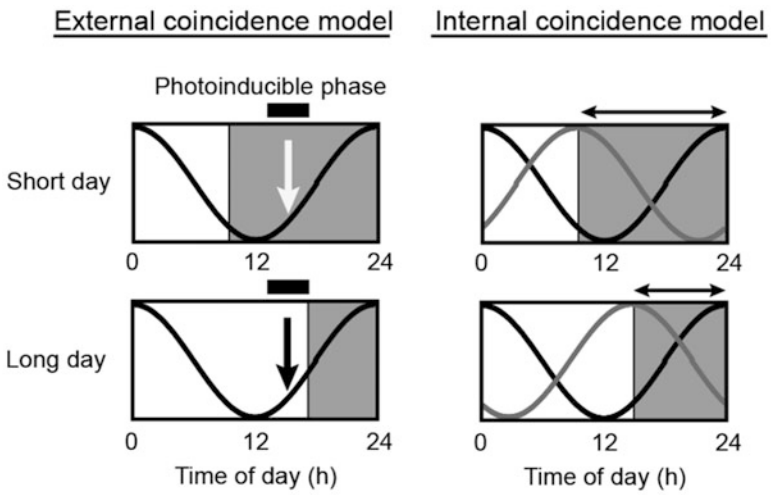


**Fig. 28.2** External coincidence model (*left*) and internal coincidence model (*right*) for photoperiodic time measurement. In the external coincidence model, a photoperiodic response is observed when light exposure occurs during the photoinducible phase (*arrow*), and the light has two effects: resetting the circadian clock and inducing the photoperiodic response. By contrast, differences in the phase angle between morning and evening oscillators cause a photoperiodic response in the internal coincidence model

### *28.3.2 Birds*

In birds, circadian pacemakers are localized not only in the SCN but also in the eyes and the pineal organ. The relative significance of the individual pacemaker is different in different species of birds. The pineal organ plays the predominant role in the house sparrow [14], while the eyes play a significant role in quail [15]. In the pigeon, the SCN plays an important role along with the pineal organ and the eyes [16]. Photoreceptors are also localized in multiple regions: the eye, the pineal organ, and the deep brain. In marked contrast to mammals, the photoperiodic response is not affected by removal of the eyes in the duck [17], by lesions around the SCN in quail [18], or by pinealectomy in quail and starlings [19, 20]. It is also interesting to note that nocturnal melatonin secretion is of little to no importance in regulating the photoperiodic gonadal response, in contrast to the situation in mammals [21]. Thus, the mechanism of seasonal reproduction is believed to have been different in birds and mammals for a long time.

In birds, injection of India ink under the scalp abolishes testicular recrudescence [22]. In addition, local illumination of the mediobasal hypothalamus (MBH) or septal region of the telencephalon induces gonadal growth [17, 23]. Therefore, involvement of deep brain photoreceptors in the photoperiodic response has been suggested. Foster and his colleagues [24] reported an action spectrum for photoperiodic responses and suggested the involvement of photoreceptors having high sensitivity at about 480 nm and at ultraviolet frequencies [24]. A number of

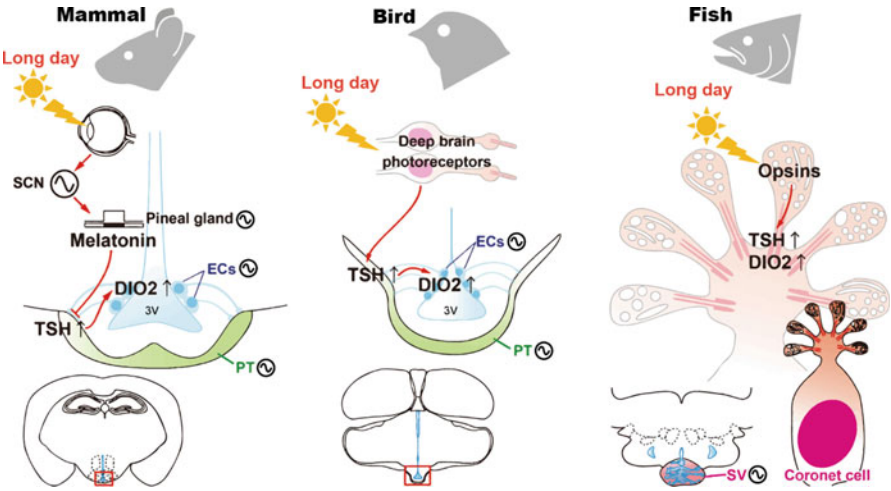


**Fig. 28.3** Photoperiodic signal transduction pathways in mammals, birds, and fish. In mammals, the eyes are the only photoreceptor organs and pineal melatonin mediates photoperiodic information. In birds, deep brain photoreceptor(s) (e.g., OPN5) directly receive light information. LD-induced TSH in the pars tuberalis (PT) acts on the ependymal cells (ECs) to induce DIO2 expression in both mammals and birds. In fish, all of the machinery required for seasonal reproduction (from photoreceptors to neuroendocrine output) is located in the saccus vasculosus (SV). Circadian clock genes are expressed in the SCN, pineal gland, PT, ECs, and SV (Modified from a previous report [30])

research groups have tried to identify the deep brain photoreceptors involved in the photoperiodic response, and the expression of several rhodopsin superfamily genes, such as rhodopsin, VA-opsin, and melanopsin, has been reported in the avian brain [25–27]. However, there has been no direct evidence for their involvement in the photoperiodic response. Recently, the expression of OPN5 (Opsin 5) was reported in the cerebrospinal fluid (CSF)-contacting neurons of the paraventricular organ (PVO) [28], and the intrinsic photosensitivity of these neurons was demonstrated using the slice patch clamp technique [29, 30]. In addition, OPN5 knockdown attenuated the photoperiodic response [30]. Therefore, OPN5 appears to be one of the deep brain photoreceptors that regulate avian seasonal reproduction (Fig. 28.3). Although the eyes have long been believed to be the only photoreceptor organ in mammals, the mammalian ortholog of OPN5 is also known to be expressed in the brain and spinal cord [31]. The functional significance of mammalian OPN5, however, remains to be clarified.

## 28.4 Signal Transduction Pathway Regulating Avian Seasonal Reproduction

Vertebrate reproduction is controlled by the hypothalamic-pituitary-gonadal (HPG) axis. Hypothalamic gonadotropin-releasing hormone (GnRH) is released to regulate the secretion of gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) from the pars distalis (PD) of the anterior pituitary gland. In seasonally breeding animals, this HPG axis is activated at a specific time of the year.

In 1925, William Rowan discovered the photoperiodicity of gonadal size changes in junco [32]. In contrast to mammals, birds have highly sophisticated photoperiodic mechanisms, and their gonadal size alters more than a 100-fold within a few weeks. This is considered to be an adaptation for flight, because it allows birds to minimize their body weight during much of the year. Follett and his colleagues [33] found that Japanese quail (*Coturnix japonica*) was an excellent model for understanding the mechanism of seasonal reproduction, because these birds show a rapid induction of serum LH and drastic testicular growth in response to changes in photoperiod [33]. In addition, classic lesion studies in quail suggested that the MBH is the center of seasonal reproduction in birds [34].

### *28.4.1 Key Genes Regulating Photoperiodism*

In recent years, functional genomics studies of birds discovered that seasonal reproduction is regulated by the ependymal cells (ECs) lining the ventrolateral walls of the third ventricle within the MBH and the adjacent pars tuberalis (PT) of the anterior pituitary gland [35, 36]. LD exposure induces the production and secretion of thyroid-stimulating hormone (TSH, thyrotropin) in the PT (Fig. 28.3). Subsequently, PT-derived TSH acts on the ECs within the MBH to induce the expression of type 2 deiodinase (DIO2) and reduce the expression of type 3 deiodinase (DIO3) via the TSH receptor (TSHR)-Gsα-cAMP signaling pathway [36]. DIO2 and DIO3 encode thyroid hormone (TH) activating and inactivating enzymes, respectively. In addition, this LD-induced DIO2/DIO3 switching locally modulates the bioactivation of TH within the MBH (Fig. 28.4). It has been demonstrated that the precursor thyroxine ($T_4$) in the CSF is transported to the ECs by the organic anion transporting polypeptide 1c1 (Oatp1c1) and then is converted into bioactive triiodothyronine ($T_3$) by DIO2 [35, 37]. TH regulates the development and plasticity of the central nervous system, and the locally activated TH induces dynamic morphological changes between the GnRH nerve terminals and the glial end feet in the median eminence (ME) where thyroid hormone receptors (THRα, THRβ, and RXRα) are expressed [38]. Under LD conditions, many GnRH nerve terminals are located in close proximity to the pericapillary space in contact with the basal lamina between the ME and the hypophyseal portal

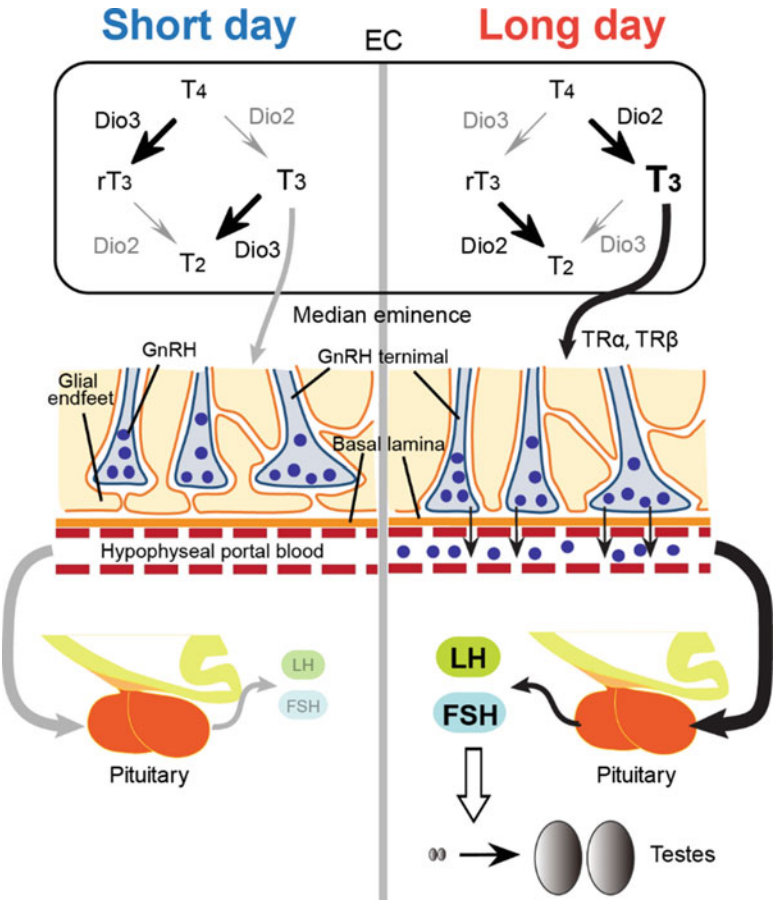


**Fig. 28.4** Local TH activation induces seasonal gonadal development. In the ECs, SD-induced Dio3 metabolizes THs, while LD-induced Dio2 converts precursor thyroxine ($T_4$) to bioactive triiodothyronine ($T_3$). In quail, LD-induced $T_3$ causes morphological changes in the GnRH nerve terminals and glial end feet processes through thyroid hormone receptors (TRα and TRβ) at the median eminence, thereby causing GnRH secretion into the hypophyseal portal blood. Photoperiodic secretion of GnRH stimulates the anterior pituitary gland to induce LH and FSH release, which leads to gonadal growth

vessel for neurohormone release. However, under SD conditions, the nerve terminals of GnRH neurons are encased by the glial end feet. Therefore, these morphological changes appear to regulate or modulate seasonal GnRH secretion from the hypothalamus to the portal capillary system (Fig. 28.4) [35, 38]. Importantly, chronic i.c.v. TSH administration to SD-exposed quail induced *DIO2* expression and led to gonadal recrudescence to the same extent as that observed in intact LD-exposed birds, suggesting that PT-derived TSH is the master factor regulating seasonal reproduction in birds [37]. In addition, since OPN5-positive CSF-contacting neurons project to the external layer of the ME adjacent to the

PT [28], it is speculated that light information, received by the OPN5-positive CSF-contacting neurons, is transmitted to the PT to induce TSH in the PT (Fig. 28.3).

### *28.4.2 Autumn Adaptation Mechanisms in Birds*

As described above, the photoperiodic signal transduction pathway that activates avian seasonal reproduction is now known. However, the endocrine and molecular mechanisms underlying seasonal gonadal regression during autumn adaptation remain unclear. In quail, low-temperature stimulus accelerates SD-induced testicular regression [39]. Although low-temperature stimulus does not influence the expression of photoperiodic key genes (*TSHB*, *DIO2*, and *DIO3*) within the PT and the hypothalamus, it does increase the amount of circulating $T_3$ for adaptive thermogenesis. This low-temperature-induced circulating $T_3$ appears to be produced by DIO2 in the liver through increased food intake, and it triggers germ cell apoptosis by activating genes known to be involved in amphibian metamorphosis (Fig. 28.5) [40, 41]. The role of TH in seasonal reproduction has been debated for several decades [42]. However, contradictory effects of TH on reproduction have often been reported; some studies reported stimulatory effects, while others reported inhibitory effects [43]. Therefore, TH has been thought to play a permissive role in seasonal reproduction. However, recent reports appear to explain these contradictory effects, showing that central activation of TH results in seasonal gonadal growth, while peripheral activation of TH for adaptive thermogenesis regulates seasonal gonadal regression. Therefore, TH has a dual role in the regulation of seasonal reproduction. It is also noteworthy that TH has two contrasting effects during the metamorphosis of the tadpole: TH induces both the outgrowth of the limb and resorption of the tail [41].

## 28.5 Regulatory Mechanism of Mammalian Seasonal Reproduction

### *28.5.1 Involvement of the Thyroid Hormone*

Mammalian gonadal mass changes approximately 10- to 15-fold seasonally, while in birds it changes more than 100-fold [43]. It has been known for several decades that TH is involved in the regulation of mammalian seasonality [42]. Photoperiodic regulation of DIO2 and/or DIO3 has been demonstrated in mammals such as hamsters [44], rats [45], and mice [46] and even in short-day breeding sheep [47] and goats [48]. Local activation of TH within the MBH is therefore considered central for the regulation of seasonal reproduction in mammals.

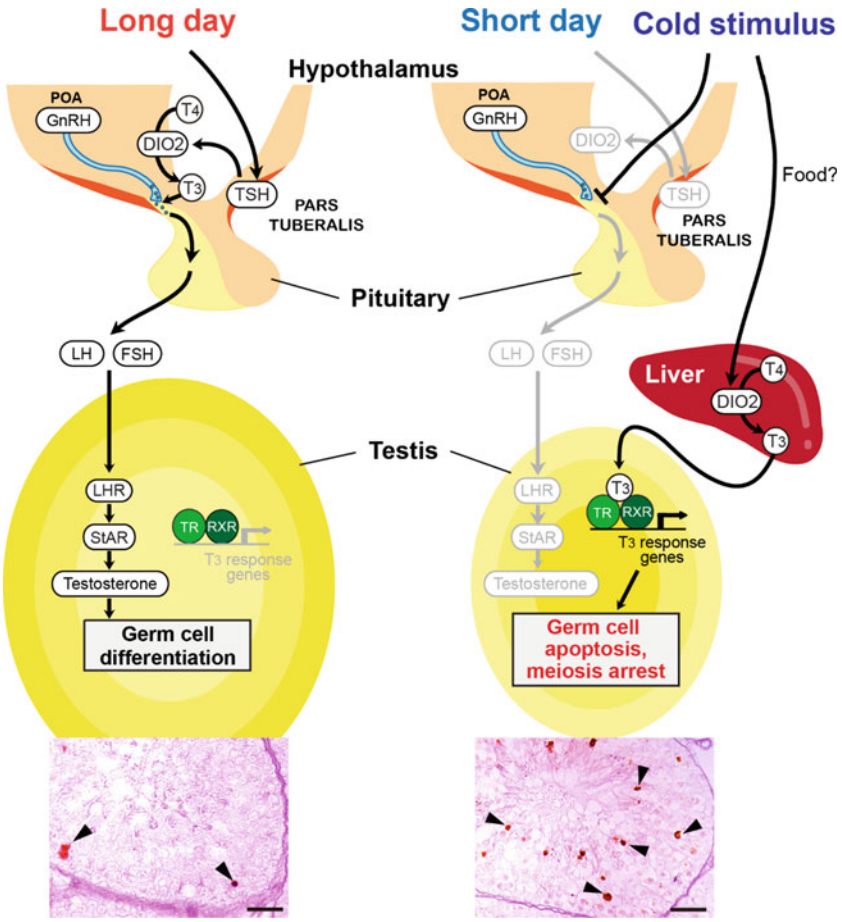


**Fig. 28.5** Mechanism of seasonal testicular development and regression in birds. In spring and summer, GnRH and subsequently LH and FSH are secreted through the TSH-DIO2 signaling pathway. LH induces testosterone production leading to germ cell differentiation. In autumn and winter, low temperature attenuates GnRH secretion and shuts down the HPG axis in conjunction with the SD stimulus. Low temperature induces hepatic *DIO2* expression and increases the level of serum $T_3$. The increased circulating $T_3$ level activates the TH receptors within the testis and induces germ cell apoptosis (see lower TUNEL-stained sections) and meiotic arrest (Modified from a previous report [40])

### *28.5.2 Mode of Melatonin Action*

Laboratory mice are generally considered to be nonseasonal breeders and therefore have not been considered appropriate models for studies of seasonal reproduction. However, many researchers have observed that mice do not breed well during winter, even though they are kept under constant light-dark and temperature conditions throughout the year. Except for the CBA and C3H strains and *Mus*

*musculus castaneus*, most strains of laboratory mice cannot produce melatonin because they lack arylalkylamine *N*-acetyltransferase (AA-NAT) and/or hydroxyindole-*O*-methyltransferase (HIOMT), which are required for melatonin biosynthesis [13, 49]. In the melatonin-proficient CBA strain, the expression of *Tshb* (TSH β-subunit) in the PT and of *Dio2*/*Dio3* in the ECs showed clear photoperiodic changes (Fig. 28.2), even though the mice had no apparent photoperiodic response in the gonads. Because melatonin plays deterministic roles in mammals, the melatonin-deficient C57BL strain failed to show photoperiodic responses in the abovementioned genes. By contrast, daily intraperitoneal melatonin injections mimicked the effects of SD conditions in the C57BL strain [46]. These results indicated that mice can be a useful model for studying the molecular mechanisms of mammalian seasonal reproduction.

Melatonin receptors are densely expressed not only in the SCN but also in the PT, but they were not expressed in the ECs where *Dio2* and *Dio3* are expressed [50]. Therefore, the mechanism of *Dio2* and *Dio3* regulation by melatonin remains unclear. In an analysis of the effects of melatonin in mice lacking the MT1 and MT2 melatonin receptors and TSHR, the role of the MT1 melatonin receptor and TSHR in the melatonin regulation underlying the *Dio2*/*Dio3* switching mechanisms were demonstrated [45, 51]. Furthermore, administration of TSH to hamsters under SD conditions induced not only gonadal development but also the expression of kisspeptin, a potent GnRH secretagogue, and RFRP3 (RFamide peptide) [52]. RFRP3 inhibits GnRH neurons in sheep, but it may both inhibit and photoperiodically activate GnRH neurons in hamster [53].

### *28.5.3 Glycosylation Diversifies the Function of TSH*

TSH is a well-known glycoprotein hormone secreted from the PD of the anterior pituitary gland; it stimulates the production and secretion of THs in the thyroid gland. In the PD, TSH expression is regulated by the hypothalamic-pituitary-thyroid (HPT) axis. Synthesis and secretion of PD-derived TSH is stimulated by thyrotropin-releasing hormone (TRH) and suppressed by TH under a negative feedback loop. In contrast to PD-derived TSH, the regulatory mechanism of PT-derived TSH does not depend on the HPT axis. PT-derived TSH is under the control of melatonin in mammals (Fig. 28.6) [54], demonstrating that mammals use the same hormone, TSH, for dual functions. It was unclear, however, how these two TSHs avoid functional cross talk within the body. A recent study demonstrated that PT-derived TSH is also secreted into the circulation, but it has little bioactivity on the thyroid gland [54]. Furthermore, structural analysis revealed that the PD- and PT-derived TSHs differ in tissue-specific posttranslational *N*-glycosylation. Glycosylation influences the half-life and bioactivity of glycoprotein hormones [55]. -PT-derived TSH had a longer half-life and higher stability when compared with PD-derived TSH. Interestingly, the bioactivities of PT-derived and PD-derived TSHs themselves are not different. Instead, PT-derived TSH forms macro-TSH

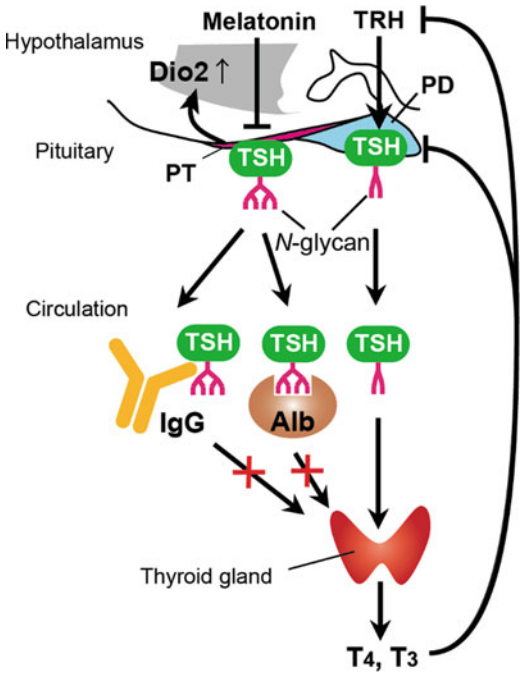


**Fig. 28.6** Glycosylation diversifies the function of TSH. The PD-derived TSH (PD-TSH) stimulates the thyroid gland to produce TH, whereas the pars tuberalis (PT)-derived TSH (PT-TSH) controls the hypothalamus to regulate seasonality. PT-TSH has tissue-specific *N*-glycan modifications and forms macro-TSH complexes with immunoglobulin (IgG) and albumin (Alb) in the circulation. Macro-TSH cannot stimulate the thyroid gland, which prevents functional cross talk between the two TSHs (Modified from a previous report [54])

complexes with immunoglobulins and albumin in circulation and therefore loses its bioactivity (Fig. 28.6) [54]. Thus, the tissue-specific glycosylation observed in the PT and PD differentiates the function of TSHs to prevent functional cross talk within the body.

## 28.6 Signal Transduction Cascade for Seasonal Reproduction in Fish

Most fish living outside tropical zones also show marked seasonal changes in physiology and behavior. Medaka (*Oryzias latipes*), which are LD breeders, develop their gonads in response to elongated day length. Salmonids, SD breeders, show migration and parr-smolt transformation. Although melatonin is also secreted from the pineal gland during the night in fish, its functional significance in the regulation of seasonal reproduction is not clear [56]. The PT of the pituitary gland is a regulatory hub of seasonal reproduction in birds and mammals, but fish do not have an anatomically distinct PT. The involvement of TH in fish seasonal reproduction has been also reported [56]. In a recent study of SD breeding, masu salmon (*Oncorhynchus masou masou*) were found to express rhodopsin family genes *TSHB*, *TSHR*, and *DIO2* in the saccus vasculosus (SV). The SV, an organ only observed in fish, is located on the ventral side of the diencephalon, caudal to the

pituitary gland. In the SV, the expression of several photopigments (OPN4 and SWS1), TSH, and DIO2 were all observed in a cell called the coronet cell (Fig. 28.3) [57]. Because all elements required for photoperiodic time measurement, from light input to neuroendocrine output, were observed in the SV, this structure was considered to play a central role in seasonal reproduction (Fig. 28.3). Indeed, the isolated SV responded to photoperiodic changes in vitro, and removal of the SV in vivo abolished the photoperiodic response of the gonads. Although the physiological roles of the SV have been a mystery for several centuries [58], recent findings indicate that the SV is a seasonal sensor in fish.

## 28.7 Circadian Clock Genes and Photoperiodism

Circadian rhythm is driven by the transcription-translation feedback of circadian clock genes. Clock gene expression is observed not only in the master pacemakers but also in almost all cells throughout the body. Clock genes and proteins are rhythmically expressed in the MBH as well as in the master pacemakers [59]. In the SCN and the pineal gland, various photoperiodic schedules affect clock gene expression profiles. However, in the MBH, photoperiod does not affect temporal clock gene expression profiles, which appear to enable animals to maintain a steady-state photoinducible phase [59].

In the mammalian SCN, temporal expression profiles of circadian clock genes were also altered under different photoperiods [60]. In addition, photoperiodic melatonin secretion profiles appear to be influenced by alterations in clock gene expression patterns. It has been reported that photoperiod changes in both birds, and mammals also affect the expression profiles of clock genes in the PT [61, 62]. *Period* (*Per*) gene expression peaks during the day, while *Cryptochrome* (*Cry*) gene expression peaks early in the night. Since different photoperiods influence the phase relationship between *Per* and *Cry*, Per/Cry protein/protein interaction has been proposed as a potential mechanism for generating the photoperiodic response (i.e., the internal coincidence model) [12, 61]. Recently, it was also reported that LD-induced transcriptional co-activator eyes absent 3 (*Eya3*) may regulate *Tshb* expression in the PT by forming a complex with the circadian transcription factor thyrotropin embryonic factor (TEF) in mammals [63]. Melatonin has been proposed to have two effects on *Eya3* expression: phase synchronization and direct suppression. These two effects of melatonin appear to trigger a morning peak of *Eya3* under LD conditions that induces *Tshb* expression. This classic "external coincidence" mechanism [11] may link the circadian system to the photoperiodic response.

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# Chapter 29
# Epigenetic Mechanisms Regulating Circannual Rhythms

**Tyler J. Stevenson and Gerald A. Lincoln**

**Abstract** This chapter presents an epigenetic model for the endogenous control of circannual timing. We propose a Dual Compartmentalization Model in which the first compartment is composed of stem/primordial cells and provides the initiation signal for the circannual cycle, and the second compartment is formed of differentiated cells that propagate and amplify the circannual cycle. The model can accommodate transgenerational and developmental variation in circannual rhythms via an *epigenetic memory*. We apply the model to our current understanding of the molecular mechanisms that regulate circannual rhythms in unicellular organisms, plants and the neuroendocrine control of circannual timing in vertebrates. Despite cell-autonomous rhythmicity, in vertebrates there is a hierarchical organisation governed by local brain regions that serves to synchronise rhythmicity in different tissue types and to the environment. The Dual Compartmentalization Model can account for circannual variation in neurogenesis derived from stem cells and may apply in all post-mitotic cells.

## 29.1 Historical Perspective: Timeline of Epigenetic Research

Epigenetic research has been an exciting and, at some periods, highly contentious field of study. The initial description of *epigenetics* was developed by Conrad Waddington, an embryologist at the University of Edinburgh who was interested in the impact of early environmental conditions on adult phenotypic traits [1]. His pivotal work established the predominant view that epigenetic modifications occur during *development* and last for the course of the organism's lifespan. He envisioned an 'epigenetic landscape' in which variation in external factors

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(e.g. climate, social context and intracellular milieu) could modify genomic function leading to differences in the individual adult phenotype [1]. Indeed there is still strong evidence for environmental programming of the adult phenotype and ultimately affecting 'fitness' [2]. One excellent example is sex differentiation, a period in which early epigenetic modifications induced by the local hormone environment in discrete hypothalamic regions in the brain lead to a spectrum of adult male and female reproductive physiology and behaviour [3].

During the 1990s, the capability to easily modify specific gene(s) or maintain genetically inbred mouse models (e.g. C57) resulted in a significant increase in the ability to examine gene function. This period is now colloquially known as the 'genetic revolution'. One significant advance was the identification of phenotypic variation despite genetic similarity [4]. For example, C57 mice were found to have marked variation in fur colour that was due to maternal variation in the gene coding for agouti-related peptide (*agrp*, [4]). These types of observations lead to the development of the second field of epigenetic research demonstrating that epigenetic effects can be *transgenerational* due to biochemical and/or structural changes in the genome. The developmental and transgenerational perspectives have dominated the research field. Both of these views posit that epigenetic modifications, once established, are permanent and are maintained throughout the lifespan, and in subsequent generations.

However, recent advances in the field of biological rhythms have revealed that epigenetic modifications are also dynamic and reversible [5–7], introducing a third perspective on epigenetics. The evidence in support of circadian and circannual rhythms in epigenetic modifications requires the reconsideration of what constitutes an 'epigenetic mechanism'. Therefore, we propose a revised view of epigenetics as:

> *A molecular process that alters the biochemical substrates that surround the genome (e.g. methylation), leading to a functional change in the transcription of genes. The regulation of modifications in the biochemical substrates are heritable, can occur in stem cells as well as differentiated cells, and have either permanent, transient or cyclical effects on physiology and behaviour. The overall outcome of the epigenetic modification at a mechanistic level leads to phenotypic flexibility and thus enhances overall genomic fitness.*

The key difference with our proposed definition is the incorporation of *endogenously regulated cyclicity* in epigenetic modifications. In the following sections, we outline what constitutes an epigenetic modification at a molecular level and then illustrate how biological rhythms, particularly circannual oscillations in key epigenetic regulated enzymes, lead to reversible changes in the adult phenotype.

## 29.2 Mechanisms and Functions of Epigenetic Modifications

Attempts to define epigenetics have been stymied over the years due to strong and contrasting approaches that seek to establish the 'level' at which modifications occur. However, key features include alterations in biochemical substrates that

attach to chromatin and to the genomic templates [8]. The epigenetic modifications in biochemical substrates are mediated by microRNA and/or long non-coding RNA. The two most commonly investigated molecular epigenetic events are DNA methylation and histone acetylation [8].

DNA methylation generally involves the binding of methyl ($CH_3$) groups onto cytosine-guanine paired nucleotides [8], and to a lesser extent, adenosine-guanine and thymidine-guanine paired nucleotides [9]. The placement of methyl groups onto the DNA template requires a family of enzymes called DNA methyltransferases (DNMTs; [10, 11]). DNMTs have been shown to have specific functional roles that are dependent on the isoform (Fig. 29.1a). In mammals, the three predominant DNMTs are DNMT1, DNMT3a and DNMT3b. DNMT1 is essential during cell division and is involved in DNA methylation maintenance [11]; whereas DNMT3a/b are involved in de novo DNA methylation [8]. The function of DNA methylation is dependent on the location in relation to the gene, specifically, the promoter, exon or intron regions. Increased levels of methylation in the promoter region act to inhibit transcription factor binding and functions to decrease gene transcription and RNA expression [10]. An alternative function for DNA methylation may involve alternative splicing of RNA transcripts due to binding in exon/intron regions [12, 13].

The mechanism responsible for the removal of methyl binding from the DNA template has long been an enigma. The recent discovery of the ten-eleven translocation (TET) family of proteins has identified a family of enzymes that catalyse active demethylation of DNA (Fig. 29.1b; [14]). To date, there are three TET enzymes (TET1, TET2 and TET3) that do not remove methyl groups but instead convert methylcytosine (mC) into 5-hydroxymethylcytosine (5mC) that is subsequently modified in a cascade of events converting cytosine into an unmethylated state [11]. Taken together, the levels of DNA methylation in the genome are dependent on a ying-yang relationship between two families of enzymes: DNMTs and TETs. The functional outcomes of increased DNA methylation are entirely dependent on geographical location within and around the coding region for a particular gene.

A complementary epigenetic process of histone acetylation is also mediated by families of enzymes known as histone acetylases (HAT) and deacetylases (HDACs) (Fig. 29.1c; [15]). Both of these families consist of several different isoforms, and the list continues to grow. What is clear is that the enzymes have tissue-dependent expression [15]. Here, HAT enzymes increase the presence of acetyl groups on histones and function to induce a conformational change in the chromatin that facilitates access of transcription binding proteins (e.g. SP1) to the DNA template. Alternatively, the removal of acetyl groups from histones requires the action of HDACs [15]. DNA methylation and histone acetylation are generally considered to act in concert to regulate the probability of RNA expression: DNMT and HDAC *inhibit*, whereas TET and HAT *permit* gene transcription [15].

The developmental and inheritance perspectives in epigenetics propose that environmental conditions result in permanent modifications in chromatin structure or genome landscape through methylation and acetylation. Recent work in the field of circadian biology has indicated, however, that epigenetic modifications exhibit

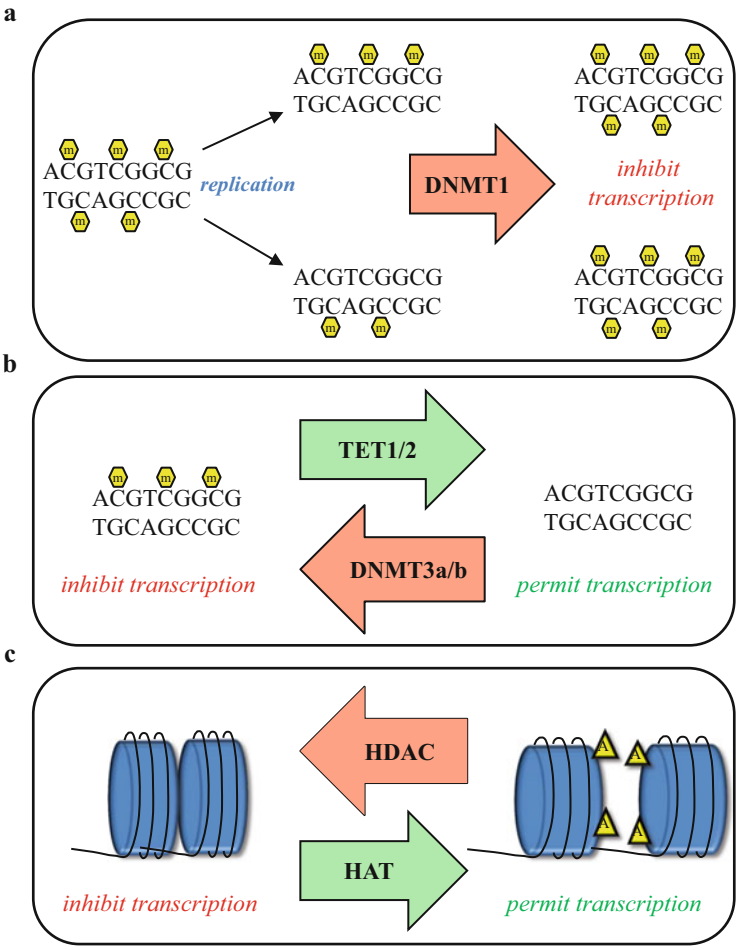


**Fig. 29.1** Epigenetic mechanisms for switching the functional state of genes. The two best characterised mechanisms are DNA methylation and histone acetylation. Increased DNA methylation and decreased histone acetylation inhibit gene transcription via conformational changes in the DNA/chromatin structure preventing transcription binding proteins from accessing the DNA template. Decreased methylation and increased histone acetylation permit gene transcription. (**a**) During cell replication, two new complementary DNA strains are formed and are devoid of methylation. DNA methyltransferase-1 (DNMT1) enzymes are essential for the reestablishment or maintenance of methylation in the newly formed sequences. Variation in methylation during cell division has long-lasting consequences on cell function and phenotype. (**b**) In differentiated cells, DNMT3a and DNMT3b function for de novo methylation, whereas ten-eleven translocation-1 and ten-eleven translocation-2 (TET1/TET2) serve to initiate the removal of methyl group from the DNA template. (**c**) Histone acetylation is established by histone acetyltransferase (HAT) enzymes, and acetyl groups are removed from histones by histone deacetyltransferase (HDAC). *Yellow* 'm' hexagons represent methyl groups, and 'A' *triangles* indicate acetyl groups. The *black line* and *blue cylinders* represent the DNA template and histone bodies, respectively

daily variation and are reversible [16]. The demonstration that reversible epigenetic modifications are involved in daily cycles in energy balance has significant and wide-ranging implications for our understanding of the molecular mechanisms that regulate biological timing.

All data suggest that DNA methylation is an evolutionarily conserved molecular system for the control of gene transcription – from unicellular [17] to complex organisms, including humans [18]. Given that DNA methylation is an ancient molecular regulator of gene transcription and that daily oscillations in RNA expression are universal, it is likely that DNA methylation is an integral component of the 'core clock' systems that govern biological timing across multiple timescales.

## 29.3 Examples of Timing by Epigenetic Control: Circadian Rhythms

Circadian rhythms have been the main focus of biological rhythms' research. In mammals these endogenous oscillations are governed by molecular and electrophysiological rhythms generated within the suprachiasmatic nucleus (SCN), the central circadian pacemaker located in the anterior hypothalamus [19]. Since the identification of circadian clock genes in the early 1990s [20], the intricate nature of the transcriptional/translational feedback loops that generate cell-autonomous circadian rhythms have been revealed [21]. The core circadian genes, such as circadian locomotor output cycles kaput (*clock*) and brain and muscle ARNT-like 1 (*bmal1*), act to drive the expression of clock-controlled genes and hence daily rhythms in hormone secretion, metabolism and energy balance [22]. How epigenetic modifications affect circadian biology has only just begun to be explored.

Genomic-wide methylation analyses of the SCN in mice have uncovered global circadian variation in promoter DNA methylation; many of the target regions include core clock genes [6]. Examination of SCN DNMT1 and DNMT3b in mice entrained to short or normal light schedules (i.e. 22T versus 24T) revealed that circadian disruption increases enzyme expression [6]. Moreover, unpublished data collected in the Stevenson Laboratory have found marked circadian rhythms in *dnmt3a* and *hdac4* expression. Analyses of the transcription binding site in the *dnmt3a* promoter reveal extensive nucleotide sequence motifs that are targeted by BMAL1. Altogether, these data suggest that DNA methylation is a component of the machinery that drives circadian biology and may be involved in transcriptional-translation feedback timing mechanisms.

In addition to DNA methylation, circadian variation in chromatin structure of specific genomic targets contributes to the daily cycles in metabolism and energy balance [5, 16, 22]. CLOCK has been implicated in the acetylation of histones to permit gene transcription, whereas REV-ERBα recruits histone deacetylase-3 (HDAC3) and subsequently deacetylates histones. One integral component of the circadian regulation of metabolism is the protein SIRT1 [23]. SIRT1 is involved in

the recruitment of a number of circadian clock protein to promoter regions of genes implicated in cholesterol metabolism (i.e. *liver X receptor*; [24]) and gluconeogenesis by deacetylation of PGC1α (PPARγ-coactivator-α) and Forkhead box O1 (FOXO1; [25]).

Taken together, these data support the hypothesis that epigenetic modifications are dynamic and reversible and occur in post-mitotic cells. How circadian variation in the regulation of methyltransferase and (de)acetylase enzymes 'fit' within transcriptional-translational feedback loop mechanisms of the circadian cycle remains a mystery. Given that gene transcription is an evolutionarily ancient process, and DNA methylation is present in all species examined to date, it is likely that epigenetic modifications are a key component for both the generation and maintenance of circadian rhythmicity.

## 29.4 Model of Epigenetic Circannual Timing

Circannual rhythms that regulate internal timing over months and years are prevalent in nature [26, 27]. The evidence in support of a role for epigenetic modifications for the generation and maintenance of these long-term rhythms is so far limited to a few seasonal rodent species [7, 28, 29]. In the Siberian hamster (*Phodopus sungorus*), there are robust changes in global DNA methylation in the hypothalamus, a key brain region that governs circannual rhythms in reproduction. Global DNA methylation is presented in much greater levels in summer compared to winter phenotypes [7], with parallel changes in *dnmt1* and *dnmt3b* expression (Fig. 29.2).

Remarkably, the opposite pattern in methylation status occurs in peripheral tissues and cells. For example, peripheral leukocytes in hamsters increase *dnmt3b* expression in winter conditions [28]. In hibernating thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*), there is a significant upregulation of *dnmt3b* in the liver and muscle tissue [29]. These patterns provide convergent evidence that cyclical DNMT enzyme expression is likely a key component in the neuroendocrine control of season rhythms. Functional manipulations of DNA methylation using a hypermethylating agent, 3-aminobenzimide (3AB), significantly delayed gonadal regression in hamsters that were transferred from summer to winter phenotypes [7]. One interpretation of these data is that hyper-methylation may lengthen the period of the circannual clock [7]. To date, there are no reports of circannual variation in chromatin structure via histone (de)acetylation. Overall, these data confirm that epigenetic mechanisms exhibit marked changes in expression across circannual time.

The current challenges in the field of circannual biology are to understand where and how rhythms are generated and/or maintained. In this chapter, we propose that all cells have the capacity for cell-autonomous circannual timing via epigenetic modifications, similar to the circadian system. Additionally, the timing mechanisms

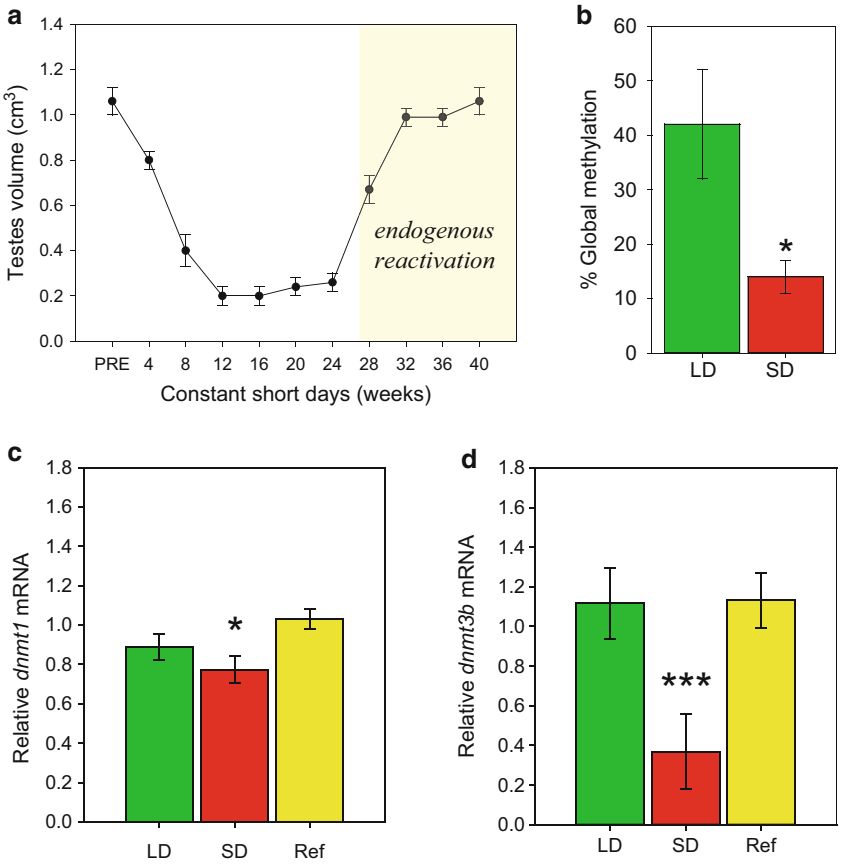


**Fig. 29.2** Cyclical variation in mammalian hypothalamic DNA methylation. (**a**) In the Siberian hamster, a simple change in day length from long days (15 h; 'PRE') to short days (9 h) causes reproductive involution and the induction of a winter phenotype. After approximately 24 weeks, endogenous reactivation is initiated, and the hamster returns to a summer phenotype (endogenous reactivation). (**b**) In parallel, the hamster hypothalamus exhibits marked variation in global DNA methylation with higher DNA methylation in the summer phenotype (*LD*) compared to winter phenotype (*SD*). (**c**) The decrease in hypothalamic DNA methylation is associated with reductions in DNMT1 and (**d**) DNMT3b expression. The endogenous reactivation (*Ref*) of gonadal development was associated with a complete reversal in DNMT1 and DNMT3b expression, back to the summer (*LD*) phenotype. These data indicate that DNA methylation in the hypothalamus is reversible and involved in the regulation of control of reproduction

may be basically the same as those governing reproductive development (e.g. puberty [30]) where epigenetic mechanisms play a major role.

Building upon the vast literature that illustrates how the regulation of methylation acts to govern gene transcription [31], we present a model of circannual timing based on reversible epigenetic control of cell function (Fig. 29.3). This proposes that there are two levels of control; the first operates in undifferentiated/stem cells

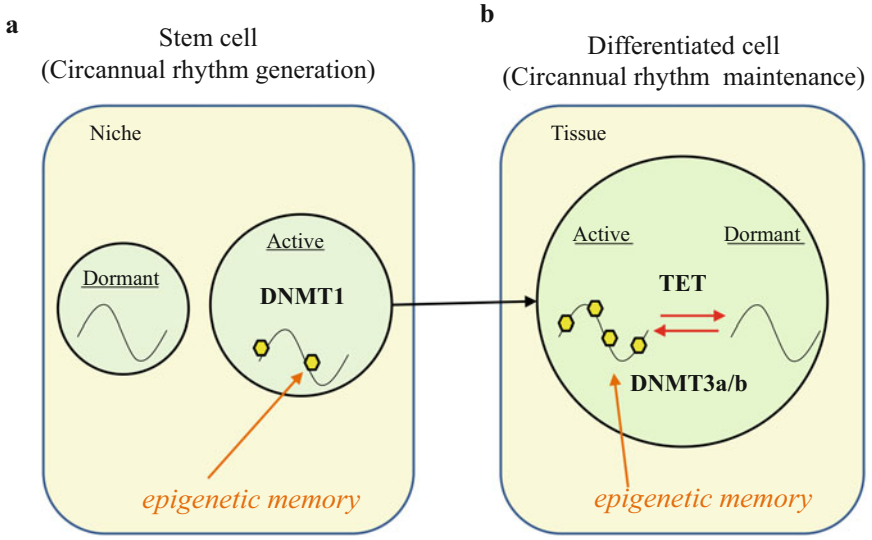


**Fig. 29.3** Dual Compartmentalization Model (DCM) for the endogenous control of circannual rhythmicity. This model proposes that epigenetic mechanisms regulate circannual rhythmicity at two levels: (**a**) undifferentiated cells (designated as Compartment 1 – seen to initiate rhythmicity) and (**b**) differentiated cells (designated as Compartment 2 – seen to propagate and amplify rhythmicity). In Compartment 1, stems cells reside in an unmethylated dormant state. During the establishment of the epigenetic landscape that derives from transgenerational and/or developmental influence, an epigenetic memory is conferred (e.g. via DNMT1; see Fig. 29.1a). For example, DNMT1 enzyme methylates the newly formed complementary DNA and establishes the active circannual rhythm (i.e. initiator). In Compartment 2, differentiated cells, specific to different tissues, will then oscillate between active and dormant states controlled by different epigenetic mechanisms. Here, post-mitotic cells switch between DNMT inhibition and TET activation of gene transcription (see Fig. 29.1b), and this process maintains circannual rhythmicity

to initiate the circannual cycle, and the second operates in differentiated cells to propagate and amplify the circannual cycle. We see this as involving two fundamentally different mechanisms/compartments and hence the name: 'Dual Compartmentalization Model' for circannual timing (Fig. 29.3).

In Compartment 1, we include germline cells or adult stem cells such as neural progenitor stem cells, whereas in Compartment 2, we include all post-mitotic cells in both brain and peripheral tissues. During gestation, the developing blastocysts become *reprogrammed* via increased DNA methylation, mediated by DNMT1 that is influenced by the foetal environment. The transition from 'inactive' to 'active' epigenetic states provides the initial circannual methylation mark. This step is fundamental to account for the maternal transmission of circannual time into the developing foetus. We propose that a similar mechanism also occurs in the adult brain. Neural stem cells are located in key hypothalamic regions that govern circannual rhythms (e.g. third ventricle), and the circannual rhythms in hypothalamic neurogenesis may be initiated by the transition from inactive to active state.

In both cases, the epigenetic marks established during the transition to the active state are in post-mitotic cells and serve as an *epigenetic memory*.

The vast majority of cells involved in circannual rhythm generation are seen to be post-mitotic. In these differentiated cells (e.g. thyrotropes of the pars tuberalis, ependymal cells of the third ventricle), reciprocal switches in epigenetic enzymes, such as DNMT-TET, cause long-term cycles in genomic methylation and act to control the expression of downstream genes (i.e. deiodinase enzymes). Recent work has indicated that thyrotropes in the pars tuberalis are in one of two binary states encoding summer or winter conditions [32]. The switch in the molecular cascade in thyrotropic cells may reflect long-term epigenetic regulation in gene transcription mediated by methyltransferases. The overall result is that epigenetic modifications provide a reciprocal switching system, between inactive and active states, at the cellular and hence phenotypic levels to govern circannual transitions in biology.

The next sections will discuss the Dual Compartmentalization Model in the context of circannual rhythms in (i) neuroendocrine control of seasonal physiology in fish, bird and mammalian species, (ii) endogenous rhythms generated in single-celled and plant species and (iii) hierarchical organisation.

## 29.5 Variation Across Vertebrate Groups

Although seasonal variation in the timing of numerous physiological and behavioural processes (e.g. birdsong, migration and hibernation) has been documented for millennia, the formal investigation of evolutionary and mechanistic questions began early in the twentieth century [33]. During the 1980s, it became apparent that the thyroid gland secreted a key hormone that permitted photoperiodic regulation of circannual rhythms in Japanese quail [34], European starlings [35] and sheep [36]. These findings indicated that the thyroid gland played a pivotal role in the photoperiodic regulation of seasonal reproduction. Over the past couple of decades, the localised conversion of thyroxine (T4) into triiodothyronine (T3) in the mediobasal hypothalamus has been shown to be a key event for circannual rhythms in reproductive physiology [37].

Unlike circadian biology, the precise neuroanatomical structures responsible for circannual timing have not been elucidated. Current hypotheses propose that thyrotropes in the pars tuberalis (PT) [32] and/or glial cells in the ependymal layer along the third cerebral ventricle provide circannual timing in vertebrate species (Fig. 29.4 [37, 38]). The general pattern is that increased thyrotropin-stimulating hormone β (TSHβ) expression in thyrotropes induced by long summer days stimulates the synthesis of deiodinase type II (DIO2) enzyme expression in the adjacent ependymal layer in the hypothalamus [38]. The increased DIO2 converts T4 into T3, leading to changes in neuropeptide release from the hypothalamus and consequent protein hormone secretion from the pituitary gland (e.g. summer phenotype-gonadal activation in rodents). During short winter days, the increased night duration reduces TSHβ and DIO2 and increases DIO3 expression. These

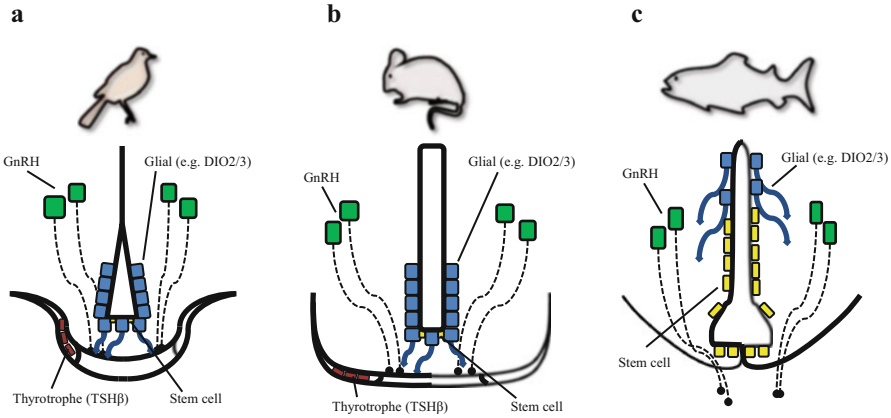


**Fig. 29.4** Neuroanatomical variation in circannual organisation in vertebrates. The pars tuberalis and ependymal cell layer lining the third ventricle of the brain is strongly implicated in the circannual regulation of reproduction in birds (**a**), mammals (**b**) and fish (**c**) [37, 38]. Several cell phenotypes are localised to the ependymal layer, including stem cells (*yellow boxes*) and supporting glial cells (*blue boxes*). The control of the reproductive axis depends on gonadotropin-releasing hormone (GnRH) neurosecretory neurons that govern the activity of the reproductive axis (GnRH, *green boxes*) and are located in the adjacent hypothalamus. To date, circannual epigenetic modifications via DNA methylation have been proposed to occur in post-mitotic cells within the ependymal layer via changes in *dnmt3b* (i.e. DIO3 [7]; Fig. 29.2d). Given the evidence in support of circannual variation in neurogenesis [25, 55], our model predicts that DNMT1 changes (Fig. 29.2c) reside in the ependymal layer stem cell niche and provide the initial circannual rhythm (Compartment 1). During cell maturation and establishment of post-mitotic specialisation, these cells retain an *epigenetic memory* for circannual rhythm (e.g. thyrotropes, tanycytes). Then, these post-mitotic cells located in the pars tuberalis/ependymal layer maintain circannual rhythm via Compartment 2-driven epigenetic mechanisms

changes result in the local catabolism of T3 and a reversal of the phenotypic response. Recent studies in fish have identified the *saccus vasculosus* as the light sensor for the integration of changes in day length and the regulation of TSHβ expression [39, 40]. This region is homologous to the infundibulum of the mediobasal hypothalamus in vertebrates, a region located dorsal to the pituitary gland [41]. Since fish lack a median eminence, these findings suggest that tanycytes are not necessary for the regulation of circannual rhythms in fish physiology. At a molecular level, it seems that *tshβ* and/or *dio2/3* form the core circannual clock mechanism in the mediobasal hypothalamus. The local change in thyroid hormone concentrations is the primary signal generating seasonal neuromorphological changes in the hypothalamus that time peripheral physiology.

*tshβ* and *dio2/3* signalling provide a molecular target to examine the role of epigenetic modifications for circannual rhythmicity. Both DNMT1 and DNMT3b are expressed in high levels in the mediobasal hypothalamus and the ependymal layer in particular [7, 42]. Given that both *dnmt1* and *dnmt3b* exhibit seasonal variation in hypothalamic expression [7], it is possible that *dio2/3* promoters may be one downstream target. One potential explanation for the annual change in gene expression could be due to neurogenesis [55]. Indeed there is strong evidence for a

neuronal stem cell niche in the ependymal layer [56] and seasonal variation in neurogenesis in the brain [57]. The timing for the maturation of neuronal stem cells into differentiated post-mitotic cells may be due to the actions of DNMT1. Here, DNMT1 serves to methylate the entire genome and provides both the trigger to initiate cell maturation and epigenetic marks that code the *time of year*.

A direct link between DNMT3a/3b and circannual variation in target or whole genome methylation has yet to be established. However, in the adult Siberian hamster hypothalamus, there are significantly greater levels of DNA methylation in the *dio3* proximal promoter in long day (LD) compared to short day (SD) phenotypic conditions. Remarkably, the endogenous reactivation of gonadal growth (i.e. refractory state) was observed to have a significant *remethylation* of the *dio3* promoter. These data indicate that one key circannual gene exhibits reversible DNA methylation; supporting the hypothesis that epigenetic modifications are a powerful long-term, time-keeping mechanism. One limitation of this work towards supporting the Dual Compartmentalization Model is that Siberian hamsters are highly photoperiodic (dependent on exogenous cues) and thus do not provide the best approach to investigate whether spontaneous reversible DNA methylation is a key component of the endogenous circannual clock.

## 29.6 Tractable Models of Circannual Rhythms

Due to the challenges of tracking circannual rhythms across multiple oscillations taking many years, very few rhythms have been characterised. Two organisms that provide a valuable means to assess endogenous circannual rhythmicity include the unicellular dinoflagellate *Alexandrium* (formerly known as *Gonyaulax*; [27, 43]) and the widely studied plant, *Arabidopsis* [44]. *Alexandrium* is a highly circannual organism whose key seasonal feature is the progression through developmental stages from resting cysts to fast-dividing vegetative cells [43]. *Alexandrium* cysts reside in water sediments and are dormant during winter periods for approximately 2–6 months. In the spring, new vegetative *Alexandrium* cells enter the open ocean and produce blooms of mass abundance in summer, before returning to dormancy (Fig. 29.5). The spring hatching is not a passive response to the external environment but is regulated endogenously by a circannual timer [43]. While the existence of an intrinsic control mechanism has been recognised for over 30 years, the identity of the genetic and molecular machinery that times the process remains a mystery. One attractive model is that epigenetic mechanisms, such as DNA methylation [7], generate the long-term cyclicity. DNA methylation occurs in *Alexandrium* similarly to eukaryotes [17], and several genes involved in DNA methylation have already been identified [47]. Given that all the necessary machinery is present, it is likely that DNA methylation either generates or maintains circannual timing in *Alexandrium* and may represent a component of an ancient clock system.

Some of the best evidence for an endogenous epigenetic long-term timer comes from studies of flowering in plants [45, 46]. *Arabidopsis* that are kept in constant

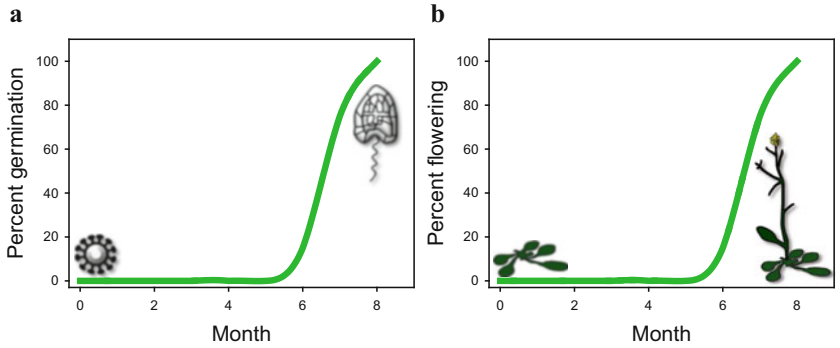


**Fig. 29.5** Cell-autonomous circannual rhythmicity. The best examples for endogenous circannual rhythmicity, operating at a simple level of evolutionary organisation, are provided by unicellular organisms such as *Alexandrium tamarense* (**a**) and the model flowering plant *Arabidopsis thaliana* (**b**). *Alexandrium* cysts reside in sediments in shallow and deep coastal water and emerge into the summer water columns as vegetative cells. Newly formed cysts remain dormant for 2–6 months during which germination into vegetative cells is not possible. Dormant cysts have an endogenous signal that triggers germination after 6 months, even when housed in unfavourable environmental conditions. *Alexandrium* that resides in water sediment exhibits a circannual pattern for several years under constant conditions. This indicates fundamental time control within a single cell, potentially under the regulation of a cyclical epigenetic memory mechanism. A similar timing mechanism is present in *Arabidopsis* in which vegetative plants remain dormant in cold environmental conditions. However, after approximately 6 months, there is an endogenous switch, and *Arabidopsis* plants will initiate flowering due to the epigenetic silencing of Flowering Locus C (FLC), even if maintained under cold conditions

cold temperatures for prolonged periods show a predictable, seasonal flowering response that develops after approximately 6 months (Fig. 29.5). The gene Flowering Locus C (FLC) in *Arabidopsis* provides an inhibitory signal that prevents flowering; however, prolonged exposure to cold temperatures leads to the gradual inhibition of FLC and the onset of flowering [44]. Inhibition of FLC is accomplished via a polycomb repressive complex 2 (PRC2) that controls epigenetic silencing via two chromatin modifications, namely, the trimethylation of histone 3 at lysine 9 (H3K9me3) and at lysine 27 (H3K27me3) [48, 49]. Recent work discovered that set domain group 7 (SDG7), a methyltransferase, acts as a negative regulator of flowering by regulating the expression of FLC [45]. Taken together, the FLC is a critical gene for *Arabidopsis* flowering, and accumulation of histone methylation markers is a critical event for circannual timing.

## 29.7 Hierarchical Organisation of Tissues and Cells

Returning to mammals, the SCN of the hypothalamus acts as a master pacemaker and coordinates circadian rhythms in both central and peripheral tissues [50]. Unlike the circadian system, the hierarchical organisation of circannual timing appears to involve a network of loosely connected tissues that act as the core pacemaker (Fig. 29.6). The

key central structures include the ependymal layer of the third cerebral ventricle and the pars tuberalis and pars distalis of the pituitary gland. The focal cell types include tanycytes, thyrotropes and lactotropes [51] in the three sites, respectively. We propose that epigenetic modifications (e.g. DNA methylation) exhibit cyclical annual oscillations in these cells and impact targets such as the *dio3* proximal promoter [7] However, it is likely that cyclical DNA methylation occurs over the entire genome causing widespread alterations in the epigenetic landscape. These key cell types provide the endogenous rhythmicity that approximates the annual period; environmental cues such as day length and social and nutrition cues then act to synchronise the molecular signalling cascades and phenotypic transitions to the seasons. In starlings, day length and social cues can impact the expression levels of *dio2/3* in the ependymal layer and serve to fine-tune the timing of reproduction [52].

The output of the core circannual pacemaker governs genomic, molecular, physiological and immunological timing in peripheral tissues (Fig. 29.6). Epigenetic enzymes, such as DNMT3a, are distributed in many peripheral tissues (e.g. gonads) and have cell-specific functions. Our model suggests that the output signal from the pacemaker cells/tissues drives slave oscillations in epigenetic enzymes in the periphery. Evidence in support of this conjecture is derived from seasonal variation in *dnmt3b* expression in peripheral leukocytes [28]. Hamster exhibits marked annual variation in multiple aspects of immune function, including innate and adaptive immunity. In summer phenotypes, immune function is significantly impaired compared to winter phenotypes [53]. *dnmt3b* signalling in leukocytes may act to regulate the DNA methylation landscape and alter the molecular signalling pathways to enhance cell function during winter conditions.

## 29.8 Infradian, Tidal and Circadian Clocks

Oscillations in gene transcription are a prevalent observation across biological rhythms. It should not be surprising then that in addition to the transcription binding factors driving gene transcription, access to the genomic template provides another layer of control. Therefore, epigenetic modifications that surround the genome, for example, DNA methylation and histone acetylation, are likely evolutionarily conserved mechanisms that govern the timing of gene expression and subsequently biological rhythms. The Dual Compartmentalization Model for circannual rhythms may equally apply at other biological timescales, such as lunar and tidal, as well as daily, where there is strong evidence for epigenetic regulation of the circadian clock [5, 6, 16, 22]. Given that circatidal rhythms can be dissociated from circadian rhythms in sea lice (*Eurydice pulchra* [54]), we speculate that there is a separate genetic mechanism that regulates tidal cycles in *Eurydice* involving epigenetic control of the timing of gene transcription. Other particularly vital reproductive rhythms are female ovarian cycles. In the human uterus, the endometrium expresses DNMT3a and DNMT3b with marked variation across the menstrual cycle with significantly lower levels during menses [58]. These data suggest that DNA

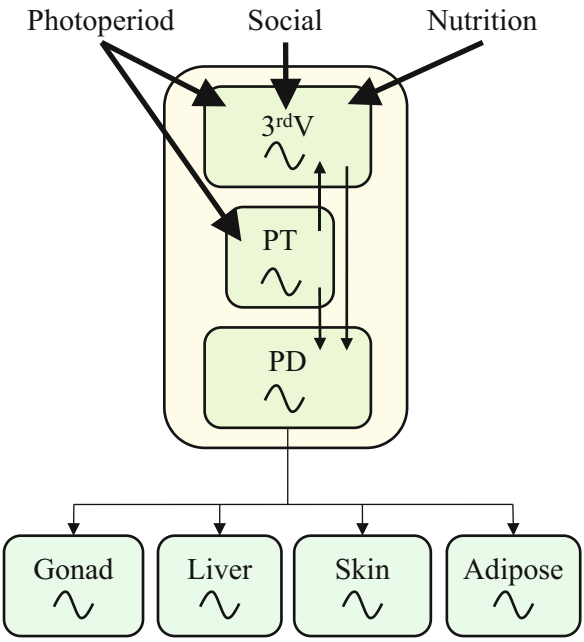


**Fig. 29.6** Hierarchical organisation for vertebrate circannual rhythms. We propose that the generation of circannual rhythms is cell autonomous dependent on stem cells and differentiated cells (Fig. 29.3) located in different tissues, but the overall circannual phenotype derives from the hierarchical organisation with the central tissues (*yellow box*; brain – third ventricle, *3rdV* and pituitary – pars tuberalis, *PT*, and pars distalis, *PD*) acting as the pacemaker for the peripheral tissues (e.g. gonads, liver, skin and adipose). We propose that environmental factors (photoperiod, social cues and nutrition) act centrally to synchronise the endogenous circannual clockwork across the seasons of the year

methylation is involved in timing relatively long-term changes (~28 days) in reproductive tissues that are vital for female fertility.

## 29.9 Conclusions

In this chapter we present a novel model for the endogenous regulation of circannual rhythms; the Dual Compartmentalization Model. Indeed our model also raises important, yet challenging, questions that are fundamental for the control of many biological rhythms, such as what mechanisms keep the cycle moving and why would stems cells be rhythmic. We speculate that epigenetic modifications, such as DNA methylation and/or histone acetylation, provide the key molecular signalling mechanism that controls circannual timing in stem cells (primary initiator sites), as well as in different differentiated cell lineages in the brain and peripheral tissues (secondary propagator/amplifier sites of circannual control). We have drawn upon evidence from the field of circadian [5, 6, 16, 22] and circannual [7, 28, 29] rhythms to support the conjecture that

cyclical patterns in the epigenetic landscape in cells govern endogenously regulated timescales of hours, months and years. Overall, there are three predictions that aim to resolve the molecular basis of circannual rhythms: (1) the timing mechanism will be cell autonomous to allow for localised autonomy, (2) long-term characteristic of circannual timing will involve epigenetic regulation of gene function – switching between 'on' and 'off' [e.g. 32] and is an ancestral trait, and (3) the timing mechanism will be fundamentally linked to the cellular energy sensing system that predicts survival.

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# Chapter 30
# Insights into the Regulation of Spring Migration in Songbirds

**Sangeeta Rani, Sudhi Singh, Shalie Malik, and Vinod Kumar**

**Abstract** Migration in songbirds (order – Passeriformes) is a periodic movement between the breeding and overwintering grounds, located apart by several 1000 km. The migrating individuals face physiological challenges to cope with the demands imposed by long travels. The particularly important challenge is to meet energy cost of flight, in-flight supply of fuel, for which they need to store energy reserves in the form of fat. Long and uninterrupted migratory flight is facilitated by seasonal reduction in different organ weights and is made more efficient by enhancing the efficiency of metabolism, particularly the tricarboxylic acid (TCA) cycle. Most diurnal birds migrate at night, and with this they undergo shifts from diurnal to nocturnal patterns both in their behavioral and neural activities. Several of these seasonal alterations are under the circadian clock. In this chapter, our focus is on the brief mechanistic explanation for physiological, behavioral, biochemical, and molecular changes that occur during transition from the nonmigratory to migratory state in songbirds.

## 30.1 Introduction

Many birds undertake long distance flight twice-a-year between the breeding and overwintering grounds. The regularity in timing and the scale at which migration occurs have fascinated humans for a long time. Most birds migrate toward the milder climes; hence the latitudinal (north–south) migrations are common, although some species do undertake the longitudinal (east–west) migrations. Regardless of the direction, birds generally cover several thousands of kilometers during their flight. Some marine birds make a round-trip of 64,000 km [1]; birds like bar-tailed godwit (*Limosa lapponica*) make it without refueling as a single nonstop flight of more than 11,000 km [2].

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The geographical boundaries within which birds migrate constitute the migration system. Six major migratory systems have been recognized, each with different landscapes and avian fauna. In one of the systems, the Palaearctic–Afrotropical migration system, each autumn the birds from Eurasia travel to their wintering areas in the tropical Africa up to India. Twenty-seven bird species with breeding grounds in mid-Palearctic regions overwinter in India [3]. The second less studied Asian–Australasian migration system hosts approximately 234 species with breeding areas extending eastwards from Central Asia to Alaska. The Nearctic–Neotropical migration system (North and South America) hosts about 250 bird species from North America overwintering partly in the Neotropics [4].

Evolution of migration has resulted in programmed seasonal (annual) life-history states (LHSs) in latitudinal migrants that are described as breeding, molt, autumnal migration, non-breeding, and spring migration (Fig. 30.1). In spite of physiologists having been interested for a long time to understand the mechanisms underlying seasonal migrations, we are still far from the complete understanding of how annual migrations are accomplished. However, the last few decades of research have made good progress, and in this chapter we shall briefly describe important events related to spring migration, mainly because of our own research bias. Our hypothesis is that the latitudinal migratory songbirds anticipate seasonal changes in the day length, which along with temperature alterations and concomitant changes in food availability help them prepare for upcoming long journey.

## 30.2 Preparation for Spring Migration

Migration is a multistep process and consists of three phases: the preparation, mature expression, and termination. Increasing photoperiods during spring trigger endogenously controlled processes linked with the preparation of migration. During preparatory phase, several physiological and metabolic changes occur at cellular and molecular levels. These are seen in changes in the hormones, viz., growth hormone and thyroid hormone [5], and changes in gene and protein levels [6–8]. The mature expression of migration is shown by morphological and behavioral changes required for energy storage and fueling the flight. In particular, birds undergo hyperphagia, lipogenesis and muscle hypertrophy, and shifts in neural and behavioral activities [7–12]. They also show directional preferences and orient themselves and navigate in the right direction (see next chapter).

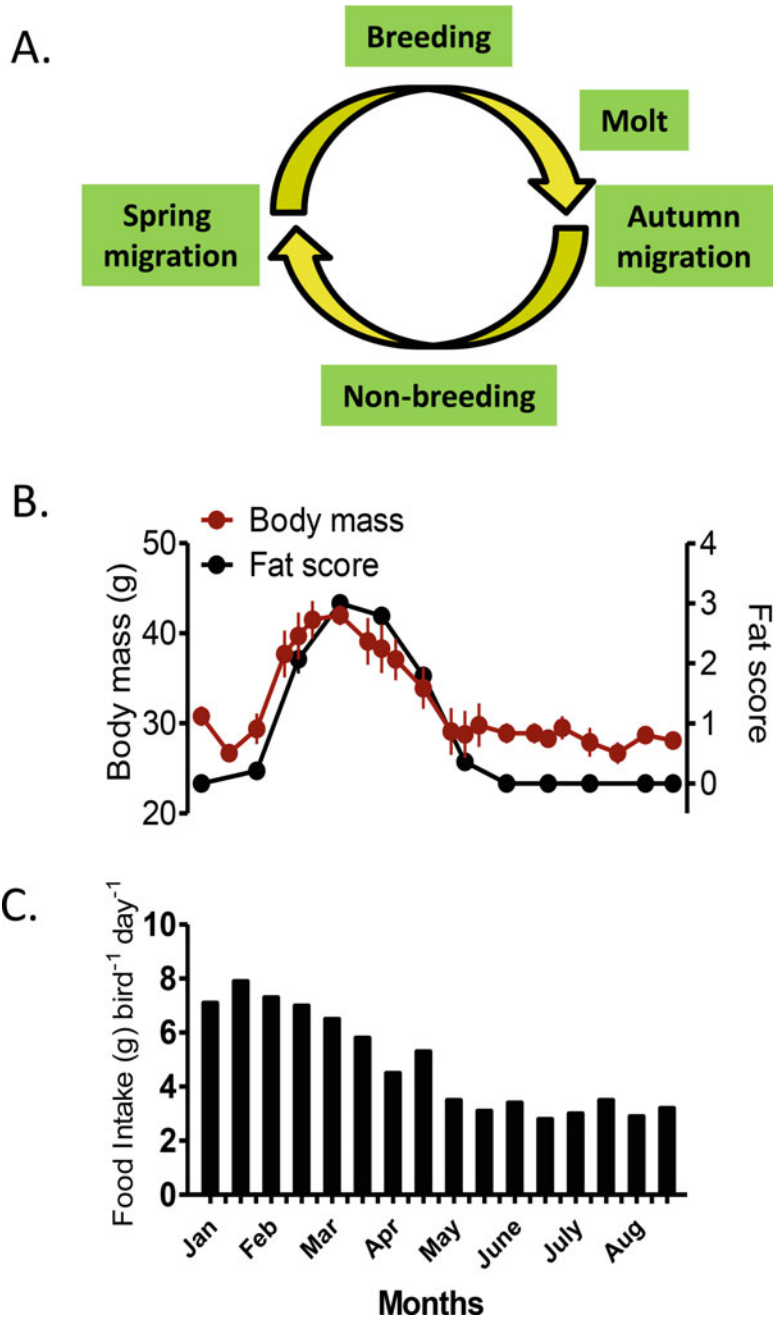


**Fig. 30.1** (**a**) Annual life-history states (LHSs) of a migratory bird. They in general show four distinct LHSs, represented as non-breeding, spring migration, breeding, and autumn migration, repeating every year almost at the same time. Molt in most birds occurs during the postbreeding period. (**b**, **c**) Changes in the body mass, fat deposition, and food intake during the premigratory (Jan–Mar), migratory (Mar–Apr), and post-migratory (Apr–Jun) periods in black-headed bunting (*Emberiza melanocephala*), based on Misra et al. [15]. The hyperphagia during the premigratory period leads to the fattening and subsequent gain in body mass. The fat is used up during the migration, and birds become lean after migration is over

### 30.2.1 Energetics of Migration: Hyperphagia and Food Preference

During preparation to migration, the most visible change in the phenotype is rapid accumulation of fat stores as the result of increased food intake, called hyperphagia [13–15]. Birds also change their food preference, with enhanced consumption of easily digestible energy-rich foods, viz., oil-rich seeds, fruits, or insects [14, our unpublished observations]. In European robins (*Erithacus rubecula*), the premigratory gain in body mass has a close relationship with the fruit consumption; the maximum gain occurs from lipid-rich fruits [16]. The rate of fat deposition and subsequent gain in body mass seem to be diet dependent; low- and high-protein diets enhance and suppress fat stores, respectively [17]. Further, the flight endurance depends on exceptional ability of birds for energy storage and fuel processing as well as its mobilization, transport, and utilization. Thus, fuel selection-based energy budgeting [18] determines the flight characteristics; a food with high ratio of energy to mass is the favored diet. Fat is the fuel of choice for migratory birds since it provides almost double the energy, as compared to that comes from similar carbohydrate or the protein diets. The near anhydrous lipid storage increases the energy efficiency of fats by about 8–10 times [18, 19].

The fat is mostly deposited subcutaneously or in the abdominal cavity and contributes about 50 % of the total body mass [13, 15]. These fat stores form a large share of unsaturated fatty acids, with functional advantage, are rapidly mobilized from adipocytes and preferentially oxidized by muscles. Generally, the fatty acid composition of body fat is similar to that of dietary fat, if birds are fed with high-fat diet, but may differ if fed with carbohydrate-rich diet, as carbohydrates are processed by the liver for de novo synthesis of fatty acids [20].

### 30.2.2 Anatomical Changes

Phenotypic flexibility is an adaptive feature of migratory birds [21]. There are dramatic anatomical and functional changes akin to the remodeling of several organs, although this seems to be largely dependent on participation of a specific organ system as per its in- and out-of-flight functional requirements [7].

#### 30.2.2.1 Flexible Remodeling of Organ Size

The pectoralis and cardiac muscles show seasonal hypertrophy and atrophy during pre- and post-migration periods, respectively, indicating changes in their functional capacity and requirement [7, 22]. Similar seasonal changes in the food quantity and/or quality are responsible for alterations in the volumetric and/or biochemical capacity of digestive organs, viz., the small intestine, gizzard, and liver [23]. Enhanced capacity of enlarged digestive organs helps in the conversion of more food into fuel and, thereby, enables the birds to meet high energy demands during the flight [24]. Short

distance migrants refuel several times during their migratory journey owing to their frequent stopovers. This results in hypertrophy of their digestive tract. However, the long distance migrants do the reverse; they do not refuel frequently, their gut atrophies, which keeps them light during flight [25]. The liver mass also increases to further increase the lipogenetic activity required for enhanced metabolism [26].

#### 30.2.2.2 Migration and Muscle Action

Intense muscular activity during the migration requires quick mobilization, transport, and oxidation of metabolic (fat) fuel [6]. As a consequence, the fatty acid transport proteins (plasma membrane fatty acid-binding protein, FABPpm, and fatty acid translocase, FAT/CD36) involved in the transport of fatty acids across muscle membranes are upregulated in the migrating birds [6, 21, 27, 28]. The heart-type fatty acid-binding protein (H-FABP), a cytosolic protein that transports the fatty acid to mitochondria for oxidation, is also upregulated [29]. The muscle myoglobin content has also been found elevated during the migratory state, in order to facilitate oxygen transport; the myoglobin has a higher affinity for oxygen as compared to the hemoglobin and is generally used for oxygen storage in aerobically active tissues [30].

### *30.2.3 Homeostatic Changes, Thermo- and Osmoregulatory Mechanisms*

Birds generally have a higher body temperature (~38–44 °C), and this can further go up to 4 °C during the flight [31]. Although a high temperature improves muscle efficiency, this may lead to an overheating during the long-distance continuous flight of without water-intake. In-flight birds require both the temperature and osmotic regulatory adjustments in order to conserve energy and water, respectively. Interestingly, the migrants lower their water levels before departure by reducing their body mass and utilize fats to cover long distances [32]. The metabolized fat produces water, which contributes to the water balance [33]. The energy expenditure is linked to the thermoregulatory costs, and, hence, birds possibly have evolved with different adaptive strategies to cope up with changes in the environmental temperature [34]. Some birds inhabiting cold environments use metabolic fuel to maintain their body temperature and flight, while others enhance their ability to store fuel by lowering their body temperature [35].

## 30.3 Regulation of Spring Migration

Migration shows both daily and seasonal changes and therefore requires the control at both the circadian and circannual clock levels [36]. Both of the clocks help the birds to assess predictable environmental changes and optimally utilize

the available resources at all the locations including departure, migratory route, and final destination [36]. An efficient timekeeping helps manage properly and most efficiently the timely decision for fuel loading as well as the orientation and navigation. The external time cues like the prevailing photoperiod and nutritional state can affect the initiation of migration in birds [14]. In general, increasing day lengths during spring months trigger the onset of migration in latitudinal migrants. Most birds migrate at night, and this can be seen as "wing whirring" activity in caged condition called as migratory restlessness or *Zugunruhe* [38].

### *30.3.1 Circannual Clocks and Photoperiodism*

There is a body of evidence showing that seasonal migration is under circannual clock control [36, see also the chapter by Helms and Lincoln]. Increasing evidence show the involvement of circannual clock in determining the timing of migration during the year. The events associated with migration, such as hyperphagia, fat deposition, and changes in nighttime plasma melatonin and other hormone levels, are under the circannual control [36]. Possibly, the completion of certain physiological states in synchrony with photoperiodic changes forms the basis of timing of annual migrations. Comparative studies on migratory stonechats (*Saxicola torquata rubicola*) show persistent circannual patterns in night activity, suggesting an evolutionary basis of behavioral shift from diurnal to nocturnal activity pattern [37]. The mechanism may involve both circannual rhythms and photoperiodic regulation (photoperiodism), and both may be mutually inclusive in the regulation of migration [15]. The cycles of *Zugunruhe* in these birds were more varied than those of the molt and reproduction [37]. This suggests that *Zugunruhe* is independent of other seasonal responses, although it remains phase linked with them. The uncoupled activities indicate the involvement of multiple oscillators in driving seasonal responses [38]. Indeed, circannual migration programs are sensitive to environmental conditions as evidenced from the studies on several bird species maintained under constant light conditions [37]. Interindividual variations in *Zugunruhe* patterns further suggest its evolutionary adjustment to the external environment. Differences in circannual rhythms between individuals or species inhabiting same latitude seem to be under genetic control. This is demonstrated by studies showing association of gene polymorphism with phenological variations in some bird species. Genetic polymorphism may play a role in shaping the migration traits and phenological variation in birds [39]. For example, barn swallows (*Hirundo rustica*), having a rare *Clock* allele, were found early in their timing. They departed early from wintering grounds and arrived early at the breeding grounds [39].

### 30.3.2 Circadian Clocks and Development of Nighttime Activity

The development of *Zugunruhe* is under circadian clock control as evidenced by its persistence under constant dim light condition in the migratory black-headed buntings (*Emberiza melanocephala*) [10, 40, 41]. The diurnal migrants undergo behavioral shift and become like a nocturnal species during the migratory state. Possibly, *Zugunruhe* is the result of interaction between different circadian oscillators, or these oscillators redefine their activity during nonmigratory and migratory periods [10, 41]. However, no change in the activity phase of medial suprachiasmatic nucleus (mSCN) was observed between nonmigratory and migratory periods in black-headed buntings [11]. Contrarily, the change in activity of mediobasal hypothalamus (MBH, see below) was observed that suggests it to be the site of timekeeping mechanism for migration in songbirds. Further, when pineal, the important clock component of avian circadian system [42], was removed, the circadian rhythms of *Zugunruhe* decayed in the white crowned sparrows (*Zonotrichia leucophrys*) [43] and red-headed buntings (*Emberiza bruniceps*) [44], and its onset was delayed in the black-headed buntings (our unpublished observation). Interestingly, removal of pineal abolished the oscillation of circadian genes in the hypothalamus but not in the retina [44]. This for the first time suggested the independence of retinal clock from the pineal clock in migratory songbirds [44]. Daily characteristics of *Zugunruhe* are shown to be influenced by the light intensity, food, and temperature [45–47], which conforms to the synchronization of circadian clock governed activity with the environmental cues.

### 30.3.3 Neural Changes Associated with Behavioral Shifts

There are changes in activity at the brain levels in parallel with the behavioral shifts. The activity of olfactory and visual subsystems shows phase inversion in the neural activity without affecting the activity of avian suprachiasmatic nucleus, the known principal circadian clock in mammals [11]. These sensory circuits play critical roles in the migration and show high immunoreactivity for fos (the protein product of c-fos immediate early proto-oncogene and marker of neuronal activation) during day and night in the nonmigratory and migratory states, respectively. This may be because of the involvement of olfactory and visual systems in the orientation and navigation, respectively [48]. The olfaction plays an important role in the migration as birds deprived of olfactory perception fail to find their home [49]. Similarly, the phase inversion of fos immunoreactivity in the visual system, with increased activity during night, suggests its involvement in processing the light-dependent magnetic compass information in nighttime migration. Perhaps, the entire thalamofugal pathway including cluster N or cluster N alone is involved in the migration [11, 50, 51].

Both migration and reproduction are coupled, photoperiod-controlled seasonal events, which involve circadian rhythms in distinguishing between short and long photoperiods. Avian mediobasal hypothalamus (MBH) rapidly responds to the changes in daylight periods. Even exposure to a single long day produces measurable effects at the levels of clock genes, thyroid hormone-regulating genes, peptides (vasoactive intestinal peptide, VIP), and proteins (gonadotropin-releasing hormone, GnRH). MBH, therefore, is the site for putative photoperiodic seasonal clock in birds. Recent study on migratory bunting has also shown that MBH but not SCN is the site of photoperiod-induced seasonal responses, and VIP (for light sensing) and NPY (for translating this information) are the candidate molecules for the neuroendocrine regulation. Increased neural activity of dorsomedial hypothalamus (DMH) in parallel with the behavioral shifts further suggests a functional linkage between the endogenous clock and overt behavior, i.e., *Zugunruhe* [12].

### *30.3.4 Endocrine Regulation*

The link between energy accumulation and takeoff decision suggests a role (direct or permissive) for metabolic hormones particularly the corticosterone, prolactin, and leptin [52]. The corticosterone has been found as main regulatory candidate for energy homeostasis in birds. It plays an important role in the induction of physical activity and transitions between the LHSs [53]. Its high levels during migration and reproduction help the birds to adjust with their external environment and promote gluconeogenesis, the biosynthesis of carbohydrates from noncarbohydrate sources, and also increase the blood glucose levels, which promote premigratory fueling [54]. The prolactin, although mainly known to be involved in the parental care, osmoregulation, and feeding behavior, has been shown to affect the food intake and body fattening in migratory birds [55]. It helps during the period of migratory preparedness by producing lipid storage enzymes and increasing the gut size (hypertrophy) [56]. Similarly, the thyroid hormones, which regulate body metabolism, also play a significant role in the migration. The removal of thyroid gland inhibits, and the administration of thyroid hormones restores nighttime activity in the migrants [57].

Before making departure decision, migratory birds deposit sufficient amount of fat fuel. Leptin secreted primarily from adipose tissue provides a reliable signal to the brain about fat content, thereby, helps birds to assess their migratory readiness. Leptin receptors are found in brain areas that regulate the feeding behavior, as leptin-treated birds show decreased food intake [58]. Another leptin like hormone, the adiponectin also produced by adipose cells affects the metabolic activity. The adiponectin levels are high in lean birds, and it stimulates glucose utilization and fatty-acid oxidation. Similar to several other candidates involved in the metabolism, the adiponectin shows circadian rhythm in its expression. Interestingly, the peaks of adiponectin secretion shift from day to night, in parallel with the behavioral shift during the migration. Also the nighttime increase in the liver adiponectin receptors suggests elevated energy utilization during nocturnal flight

[59]. Furthermore, melatonin levels have been found lower at night during the migration than at other times of the year [60, 61]. This may reflect a rise in the body temperature and metabolic rate during flight [62] and/or adaptation that allows for rapid adjustment to changes in photoperiods experienced en route [36]. As of now, there is no definitive evidence for melatonin acting alone in the stimulation of *Zugunruhe*; therefore, most likely, melatonin interacts with a suit of hormones in regulating nocturnal migration in the songbirds [63, 64].

### *30.3.5 Molecular Regulation*

In the last few years, evidence has accumulated on molecules involved in regulation of timing and physiology of migration at various levels including the clock control and metabolism.

#### 30.3.5.1 Circadian Clock Control

The circadian clock control effects through a set of genes, which are functionally arranged in a transcriptional–translational feedback loop. The positive limb of this loop comprises *Clock*/*Npas2* and *Bmal1*, whereas, the negative limb is mainly constituted by *Per* and *Cry* genes [65, 66]. Additional loop of nuclear orphan receptor genes, *Rors* and *Rev.-erbs*, connects the two limbs. These circadian clock genes are shown to be involved in energy homeostasis and regulate the carbohydrate and lipid metabolism, the major source of metabolic fuels [67]. *Bmal1* contributes to the secretion of insulin and lipogenesis and adipogenesis [68]. Similarly, *Rev-erbα* affects the lipid and cholesterol biosynthesis in liver, as well as, the fatty acid and carbohydrate oxidation [69]. *Rorα* affects the steroid metabolism [70]. Also *Sirt1* gene has both the circadian and clock-mediated functions and regulates glucose levels by modulating the insulin sensitivity through *FoxO1* gene [71]. In addition, there are other genes involved in the carbohydrate and fat metabolism [72].

#### 30.3.5.2 Carbohydrate and Fat Metabolism

The genes involved in the carbohydrate (*FoxO1, Sirt1, Glut1, Pygl)* and lipid metabolism (*Hmg-CoA, Ppars, Fasn*) are differentially expressed during different life-history stages [7]. Hypothalamic *Sirt1* and *FoxO1* are upregulated during the premigratory state in order to induce hyperphagia and meet the high energy demands during migratory state. *FoxO1* stimulates the synthesis of orexigenic peptides (neuropeptide Y and agouti-related peptide, AgRP) but suppresses the genes coding for anorexigenic candidate molecules, viz., proopiomelanocortin, POMC [73]. Whereas *Sirt1* plays a key role in energy homeostasis, the POMC

and AgRP neurons are major regulators of feeding. The *Sirt1* regulates the function of these neuronal populations via *FoxO1* [74]. Decrease in *Sirt1* activity increases acetylation of *FoxO1*, which results in increased POMC and decreased AgRP expressions, thereby decreasing the food intake.

Thus, an apparent functional relation between *Sirt1* and *FoxO1* in birds during the migratory state may implicate the liver glycogen as a possible alternative flight fuel [71]. Similarly, there occurs seasonal change in the expression pattern of genes regulating lipid metabolism. The expression of *Hmg-CoA* follows feeding pattern and shows diurnal variations in the liver and brain [75]. During the premigratory and migratory states, enhanced levels of *Pparα* and *Fasn* suggest the involvement of fatty acid oxidative pathway in the energy generation, and those of *Pparγ* reflect an increased adipogenesis required for the storage of fat fuel [7, 76]. Studies have shown that high demand of energy is also met by de novo lipogenesis [77].

Some genes can mediate multiple metabolic pathways; for example, *Sirt1* connects the circadian clock with the metabolism, whereas *FoxO1* with *Sirt1* is involved in the energy homeostasis [71, 72]. There may be several unreported genes that might link carbohydrate and lipid metabolism, and this could be of help to seasonal migrants in order to switch from carbohydrate to lipid fuel or vice versa, as per body demand.

#### 30.3.5.3 Cellular Metabolism: Tricarboxylic Acid (TCA) Cycle

Migratory birds use glycogen or fat as metabolic fuels by cellular respiration during in-flight periods of starvation. As a result, the mitochondrial density and activity of key oxidative enzymes, viz., citrate synthase, cytochrome oxidase, 3-hydroxy-acyl-CoA-dehydrogenase, and carnitine palmitoyl transferase, are altered in and out of the migratory periods [78, 79]. The required energy is produced by the oxidative phosphorylation of metabolic fuels via the tricarboxylic acid (TCA) cycle (Fig. 30.2). In general, high free fatty acid levels indicate an enhanced metabolic reserve. Increased levels of citrate, an intermediate substrate that is used to start TCA cycle, and malate dehydrogenase (mdh) enzyme that regulates production of the oxaloacetate (OAA) also show enhanced cellular metabolism during the migration [8]. Further, citrate synthase (cs) activity suggests enhanced energy generation, since cs knockdown cells have severely disrupted ATP production [80]. The other dehydrogenases, viz., α-keto glutarate (odgh) and succinate dehydrogenase (sdh), are also upregulated during the migratory state (Fig. 30.2). Odgh possibly controls metabolic flux through TCA cycle in the brain and peripheral tissues and, thus, contributes to the energy homeostasis [8, 81]. A high liver sdh and mdh mRNA could be linked with increased energy demand and increased endurance and contractile muscle strength, respectively. Increased mdh also reflects increased adipogenesis during the premigratory/migratory states. Thus, transitions in metabolism between the seasonal LHSs appear to be regulated at the transcriptional levels, particularly involving genes coding for enzymes of the oxidative phosphorylation pathway of the carbohydrate and lipids metabolism [8].

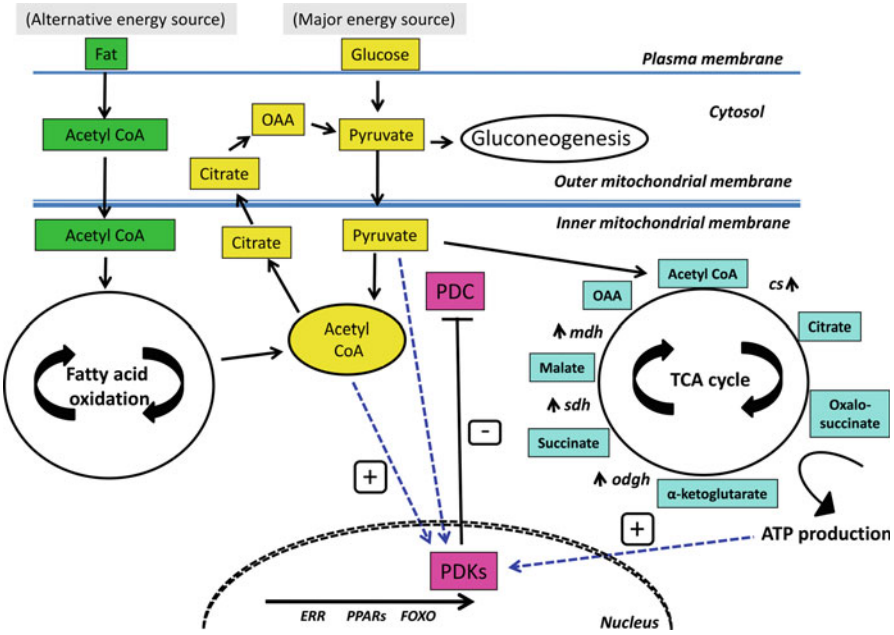


**Fig. 30.2** A generalized scheme of biochemical and molecular pathways involved in the regulation of fuel metabolism in migratory birds. Glucose is the main fuel and fat is the alternative fuel in energy production. Pyruvate is converted to glucose and glycogen via gluconeogenesis or oxidized to acetyl-CoA. The acetyl-CoA oxidation via TCA cycle is mediated by different enzymes that are upregulated during high energy demand. The two energy-producing pathways compete with each other for oxidation by PDC. The action of PDC links glucose and fatty acid metabolism via oxidative decarboxylation of the pyruvate to acetyl-CoA. PDKs phosphorylate PDC, and this is under the control of mitochondrial acetyl-CoA, pyruvate, energy production, and nuclear transcription factors. *cs* citrate synthase, *odgh* α-ketoglutarate, *sdh* succinate dehydrogenase, *mdh* malate dehydrogenase, *FoxO* forkhead box protein O, *PDC* pyruvate dehydrogenase complex, *PDKs* pyruvate dehydrogenase kinases, *PPARs* peroxisome proliferator-activated receptors, *TCA* tricarboxylic acid (Adapted from Zhang et al. 2014 [82])

The key regulators of cellular energy metabolism are pyruvate dehydrogenase complex (PDC) and pyruvate dehydrogenase kinases (PDKs; Fig. 30.2). PDC catalyzes oxidative decarboxylation of pyruvate to form acetyl-CoA, thereby linking the glucose and fatty acid metabolism and the TCA cycle. This makes glucose and fatty acids to compete for oxidation at the level of PDC. The PDC is normally active in fed state; however, during starvation its levels are suppressed by PDK, which catalyzes the phosphorylation PDC and inactivates it. This step is crucial in maintaining the energy homeostasis under condition of nutritional stress. In the experimental condition of starvation, which is akin to in-flight condition of a migrant, PDKs are induced, and PDC is inactivated which can switch glucose catabolism to fatty acid utilization. Thus, the reciprocal switching between the PDC and PDK expression during the nonmigratory and migratory states explains their role in the regulation of seasonal changes in the body metabolism [8, 82].

## 30.4 Physiological Conflicts: Trade-off Between Spring Migration and Reproduction

The cost of spring migration may trade off with preparations for breeding. Migrating birds are under stress to reach their breeding grounds, since arrival on time indicates their reproductive fitness. The early arrivals in general have a higher reproductive success than the late arrivals. Therefore, birds need to avoid the physiological conflict between the events of migration and reproduction [83, 84]. Whereas high mobility, hyperphagia, and body fattening are the key points of preparation for migration, initial decline in food intake and reduction of fuel stores mark the initiation of breeding.

In long-distance migrants, the gonadal development initiates during the migration, but in short-distance migrants, it commences only when migration is over [85]. In high-latitude species, both male and female birds initiate gonadal growth while they are still in migration, perhaps because of very short periods of favorable conditions at their breeding grounds. This is particularly seen in alpine- and arctic-breeding birds [86]. These birds have large gonads while they retain the ability to fly-off to avoid any unpredictable environmental condition like deep snow cover or totally snow-free grounds. Though the events underlying migration and reproduction may overlap, the onset of two may never coincide. This is because the breeding birds become temporarily sedentary, establish their breeding territories, and initiate reproduction [87].

Also, the two events are separately controlled by photoperiod, albeit with different thresholds, which helps them to avoid the physiological conflict [84]. Though the gonads may grow during migration, they are not involved in its induction, since castrated birds exhibit premigratory and migratory phenotypes, as do the intacts [88]. However, an understanding of the "switch on and off" mechanisms of the hypothalamo–hypophyseal–gonadal axis during premigratory and migratory states will help us understand the temporal spacing of spring migration and reproduction in songbirds.

## 30.5 Conclusion and Perspective

Migratory birds complete their different life-history states in geographically different areas. Their seasonal interaction with different habitats results in multiple phenotypes and/or flexibility in seasonal phenotypes during the year. Figure 30.3 summarizes various components of the spring migration. The migratory journey depends on the integration of multiple adjustments in the morphology, physiology, behavior, and metabolism, and in fact migratory birds seem to represent a well-orchestrated unit of coordinated yet distinct activities.

In general, migrants exhibit unique behavioral and physiological traits associated with the long enduring flights, seasonal behavioral shifts from diurnal to the nocturnal, internal organ remodeling, usage of fat as the more efficient fuel, and

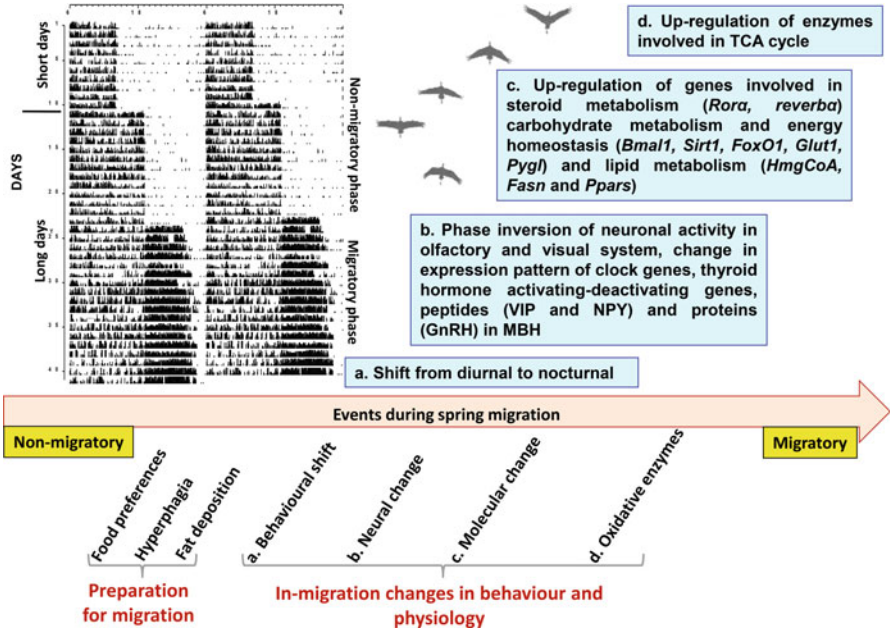


**Fig. 30.3** The actogram (double-plotted activity behavior of an individual bird housed in a cage over successive days under sequential short and long photoperiods) shows behavioral shift from diurnal in nonmigratory state to the predominantly nocturnal during the migratory sate. Summary of events during the transition from nonmigratory to migratory LHS is shown along an *arrow*. The preparatory and behavioral and physiological changes that occur (*below* the *arrow*) have been summarized above the arrow in steps (**a–d**)

most significant seasonal changes in body mass (lean to obese and back to normal) without showing any metabolic disease symptoms and adaptation to survive through a period of less sleep or total sleeplessness when in-flight. Interestingly, despite exhibiting a condition akin to the "metabolic syndrome" during the migration time, these birds lose weight and downregulate many factors and revert to normal physiology and behavior. The fat depots determine distance to travel; heavier birds travel longer distances. Taking off of the ground with heavy load is possible only by efficient muscles and heart condition and reduction in weight of less used organs (e.g., digestive organs) during flight. These birds use fat as fuel that gives more energy when oxidized compared to the carbohydrates. This is in contrast to the human athlete, who would not carry excessive weight, but should sustain endurance exercise. By human standards, equivalent fat deposition and higher blood glucose and lipid levels will be diagnosed as obesity and will have an increased risk of diabetes and cardiovascular disease. The nighttime migration in the birds with sleeplessness for days and/or weeks, without losing any cognitive function, could be of interest to human who lose one IQ point per hour of sleep deficit [59]. How migratory birds achieve all these physiological and metabolic adaptations is of great significance and is open to intense research in future years.

However, various physiological adjustments shown by migrants indeed provide a unique opportunity to understand the mechanisms and the cure underlying lifestyle diseases such as obesity, diabetes, and sleep disorders and help improve cognitive abilities in humans.

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# Chapter 31
# Orientation in Migrating Animals: Role of Biological Clocks

**Neelu Jain Gupta, Vatsala Dwivedi, Bhanu P. Singh, and Sanjay K. Bhardwaj**

**Abstract** How migrating animals find their direction to reach migratory destination is an important question of wildlife migration. Animals use a variety of geophysical cues such as the sun compass, stellar constellation, and geomagnetic field of the Earth to accomplish this feat. Endogenous clocks facilitate, to some extent, the challenge of heading toward the right direction. Whereas extensive body of research has focused on the biophysical and neurobiological mechanisms, relatively less is known of the extent of involvement of biological clocks in the migratory orientation. Studies on the innate capability of first year migrants and experimentally displaced experienced migrants to correctly reach their destination indicate that an endogenous time program controls spontaneous changes during the course of migratory journey. Here, we intend to briefly summarize the orientation studies in animals, with emphasis placed on the role of biological clocks in the avian orientation.

## 31.1 Introduction: Orientation in Migrating Animals

Many animal taxa including insects, crustaceans, fishes, amphibians, reptiles, birds, and mammals exhibit seasonal migrations. Massive to-and-fro seasonal movement of a species constitutes the migration, an evolutionary strategy to enhance the survival of species by skipping unfavorable conditions of the breeding grounds. There are three critical components of the successful migration: "where to fly,"

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"how to find way," and "how to return home." The decision "where to fly," i.e., the direction to migratory destination, is the most significant component, since any wrong decision will have huge costs to the migrating individuals. Hence, the orientation has been of significant interest of scientific investigation for many decades. Researches have ranged from the large-scale displacement experiments [1] to laboratory simulations [2].

It has been suggested that endogenous circannual clocks in interaction with the environmental day length are involved in both the initiation and orientation of migration at the "correct" time during the year [1, 3]. At this time, a migrating individual faces at least three challenges: it needs to know its spatial boundaries [present geographic location], which direction to start the movement, and how far to go. The latter is linked with the speed of flight, duration of travel, and the distance animals are required to navigate. Hence, migrating animals need to be oriented at correct angle and follow the direction toward the selected destination utilizing a confluence of information from endogenous or exogenous environmental cues.

## 31.2 Resource for Directional Information

Migrating animals acquire sensory information from both the external and internal environment. Internally derived resource includes proprioceptive genetic information as well as the biological measures, viz., physiological changes, locomotion patterns, motivation, etc. They may switch from one resource to another and/or may use multiple modalities (guiding factors) during the journey in order to optimize the resource availability. Animals can also use a variety of sources in the hierarchical fashion, although relative contribution of each resource during multiple modalities has not been well understood [4].

### *31.2.1 Geophysical Cues*

Geophysical cues include geomagnetic and celestial cues, viz., sun compass, polarized light, and position of stars. An animal could infer its subjective location only if it had some variation from the contiguous areas, and Earth's magnetic field provides a suitable choice both in space and time because of variation in the neighboring magnetic parameters. Geomagnetic field is ubiquitous; it is available all over the globe, at all times, under all climatic conditions. More so, magnetic field is perceived even in the absence of visual or olfactory senses. However, to sense the magnetic field, the animal possesses structures that are biophysically enabled to find its position (map) and direction (compass). On the other hand, gradient of Earth's magnetic field enables animals to recognize its own position, and its polarity compass contributes to the animal's navigational capability and helps to decide

the correct direction. Geomagnetic polarity compass is different from the polarity of the Earth, which is poleward from the equator rather than the north from south. The total strength of magnetic field increases from the equator to magnetic poles, hence the latitudinal gradient. Intersection of magnetic field lines with Earth's surface forms the angle of inclination. This angle changes from the equator to magnetic poles, therein providing a bi-coordinate grid due to location-specific combination of the magnetic field strength and inclination; this gives the "map" information. Thus, an individual with derivation of the positional information from the magnetic map can correctly navigate utilizing the geomagnetic cues. This way the decision-making involves the adequate spatiotemporal orientation that comprises animal's ability to utilize Earth's magnetic field as a source of directional information.

Most migratory animals including butterflies, bees, ants, sand hoppers, fish, sea turtles, reptiles, and birds derive the directional information using one or multiple celestial cues like the sun, skylight polarization, and star constellations. Sun compass depicts the position or movement of the sun in the sky and delivers solar information in the azimuth and/or zenith angles. The azimuth angle defines the direction of the sun, while the zenith angle defines the height of the sun from the Earth. Sea turtles use sun's azimuth as guide to locate a suitable breeding ground each year [5]. Monarch butterflies use sun as a compass to guide their southwesterly autumn migration from Canada to Mexico [6]. Many animals like the dung beetle exhibits preference for sun compass over the skylight compass [7].

Juvenile and adult migratory birds prominently use celestial information during the premigratory season, to recalibrate their magnetic compass. They use skylight compass as the reference, i.e., sunset polarized light patterns near the horizon of the sky. Savannah sparrows, *Passerculus sandwichensis*, and white-throated sparrows, *Zonotrichia leucophrys gambelii*, use polarized light cues near the horizon at the sunrise and sunset times as primary calibration reference during the initiation and end of migration [8, 9]. Interestingly, the ability to discriminate between polarization patterns varies with species [10]. For example, savannah and white-throated sparrows recalibrated the magnetic compass on the basis of polarized skylight, while other species tested at different geographical regions such as Tasmanian silvereyes, *Zosterops lateralis*, in Australia [11]; European robins, *Erithacus rubecula*, in Germany; pied flycatchers [12], *Ficedula hypoleuca*, in Italy; song thrushes, *Turdus philomelos*, in Russia [13]; and northern wheatears, *Oenanthe oenanthe*, in Germany [14] did not elicit similar response by recalibrating their migratory orientation flight to artificially shifted polarization pattern.

The supernatural expertise of nocturnal migrating birds in using the star patterns in the night sky was recognized few decades ago [9, 15, 16]. Emlen used simulated stellar cues projected on a planetarium ceiling to test if birds (indigo bunting, *Passerina cyanea*) still oriented toward the expected migratory direction [17]. A shift in the direction of the planetarium ceiling caused parallel shift in birds' orientation. He proposed that during the juvenile stage, birds had learned "sky map" based on the stellar constellation of the sky. The asterism of seven stars, the big dipper with lips pointed toward the North Star, is probably an important

guidepost for the migrants. Indigo bunting's exposure to the false sky rotating around the star Betelgeuse in the constellation Orion made them follow Betelgeuse as if it were the North Star [17].

### *31.2.2 Inherited Program*

Avian studies on association of genetic determinants involving the crossbreeding and selective breeding experiment have shown directionality as a predominantly non-sex-linked heritable character [18]. F1 progenies of a crossbreed of differently oriented blackcaps (*Sylvia atricapilla*) exhibited phenotypically intermediate directional preferences, significantly different from the parent birds [19]. Also, displacement experiments of first year migrants showed their ability to perform their migratory journey to wintering grounds in the absence of conspecifics in many bird species. Even hand-raised birds were able to choose a direction close to population-specific route [19]. Purposive migratory direction of young migrants seems to be an outcome of the evolutionary processes, such that natural selection favors the most adaptive direction for a population that eventually becomes genetically fixed.

### *31.2.3 Other Cues*

Adult animals in their second migratory journey seem to rely on the remembered external landmarks. A remembered landmark is memory-based location-specific information received as previous experience of sensory input from the bird's eyes. Bingman [20] suggested that most autumn night migrants passing through the Hudson River area in New York preferred "known" track direction to closely parallel the river in partial compensation for wind drift.

Emlen funnel experiments were performed on pied flycatchers, *Ficedula hypoleuca*, to test the hypothesis stating the moon as an orientation cue [21]. In general, migratory activity of test birds increased under the moonlight, and the fact that one-half of testing funnel received better illumination than the rest resulted into artifact as phototactic response took over the directionality exhibited by bimodal activity of pied flycatcher. But this opinion was also refuted because of striking species differences among night migrants under moonlight. For example, in garden warbler's (*Sylvia borin*) Emlen funnel testing, the concentrated distribution in right migratory direction was because of the tendency to move away from the moon rather than phototactical response or taking directional cue from the moon's position in the sky [22].

Homing pigeons use olfactory cues, and so it was proposed that the olfactory cues could be involved in the migration as well [23]. Field studies at Max Planck Institute for Ornithology have established that odors considerably facilitate bird

migration and sometimes act with greater potential as navigational cue than geophysical cues [24].

Migrants may also use wind direction (anemotaxis) as a directional cue at different stages of migration. Wind direction and speed have been found to play roles in shaping individual flights but it is unclear whether they are directly involved as the direct reference cue [25].

## 31.3 Magnetic Orientation: Role of Magnetoreceptor

Animals inhabiting different habitats can perceive one or more of the three possible modalities, viz., electromagnetic induction, chemical magnetoreception, and perception using biogenous magnetite. Evidence for magnetic perception is rather obscure in lower vertebrates. Electrosensitive marine fish such as sharks detect Earth's field through electromagnetic induction and exhibit well-developed electroreception utilizing seawater as a conductive medium for the electromagnetic induction.

Studies in other animals, viz., certain insects, homing pigeons, migratory birds, and some mammals, support the use of Earth's magnetic field as a guiding cue based on two biogenous magnetite and chemical reactions which are modulated by weak magnetic fields. Over the last 30 years, both the theoretical and experimental evidence based on spin chemistry knowledge have added enormous information to magnetic properties, kinetics, and dynamics of radicals, supporting the hypothesis of "radical pair mechanism." The direction and intensity of Earth-strength magnetic fields modify molecular reactions in the chemical magnetoreception. These reactions involve electron spins in pairs of radicals. But there is a lack of direct evidence identifying radical pair reaction to a weak magnetic field like that of the Earth [26]. Existing evidence is based on the alteration of orientation in response to radical pair modulation either by light or radio frequency fields. Ritz and colleagues [27] have emphasized for the molecular, genetic, and neurobiological characterization magnetoreceptor. The magnetite hypothesis is based on neurological detection crystals of the magnetic mineral magnetite and the ability of magnetite to transduce the magnetic field energy.

Among vertebrates, the biogenetic structure implicated for geophysical resource utilization ranges from ampullae of Lorenzini in fish to magnetite crystals in birds. The ampullae of Lorenzini have specialized cells in electrosensitive fishes, supporting their implication in electromagnetic induction aquatic environments. However, in trout, fish of family Salmonidae, confocal microscopy analyses of olfactory lamellae revealed cells that contain single-domain magnetite crystals in the nose innervated by a branch of the fifth trigeminal nerve of the cranium [28]. Electrophysiological recordings also revealed that abrupt changes in field intensity could stimulate magnetite-containing cells. But it is not straightforward to extrapolate single-domain magnetite detection in fish to higher nonaquatic vertebrates, although there exists striking similarity between fish and birds in the

anatomical site containing the magnetite, being innervated by the ophthalmic branch of the trigeminal nerve [29]. These ferromagnetite-based mechanisms surmise in birds as putative magnetoreception sensory structures because of unique properties of iron that allow its detection and structural characterization. Solov'yov and Greiner demonstrated that the connection of magnetite assemblies to the cell membrane upper beak area could be excited to stimulate specific mechanoreceptive membrane channels in the nerve cell [30]. Another doctrine of photo-magnetoreception based on the radical pair mechanism [27] is suggested to underpin the need for a light-sensing molecule capable of changing its state of oxidation upon light absorption. To date, electrophysiological studies in birds have succeeded to reveal ophthalmic nerve specific neurons in the trigeminal ganglion respond to changes in vertical field intensity as small as about 0.5 % of the Earth's field; a possibility to manipulate magnetic sensitivity by genetic maneuver of cryptochrome in a model organism is yet to be achieved. The scope of this investigation clearly lies in bioinformatics-based approaches to deduce gene sequences of magnetite production from vertebrate genomic libraries and investigate if these sequences were conserved during evolution. Another aspect yet to demonstrate is Earth-strength magnetic field effects on cryptochromes in vivo.

It is still unclear how information from the magnetoreceptor is transduced to the brain. Quantitative genetics analysis studies suggest that migratory orientation has a strong genetic component. A recent publication [31] has outlined the advantage of high-resolution tracking and sequencing techniques to characterize genomic architecture of the migratory orientation.

## 31.4 Migratory Orientation: Role of Biological Clocks

In an interesting discovery [32], Kramer suggested the involvement of the sun compass orientation in directional migration of day-migrating starlings; Hoffman [33] argued that starlings could not achieve the knowledge of the sun's apparent movement without having an internal chronometer. His argument was supported by a 15° shift in directional orientation in training apparatus every hour of counter-clockwise rotation of birds' biological clock. Further, Gwinner and Wiltschko [34] hypothesized that characteristic changes exhibited by garden warblers in the flight direction of fall migration were dependent on endogenous timing processes. They hand-raised 59 garden warblers under constant temperature and photoperiodic conditions during the fall migratory season. Periodically, these birds during nocturnal migratory restlessness state were tested in a circular orientation cage for directional preferences. In the orientation cages, the birds had no view of the sky, but were naturally exposed to the Earth's magnetic field. Garden warblers showing migratory restlessness initially exhibited significant concentration of mean direction in the southern sector but after sometime they showed a counterclockwise shift in directional preference from south to southwest. This change in directional preference corresponded with that in the free-living conspecifics suggesting thereby

that spontaneous endogenous changes mediated directional preference relative to external orienting cues, probably of the Earth's magnetic field [34]. These spontaneous directional changes were controlled by endogenous time program; the location of the spontaneous drift was coded as time span required to reach that location [35].

A possibility of involvement of circadian rhythms in migratory orientation could be investigated by tweaking circadian rhythm markers such as dark hormone, melatonin, and observing consequential change in migratory orientation of birds, if any. Schneider and colleagues [36] removed the pineal gland in pied flycatchers (*Ficedula hypoleuca* Pallas) and tested them for magnetic compass orientation. The pinealectomized birds were found disoriented. However, exogenous administration of melatonin restored the ability of birds to use their magnetic compass to orient correctly. Therefore, measuring melatonin levels in night migrants might contribute to better understanding of magnetic orientation. Change in melatonin levels under light regimes could be replicated pharmacologically to investigate its possible effects on orientation behavior.

In the present millennium, the role of biological clocks in migratory orientation has gone beyond physiological mechanisms with experimental possibilities suggesting a molecular mechanism underlying magnetic compass orientation in garden warblers [37]. The association of magnetoreception with photoreception supports the possibility of the process involving cryptochromes, photosensitive proteins implicated in the circadian systems of many animals. Mouritsen and colleagues [37] hypothesized that CRYs (involved in the inhibitory branch of the autoregulatory transcriptional loop controlling the circadian clock) could mediate magnetic field reception. Cryptochrome expression in retinal ganglion cells (RGCs) in a night-migrating bird co-localizes with neural activation during magnetic orientation preceding migratory flight. But the extent of association of cryptochromes with magnetoreception is yet to be ascertained.

### *31.4.1 Light-Dependent Changes in Magnetic Orientation*

Seasonal migration is intimately connected to the annual cycle of photoperiod that plays a crucial role in triggering migratory behavior in suited season. It is likely that some light-dependent changes in magnetic orientation [38] arise as signal from circadian pacemaker system, which is already implicated in timing orientation behavior. In vertebrates, the photopigment melanopsin, that absorbs light to entrain of circadian rhythms, has been localized to some retinal ganglion cells [39]. Its sensitivity to blue light with blue–yellow opponency [40] might result into varied spectral composition of light, capable of invigorating the pacemaker system in complex ways [reviewed in 28].

Studies on magnetic orientation that kept one eye of bird covered to investigate apparent lateralization of the magnetic sense suggested the lateralization of the circadian pacemaker [41].

### *31.4.2 Spring vs. Autumn Avian Migrations*

First year autumn migration is innately programmed so that the bird is able to utilize the directional cue resources in suitable manner [19]. The spring migration however brings the birds to breeding site, which the bird has experienced before. There are two possibilities: (1) the birds might be utilizing the same orientation mechanism in both spring and autumn migration, for example, birds recalibrated stellar compass according to magnetic north, when faced with a conflict under simulated conditions during spring [European robins, 42] and autumn (warblers, bobolink) migration, and (2) they may be using the separate mechanisms to accomplish the two travels as suggested by different routes during the two travels. Gwinner and Wiltschko [34] kept hand-raised garden warblers under constant conditions over the winter such that test birds had only geomagnetic cue resource. The birds exhibited southward preference in autumn and northward preference in spring suggesting reversal of the migratory direction [please also see Sect. 31.4; 43]. The normal orientation of savannah sparrows [10] in spring after manipulation during autumn migration also supports the view that spring course is available independent of autumn experiences.

## 31.5 Orientation in Migrating Birds

Techniques for studying orientation in migrating animals range from simple counts to the sophisticated computerized devices. In the wild, the technique ranges from capture–recapture to the use of GPS tracking with adequate simulation of conditions such as altered magnetic field, night starry sky, photoperiod, etc. Mechanistic studies under laboratory conditions involve controlled manipulation of confounding factors through hi-tech engineering and calibration devices. Some recent laboratory device setups include flight chambers to study performance and directional preference in free-flying animals, for example, wind tunnels and flumes.

### *31.5.1 Field and Laboratory Studies*

Longitudinal surveys involve physical counting of animals at topographical sites. This method is inexpensive and gives primary information on long-term trends of the populations and seasonal timing at that particular location. This technique is unsuited for flying migrants, however. Trapping live or dead was another method to study migrating insects, fishes, and small mammals. Banding–recapture and seasonal censuses were the methods for constructing bird maps. The method involved tying an identification tag/band in bird's leg before releasing. Many migratory route displacement experiments on starlings [44] and white-crowned sparrows [45] used

banding method. This method needed large sample size for effectiveness thus was often replaced by mass-marking and larger numbers of observing location and personnel.

Miniaturized detectors and satellites have enriched field studies with real-time mapping tracking the flight animals at a given site of expected route allowed sequential tracking of flocks and swarms. In the 1970s, an effective way of following migrants over their journey was radiotelemetry. A small radio transmitter enabled to release a unique signal was fitted to the body of the bird to be tracked. Cochran [46] fitted a transmitter to the body of Swanson thrush (*Catharus ustulatus*) and followed them by car, thus revealing about flight performance and direction of night migratory songbird. Thorup [47] tracked white-crowned sparrows using a small aircraft and short-range transmitters. Eco-ranging RADAR (radio detection and ranging) systems as a combination of detector and directional antenna have also been effective in studying migration. The satellite technology has allowed the tracking of individual animals during migration over intercontinental distances.

Avian migration studies in the laboratory involve the use of specially designed circular funnels, activity-monitoring devices, or videography. Introduced in 1966 by Emlen and Emlen [48], Emlen funnels are a frequently used device to determine orientation and navigational capabilities under controlled conditions. These are round nonmetallic funnel-shaped cages provided with blotter paper that is primarily used in studies involving avian migratory behavior. It records birds' footprints, which are assessed to obtain a mean direction and a measure of vector, r, of the activity using circular statistics. Modern tools involve videography and computerized analysis.

In 1995, Busse [49] proposed another technique in the form of a flat, round, cylindrical cage, popularly called as Busse's cage. He suggested that nocturnal migrants tested in daylight and at night displayed similar distributions of their preferred directions. This study, for the first time, compared results of the same individuals tested in the two types of orientation cages during the day and night. Large angular dispersion suggesting multi-heading behavior of birds was similar to that in Emlen funnel, but the flat cage recorded more activity in daytime tests.

### *31.5.2 Differences Between First Year and Experienced Migrants*

Orientation experiments involving displacement of first time migrants were initiated by Thienemann [50], who interpreted that young storks do not need the support of adults for first autumnal migration and depend on inherited program. This theory was, however, soon contradicted to that juvenile storks prefer to follow experienced conspecifics than innate directional information [51]. Another opinion suggested that spatiotemporal programming of migration of inexperienced birds primarily depended on compass direction, to bring them from breeding to population-specific

wintering areas [34], but if the birds were displaced, they may alter the route to come back to desired trajectory (directions and distances). The debate continued with the suggestions from Helbig as stated in Sect. 31.2.2 and experiments began to include delays besides displacements. The delayed and displaced migrating storks preferred to travel alone suggesting a utilization of innate program and continued to correct their respective route even if they were delayed as compared to their non-manipulated conspecifics [52].

Thorup and coworkers [53] tested the navigational capabilities of experienced nocturnal migratory cuckoos, *Cuculus canorus*, by experimentally displacing solitary birds from breeding grounds to unknown locations just before the ensuing migration. The birds exhibited uncanny skill by compensating for the translocation by moving toward population-specific staging areas, featuring true navigation capabilities. Although the stage of translocation influenced their respective choice of staging area to join, cuckoos possessed spatial knowledge far beyond their population-specific flyway scale and were able to assess the alternative route options in terms of anticipated loss or gain. The abovementioned bird relocation study differed from the earlier ones in the use of satellite technology which enabled tracking of the individual bird's complete migration following relocation.

The phenomenal ability of adult migrants to return to the breeding/wintering grounds following displacements is explained as true navigation. Two important questions arise in the wake of this explanation: one, how pervasive is true navigation among different species of experienced migrants, and, second, whether all displaced birds exhibit goal-directed reorientation or they exhibit search behavior before reorienting. An answer to these questions is partly addressed by analyzing flight path and comparing them with predictions from specific migration strategy models [54]. Techniques enabling route reconstructions using satellite technology add to current understanding of corrective navigation.

## 31.6 Lessons Learned from Non-avian Systems

Besides birds, animals from other taxa also perform astonishing feats of migration including east–west journeys, complex land and ocean round-trips, up–down (altitudinal, mountain) journeys, and vertical movements in the water column of oceans and lakes with a similar challenge of initiating movement in the right direction. The diverse range of migrating animals, from zooplankton, sized 1–2 mm (e.g., crab larvae, copepods), to blue whale, *Balaenoptera musculus*, sized 24–27 m, might sometimes involve intergenerational relay as in monarch butterfly (*Danaus plexippus*). Luschi [55] reviewed the findings of studies on directionality in marine animals during interhemispheric flights (seabirds) and transoceanic (pelagic fish, turtles, pinnipeds, and whales) migrations. It is suggested that oceanic migrants rely on biological compasses to maintain a direction in the open sea. Displacement experiments support evidence for position-fixing mechanisms in seabirds and

turtles suggesting involvement of magnetic and/or olfactory cues besides other cues for sensing direction.

### *31.6.1 Underwater and Terrestrial Crustacean*

Marine crustaceans migrate from oceanic environments to oceanic islands or offshore areas like reef, offshore atolls. For example, western Atlantic spiny lobster *Panulirus argus* migrates annually and has the capability of homing to specific dens in its coral reefs. Lohmann and colleagues [56] investigated whether lobsters could orient to the Earth's magnetic field, using magnetic coil systems submerged offshore. The coil was used to reverse either the horizontal or vertical component of the Earth's field. Tethered lobsters walking inside the coils were able to establish and maintain consistent courses toward specific directions, but each individual lobster walked in a direction of its own choosing, apparently unrelated to that chosen by others.

### *31.6.2 Insects*

Many insects undertake to exploit temporary breeding sites hundreds or thousands of kilometers apart, but the behavioral adaptations that facilitate these movements remain largely unknown. Entomological radar is used to track long-range seasonal migrations in insects. It is suggested that insects adopt optimal flight headings that partially correct for crosswind drift maximizing distances traveled. Insect flight trajectory studies indicate that insect migration matches sophistication of migrating birds [57]. These also throw light on some evolutionarily conserved mechanisms in nature. Night migration of silver Y moths is assumed to depend on wind displacements, and their direction of movement might include magnetic sense when in flight [58].

### *31.6.3 Fishes*

Tagging experiments of fishes date back to 1926 [59]. Pacific trouts tagged in feeding areas 100 miles away from natal streams successfully returned to their breeding streams. Such "homing" behavior studies are of importance to migration studies because they pertained to open sea rather than migration in streams. The orientation was suggested to be guided by olfaction/olfactory senses followed by the ability to recognize vicinity of home stream [60].

The multitude of cues is much enhanced with the realization that orientation differs in open water and fluvial habitats. Open water migrations involve solar cues,

the information being derived from changes in the sun's azimuth (angle of the sun with Earth's surface in horizontal plane and vertical planes) and even by detecting and discriminating polarized light in case the sunlight is hazy or indeterminate. But as the sun's angles vary on seasonal and daily basis, a counteracting (compensatory) mechanism involves endogenous circannual rhythm entrained using a temperature-dependent strategy [61]. In open water, large-scale currents also influence the direction of some migrations.

The support for geomagnetic cues for migratory orientation is overwhelming recently. It was suggested that when water moves across the Earth's magnetic field, it induces a weak electric current possibly detected by discrete magnetoreceptor cells (containing magnetite crystals, biogenic Fe3O4) located in the olfactory epithelium. But this suggestion could not explain a magnetic map in the wake of temporal and spatial variations in the magnetic field and whether fishes had the ability to memorize such a map.

The strongest evidence on juvenile Chinook salmon (*Oncorhynchus tshawytscha*) with no prior migratory experience emerged recently [62]. They experimentally demonstrated that the salmons responded to magnetic fields like those at the latitudinal extremes of their ocean range by orienting in directions that would, in each case, lead toward their marine feeding grounds and showed that fish used the combination of magnetic intensity and inclination angle to assess their geographic location. The "magnetic map" of salmon appears to be inherited, as the fish had no prior migratory experience. The study results pointed toward the extraordinary navigational abilities in long-distance underwater migrant fishes. Another study on eels suggested that eels have a magnetic compass, which they used to orient in a direction they had registered moments before they were displaced [63].

### *31.6.4 Amphibians and Reptiles*

Water balance demand and thermoregulation restrict migratory range of amphibians; spatial separation of important habitat resources however sometimes compels migration. Amphibians use many sensory systems for perception of orientation stimuli: the auditory, the olfactory, and the visual system as well as the neural system of magneto-perception [64]. A variety of sources such as vocalization of conspecifics; odors of ponds; landmarks; the positions of the sun, moon, and stars; polarization patterns of the sky; and the Earth's magnetic field may serve as orientation cues. The relative availability of the potential cues in a given habitat and animal's sensory ability to process the information in hierarchical order determines initial orientation displacement. The map–compass orientation is shown to be applicable to anurans, although there is evidence for redundancy in multisensory system: olfactory and some visual cues are probably related to the map, magnetic and celestial cues to the compass orientation, and acoustic cues and fixed visual landmarks might be used for piloting. For example, short-distance

palmate newts rely on acoustic cues for orientation to breeding ponds, while for long-distance homing, they use magnetic compass as the sole source of directionality. Tadpoles of European common frog and Iberian green frog use wavelength-dependent effect of light on magnetic compass along the shore-deep water axis [65–67]. True navigation has been shown in only one species, the aquatic salamander *Notophthalmus viridescens*.

Migratory movement of reptiles is often associated with egg-laying sites or common hibernacula [68]. Among chelonians, turtles exhibit long-distance migrations from nesting beaches to foraging grounds often using celestial, geomagnetic, olfactory, auditory, thermal, wave, and current pattern signal cues besides map–compass orientation discussed earlier.

### *31.6.5 Mammals*

Mammalian migrations tend to depend on large contiguous habitats across regional environmental gradients, but the same has witnessed a global decline due to increased human pressures and invasion. But large-herbivore migrations are shaped by exposure to predation and interspecific competition although these are also affected by underlying geographic patterns in rainfall, elevation, or latitude. The movement trajectories of wildebeest in relation to grass biomass, high-quality food patches, and predation risk suggest food quality as the primary cue regardless of predation. However, zebra migratory movements revolve around striking a balance between predation risk and the high-quality food access [69].

With the recent added advantage of advanced technologies like wind tunnel and online monitoring both in field and laboratory, there is enhanced scope for understanding the migration mystery at mechanistic level including understanding underlying sensory systems and the possible role of the endogenous program of biological clocks.

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