Healthy Ageing and Longevity 18
*Editor-in-Chief:* Suresh I. S. Rattan

Anita Jagota *Editor*

# Sleep and Clocks in Aging and Longevity

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# Healthy Ageing and Longevity

Volume 18

Rapidly changing demographics worldwide towards increased proportion of the elderly in the population and increased life-expectancy have brought the issues, such as "why we grow old", "how we grow old", "how long can we live", "how to maintain health", "how to prevent and treat diseases in old age", "what are the future perspectives for healthy ageing and longevity" and so on, in the centre stage of scientific, social, political, and economic arena. Although the descriptive aspects of ageing are now well established at the level of species, populations, individuals, and within an individual at the tissue, cell and molecular levels, the implications of such detailed understanding with respect to the aim of achieving healthy ageing and longevity are ever-changing and challenging issues. This continuing success of gerontology, and especially of biogerontology, is attracting the attention of both the well established academicians and the younger generation of students and researchers in biology, medicine, bioinformatics, bioeconomy, sports science, and nutritional sciences, along with sociologists, psychologists, politicians, public health experts, and health-care industry including cosmeceutical-, food-, and lifestyle-industry. Books in this series will cover the topics related to the issues of healthy ageing and longevity. This series will provide not only the exhaustive reviews of the established body of knowledge, but also will give a critical evaluation of the ongoing research and development with respect to theoretical and evidence-based practical and ethical aspects of interventions towards maintaining, recovering and enhancing health and longevity.

Anita Jagota
Editor

# Sleep and Clocks in Aging and Longevity

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# Preface

Sleep is organized through intricate interactions between various brain regions responsible for normal neural functions, such as cognition, attention, memory and emotions influencing appetite, libido, mood and behavior. The circadian timing system (CTS) regulates the timing and duration of sleep keeping an approximately 24-h internal rhythm that entrains to environmental stimuli and the sleep homeostat guiding sleep drive. Aging is linked with the progressive deterioration of the behavioral, biochemical, physiological, morphological and anatomical aspects of an organism. Both sleep and circadian rhythms show robust perturbances with age affecting sleep and wakefulness "flip-flop" switch.

Modern lifestyle, however, poses a paradox: on the one hand, there is an increase in lifespan, and on the other, demanding social pressures result in insufficient sleep. While sleep is essential for human health and longevity, changing sleep patterns with age significantly affect the daily functioning and quality of life by altering key homeostatic processes, resulting in neurodegeneration and a variety of diseases.

This book is a compendium of 25 chapters contributed by leading researchers from across the globe. Each chapter is designed to offer a comprehensive and critical review of the topic. The book is divided into seven parts: Part I: Understanding Sleep and Clock Interlink in Health and Longevity, deals with basic understanding of sleep homeostasis and circadian timing system and how these interactions change with aging; Part II: Sleep, Aging and Longevity, deals with changing sleep physiology influencing aging and longevity; Part III: Clock, Aging and Longevity, deals with alterations in the circadian clock and pineal gland physiology with aging and role of nonphotic cues in healthy aging; Part IV: Melatonin, Sleep and Clock, deals with multitasking hormone melatonin changes with aging and its role in restoring such changes toward healthy aging and longevity; Part V: Genetic Regulation of Sleep and Clock, deals with chronotypes, epigenetic regulation and role of CNS insults in restoration of behavior with aging; Part VI: Therapeutic Interventions in Sleep Disorders and Clock Misalignment, deals with restoring age-induced misaligned clocks with physical exercises, specific chrononutrition methods, achieving healthy aging in light polluted modern world, neurodegeneration linked with circadian rhythm disruptions and sleep cycles as well insomnia in elderly and treatment; and Part VII:

Experimental Models to Study Sleep and Clocks in Aging and Longevity, deals with various experimental systems used in research for circadian dysfunction and sleep disorders, and possible interventions.

It is my hope that these expert insights into the changing harmony of the interplay between sleep and circadian rhythms with aging, responsible for a variety of diseases and associated therapeutic interventions, will make an important step toward driving and underpinning novel advances in treatments for sleep disorders and clock misalignment. The target readership is advanced undergraduate and graduate students, postgraduate researchers and medical practitioners.

I wish to extend my deep gratitude to Prof. Suresh Rattan, editor of the book series *Healthy Ageing and Longevity*, for his constant support and encouragement, and also my heartfelt thanks to my family for their affection and support.

Hyderabad, India Anita Jagota

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# Part I
# Understanding Sleep and Clock Interlink in Health and Longevity

# Chapter 1
# Sleep and Circadian Clock: Novel Players in Health Impacts and Aging


**Anita Jagota**

## 1.1 Introduction

Sleep is thought to be a homeostatically regulated process. This homeostatic process tracks the buildup of sleep need as a function of time spent awake. Sleep is essential for proper brain function in mammals. During sleep, animals are disconnected from the external world; they show high arousal thresholds and changed brain activity. Thus, sleep (fascinating behavioral state) despite these risks to organisms must have evolved with vital benefits to the organism whose function still needs to be completely understood. Sleep deprivation results in a sleep rebound (Siegel 2005; Deboer 2018). The primary function of sleep appears to be the downscaling of synapses that have been built up during wakefulness. Thus, brain homeostasis is maintained, and learning and memory are assured through sleep. Wakefulness and sleep are regulated by multiple brain regions such as the ventrolateral preoptic area (VLPO) of the anterior hypothalamus, the locus coeruleus (LC) of pons and the lateral hypothalamus (LH). Of these, VLPO plays most prominent role. Genes involved in sleep control code for ion channels, factors influencing neurotransmission and neuromodulation, and proteins involved in the circadian clock. The neurotransmitters/neuromodulators such as GABA, dopamine, acetylcholine, serotonin and several neuropeptides involved in sleep control (Crocker and Sehgal 2010).

The adaptation of organisms to a rhythmic environment is mediated by an internal timing system termed as circadian (circa = about and dies = day) clock. Such a circadian timing system (CTS) regulates timing and duration of sleep keeping an approximately 24 h internal rhythm that entrains to environmental stimuli, and the sleep homeostat, which rises as a function of time awake, guides sleep drive (Cirelli 2009). In mammals, circadian clock network is found in all tissues and organs.

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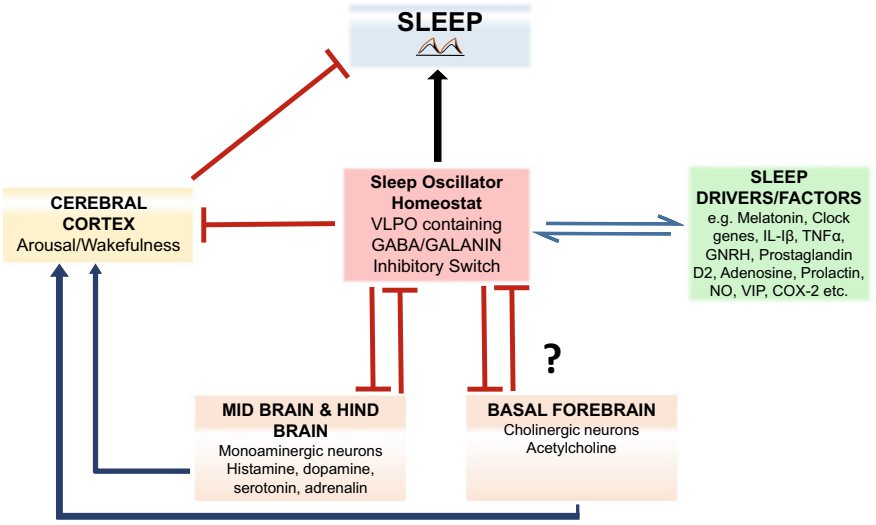


**Fig. 1.1** Schematic drawing showing various brain centers and the various neurotransmitter and molecular components involved in regulation of sleep

These networks regulate the release of many hormones, which regulate several physiological functions. Extensive physiological and behavioral studies have indicated that the circadian pacemaker is localized to discrete sites in central nervous system (CNS) and, in mammals, to the bilaterally paired suprachiasmatic nucleus (SCN) in hypothalamus just above the optic chiasm (Jagota et al. 2000; Welsh et al. 2010). The interplay between sleep homeostasis and the circadian clock regulates sleep. Putative sleep-wake centers are located in higher-order brain centers that are indirectly connected to the circadian clock network (Fig. 1.1).

Aging is the progressive deterioration in the behavioral, biochemical, physiological, morphological and anatomical aspects of an organism (Jagota 2012; Panagiotou et al. 2021). The aging process is linked with profound disruption of an individual's daily sleep-wake cycle with increased daytime napping, advanced sleep timing, reduced overall sleep, fragmented sleep and prolonged sleep-onset latency, etc. Disruption of sleep and circadian rhythms with aging causes many diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), cancer, inflammatory bowel disease (IBD) etc. Aging and circadian rhythms were linked initially by studies on circadian rhythms of release of hormones such as cortisol, thyroid stimulating hormone (TSH), melatonin, prolactin, growth hormone (GH) and sleep pattern of young and healthy elderly men. Sleep-wake cycles are under strict circadian control with strong influence of rhythmic hormones such as melatonin (Koop and Oster 2021). The mechanisms of sleep homeostasis and the circadian system become less robust with normal aging.

Sleep and circadian rhythms show perturbances with age. The alterations in sleep and circadian dysfunction are linked to premature aging and appear as overt hallmark

for several underlying diseases. It is therefore very important to understand link between sleep and circadian rhythms.

## 1.2 Sleep

Sleep appears to be an essential part of animal life. Organisms are disconnected from the external world during sleep state due to elevated sensory thresholds, at considerable potentially life-threatening risks and costs to the individuals. Further, during sleep, animals cannot forage or take care of their young ones.

### *1.2.1 What is Sleep?*

Sleep is a fundamental and evolutionarily conserved biological phenomenon. Humans spend about one-third of their life sleeping, and many other mammalian species such as (o)possums (*Didelphis marsupialis*, *Lutreolina crassicaudata*, *Trichosurus vulpecula*), kangaroos (*Megaleia rufa*) or kangaroo rats (*Potorous apicalis*), tree shrews (*Tupaia glis*), hedgehogs (*Erinaceus europaeus*, *Paraechinus hypomelas*), bats (*Eptesicus focus*, *Myotis lucifugus*), beavers (*Aplodontia rufa*), chipmunks (*Tamias striatus*), golden hamsters (*Mesocricetus auratus*), gerbils (*Meriones unguiculatus*) and rats (*Rattus norvegicus*) spend more than half of their life sleeping. Some animals such as elephants (*Elephas maximus*, *Loxodonta africano*), horses (*Equus caballus*), donkeys (*E. asinus*), tapirs (*Tapirus terrestris*), cows (*Bos taurus*) and sheep (*Ovis aries*) have been reported to be short sleeper with consolidated sleep periods of only 3–5 h per 24 h cycle. Thus, the function of sleep must be very important for the organism. There is impaired cognitive performance observed after only one day of sleep deprivation, and longer sleep deprivation results in more complex brain dysfunction resulting in hallucinations and alalia (speech delay), etc., indicating the importance of sleep as reported by several researchers (Helfrich-Forster 2018; Garbarino et al. 2021). Sleep, though seemingly passive, is actually a critically active stage of the day. It is a period essential for growth, differentiation and renewal of cells, and it plays an important role in immunity (Irwin 2019; Zielinski and Gibbons 2022). Adequate sleep is pivotal for human health, and inadequate sleep contributes to the development of disease. This is demonstrated by numerous studies on the consequences of inefficient sleep, in which impaired sleep is associated with infectious disease, increased risk of cardiovascular disease, mental illness and cancer (Kecklund and Axelsson 2016). Inadequate sleep not only includes insufficient duration, poor sleep quality or the presence of sleep disturbances, but may also be due to inappropriate sleep timing (Roenneberg and Merrow 2016).

Sleep is characterized by three main behavioral criteria: first, a period of quiescence associated with a species specific posture and/or resting place, which is typically accompanied by reduced motor activity; second, an elevated response threshold

(i.e., a stronger threshold is needed to produce a response); third, a homeostatic regulatory mechanism which is manifested in a sleep rebound after periods of sleep deprivation. Sleep is distinguished from quite wakefulness by the reduction in the ability to react to stimuli, while the reversibility to an awake state distinguishes sleep from coma (Siegel 2005).

### *1.2.2 Physiological Basis of Sleep*

Sleep is ubiquitous and associated with a set of pharmacological, electrophysiological and molecular characteristics. Though ubiquitous, the adaptive value of sleep function is linked to energy conservation and normal neural function, including neural maintenance, neurogenesis, restoration at the cellular and network levels, memory consolidation and synaptic homeostasis, including synaptic plasticity and homeostatic synaptic downscaling, etc. Further, in mammals, sleep is realized as important and necessary as it not just clears toxins from the brain, cherishes the body cells, helps learn and memorize but also plays vital role in regulating appetite, libido, mood and behavior (Vyazovskiy et al. 2008).

It has been reported by some workers that various types of behavior and memory formation/storage involve transcriptional and translational processes that kicks in occur within a specific time window following a learning event (Peixoto et al. 2015).

The measure of the electrical activity of the cerebral cortex called electroencephalogram (EEG) provides the primary electrophysiological characteristics that are used to define different stages of sleep as well as to distinguish sleep from wakefulness. EEG activity is the product of intrinsic electrical rhythms generated within the cortex and a dynamic interplay between the thalamus and the cortex (Steriade 2006). In contrast, the transitions between, and duration of, different sleep and behavioral states are regulated by subcortical waking and sleep active brain regions. These structures include the orexin/hypocretin-containing (Hcrt) neurons in the tuberal hypothalamus, histaminergic tuberomammillary nuclei (TMN), noradrenergic LC, serotonergic raphe nuclei, cholinergic basal forebrain (BF) and GABAergic VLPO (Saper 2013; Schwartz and Kilduff 2015), as well as the circadian pacemaker in SCN (Figs. 1.1 and 1.2). The homeostatic mechanism reflects the need for sleep that accumulates during prolonged periods of wakefulness (Fig. 1.3).

Sleep is regulated by circadian and homeostatic mechanisms which are partly independent. The circadian system plays an important role in the timing and consolidation of sleep to an ecologically appropriate period such that diurnal animals sleep during the night and nocturnal during the day (Roenneberg and Merrow 2016). The chronotype also plays very important role often categorized by the terms early (larks) or late (night owls). The larks and night owls interact with the environment differently and may therefore experience different degrees of circadian rhythm disruption during sleep (Harfmann et al. 2020).

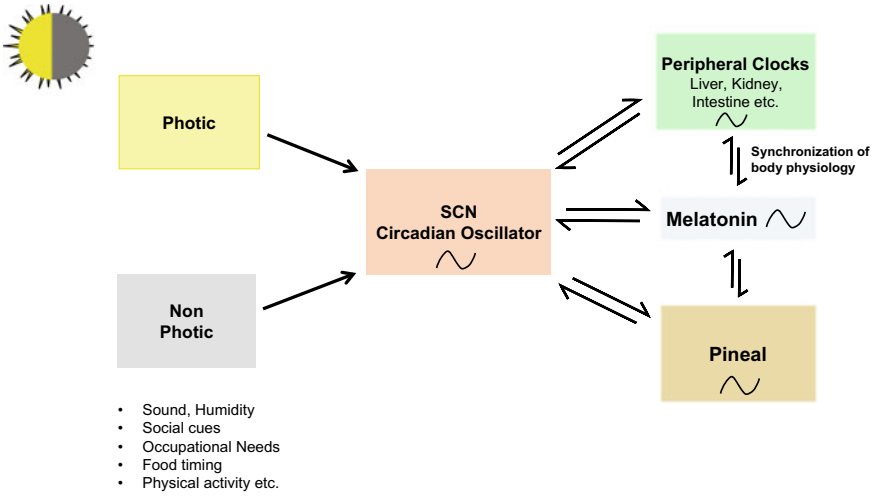


**Fig. 1.2** Schematic drawing showing suprachiasmatic nucleus regulating the peripheral clocks through photic and non-photic signals

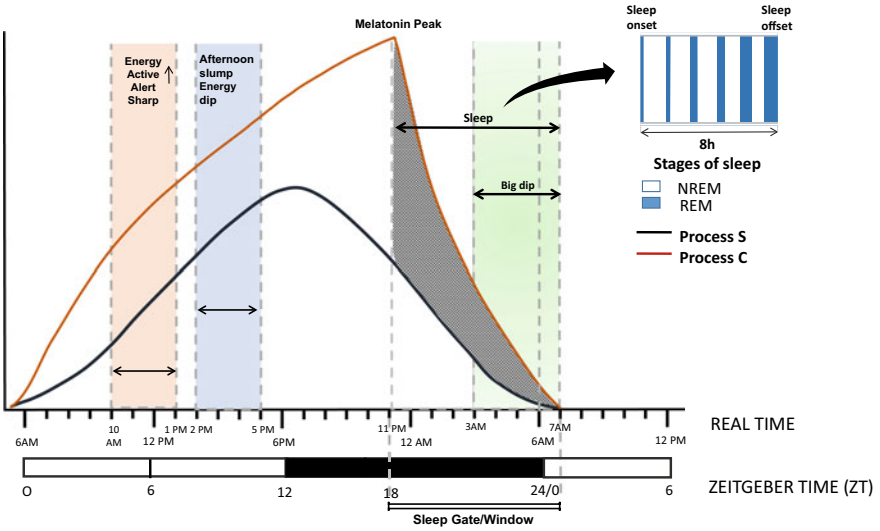


**Fig. 1.3** Diagram showing interplay between sleep and circadian clock: interaction of Process S and Process C during the 24 h day

## *1.2.3 Types of Sleep/Different Stages of Sleep*

The sleep is inhomogeneous characterized by two behaviorally and physiologically distinct phases. Distinct neural circuits have been involved in the synchronization

and desynchronization of cortical activity that distinguish non-rapid eye movements (NREM) sleep from wakefulness and rapid eye movements (REM) sleep. Input from the BF, likely from both cholinergic and non-cholinergic neurons, is critical for the desynchronized EEG characteristic of wakefulness and REM.

EEG activity reflects the aggregate firing of large neuronal ensembles and is explained by various bandwidths with the specific frequencies: such as alpha (9–12 Hz), beta (12–30 Hz), delta (0.5–4.0 Hz), low (30–60 Hz) and high (60–100 Hz) gamma and theta (5–9 Hz) (Schwartz and Kilduff 2015). Sleep occurs as wake, NREM and REM. Thus, first phase is characterized by NREM sleep, i.e., deep or slow-wave sleep. Within NREM, there are four stages, ranging from Stage 1 (the lightest level with low voltage and spindles) to Stages 2 and 3 and 4, i.e., deep or slow-wave sleep. After deep sleep, the body moves into a period of REM sleep and brain activity is close to the same as when awake stage, but muscles cannot move, except for eyes and diaphragm. This is the stage where dreaming occurs (Patel et al. 2021).

### *1.2.4 Sleep Duration/Need*

It is not yet clearly understood how much sleep is actually required. Though, sleep duration/need varies across the population and is recommended as 7–9 h (Culnan et al. 2019). Interestingly, the sleep EEG is remarkably stable for an individual and is like a trait-like pattern (Tucker et al. 2007). Based on the rebound, or compensatory sleep, that follows sleep deprivation, sleep is thought to be an essential process whose amount is controlled by a homeostatic system (Dauvilliers et al. 2005).

#### 1.2.4.1 Sleep is Said to Be Cumulative

To a limited extent, yes. Several workers have reported that after an ordinary night's sleep, subjects taking an extra nap/siesta in the afternoon worked through the night with greater alertness (Duval and Haupais 2019). Additionally, extended sleep on weekends may be helpful in fighting insufficient sleep syndrome (ISS) (Baugmann-Vogel et al. 2021).

#### 1.2.4.2 Sleep Debt

It is the difference between the amount of sleep required and actually one gets. It is a deficit that grows every time one skims some extra minutes off one's nightly slumber. Interestingly, sleep debt can be repaid—though it will not happen in one extended snooze session. Tacking on an extra hour or two of sleep a night is the way to catch up (Schwartz and Kilduff 2015).

#### 1.2.4.3 Sleep Bank Theory

According to Sleep Bank theory, the "banking" of sleep prior to sleep loss may help sustain performance and alertness in operational environments and speed recovery. After a second consecutive night without sleep, all of the subjects performed equally badly, regardless of how much sleep they had initially. It may be that normally everyone is slightly sleep-deprived and one really needs good night's sleep to bring one back up to 100%, and that the "tank/bank" is not big enough to buffer us against more than one all-nighter (Rupp et al. 2009).

### *1.2.5 Regulation of Sleep*

Sleep impacts every aspect of brain functions. It is therefore essential to understand sleep and wakefulness "flip-flop" switch at various levels that ensures behavioral-state stability with a perfect and coordinated equilibrium in the mental, emotional and physiological activities.

#### 1.2.5.1 Sleep Regulation at Neurotransmitter Level

Several chemical mediators have been identified in driving sleep. However, sleep induction is driven by rapid reduction in arousal which has been linked to primarily an inhibitory switch controlled by GABA/Galanin-containing neurons in the VLPO region in hypothalamus (Fig. 1.1). Various monoamine neurotransmitters such as histamine, dopamine, noradrenalin and serotonin as well as cholinergic neurotransmitter acetylcholine promote wakefulness. The awakening process is further activated by orexin/hypocretin-producing neurons in LH (Cirelli 2009). Within the preoptic hypothalamus, various other neuronal subtypes glutamate/NOS1 induce NREM sleep in addition to GABA/Galanin (Ma et al. 2019). Additionally, adenosine has been identified as a strong marker candidate regulating sleep. Adenosine increases during the waking hours as well as after sleep deprivation (Saper 2013).

#### 1.2.5.2 Sleep Regulation at Gene Level

There are multiple genes, including *per*2 and *dec*2 clock genes which have been identified to regulate sleep homeostat and clock pacemaker (Chang et al. 2016). Various sleep-regulating molecules have been identified (Table 1.1). Many researchers are focusing on genetic screens as well as genetic manipulation of candidate genes so as to understand changes in sleep amount as a readout of sleep homeostasis and therefore to assess how loss or gain of a specific function affects sleep quantity (Crocker and Sehgal 2010).

**Table 1.1** Various genes involved in the circadian timing system

| Clock genes | Function | References |
|---|---|---|
| *Per1* | Negative regulator | Takahashi (2017) |
| *Per2* | Negative regulator | Takahashi (2017) |
| *Per3* | Negative regulator | Cox and Takahashi (2019) |
| *Cry1* | Negative regulator | Takahashi (2017) |
| *Cry2* | Negative regulator | Li et al. (2022) |
| *Bmal1 (Arnt1)* | Positive regulator | Okamura et al. (2002) |
| *Clock* | Positive regulator | Gul et al. (2022) |
| *Rorα* | Positive regulator | Sato et al. (2004) |
| *Rev-erbα* | Negative regulator | Sato et al. (2004) |
| *Few other players* | | |
| CK1 | Regulator | Zhang et al. (2021) |
| PP1, PP4 & PP5 | Regulator | Klemz et al. (2021) |
| NPAS2 | Regulator | Mosig et al. (2021) |
| PK2 and AVP | Regulator | Samoilova et al. (2021) |
| *Various clock genes involved in sleep dysfunction* | | |
| *Per2* | Sleep deprivation | Hou et al. (2019) |
| *Per3* | DSWPD, poor sleep quality | Peng et al. (2022) |
| *Per* | Duration of sleep in *Drosophila* | Fropf et al. (2018) |
| *Cry2* | Sleep latency, sleep disturbance | Lou et al. (2021) |
| *Bmal1* | ↓ REM sleep | Niu et al. (2022) |
| *Clock* | ↓ Sleep duration upon ageing | Lou et al. (2021) |
| *Rev-erb α & β*(*Nr1d1* and *Nr1d2*) | DSWPD | Haraguchi et al. (2019) |
| *NPAS2* | ↑ Sleep deprivation | Bolsius et al. (2021) |
| *DEC2* | ↓ Sleep duration | Ashbrook et al. (2020) |
| *Ck1 δ* | ASWPD | Xu et al. (2005) |
| *PK2* | ↓ Total sleep time | Hu et al. (2007) |

(continued)

**Table 1.1** (continued)

| Clock genes | Function | References |
|---|---|---|
| *Hcrtr1 & Hcrtr2*(*OX1 or OX2*) | Narcolepsy | Schwartz and Kilduff (2015) |

#### 1.2.5.3 Sleep Regulation at Epigenetic Level

Both SCN and peripheral tissues are subject to epigenetic modulations. The "circadian epigenome", created by the action of the clock, temporally as well as locally drives the DNA to become permissive to a rhythmic transcription, thus creating a "circadian transcriptome". Several workers have reported that there are highly dynamic rhythmic changes in chromatin transitions. The epigenome plays a critical role in regulating gene expression in the context of memory storage and long-term potentiation toward formation of memories occurring during sleep. Changes in histones can mediate epigenetic gene regulation. Further, there is substantial evidence to suggest that DNA methylation is critically affected by sleep (Narwade et al. 2017). There is also substantial evidence linking the methylation status of circadian clock genes and sleep loss (Tabibzadeh 2021).

Much of the interindividual variability in the sleep EEG has been reported to be driven by genetics (Landolt 2011) and is linked to PER2 variant. Additionally, polymorphism at rs4753426 in a melatonin receptor is linked with more time in bed on weekends and another variant rs7942988 is linked to impact duration of melatonin (Silva et al. 2019).

## 1.3 Biological Clock: Circadian Timing System (CTS)

In mammals, SCN contains the central clock that synchronizes physiology, behavior and metabolism to the external environmental cues (zeitgebers). These clocks are periodically synchronized to the geophysical time. Photoperiod is the most dominant environmental zeitgeber (time giver) for the phase entrainment of the circadian oscillator (Fig. 1.2). There are various review articles giving details of components of CTS (Takahashi 2017).

### 1.3.1 SCN: Neurotransmitters in Input and Output Pathways

SCN is a cluster of about 20,000 neurons that receive information of time cues from the external surroundings via three major afferent or input pathways: the retino-hypothalamic tract (RHT), the geniculo-hypothalamic tract (GHT) and the retino-raphe pathway (RRP). Among these three pathways, RHT mediates photic signals, whereas GHT and RRP mediate non-photic signals (Dibner et al. 2010).

SCN receives light exposure directly from the eyes via melanopsin-containing ganglion cells in retina through the retino-hypothalamic tract (RHT) by releasing glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) which causes entrainment of clock gene expression in the SCN. The communication from the SCN is majorly via neurotransmitters such as GABA and glutamate; however, there are number of other molecules such as AVP, VIP, prokineticin 2 (PK2), cardiolipin like cytokine and transforming growth factor α (TGFα) which are output signals of the SCN (Roenneberg and Merrow 2016; Takahashi 2017).

### 1.3.2 SCN: Relay Center for Information

In mammals, the circadian system is comprised of a hierarchy of oscillators, in which SCN of the hypothalamus is regarded as the master clock regulating downstream oscillators in peripheral tissues called as peripheral clocks such as liver, kidney, intestine (Ko and Takahashi 2006). The master pacemaker coupled population of neuronal circadian oscillators perceives the external photic cues (light) and in turn synchronizes the peripheral clocks.

Every mammalian cell is autonomous and has its own clock machinery constituting the peripheral clock system controlled by the SCN through both sympathetic and parasympathetic pathways (Schibler et al. 2015). Thus, SCN plays a major role in orchestrating cellular and metabolic processes by relaying temporal information to the entire body via humoral and neural communication (Hastings et al. 2018; Walker et al. 2020).

### 1.3.3 Melatonin: Messenger of Darkness

Melatonin (internal zeitgeber, hormonal message of darkness) biosynthesis and secretion from pineal gland are directly regulated via a multi-synaptic pathway by the SCN (Majidinia et al. 2018). The electrical information is converted into chemical information that alters the phase of clock gene expression in a subset of SCN neurons which then regulates both synthesis and release of melatonin (hormonal message for darkness) from pineal (Jagota 2012). In this multi-synaptic neural circuit, GABAergic axons of SCN neurons project to hypothalamic PVN. Efferents from PVN descend

via the brain stem to spinal cord and synapse with preganglionic sympathetic neurons of the inter-mediolateral cell column (ILCC). Axons of these nerve cells project onto a set of cells in superior cervical ganglion (SCG) eventually terminating in pinealocytes. Release of norepinephrine from superior cervical post-ganglionic neurons stimulates melatonin's synthesis and release (Jagota 2012; Majidinia et al. 2018).

This pathway is actually activated during night without light stimuli. In pinealocytes, tryptophan is converted into neurotransmitter serotonin (5-hydroxy tryptamine; 5-HT) via 5-hydroxytryptophan. Then *N*-acetylation of serotonin by arylalkylamine *N*-acetyltransferase (AANAT) followed by methylation of the 5-hydroxy moiety by hydroxyindole-O-methyl-transferase (HIOMT) results in melatonin synthesis. Melatonin is then secreted into circulation and also to cerebrospinal fluid (CSF) of the third ventricle that influences the master clock via melatonin membrane receptors (Jagota 2006; Majidinia et al. 2018). Activation of AANAT results in a ten-fold increase in melatonin synthesis and secretion, approximately 5–6 h after the onset of night. Melatonin receptors are G protein-coupled receptors with two types of G proteins (Gi (inhibitory—activates $K^+$ channels, inhibits adenylate cyclase) and Go (inhibits $Ca^{2+}$ channels)). G proteins activate AC which in turn activates second messenger molecules for regulation of various physiological functions. In mammals, three types of melatonin receptors have been identified: MT1 (or Mel1A or MTNR1A), MT2 (or Mel1B or MTNR1B) and MT3 (or Mel1C or MTNR1C) (Sugden et al. 2004). Further, SCN is rich in CaMKII, and it is known to be involved in transmission of photic information and phase resetting of the circadian clock upon light exposure. Phosphorylation of CaMKII is rhythmic both under free-running and entrained conditions with peak levels during the subjective day (Agostino et al. 2004).

In addition to pineal gland, extra pineal sites including retina, lacrimal gland, skin, Harderian gland, ovary, lymphocytes, bone marrow and most importantly gastrointestinal tract are known to synthesize melatonin. Based on the site of its synthesis and the organ/tissue of target, melatonin can function as a biological modulator, neurotransmitter, hormone or cytokine (Slominski et al. 2012).

### *1.3.4 Molecular Components of CTS*

Circadian rhythms impose daily cycles to many behaviors and physiological processes in a wide variety of organisms. In mammals, such rhythms are regulated by SCN (Hardin 2011). It involves various core clock genes such as CLOCK, BMAL1, Periods, Cryptochromes, Rev-erbα, Rorα whose expression is orchestrated by transcriptional and translational feedback loops that eventually result in 24 h periodicity (Table 1.1).

Thus at cellular level, the transcriptional activators such as CLOCK (and its paralogue NPAS2) and BMAL1 (also referred as ARNTL) along with several other dedicated transcription factors exist at the core of these feedback loops. At the beginning of a subjective day, BMAL1-CLOCK heterodimer binds to the E-box elements

of *Period (Per1, 2, 3), Cryptochrome (Cry1, 2)* along with several clock-controlled genes (CCGs) initiating their transcription. Upon reaching critical levels toward the end of subjective day, PER-CRY proteins interact, hetero-dimerize and translocate to the nucleus during the subjective night to block BMAL1-CLOCK activity. This leads to the repression of their own transcription and also of other CCGs (Takahashi 2017).

Phosphorylation of PER-CRY heterodimer by serine/threonine kinases such as casein kinase 1ε (CK1ε) and casein kinase 1δ (CK1δ) plays key role by governing the stability and localization of these clock elements. Phosphatases PP1 and PP5 are in turn regulated by casein kinases. The E3 ubiquitin ligase complexes regulate turnover of PER and CRY proteins by targeted ubiquitylation resulting in proteasome-mediated degradation. As the repression on CLOCK-BMAL1 gets relieved upon degradation of repressor complex, the cycle commences again with a periodicity of 24 h. Along with the core CLOCK-BMAL1/PER-CRY loop, auxiliary loops involving *Retinoic acid receptor-related orphan receptor alpha (Rorα), Rorβ* and *Rev-erbα* also known as *Nr1d1* (nuclear receptor subfamily 1, group D member 1), *Rev-erbβ* (*Nr1d2*) further function to stabilize the clock mechanism. RORs and REV-ERBs are directly under the transcriptional regulation of CLOCK-BMAL1. ROR-responsive elements (RREs) present in the *BMAL1* promotor region are the targets for these factors wherein REV-ERBs suppress and RORs activate *BMAL1* transcription. This leads to the rhythmic expression of BMAL1 in antiphase with rhythmic PER expression. In addition to these, RORs and REV-ERBs rhythmically regulate the repressor nuclear factor interleukin 3 (*Nfil3*) contributing to another auxiliary loop. NFIL3 in turn represses D-box binding protein (DBP) to modulate rhythmic ROR expression. These three interlocked TTFLs together generate robust transcriptional rhythms underpinning the 24 h circadian machinery (Takahashi 2017; Honma 2018).

Interestingly, sirtuin 1 (SirT1) has been linked recently to the circadian rhythm machinery through direct deacetylation activity as well as through the NAD + salvage pathway (Majidinia et al. 2018). In addition, the post-translational modifications of clock proteins are important for ensuring the maintenance of circadian rhythms, as they can modulate the activity and turnover of major clock components (Bellet and Sassone-Corsi 2010).

Further, such transcriptional machinery/core circadian clock is present in most cells in the body including neurons and astrocytes in SCN and brain. The core circadian clock regulates the circadian expression of thousands of genes in tissue-specific manner and is a major regulator of cellular metabolism, stress response and many other functions (Musiek 2015).

## 1.4 Sleep and Circadian Rhythms: Interplay

The sleep–wake cycle, an evolutionary conserved neurobiological phenomenon, is a prominent manifestation of the biological clock (Sehgal and Mignot 2011). SCN

consolidates the sleep–wake cycle by generating a signal of arousal during the active period and thus can alter baseline sleep amount. The role of the circadian clock in the regulation of sleep has been extensively studied, but the role of sleep in the regulation of circadian clock rhythms is not much understood. Sleep and clock dysfunction is linked with several disorders (Table 1.2a–c).

Recent data suggests that clock genes outside the SCN are involved in fundamental brain processes such as sleep/wakefulness, stress and memory. The role of clock genes in these brain processes are complex influencing sleep, stress, memory, etc.

### *1.4.1 The Two-Process Model: The Interaction of Circadian Forces and Sleep Homeostasis*

The two-process model, first described by Borbely (1982), puts forth a description of sleep regulation that relies on both the circadian system (termed Process C) and sleep homeostasis (termed Process S) (Fig. 1.3). Process C is dependent on the ~24 h rhythmic variation of propensity to sleep, and this is balanced with Process S, which increases as a function of time awake. Process S is estimated by EEG slow-wave activity and has an exponential decline during sleep. The model posits that it is the interaction between Process C and Process S that determines time to wake and time to sleep. It explains that circadian factors help stay awake throughout the day as sleep pressure, modeled by Process S, builds up and also helps stay asleep in the latter part of the night once this sleep pressure has largely declined. Sleep need continuously increases during wakefulness and is reset to time zero only after proper sleep. Sleep pressure also explains why more time awake can lead to more and deeper sleep (Ashbrook et al. 2020).

### *1.4.2 Sleep Gate*

The interaction between Process S and Process C has been named as “sleep gate”. Sleep pressure within the sleep gate will be highest during the initial phase of night but is increasingly reduced as homeostatic drive for sleep is dissipated. The melatonin-concentrating hormone/GABAergic cells in LH “gate” REM sleep. The temporal distribution of sleep and wakefulness is due to interaction between the circadian system and the sleep homeostatic system (Schwartz and Kilduff 2015; Wang et al. 2021).

**Table 1.2** **a** Various disorders linked to sleep dysfunction. **b** Age induced disorders linked to circadian dysfunction. **c** Age related disorders showing both sleep and circadian dysfunctions

a

| Disorders | Sleep dysfunction | References |
|---|---|---|
| CRSDs | Misaligned endogenous CTS; perturbed homeostasis; ASWPD; DSWPD; sleep fragmentation irregular sleep, SDB, PLMS, RLS, Narcolepsy etc. | Culnan et al. (2019) |
| *Neurodegenerative diseases* | | |
| PD | Perturbed sleep architecture; RBDs; ESD; insomnia ↑; SBDs; OSA; RLS; arousal and orexin system ∿; sleep time ↓, total wake time ↑; sleep efficiency↓, awakenings ↑; hypersomnia | Yang et al. (2018) |
| AD | Nocturnal awakenings ↑ daytime sleep bouts ↑; REM sleep; NREM sleep ∿, sleep fragmentation, SDBs, insomnia, EDS; orexinergic, glutamatergic, γ-aminobutyric acid systems and the circadian rhythm ∿ | Spinedi and Cardinali (2019) |
| HD | Insomnia; fragmented sleep; SWS ∿; NREM sleep γ-frequency↑; daytime sleepiness ↑; sleep efficiency ↓; sleep latency ↑ | Ogilvie et al. (2021) |
| Insomnia | Sleepiness ↑; circadian and homeostatic sleep ∿; hypothalamic–pituitary–adrenal (HPA) axis ∿ | Van Someren (2021) |
| Dementia | Day nap ↑; night awakenings ↑; sleep time ↓; sundowning ↑; insomnia; OSA; RBD; EDS; circadian dysregulation of sleep | Shi et al. (2018) |
| Mood disorder and depression | SWS ↓; REM sleep distribution ∿, REM latency ↓, REM density ↑; total REM sleep time ↑; insomnia; OSA; circadian rhythms and sleep wake architecture and mechanism ∿ | Riemann et al. (2020) |
| *Other disorders* | | |
| Metabolic disorder: IBD | Sleep characteristics ∿; rest-activity rhythms ∿; sleep/wake activity ∿; IBD severity ↑ with insomnia ↑; night time awakenings ↑; polysomnographic patterns ∿ | Sobolewska-Włodarczyk et al. (2021) |
| Cancer | Latent, moderate and severe insomnia; RLS; sleep apnea; PSQI ↑; daytime sleepiness ↑ | Starreveld et al. (2021) |

(continued)

**Table 1.2** (continued)

a

| Disorders | Sleep dysfunction | References |
|---|---|---|
| Cardiovascular disease | Narcolepsy; sleep apnea; cardiac arrhythmias during sleep ∿; RLS; inflamation | Wang et al. (2021) |
| Immune dysfunction | Fragmented sleep–wake cycles and premature REM episodes; REM sleep fragmentation; sleep stages ∿; SWS ∿; EDS; RBD; narcolepsy; susceptibility to infections ↑ with sleep deprivation/reduction | Irwin (2019) |
| Respiratory diseases | Sleep efficiency ↓; fragmented sleep; REM sleep ↓; NREM sleep episode ↓; SWS ∿; daytime sleepiness ↑; restless sleep; OSA; SDB; insomnia; sleep latency ↑, total sleep time | Adir et al. (2021) |

b

| Aging disorders | Circadian dysfunction | References |
|---|---|---|
| *Sleep disorders* | | |
| DSWPD | Intrinsic circadian period length ↑; nocturnal light exposure hypersensitivity; melatonin ↓ | Auger et al. (2015) |
| PLMS | Circadian rhythm ∿ | Duffy et al. (2011) |
| RLS | Circadian impairment; daytime dysfunction ↑; dynamic perturbations in dopamine system during circadian cycle; circadian blood pressure ∿ | Romigi et al. (2014) |
| SDB | Circadian clock ∿; circadian rhythm of distal skin temperature (DST) | Martinez-Nicolas et al. (2021) |
| ASWPD | Advanced sleep–wake phase; chrono-disruption | Basit et al. (2021) |
| Narcolepsy | Altered circadian autonomic function; circadian rhythm ∿ | Sorensen et al. (2013) |
| *Neurodegenerative disorders* | | |
| PD | Nocturnal core-body temperature ↓; diurnal activity ↓; nocturnal activity ↑; daytime motor deficits; reversed circadian rhythm; sympathetic and autonomic system ∿; circadian and sleep regulation ∿; clock gene expression ↓; *Bmal-1*and *Bmal-2* levels ↓; melatonin and cortisol ∿ | Yang et al. (2018) |
| AD | Rest–activity ∿; oxidative stress ↑; inflammation ↑; cerebral blood-flow rhythm disorder; circadian metabolic dyshomeostasis ↑; glymphatic system ∿; BMAL1 circadian oscillation ∿ | Uddin et al. (2021) |

(continued)

**Table 1.2** (continued)

b

| Aging disorders | Circadian dysfunction | References |
|---|---|---|
| HD | Diurnal and circadian locomotor activity rhythms ∿; CRSDs; EDS; circadian clock gene expression ∿; *Per2* and *Bmal1* expression ↑; autonomic dysfunction; core body temperature ∿ | Diago et al. (2018) |
| Dementia | Circadian amplitude ↓, acrophase ↓, inter-daily stability ↓ and intra-daily variability ↑; circadian clock genes ∿; body temperature rhythm (advanced); day-night activity and fractal activity patterns ∿ | Maiese (2021) |
| Insomnia | CRSDs; altered circadian sleep; light and dark cycle ∿; feeding-fasting cycle ∿; rest-activity cycle ∿ | Nobre et al. (2021) |
| *Other disorders* | | |
| Metabolic disorder: IBD | Rest-activity cycles ∿; colon tissue core clock genes ∿; circadian associated mitochondrial dysfunction; phase shift exacerbated colitis; expression of circadian genes ↓; rest-wake activity ∿ | Gombert et al. (2019) |
| Cancer | Daily sleep-activity cycles ∿; cortisol rhythm ∿; circadian clock ∿; PER2 proteins loss alters chemotherapy drug efficacy | Jensen et al. (2021) |
| Cardiovascular disease | Circadian clock ∿; 24-h rhythm repolarization variation ↑; heart rate and blood pressure ∿ in *Bmal1*-knockout mice; endothelial dysfunction in *Per2*-knockout mice | Crnko et al. (2019) |
| Immune dysfunction | Circadian oscillations of the immune genes ∿; systemic circadian control of the immunity ∿ | Zielinski and Gibbons (2022) |
| Respiratory diseases | Phasic responsiveness to inflammation ∿; circadian rhythms allergic and non-allergic asthma; clock gene expression ∿; free-running periodicity of daily activity ↓ | Nosal et al. (2020) |

c

| Disorders | Sleep and circadian dysfunctions | References |
|---|---|---|
| *Neurodegenerative disorders* | | |
| PD | Circadian clock ∿; peripheral clocks ∿; sleep ∿; REM sleep ↑; insomnia and RBD | Gros and Videnovic (2020) |
| AD | Sleep deprivation ↑; circadian clock ∿; sleep cycle ∿; diurnal activity ↓; nocturnal activity ↑; sleep duration ↓; wakefulness ↑; hypothalamic dysregulation ∿ | Uddin et al. (2021) |
| HD | Delayed sleep onset; fragmented sleep; sleep efficiency ↓, frequent awakening ↑, delayed REM sleep onset | Voysey et al. (2021) |

(continued)

**Table 1.2** (continued)

c

| Disorders | Sleep and circadian dysfunctions | References |
|---|---|---|
| *Other disorders* | | |
| Metabolic disorder: IBD | Leptin rhythm ∿; sleep deprivation, sleep homeostasis ∿, circadian rhythm ∿ | Gombert et al. (2019) |
| Cancer | Orexin neurons night activity ∿; MCH neuron day activity ∿; cortisol ↑; melatonin ↓; daily sleep behaviour variations; day levels ↑: IL1β, IL6, TNF-α; night levels ↑: IL4, IL10, TGFβ; circadian clock genes mutations | Jensen et al. (2021) |
| Immune dysfunction | Pro-inflammatory cytokines trafficking rhythms ∿; sleep and circadian regulation ∿ | Zielinski and Gibbons (2022) |
| Cardiovascular diseases | Sleep deprivation; insomnia with hyper-arousals; OSA; sleep fragmentation; hypothalamic pituitary adrenal axis ∿; circadian clock and sleep cycle ∿ | Nobre et al. (2021) |
| Respiratory diseases | Insomnia; SDBs; circadian disruption ∿ | Yang et al. (2020) |

*CTS* circadian time-keeping system; *RLS* rest leg syndrome; *PLMS* periodic limb movements in sleep; *PD* Parkinson's disease; *AD* Alzheimer's disease; *HD* Huntington's disease; *ASWPP* advanced; sleep–wake phase disorder; *DSWPD* delayed sleep–wake phase disorder; *REM* rapid eye movement; *NREM* non-rapid eye movement; *RBD* REM sleep behavior disorder; *ESD* excessive daytime sleepiness; *OSA* obstructive sleep apnea; *SBD* sleep related breathing disorders; *SWS* slow-wave sleep; *PSQI* Pittsburgh sleep quality index; *SDB* sleep-related breathing disturbances; *CRSDs* circadian rhythms sleep disorders; *CRSWDS* circadian rhythm sleep wake disorders; *PER* period; *CRY* cryptochrome; *BMAL1* muscle ARNT-like protein; *MCH Neurons* melanin concentrating hormone; *IL-1β* interleukin-1beta; *IL-6* interleukin-6; *IL-4* interleukin-4; *IL-10* interleukin-10; *TNF-α* tumour necrosis factor-alpha; *TGFβ* transforming growth factor-beta

## 1.5 Sleep and Clock Misalignment with Aging

Aging is an inevitable phenomena characterized by progressive decline in physiological functions and cognitive impairments. It is an unidirectional process which is associated with decrease in "buffering capacity" or "homeodynamic space" that eventually leads to the progressive decline of metabolism, physiology and behavior, ultimately leading to death (Rattan 2008; Jagota et al. 2019). Aging process is a multifactorial process and modulated by many molecular and cellular events. Various hallmarks of aging are reduced genomic stability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (Lopez-Otin et al. 2013; Majidinia et al. 2018).

Interestingly, about 80% of subjects in the 50–80 year age group have been found to show spontaneous internal desynchronization of rhythms that may affect sleep patterns and other aspects of aging (Wu et al. 2007). The phase advance in various rhythms with aging in older people such as sleep wake, body temperature and hormone rhythms has been reported. Sleep disturbance is also a frequent

symptom in patients with age-associated neurodegenerative diseases such as PD, AD and Dementia (Musiek 2015).

The age-associated decline in physiological functions is linked with malfunctioning of the various autonomic systems in the body, like CNS in which aging causes a diminished function accompanied by changes in various neurotransmitter levels. Aging also involves neuronal and synaptic loss of function which depends on adaptations in cellular responsiveness (Slotkin et al. 2005). The age-related decline of pineal melatonin production is due to the degenerative changes of the neural structures (serotonergic and noradrenergic neuron systems) innervating the pineal gland and the SCN rather than to the degeneration of the pineal tissue itself. Decreased and perturbed melatonin levels influence the circadian function.

### *1.5.1 Alterations in Sleep Structure in Old*

Aging is linked to decreased amount and quality of sleep in about 40% of elderly people. Sleep disorders have been considered to be major symptoms and problems of aging which in turn may cause disruption in other functions such as digestion, mood, fatigue and decrease in alertness. Sleep disturbances in older adults are often overlooked but can have a significant negative impact on daily functioning and quality of life. These changes may lead to changes in basic homeostatic processes with age.

Aging is associated with numerous changes, including changes in sleep timing, duration and quality. The CTS interacts with a sleep–wake homeostatic system to regulate human sleep, including sleep timing and sleep structure. Interestingly, sleep is one of the various processes that invariably undergoes change with age (Fig. 1.4). The increased sleep and circadian disturbances in the elderly result in various physiological, metabolic and behavioral disorders (Hatori et al. 2017). Such disturbances are primarily due to age-related deterioration as well as secondarily due to physical illness and as a side effect of medications especially use of sedatives (Schwartz and Klerman 2019).

Sleep is one of the many biological processes that invariably undergoes change with age. The aging process is linked with profound disruption of an individual's daily sleep–wake cycle.

Few age-induced changes in sleep architecture have been demonstrated by some researchers using sleep tracking/polysomnographic (PSG) studies in full adult life span (ages 19–102 years). Interestingly, some researchers have demonstrated that, most of the age-related changes in sleep progress steadily across the adult human life span actually occur from age adult 19 up to age 60 and individuals over 60 who remain healthy can expect their sleep quality to remain relatively stable as they age as it becomes asymptote, declining minimally from age 60-age 102 (Ohayon et al. 2004).

The most important among age-induced changes in microarchitecture being changes in nighttime sleep quality: decrease in total sleep time (TST), decreases in sleep efficiency (SE), reductions in slow-wave sleep (SWS), increases in waking

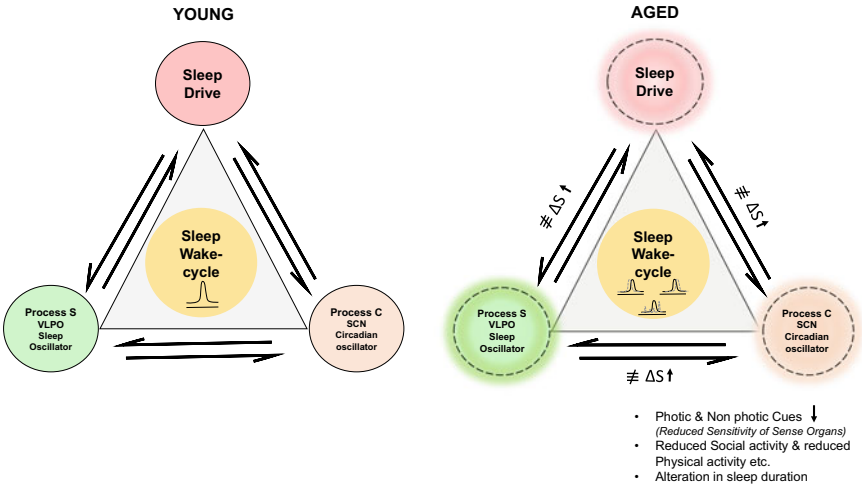


**Fig. 1.4** Age-induced changes in the interaction patterns between sleep oscillator: VLPO (Process S) and circadian clock: SCN (Process C) in regulation of sleep drive. $\neq \Delta S\uparrow$ unequivalent increase in disorder in interaction pattern

after sleep-onset (WASO) in addition to sleep-onset latency SLAT as well as REM latency (REMLAT), Stages 1 and 2, and REM (Duffy et al. 2011).

### *1.5.2 Desynchronization of Circadian Rhythm Patterns in Old*

Several evidences have suggested that aging is associated with dysfunction in 24 h circadian rhythms due to loss of synchronization between the master clock and peripheral clocks leading to a myriad of complications in cellular, hormonal and metabolic processes (Table 1.2b). The circadian clock properties and functioning are altered with aging with the desynchronization of rhythms and the efficacy of input and output pathways to and from the SCN. The diurnal rhythm of α1 adrenergic receptor expression, characteristic of young rats, disappears by middle age (Smith et al. 2005).

Aging results in neuronal deterioration, reduction of dendritic surface, decrease in protein levels as well as changes in the glucose rhythms. These changes lead to the aperiodic pattern of firing in the SCN neurons. Circadian disruptions associated with aging lead to poor health consequences and hastened senescence in elderly people. The decline in physiological function with aging may be associated with malfunctioning of various autonomic systems in the body. Chronotype gets shifted earlier as people grow older (Taillard et al. 2021).

Such alterations can be related to modification in the kinetics of the activation of signaling pathways in the SCN as well as age-related changes within the clock mechanism of the SCN itself (Mishima et al. 2001). Aging is linked with the decreased robustness in the functioning of the circadian system in humans with reduced sensitivity of the SCN to retinal stimulations, loss of temporal coordination among bodily systems, leading to deficits in homeostasis and suboptimal functioning of the physiology. Such alterations accelerate the aging process and contribute to senescence with neuronal degeneration of SCN leading to organic deterioration of the circadian oscillator and are characterized by loss of precision, decreased synchronization, a shorter period of the endogenous oscillator, reduced exposure to synchronizing stimuli such as light or altered responsiveness to zeitgeber (Gibson et al. 2009). Various studies on human aging show a decrease in pacemaker output and that the symptomatic expression of this abnormality is circadian rhythm sleep disorders (CRSD) responsible for decrease in daytime alertness with a decline in actual sleep time in addition to changes affecting physiology, e.g., digestion, mood and fatigue (Jagota 2005; Gibson et al. 2009).

Reports from our laboratory suggest that there are alterations in daily rhythms of serotonin (Jagota and Kalyani 2008, 2010), antioxidant enzymes (Manikonda and Jagota 2012), leptin (Reddy and Jagota 2014), clock genes (Mattam and Jagota 2014), immune genes, *Sirt1* and nuclear factor erythroid 2-related factor 2(*Nrf2*) (Kukkemane and Jagota 2019, 2020; Thummadi and Jagota 2019) serotonin metabolism (Reddy and Jagota 2015), NO and Socs expression rhythms (Vinod and Jagota 2016, 2017), etc. with aging.

### *1.5.3 The Circadian and Sleep Perturbances*

Sleep and circadian rhythms patterns show alterations with age. The homeostatic and circadian rhythm processes regulating the sleep cycle become more fragile with age. Cellular senescence has been reported to impair circadian expression of clock genes due to decrease in ability of cells to transmit circadian signals to their clocks. Such impairment is associated with decreased responsiveness of CREB-dependent signaling (Kunieda et al. 2006). Altered sleep/activity patterns can affect the function of the central and peripheral oscillators leading to alterations in metabolism. Altered sleep patterns can lead to arrhythmic exposure to light and thus constant resetting of the central oscillator which in turn may alter normal feeding patterns and desynchronize peripheral oscillators in metabolic tissues, such as liver and pancreas (Bellet and Sassone-Corsi 2010). The biological rhythms appear compromised by the middle age. We have also previously reported age-induced alterations in daily rhythms of serotonin in brain as well as SCN starting at middle age (Jagota and Kalyani 2008, 2010).

There is large and important heterogeneity with normal aging even in the absence of clinically significant sleep disorders linked to circadian rhythm disruption and

consequent sleep abnormality with decline in nighttime sleep quality and duration, decreases in sleep depth, sleep intensity and sleep continuity leading to cognitive decline. These have been identified as common features of the most prevalent neurodegenerative diseases, such as AD, PD and Huntington's disease (HD) (Musiek 2015).

Concomitantly, a reduced amplitude of circadian rhythm output signals has been shown in older participants, suggesting that age-related changes in sleep may be partially due to a weaker circadian regulation of sleep and wakefulness. Interestingly, it has been reported that older people may need less sleep (Dijk et al. 2010) suggesting that in spite of marked changes in sleep physiology, excessive daytime sleepiness is not common during healthy aging (Duffy et al. 2011).

Further, aging is linked to more disruption in sleep and circadian rhythm outputs and increased disease susceptibility (Hastings et al. 2018). However, with increasing age, circadian and sleep–wake-related neural areas or the connections within the functional neuroanatomical networks may compensate for initial dysfunction (van Someran et al. 2021). The age-related decline in absolute levels of SWS represents one of the most common reported features in the aging with decline in homeostatic sleep pressure (Dijk et al. 2010).

One major cue, the presence of light, is reported to the cells of the SCN using a type of glutamate receptor called an NMDA receptor. This receptor becomes less effective with aging. There is loss of some of its ability to adjust circadian rhythm according to the presence of light. The loss of effectiveness appears to be related to an age-related decline in the expression of a critical subunit in the receptor.

There is evidence for age-related changes in many aspects of circadian rhythmicity, including the TTFLs involved in circadian rhythm generation, the neuroanatomical structures, the transmission and responsiveness to light and the timing and amplitude of output rhythms (De Nobrega and Lyons 2020).

## 1.6 Interventions to Improve Sleep and Clock Function: A Step Toward Healthy Aging and Longevity

The robustness of sleep and circadian rhythms declines with age. Reduced sensitivity toward reception of photic/non-photic cues is linked to aging. Therefore, several scientists and researchers are working toward restoring stoichiometric interactions and treatment methodologies for aligning the various internal physiologies at molecular, cellular and tissue level so as to synchronize parameters to have healthy aging.

Although sleep quality and circadian rhythms appear to be well preserved in healthy older, there are the negative impacts of age-related other comorbidities on sleep and circadian rhythms. It has been demonstrated by careful screening of old individuals by some researchers that older individuals who have no or minimal

medical burdens show the changes when compared to young adults thus demonstrating aging process is independent of any medical or psychiatric illnesses or primary sleep disorders and is just age-induced sleep change.

Adequate sleep assessment is critical in clinical and research settings; however, current sleep assessment protocols fail to account for circadian rhythms, despite the fact that sleep is a well-recognized circadian process.

### *1.6.1 Sleep Hygiene*

Sleep disturbances are common in elderly patients. Attention must be paid to the treatment of comorbid disease. Inadequate sleep not only includes insufficient duration, poor sleep quality or the presence of sleep disturbances, but may also be due to inappropriate sleep timing (Erren and Reiter 2015). The circadian rhythm tends to advance with age, causing older people to awaken early in the morning.

Our modern lifestyle and artificial nocturnal light delay our bedtime, make us wake up and lead to a greater intraindividual variability in sleep timing. Depending on the constraints that social time places, sleep timing may be in or out of phase with the internal circadian timing determined by the circadian clock (Wong et al. 2015). When a person's social time is out of phase with their circadian time, they may be considered to suffer from circadian disruption or "social jetlag" (Taillard et al. 2021).

### *1.6.2 Light Therapy*

Phototherapy is one treatment of circadian sleep–wake disorders which is linked to scientific and clinical evidences. The several light characteristics determine treatment strategies such as intensity, length of exposure, time of exposure and wavelength. Phototherapy is potentially indicated in several age-related circadian dysfunction sleep disorders such as ASWPD, DSWPD, non-24-N24SWD as well as in social jetlag due to aging (Leger et al. 2018).

### *1.6.3 Administration of Exogenous Melatonin*

Melatonin appears to be firstly an effective molecule in helping align misaligned clocks and secondly an effective antioxidant, a defense tool against biologically damaging free radicals. Melatonin may slow aging process by removing free radicals and enhancing immunity (Majidinia et al. 2018). Melatonin has been identified as a prime regulator of human chronobiological and endocrine physiology and is highly reputed as an antioxidant, immunomodulatory, antiproliferative, oncostatic and endocrine-modulatory molecule. Interestingly, several recent reports support

melatonin as an anti-aging agent whose multifaceted functions may lessen the consequences of aging (Moretti et al. 2020; Gimenez et al. 2022).

Reports from our laboratory suggested that with exogenous melatonin administration, there was differential restoration in various age-altered parameters in animal models in daily rhythms of serotonin (Jagota and Kalyani 2008, 2010), antioxidant enzymes (Manikonda and Jagota 2012), clock genes (Mattam and Jagota 2014), serotonin metabolism (Reddy and Jagota 2015), NO and Socs expression rhythms (Vinod and Jagota 2016, 2017).

### *1.6.4 Herbal and Other Interventions*

Use of herbal interventions, availability of food or food restriction is one of the strongest non-photic stimuli that can entrain the principal oscillator. Hence, various strategies to align food consumption with circadian clock system establishing achieving restoration of sleep and clock alignment are emerging in recent years.

Further, we have also reported various herbal therapeutic interventions such as curcumin and hydro-alcoholic leaf extract of *Withania somnifera* toward the restoration of various clock genes, immune genes, *Sirt1* and nuclear factor erythroid 2-related factor 2(*Nrf2*) upon aging (Kukkemane and Jagota 2019, 2020; Thummadi and Jagota 2019).

Due to complex interactions with circadian clock and metabolism, meal timings are considered as critical modulators and approaches to align food consumption with endogenous circadian rhythms are emerging in recent years (Queiroz et al. 2021). We had reported, time restricted feeding (TRF) as a non-photic potential cue in entraining age-induced misalignment in leptin and locomotor rhythms (Reddy and Jagota 2014). The challenge still remains to translate such findings from circadian clocks studies to be exploited for establishing therapeutic role in restoration of age-related sleep disorders.

## 1.7 Goals and Conclusions

Sleep is absolutely important for human health as insufficient sleep has been associated with a plethora of diseases. Due to robust increase in life span in twenty-first century, there is a pressing need to understand risk factors for age-induced neurodegeneration and age-related human diseases. In humans, however, social time and nocturnal artificial light modify sleep timing leading to "social jetlag" due to alterations in internal CTS regulation. It is absolutely essential to understand sleep and wakefulness "flip-flop" switch so as to ensure the reduced ability of getting restful sleep in elderly can be better addressed, as sleep impacts memory, learning, mood, behavior, immunological responses, metabolism, hormone levels, digestive process and many more physiological functions. Investigation into core mechanisms may

provide therapies to reset or amplify circadian signals. A mechanistic understanding of the link between the clock and sleep toward healthy aging can be leveraged through identification of the appropriate timing of therapies, as well as new treatment targets. Healthy aging and wellbeing are common goals in this complex process in the present scenario.

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# Chapter 2
# Cells and Circuits of the Suprachiasmatic Nucleus and the Control of Circadian Behaviour and Sleep


**A. P. Patton, M. H. Hastings, and N. J. Smyllie**

## 2.1 Introduction: Mammalian Circadian System Overview

Circadian clocks are self-sustaining biological timing mechanisms with an intrinsic period of approximately one day (hence, *circadian*). They are widely distributed across all forms of life (Edgar et al. 2012) because they confer adaptive value by facilitating the anticipation of, and thus preparation for, the alternating challenges and opportunities presented by daily and seasonal environmental cycles. Consequently, their influence impinges on virtually all aspects of metabolism, physiology and behaviour. In humans, the daily cycle of sleep and wakefulness is the most obvious output of the circadian system, but it is accompanied by equally dramatic cycles of autonomic function and endocrine status that maintain internal temporal coherence. In modern societies, this temporal coherence is compromised by factors such as rotational shift work, exposure to irregular lighting environments and increasingly prevalent age-related diseases, most notably neurodegenerative conditions. The principal organiser of our circadian life is the suprachiasmatic nucleus (SCN) of the hypothalamus, a cluster of ca. 20,000 cells sitting immediately above the optic chiasm, on either side of the midline third ventricle (Hastings et al. 2018) (Fig. 2.1a). It receives direct photic input via the retinal hypothalamic tract (RHT), which consists of the axonal projections of retinal ganglion cells (RGC). Many of these RGCs express melanopsin and are thus intrinsically photoreceptive (LeGates et al. 2014). Under normal circumstances, this input allows the SCN to align its internal representation of circadian time to external solar time. Importantly, however, the SCN will continue to maintain and generate a coherent representation of circadian time in the absence of environmental input, for example, when an animal is placed into constant darkness and even when the SCN is removed from a mouse and cultured as

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an explant. The discovery of circadian clock genes (see below) brought a totally unexpected level of circadian organisation into view. It is now clear that all major organ systems and many cell types, not least fibroblasts, have intrinsic circadian clocks (Reppert and Weaver 2002). This means that the role of the SCN is not as a driver of rhythms in a passive periphery, but rather that of a synchroniser of these innumerable, distributed local clocks (Fig. 2.1b). This dynamic interaction with the periphery highlights even further the power and sophistication of the SCN as a central timekeeper, and underscores the potential fragility of the overall system to genetic, physiological and environmental insults. The purpose of this review is, first, to consider the **molecular-genetic and cellular basis of circadian timekeeping** in mammals. Second, we discuss the **circadian properties of SCN neurons** followed by examination of the **circuit architecture of the SCN as a cellular network**. Fourth, we review the **role of astrocytes in the SCN**, before considering **SCN output pathways and their control over behaviour**, including the cycle of sleep and wakefulness. Finally, we note potential **future directions**.

## 2.2 Molecular-Genetic and Cellular Basis of Circadian Timekeeping in Mammals

### *2.2.1 The Core Feedback Loop—Genes and Molecules: Discoveries Through Mapping and Mutagenesis Screens*

The possession of a cell-autonomous circadian clock is not unique to mammals. In the genetically tractable *Drosophila* and *Neurospora*, forward mutagenesis screens uncovered the first genetic components of eukaryotic circadian clocks: *period* (*per*) and *frequency* (*frq*), for each organism, respectively (Dunlap 1999). These were soon followed by the discoveries of *timeless* (*tim*), *clock* (*clk*) and *cycle* (*cyc*) in *Drosophila.* In these lower organisms, a common organisational feature emerged: transcriptional-translational feedback loops (TTFLs) were central to circadian timekeeping. Here, positive regulators transactivate transcription of negative regulator genes, whose protein products then, in turn, inhibit their own transcription. Importantly, these self-sustaining oscillations are entrained by light. In *Drosophila,* this is mediated by cryptochrome (CRY) proteins (Emery et al. 1998; Yuan et al. 2007), which are related to photolyase DNA repair enzymes, and in *Neurospora* by photosensitive white-collar complexes (WC-1, 2) (Froehlich et al. 2002). Although the *Tau* mutation, discovered in the Syrian hamster, was the first identified circadian clock mutant in mammals (Ralph and Menaker 1988), the turning point for assembling the mammalian molecular clock mechanism came with the discovery of the *CLOCK* gene, through a forward mutagenesis screen in mice (King and Takahashi 2000). A mutant allele that lengthened circadian period was positionally mapped (a heroic

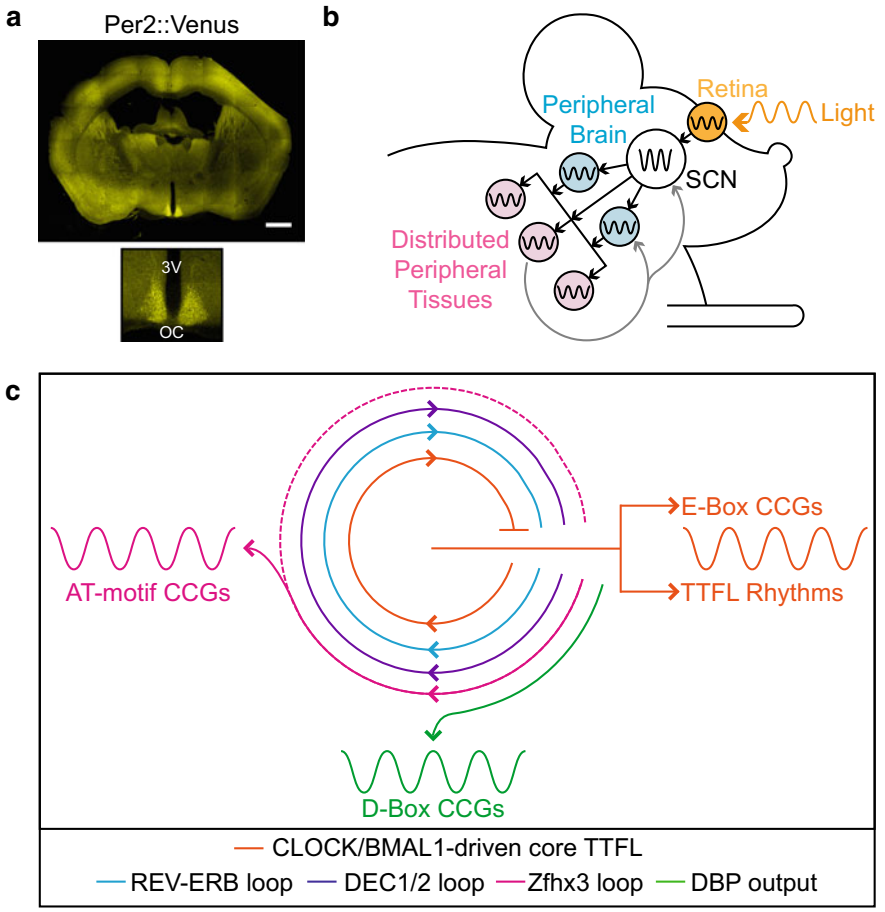


**Fig. 2.1** The SCN and re-entrant motifs in the core clock and additional TTFLs. **a** Coronal section of mouse brain revealing location of the SCN by high expression level of endogenous PER2::Venus fluorescent protein. Scale 1 mm, inset higher power view to show nuclear localisation of PER2. 3 V: third ventricle, OC: optic chiasm. **b** Schematic view of the role of the SCN as the dominant, light-entrained circadian clock in mammals, orchestrating peripheral clocks in brain regions and major organs. **c** Schematic view of the inter-nested TTFL loops of the cell-autonomous clock. The core BMAL1:CLOCK-driven loop (centre) additionally drives rhythmic output from E-boxes in clock-controlled genes (CCGs) (orange). Robustness is conferred to this loop by the interwoven REV-ERB-driven (blue), DEC1/2-driven (purple) and ZFHX3-driven (magenta) additional loops. In addition to modulating the core clock TTFL, ZFHX3 also controls rhythmic transcriptional outputs via AT-motifs in CCGs. Finally, DBP-driven targets (green) are important for regulation of rhythmic outputs regulated by D-box regulatory elements in CCGs, but do not impinge on core clock function

undertaking in the pre-genome era) and then subsequently a series of rescue experiments showed that the mutation was mapped to exon 19 of the gene. The resulting exonal deletion (*CLOCK*$^{delta19}$) compromised the transcriptional activity of CLOCK protein as a core clock component. This was complemented by the discovery of mammalian homologues to *Drosophila per* and *cry* as negative transcriptional regulators (Tei et al. 1997; van der Horst et al. 1999). Interestingly, the mammalian *TIMELESS* gene is not closely related to the *Drosophila timeless* gene and is not a component of the mammalian clock (Gotter et al. 2000).

With the discovery of MOP3 (also known as BMAL1) (Bunger et al. 2000), the final piece was in place identifying the core factors in the mammalian TTFL clock: CLOCK and BMAL1 are basic helix-loop-helix (bHLH) transcriptional activators, which heterodimerise via PAS domains and activate the transcription of negative regulator genes *PERIOD* (*PER1, 2*) and *CRYPTOCHROME* (*CRY1, 2*). The production of PER and CRY proteins increases through the circadian day, during which they heterodimerise and translocate into the nucleus to inhibit their own transcription. PAS domains in PER likely facilitate interaction with CLOCK:BMAL1. The PER and CRY proteins are degraded through the subjective night, until the feedback inhibition is finally alleviated, after which the cycle can begin again. The dynamics of transcription, translation and protein stability provide a regulated delay within this feedback loop, which consequently takes approximately 24 h to complete (Fig. 2.1c). Importantly, there is redundancy inherent to the system. First, there are two PER and two CRY proteins, which have overlapping functionality and provide resilience to the negative limb of the clockwork, and second, NPAS2, a paralog to CLOCK, can also heterodimerise with BMAL1. This redundancy is clearly demonstrated by the fact that animals only become arrhythmic in their behaviour when both Clock and NPAS2 are deleted (DeBruyne et al. 2007). Interestingly, BMAL1 is the only component of the mammalian molecular clock that is irreplaceable. Global or SCN-restricted loss of the *Bmal1* gene alone results in arrhythmia in mice. The simple TTFL motif is that of a re-entrant loop whereby output becomes input and thus, by incorporating a delay, it establishes a self-sustained oscillation. This re-entrant loop motif lies at the heart of clock function at multiple levels (Fig. 2.1c). Finally, to be effective, the oscillatory TTFL must control cellular functions and the most direct way is for the periodic activation and repression of E-boxes across the genome to create circadian waves of transcription of "clock-controlled genes" (CCGs) (Akhtar et al. 2002; Koike et al. 2012). Surprisingly, across all tissues examined, the circadian clock controls the expression of 43% of genes (Zhang et al. 2014) and so any loss of circadian competence will inevitably disrupt physiology and thereby aggravate ageing and compromise longevity (Lowrey and Takahashi 2004).

### 2.2.2 Additional Feedback Loops Support the TTFL

The discovery of the TTFL provided a range of new approaches, most notably real-time recording of circadian gene expression using bioluminescent and fluorescent reporters, to understand both cell-autonomous and tissue-based clock functions. Remarkably, not only the SCN but also peripheral tissues exhibits cell-autonomous TTFL cycles (Stokkan et al. 2001; Yoo et al. 2004). These discoveries revealed a completely unanticipated level to the sophistication of circadian co-ordination across the organism. This systemic complexity was complemented by the discovery at a molecular level of a series of additional feedback loops that stabilise and amplify the core TTFL. First, the transcription of nuclear receptors *ROR α*, *REV-ERB α* and *REV-ERB β* is activated by CLOCK:BMAL1, and these factors in turn act back on the TTFL by regulating the transcription of *BMAL1* itself, which carries ROR response elements (ROREs) (Preitner et al. 2002) (Fig. 2.1c). The *staggerer* mutation of *Ror α* results in the reduction of BMAL1 expression in mice and shortened circadian period (Akashi and Takumi 2005), whilst loss of both *Rev-Erb α and Rev-Erb β* is accompanied by arrhythmia (Cho et al. 2012). These factors are also important in sculpting circadian output because the circadian cycle of activation to ROREs across the genome drives further waves of CCG transcription, particularly metabolically relevant genes, thereby complementing control by E-boxes. An additional loop incorporates the circadian E-box and light-driven DEC1 and DEC2 bHLH transcriptional regulators. DEC1 in particular can repress CLOCK:BMAL1-mediated activation at E-boxes, including those of *PER1* (Honma et al. 2002), again closing a re-entrant loop around E-boxes. A third regulatory output of the TTFL pivots around the basic leucine zipper transcription factors DBP, TEF and HLF, which are expressed in a highly circadian manner in many tissues, including the SCN, where they further co-ordinate daily cycles of CCG expression, (Gachon et al. 2006), although their influence on the TTFL is minimal.

Forward mutagenesis screening in mice uncovered an additional loop centred on the transcription factor ZFHX3 which acts via "AT-box" regulatory elements to activate gene expression (Parsons et al. 2015). Mice carrying the dominant *Shortcircuit* autosomal mutation of *Zfhx3* (*Zfhx3*$^{Sci}$) have a significantly shorter circadian period (~ 0.6 h for each mutant allele), which accompanies a reduced transactivational potency of the protein at AT-boxes, whilst deletion of ZFHX3 in adult mice shortened behavioural period by ~ 1 h and in ~ 30% of mice caused arrhythmia (Wilcox et al. 2017). Not only does ZFHX3 therefore feed into the TTFL, but the AT-box axis is also under circadian regulation and so constitutes a downstream output of the TTFL (Parsons et al. 2015): again, a re-entrant loop motif (Fig. 2.1c). The contributions of cell-autonomous and network-level actions of ZFHX3 remain unclear, although the transactivational compromise in the mutant is reflected in lowered levels of expression of SCN neuropeptides (see below), several of which carry AT-boxes regulatory elements. The shortened period may therefore arise in part from changes in the interneuronal neuropeptidergic signalling network. More significantly, ZFHX3 regulates the expression of numerous genes in the SCN and so, alongside E-boxes,

and ROREs, it will be able to play a significant part in sculpting the circadian transcriptome that underpins the output signalling by the SCN. Finally, ZFHX3 plays a developmental role because SCN specification fails in mice carrying a conditional null allele from embryonic stages and so are behaviourally arrhythmic, even though circadian competence is retained outside the SCN (Wilcox et al. 2021). This argues further that the "re-entrant" actions of ZFHX3 are expressed at the level of the SCN circuit, whereas those of RORs and DEC are cell-autonomous (Fig. 2.1). In all cases, however, the net effect of these additional loops is to enhance robustness and amplitude of the core TTFL and to broadcast its timing cues via downstream transcriptional cascades.

### 2.2.3 Control of the Stability of Clock Proteins and Effect on Behaviour

It is implicit in the structure of the TTFL that changes to the rate of expression and/or stability of the mRNAs and proteins within it will alter its dynamics and therefore the period of overt measurable rhythms. Indeed, this relationship was foundational to the success of the forward genetic screens that identified clock genes, not least the *CLOCK*$^{delta19}$ mutation that revealed CLOCK as a positive regulator in the TTFL of mammals (King and Takahashi 2000). The analysis of period mutants in rodents has revealed the post-translational modifications that determine the activity and stability of PER and CRY proteins. The *Tau* mutation provided the first evidence for the single-allele (as opposed to multigenic) control of circadian period in mammals, as well as identifying the SCN as the source of this control (Ralph et al. 1990). This mutation was later mapped to the *Casein Kinase 1 Epsilon* (*Ck1 ε*) gene (Lowrey et al. 2000) and re-engineered in mice to generate a comparable phenotype (Meng et al. 2008). Both in hamsters and mice, the mutant allele accelerates wheel-running rhythms by 2 h per copy. Biochemical studies in tissues and primary cells from *Tau* mutant mice indicated that this was a gain-of-function (GOF) mutation in CK1 $\varepsilon$ that destabilised PER proteins, thereby accelerating their clearance and, therefore, the speed of the clock (Meng et al. 2008). Consistent with the gain-of-function mutation, genetic deletion of CK1 $\varepsilon$ does not have a pronounced circadian phenotype: rather, CK1 $\delta$ is the principal endogenous regulator of PER stability and circadian period under normal conditions (Etchegaray et al. 2010), a conclusion confirmed by the contrasting effects of selective pharmacological inhibition of either CK1 $\delta$ or CK1 $\varepsilon$ (Meng et al. 2010). It is now clear, however, that circadian period is tuned by the balance between kinase and phosphatase activity on PER proteins (Lee et al. 2011). Indeed, multiple CK1-dependent phosphorylation sites on PER can competitively stabilise or destabilise the protein (Philpott et al. 2020) by providing or denying access to the degron sequences that target it for ubiquitinylation by the ubiquitin ligase beta-TRCP and thus proteasomal degradation (D'Alessandro et al. 2017). Consequently, the *Early doors* mutation of PER2, which compromises packing of the PAS domain

and thus provides greater access to the degron sequences, destabilises the protein and shortens circadian period. In combination with the CK1ε Tau allele, it can accelerate SCN and behavioural rhythms to extremely short periods of below 19 h (Militi et al. 2016). Remarkably, these ultra-fast clocks remain stable and precise.

The period of the SCN and thus behavioural rhythms is also influenced by the activity of CRY proteins. Loss of CRY1 shortens period whilst loss of CRY2 lengthens it, and loss of both causes arrhythmia (van der Horst et al. 1999). Mutagenesis screens (Godinho et al. 2007; Siepka et al. 2007) revealed that stability of both proteins is regulated by the E3-ubiquitin ligase, FBXL3. Loss-of-function mutations (*Afterhours* and *Overtime*) of *Fbxl3* correspondingly increase the stability of CRY proteins and thereby lengthen SCN and behavioural periods in wild-type, *Cry1*-null and *Cry2*-null mice (Anand et al. 2013). Consequently, when combined with CRY2 deficiency, the *Afterhours* mutation lengthens SCN and behavioural periods to over 29 h (Anand et al. 2013). A second ubiquitin ligase, FBXL21, counterbalances the action of FBXL3: it is localised predominantly in the cytoplasm (in contrast to the nuclear FBXL3), stabilises CRY proteins (in contrast to the destabilising actions of FBXL3), and loss of FBXL21 attenuates the period lengthening caused by the absence of FBXL3 (Hirano et al. 2013). AMP kinase (AMPK) is an important upstream regulator of the ubiquitinylation of CRY proteins (Lamia et al. 2009), with loss of different catalytic isoforms shortening or lengthening TTFL period (Um et al. 2011). Given that AMPK is nutrient-responsive and the metabolic state of the cell is circadian, this provides an additional example of a cell-autonomous re-entrant loop that stabilises and tunes the TTFL. Finally, ubiquitinylation-dependent stability of REV-ERB proteins allows this additional loop to tune the levels of BMAL1 and therefore modulate the core TTFL (Stojkovic et al. 2014).

These discoveries in rodent models provide a satisfying confirmation of the elegant dynamics of the TTFL. They also offer insight into potential therapy and management of circadian disturbances, because the very same mechanisms direct the human TTFL and their compromise is at the heart of familial sleep disturbances. For example, mutations in human CK1 $\delta$ or at the kinase target sites of PER2 are associated with advanced sleep phase disorders (Chong et al. 2018). Equally, mutation of the human CRY1 that enhances its transcriptional repression leads to familial delayed sleep phase disorder (Patke et al. 2017). Agents that modify the molecular interactions within the TTFL therefore hold promise for therapeutic intervention. For example, small molecules that target the interaction between CRY and FBXL3 can lengthen period in cell assays (Hirota and Kay 2015), whilst RNAi screening in human cells has identified many potential circadian-regulatory targets (Zhang et al. 2009). Chronotherapy directed at the TTFL or at its innumerable outputs remains in its infancy, but its promise for therapeutic benefit is now evident (Zhang et al. 2014).

## 2.3 Circadian Properties of SCN Neurons

### *2.3.1 Observing Clock Proteins at the Cellular Level*

The molecular clockwork does not operate in isolation—it oscillates within its cellular setting. Each component of the TTFL and auxiliary loops has its own distinct set of intracellular dynamics necessary for progression of the SCN clockwork. This is in part imposed by the compartmentalisation of the cell, where translocation from cytoplasm to nucleus is essential for PER and CRY proteins to inhibit their own transcription. Early over-expression studies in cell lines and immunostaining in mouse tissues made a link between PERs and CRYs and the regulation of their own nuclear translocation. More recently, RNAi screening in human cells revealed genes associated with nucleocytoplasmic translocation, including both canonical (beta importin-mediated) and novel (Transportin 1 (TNPO1)) pathways, knockdowns of which variously lengthened or shortened circadian period in line with altered protein localisation (Korge et al. 2018). Nevertheless, over-expression systems and "snap-shot" type single-timepoint imaging approaches carry limitations in terms of deriving quantitative, physiologically relevant measures. The recent creation of knock-in cell lines (Gabriel et al. 2021; Koch et al. 2022) and mice (Smyllie et al. 2016b; Yang et al. 2020) expressing fluorescently tagged versions of endogenous clock proteins has begun to give mechanistic insight and "put numbers" on to these intracellular dynamics. Real-time imaging of PER2::Venus revealed that although its abundance oscillates, when present, it was present in the nucleus throughout the circadian day and this nuclear retention is dependent on CRY proteins (Smyllie et al. 2022). Importantly, the mere presence of the CRY proteins is not sufficient: they themselves must also be able translocate into the nucleus for PER2 to also keep its nuclear localisation. This may be because CRY proteins reduce the mobility of PER2 molecules in nucleus and cytoplasm by a factor of ~ 2. Equally, PER2 slows down the mobility of CRY1.

Fluorescence correlation spectroscopy (FCS) measurements in fibroblasts revealed that at the peak times of expression, between 3000 and 10,000 molecules of either PER2, BMAL1 or CRY1 are present in the nucleus. This relatively low molecular abundance may be an important feature of the TTFL because it sustains large-amplitude changes in TTFL phase in response to small changes in abundance. Importantly, the dynamics of endogenous PER2, BMAL1 and CRY1 proteins are very different: PER2::Venus has the highest rhythm amplitude of ~ tenfold over a relatively low baseline of fluorescence, whereas BMAL1 has a very low-amplitude rhythm of ~ 5%, with a very high baseline. Perhaps related to this, there is a negative correlation between rhythm amplitude and the half-lives of these proteins, whereas endogenous PER2 has a half-life of a few hours, BMAL1, which had the lowest amplitude oscillation, was found to be surprisingly stable and was not fully cleared after 3 days of cycloheximide treatment. Between PER2 and BMAL1, endogenous CRY1 oscillates with a high (~fivefold) amplitude but even at its nadir, there are sufficient molecules to effect nuclear translocation of PER2 (Smyllie et al. 2022). Surprisingly, in terms of molecular mobility, there is no evidence of circadian control

of these proteins: where measured in wild-type cells, the mobility of PER2 and BMAL1 did not vary between peak and trough of the cycle. Phases of expression do, however, vary markedly. Real-time imaging in SCN slices confirmed that endogenous PER2 peaks at CT12, but surprisingly, CRY1 peaks 6 h later at CT18 (Smyllie et al. 2022; Koch et al. 2022), followed by BMAL1 a few hours after that, at CT20 (Yang et al. 2020). This late expression peak of BMAL1 positions it temporally "poised" to be ready to transactivate E-box regulated *Per* and *Cry* genes to start a new cycle (Koike et al. 2012). The precise composition, and presumably the activity, of E-box-bound complexes containing CLOCK, BMAL1, CRY and PER proteins will therefore evolve through circadian time as the TTFL progresses. The temporal segregation of negative and positive regulators will enhance robustness and will also direct distinct, phase-appropriate waves of differential gene expression.

Finally, quantitative imaging in cells and associated modelling has indicated dual modes of action of PER2:CRY1 complexes. First, they conventionally repress transactivation via displacement of CLOCK:BMAL1 from target sites, but this in turn facilitates mobility of CLOCK:BMAL1 and binding to new target sites (Koch et al. 2022). Thus, PER2 acts both as part of a transcriptional repressor complex and as a facilitator of CLOCK:BMAL1 mobility to explore the genome. Given that CLOCK:BMAL1 will epigenetically mark its sites, such brief "visits" by relatively few molecules may nevertheless be sufficient to maintain genome-wide circadian co-ordination of the many CCGs (Koike et al. 2012).

### *2.3.2 SCN Neural Activity and Transcriptional Cycles*

Beyond the TTFL, multi-channel and multi-modal recordings have enabled temporal "phase-mapping" to define the cell-autonomous programme of SCN neurons, mapping to the TTFL electrical activity and cytosolic signalling (Brancaccio et al. 2017). All cellular activities can be aligned to the well-characterised peak of PER2 expression, at circadian time 12 (CT12). Briefly, this programme begins in the subjective morning, when intracellular calcium levels $[Ca^{2+}]_i$ monitored with GCaMP fluorescent reporter, peak at CT7 (Brancaccio et al. 2013; Noguchi et al. 2017). This coincides with a circadian peak in levels of cAMP (O'Neill et al. 2008) and precedes gene transcription driven through cAMP/calcium response elements (CREs), monitored by lentiviral bioluminescent reporter, which peaks shortly afterwards at CT9 (Brancaccio et al. 2013). As *Per1* and *Per2* themselves carry CREs (Travnickova-Bendova et al. 2002), this provides a further example of rhythmic circadian outputs, here calcium and cAMP levels becoming inputs to the TTFL. Indeed, *Per1* and *Per2* expression peaks soon afterwards, followed by E-box-driven but CRE-independent CRY expression (Maywood et al. 2013). The outcome is a pronounced circadian cycle of metabolism and electrical activity in the SCN, with depolarisation and peak firing in the middle of circadian day, leading to the peak in $[Ca^{2+}]_i$. Electrical activity declines in circadian night as the neurons hyperpolarise and their metabolic redox state changes, as evidenced by increased super-oxidation of peroxiredoxin, a

highly conserved maker of circadian redox state (Edgar et al. 2012). These intracellular changes are accompanied by oscillations within the SCN extracellular milieu, including paracrine glutamate ($[Glu]_e$) and synaptic γ-aminobutyric acid (GABA) ($[GABA]_e$). Surprisingly, both of these neurochemical signals peak in circadian night, when neurons are electrically silent (see below). At a cell-autonomous level, a circadian output, in this case neural electrical activity, is again a re-entrant input to the TTFL. Consequently, compromise of the electrical state of the neurons, for example by pharmacological blockade of the firing of action potentials, in turns weakens the TTFL (Colwell 2011).

### *2.3.3 The Importance of Coupling Between SCN Neurons*

The importance of electrical activity for stable progression of the TTFL is also evident at the level of the SCN circuit. Each SCN consists of ~ 10,000 neurons and ~ 3000 glial cells, the latter being principally astrocytes, but with single-cell RNA sequencing (scRNA-seq) revealing populations of ependymocytes, radial glia, oligodendrocytes and microglia (Wen et al. 2020; Morris et al. 2021). Conventionally, the SCN has been sub-divided into the retinorecipient "core", which is the termination zone of the RHT, and the surrounding "shell". Core and shell also have distinct patterns of efferent output and afferent input, as well as distinct neuropeptide expression (Abrahamson and Moore 2001). Vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP) are expressed in the core whilst arginine vasopressin (AVP) is in the shell and Prokineticin 2 (PROK2) and Neuromedin-S (NMS) straddle both domains (Lee et al. 2015; Cheng et al. 2002; Masumoto et al. 2006). The localised expression of their various cognate receptors sustains both intra- and inter-sub-divisional signalling (Wen et al. 2020; Morris et al. 2021). The importance of such intra-SCN communication is evident in several ways. First, both in vivo and in the SCN slice ex vivo, the expression of *Per* and *Cry* genes traces a spatiotemporal wave across the nucleus, starting in the dorsomedial lip of the shell, and progressing ventrally to the core and then dorsally and laterally before activity is curtailed, to be reinitiated on the next cycle (Hastings et al. 2018) (Fig. 2.2a). This spatiotemporal wave is additionally reflected in a wave of neuronal activity (represented by $[Ca^{2+}]_i$) that passes across the nucleus with the same trajectory as, but phase advanced to, gene expression. The spatiotemporal wave is therefore a clear demonstration of the flow of information through the circuit, and indicative of regional functionality. Its role is less clear, although one possibility is to segregate temporally distinct efferent signals in different neuroanatomical pathways, i.e. early and late timing cues to regulate appropriate output.

The second demonstration of the importance of coupling comes from the observation that in dispersed cultures, although SCN neurons can remain individually rhythmic in terms of gene expression and neural activity, these oscillations are less robust, less frequent and are poorly defined compared to those of intact SCN

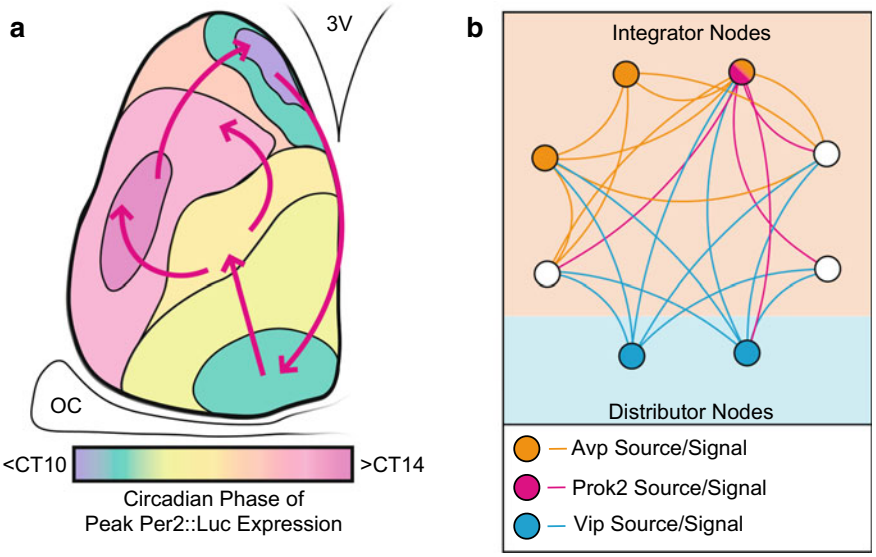


**Fig. 2.2** Spatiotemporal dynamics of gene expression across the SCN indicate regional functionality and form a network-level re-entrant motif. **a** Schematic showing the spatiotemporal progression of peak PER2 gene expression passing across the coronal surface of the SCN indicated by differently phase-mapped regions. Arrows indicate the directional progression of the wave emanating from the dorsomedial lip of the SCN, adjacent to the 3rd ventricle and progressing sequentially: ventrally into the retinorecipient core of the SCN, dorsolaterally into the shell of the SCN, and finally into the dorsomedial region for the cycle to begin anew. **b** Neuropeptidergic topology of the SCN determined from scRNA-seq data (redrawn from Morris et al. 2021) and organised to represent the flow of information from and across distributor and integrator nodes in circadian day. Coloured circles indicate neuropeptide source nodes (cell populations) and coloured lines indicate inferred connectivity via that neuropeptide for VIP (blue), PROK2 (magenta), AVP (purple) and undetermined (white). Note that the PROK2 node is also a source node for AVP. This network is transcriptionally dismantled at night

slices (Noguchi et al. 2017). Moreover, mutations of the TTFL that do not compromise circadian behaviour of the animal nor timekeeping the SCN slice nevertheless disrupt further the rhythmicity of dispersed SCN neurons (Liu et al. 2007). Finally, compromise of electrical signalling across the circuit with reversible pharmacological (Yamaguchi et al. 2003) or genetic (Lee et al. 2015) means not only desynchronises the circadian cycles of the cells, but also causes them to lose amplitude and precision. The SCN circuit therefore creates a mutually reinforcing network, established by coupling between individual clock cells. In doing so, they establish additional emergent properties, including ensemble phase, ensemble period and phase-spread, as evidenced by the stereotypical spatiotemporal wave (Hastings et al. 2018). The re-entrant loop motif is therefore seen again, this time at the level of the SCN circuit.

## 2.4 Circuit Architecture of the SCN as a Neuronal Network

### *2.4.1 Entrainment of the SCN Network: Photic and Non-photic Cues*

The powerful role of intercellular coupling within the SCN immediately begs the question of how SCN cells communicate within the circuit and how the circuit as a whole is entrained by inputs conveying cues regarding external time (i.e. retinal) and internal state. Entrainment to solar time is mediated by glutamatergic signals from the RHT, which act on the SCN via AMPA- and NMDA-type glutamate receptors expressed on retinorecipient neurons, including those that express VIP and GRP (Colwell 2011). The NMDA receptors all contain NR1, in combination with NR2A or NR2B sub-units that confer specific properties, such as calcium channel kinetics. These in turn determine the responses of retinorecipient neurons, including enhanced firing rates that ultimately lead to changes in the TTFL and thus entrainment to light (Mazuski et al. 2018). This control of the TTFL is achieved principally via induction of *Per* gene expression as it spontaneously declines or increases in early and late circadian night, respectively. Although it occurs initially in the core neurons, induction of *Per* expression spreads rapidly to the SCN shell, mediated by non-glutamatergic signals. Consequently, the spontaneous oscillation of the entire SCN is delayed by light after dusk (when induction opposes the spontaneous decline) or advanced by light before dawn (when induction accelerates the spontaneous increase) (Shigeyoshi et al. 1997). In nature, such shifts are small, of the order of minutes, as the SCN tracks solar dusk and dawn. Experimentally, however, the presentation of artificial light in the middle of night can shift the SCN and circadian behaviour by several hours. This perturbation also transiently uncouples the TTFL oscillations in the core, which responds rapidly, and the shell, which lags behind the core because of its indirect regulation by the RHT (Nagano et al. 2003). In modern society, nocturnal exposure to bright artificial lighting for recreational or work-related reasons is a cause of circadian and sleep disruptions that compromise well-being (Chang et al. 2015). Conversely, progressive decline in these entraining pathways at all and any level can lead to poor circadian coherence and sleep disturbance during ageing that can be associated with cognitive and other health-related difficulties (Duffy et al. 2015; Robbins et al. 2021).

Complementing photic entrainment by the induction of *Per* gene expression, the suppression of *Per* gene expression during circadian day, when it is high, can also reset the TTFL by advancing the spontaneous decline. This is best understood in experimental rodents, in which acute behavioural arousal is signalled to the core SCN by thalamic, brain stem and basal forebrain centres via cholinergic, neuropeptide Y- and serotonin-mediated cues (Mistlberger and Antle 2011; Yamakawa et al. 2016). Furthermore, photic and arousing cues can interact at the SCN, modulating and even cancelling out the entraining effect of the other, depending on the balance of *Per* expression (Maywood et al. 2002). The basic sequence of information flow is therefore core to shell and then to targets outside the SCN, with recurrent feedback

from those targets that reflects activity levels. This is important because it allows the stereotypical control of behaviour by the SCN to be over-ridden, and modified to match circumstances. A striking example of this is the recent finding that in mice fed a high-calorie diet, dopamine receptors expressed in the SCN mediate a decrease in neuronal excitability in response to extra-SCN dopamine. This reduction in SCN neuronal electrical activity promotes food intake at inappropriate phases, leading to severe metabolic dysfunction (Grippo et al. 2020). Similar neurochemical pathways exist to the human SCN and so may mediate the entraining effect of altered schedules of activity (e.g. shift work, meal timing) on the circadian system. In addition, from a therapeutic perspective, non-photic behaviourally mediated cues may sustain circadian entrainment when retinal signalling is compromised, most obviously following damage to the eye (Lockley et al. 2007), but also with progressive ageing.

### *2.4.2 SCN Network Synchrony: GABA and Neuropeptides*

In the context of within-circuit coupling, SCN neurons constitute a homogeneous population that ubiquitously synthesises and utilises the inhibitory neurotransmitter GABA, and both ionotropic $GABA_A$ receptors and Gi-coupled metabotropic $GABA_B$ receptors are expressed in the SCN alongside ancillary proteins involved in GABA metabolism and transport (Albers et al. 2017). It is therefore reasonable to infer that GABA plays a role in determining network synchrony. Indeed, exposure of dissociated SCN neurons to exogenous GABA suppresses their electrical firing, elicits phase delays when applied in late circadian day/early circadian night and daily treatment with GABA synchronously entrains their firing rhythms (Evans et al. 2013; Liu and Reppert 2000; Rohr et al. 2019). These effects are dependent on the $GABA_A$ receptors, as the $GABA_A$-specific agonist muscimol recapitulates them, whereas the $GABA_B$-specific agonist baclofen cannot (Liu and Reppert 2000). Notwithstanding these observations, pharmacological or genetic loss of GABAergic signalling in intact SCN explants does not disrupt aggregate SCN circadian timekeeping: a counter-intuitive observation (Aton et al. 2006; Freeman et al. 2013a, b; Ono et al. 2019; Patton et al. 2016). SCN explants from mice deficient in the vesicular GABA transporter (vGAT) do have elevated electrical activity, associated with synchronous burst firing across the network, which reveals a reduced GABAergic tone, but ensemble PER2::LUC rhythms are unaffected (Ono et al. 2019). Pharmacological GABA antagonism does have a subtle network-level effect, increasing the amplitude of cellular *Per1*-LUC oscillations and reducing the period distribution of individual SCN neurons (Aton et al. 2006; Freeman et al. 2013a). This indicates that within the SCN network, GABAergic transmission appears to "repulsively couple" neurons. A consequent decrease of network precision could potentially make the network more susceptible to phase-shifting stimuli, and thereby responsive to seasonal changes in photoperiod, a response thought to be mediated by GABAergic signals (Meijer and Michel 2015). Thus, although implicated in coupling of SCN neurons and the encoding of photoperiodic information, the definitive role(s) of GABA within

the SCN remains unclear (Albers et al. 2017). Indeed, GABA signalling may be more important for the time-dependent inhibitory control by SCN neurons of their extra-SCN targets (Paul et al. 2020; Ono et al. 2021).

SCN factors beyond GABA must, therefore, sustain its potent ensemble timekeeping. Dense core vesicles (DCV) for neuropeptide release are found in the terminals of SCN GABAergic neurons (Albers et al. 2017) and to some extent, therefore, GABAergic and neuropeptidergic signalling presumably work in concert in the SCN. One model is that the repulsive coupling mediated by GABA is counterbalanced by the attractive coupling of neuropeptides such as VIP. Certainly, $GABA_A$ antagonism can induce synchronous rhythmicity within previously asynchronous VIP-null SCN explants (Freeman et al. 2013a). In such a scenario, where neither repulsive nor attractive cues are active, other signalling axes are able to sustain circuit function. Under normal circumstances, by balancing destabilising GABA against neuropeptidergic synchronisation, the network is able to generate correctly timed spatiotemporal dynamics. It is likely that GABA and neuropeptides interact together at the network level to help SCN neurons (alongside their cell-autonomous electrical programme) reach a synchronous happy point of depolarisation that sustains firing and quiescence by clamping resting membrane potential within a permissive range.

Although most attention has focused on VIP, a range of neuropeptides and their cognate receptors is expressed in the SCN, including AVP, GRP, PROK2, cholecystokinin (CCK), Neuromedin-S (NMS) and –U (NMU), Neurotensin (NT), Angiotensin II (AII), methionine enkephalin (mENK), somatostatin (SST) and substance P (SP) (Abrahamson and Moore 2001; Karatsoreos and Silver 2007; van den Pol and Tsujimoto 1985). Notwithstanding variation between species, in unbiased transcriptomic analysis of single cells from the mouse SCN, these genes have been used to define neuronal clusters across the population (Wen et al. 2020; Morris et al. 2021) (Fig. 2.2b). Thus, it is important to consider the SCN network organisation on three levels: the active neuropeptide, its cognate receptor and the distinct cellular populations expressing these factors.

### *2.4.3 VIP Axis: Mediator of SCN Photic Entrainment and Neuronal Synchrony*

VIP is the best characterised neuropeptide in the SCN. Expressed in the SCN core in ~ 10% of SCN neurons, it forms a paracrine signalling axis with VPAC2 receptor-expressing neurons in the SCN shell (~ 35% of SCN neurons) (Morris et al. 2021; Patton et al. 2020). SCN VIP expression is rhythmic, regulated by clock-driven upstream E-box elements (Silver et al. 1999), and AT-motifs, driven by ZFHX3 (Parsons et al. 2015). VPAC2, a Gs-coupled GPCR is also rhythmic, peaking in circadian day at ~ CT4 (An et al. 2012; Doi et al. 2016). Consistent with the retinorecipient character of VIP cells, VIP is a potent regulator, phase-shifting SCN slices ex vivo and animals in vivo, in a phase-dependent mimic of light pulses, with delays

and advances in early and late circadian night, respectively (An et al. 2011; Hamnett et al. 2019; Piggins et al. 1995). It can also accelerate the speed of re-entrainment of mice to a new lighting schedule (An et al. 2013). In steady-state oscillation, VIP synchronises and maintains SCN neuronal and behavioural rhythms, which are disorganised in VIP- or VPAC2-null mice (Harmar et al. 2002; Colwell et al. 2003; Hughes et al. 2004; Aton et al. 2005). This is caused by a loss of network synchrony and reduced amplitude of SCN neuronal rhythms (Atkinson et al. 2011; Aton et al. 2005; Maywood et al. 2011, 2006) and can be restored acutely in VIP-null SCN by treatment with VIP or a VPAC2 agonist (Atkinson et al. 2011; Aton et al. 2005; Maywood et al. 2006) or chronically by co-culture with a VIP-proficient SCN. This is effected by paracrine release of VIP (Maywood et al. 2011; Ono et al. 2016). Activation of the VPAC2 receptor by VIP stimulates intracellular cAMP- (An et al. 2011; Hamnett et al. 2019) and kinase-dependent signalling cascades, acting through the extracellular-signal regulated kinase 1/2 (ERK1/2) pathway and dual specificity phosphatase 4 (DUSP4) (Hamnett et al. 2019) to tune TTFL phase. It also increases electrical activity of SCN neurons by activation of a fast-delayed rectifier (FDR) current (Kudo et al. 2013).

VIP cells peak in electrical activity during mid-circadian day at ~ CT6.5 (Enoki et al. 2017; Hermanstyne et al. 2016; Mazuski et al. 2018; Patton et al. 2020; Paul et al. 2020), while VPAC2 cells peak ~ 1.5 h later (~ CT8) (Patton et al. 2020). This serial activation from VIP to VPAC2 cells within the network-level spatiotemporal wave is also evident in their TTFL and cytosolic calcium rhythms (Patton et al. 2020). Both cell types are electrically quiescent in circadian night, when acute optogenetic stimulation of VIP cells (mimicking their activation via the RHT by nocturnal light) can shift the phase of circadian behaviour or ensemble molecular rhythms (Jones et al. 2018; Mazuski et al. 2018; Patton et al. 2020). VPAC2 receptor antagonism blocks such optogenetically induced shifts (Jones et al. 2015), whilst sustained chemogenetic activation of VIP cells phase-shifts the SCN and irreversibly re-programmes the spatiotemporal wave of gene expression in the SCN (Brancaccio et al. 2013). In contrast, optogenetic stimulation of VPAC2 cells is not sufficient to shift SCN phase (Patton et al. 2020) suggesting that VIP cells entrain the TTFL of VPAC2 cells by controlling activity-independent signalling pathways (which likely include ERK1/2 and DUSP4) (Hamnett et al. 2019). Ablation of VIP cells in adult mice has behavioural effects ranging from minor loss of precision and shortening of free-running period (Mazuski et al. 2020) to behavioural arrhythmia (Todd et al. 2020). Ablation of VIP or VPAC2 cells in SCN slices severely attenuated the amplitude of molecular rhythms (Mazuski et al. 2020; Patton et al. 2020), with less severe effects following VPAC2 cell ablation (Patton et al. 2020). Thus, loss of the cells that express the neuropeptide source or receptor is not as detrimental to circadian time-keeping as the loss of the neuropeptide or receptor itself. This indicates that the VIP or VPAC2 cells release other factors, and focussed loss of VIP-VPAC2 signalling causes a neurochemical imbalance that desynchronises the SCN network. As noted above, unopposed synaptic GABAergic communication may cause this (Fan et al. 2015; Paul et al. 2020; Freeman et al. 2013a).

Finally, beyond acute phase-shifting and sustained cellular synchrony, the VIP-VPAC2 cellular axis acts as pacemaker to the SCN circuit. AAV-mediated genetic complementation in CRY-deficient mice to alter the properties of the cell-autonomous TTFLs of VIP and/or VPAC2 cells can change the ensemble period of the SCN to match that of the VIP-VPAC2 cellular axis. It can also establish robust TTFL rhythms across otherwise arrhythmic SCN when only VIP and VPAC2 cells, together, have competent TTFLs. The initiation of circadian competence in the VIP-VPAC2 cellular axis also initiates behavioural rhythms in previously arrhythmic CRY-null mice (Patton et al. 2020). Indeed, the cell-autonomous TTFL of VPAC2 cells, the cellular output of this neurochemical axis, determines the period and circadian competence of behavioural rhythms in mice (Hamnett et al. 2021).

### *2.4.4 GRP Axis: An Accessory Entrainment and Synchronisation Pathway*

GRP is expressed in the SCN core, in retinorecipient cells that constitute in ~ 5% of SCN neurons, with some overlap in a subset of the VIP cells (Morris et al. 2021; Wen et al. 2020). Its expression is circadian (Dardente et al. 2004), driven at least in part by AT-motifs activated by ZFHX3 (Parsons et al. 2015). The GRP receptor, GRPR (also known as $BB_2$) is a Gq-coupled GPCR expressed in the SCN shell (Karatsoreos et al. 2006). Both in mice in vivo and in SCN slices ex vivo, exogenous GRP can trigger phase-shifts and acute gene expression comparable to the effect of light pulses (Piggins et al. 1995; Gamble et al. 2007). GRP therefore acts in parallel to, and converges with, the VIP-VPAC2 axis by activating the TTFL of shell AVP neurons. Correspondingly, mice lacking GRPR have reduced responses to photic cues (Aida et al. 2002), whilst GRPR antagonists can suppress peak firing rate (Brown et al. 2005). Moreover, in the absence of effective VIP-mediated signalling, GRP can restore cellular synchrony to SCN (Maywood et al. 2006, 2011).

### *2.4.5 AVP Axis: Within-Shell Coupling and Circadian Output*

AVP is enriched in 15–20% of SCN neurons located in the shell. Its transcription peaks in circadian day, controlled by TTFL E-box elements (Jin et al. 1999), cAMP-response elements (CREs) (Arima et al. 2002) and ZFHX3-driven AT-motifs (Parsons et al. 2015). AVP receptors (AVPR) V1a and V1b are Gq-coupled GPCRs that are expressed rhythmically in the SCN shell (Bedont et al. 2018; Morris et al. 2021) (Morris et al. 2021) peaking during circadian night. Consistent with this, V1a/1b agonists induce phase delays when administered to SCN explants during circadian night (Rohr et al. 2021). Genetic deletion of the V1a and V1b receptors creates a more loosely synchronised circuit, which allows both the SCN and animal to entrain more

rapidly to external perturbation (Yamaguchi et al. 2013), and in the absence of VIP-signalling, AVP can act as a synchronising factor (Maywood et al. 2011; Ono et al. 2016). Additionally, treatment with V1a/V1b antagonists lengthened the period of SCN explants and decreased spatiotemporal phase dispersion (Bedont et al. 2018). It also prevented initiation of de novo SCN oscillation following CRY1 complementation, suggesting that AVP is required to couple previously desynchronised oscillatory cells (Edwards et al. 2016).

AVP neurons are highly rhythmic, with calcium peaking in advance of voltage (Enoki et al. 2017) and both leading PER2::LUC (Shan et al. 2020). Rhythmicity requires the BMAL1-dependent cell-autonomous TTFL, although loss of rhythmicity in AVP cells does not compromise the rest of the SCN, which remains synchronously rhythmic (Mieda et al. 2015). Interestingly, in contrast to the VPAC2 cells (Hamnett et al. 2021), which encompass 85% of the AVP cells (Patton et al. 2020), conditional ablation of BMAL1 in AVP neurons lengthened the period of locomotor activity, but did not induce arrhythmia (Mieda et al. 2015; Shan et al. 2020), indicating that AVP neurons only play a role in coordinating some SCN outputs, while additional VPAC2-expressing cells also function to sustain network rhythmicity (Hamnett et al. 2021). AVP cells can, however, act as behavioural pacemakers: conditional deletion or over-expression of CK1 δ into AVP cells lengthens or shortens behavioural period, respectively, but again without altering intrinsic SCN explant period (Mieda et al. 2016). Thus, AVP neurons form at least part of the output from the SCN and when GABAergic transmission from AVP neurons is compromised, activity time is lengthened independent of behavioural period (Maejima et al. 2021). This is consistent with the phenotype of the AVP BMAL1 knockout where in addition to a lengthening of period, circadian activity time also lengthens (Mieda et al. 2015).

### *2.4.6 Prokineticin-2 Axis: More Than an SCN Output?*

PROK2 is enriched in ~ 12% of SCN neurons, straddling the core–shell division and overlapping to varying degrees other neuropeptidergic populations as well as cells that express its cognate receptor, PROKR2 (Cheng et al. 2002; Masumoto et al. 2006). PROK2 transcription is highly rhythmic, driven by TTFL E-box elements (Cheng et al. 2005) and AT-motifs controlled by ZFHX3 (Parsons et al. 2015). It peaks during circadian day and is correspondingly low during circadian night, when it can be induced by light. PROKR2 is a Gs-coupled GPCR enriched in ~ 16% of SCN neurons, 10% of which co-expresses PROK2 (Morris et al. 2021). Within the SCN, PROKR2 expression is also rhythmic at the transcript level following the pattern of PROK2 expression: peaking during the early circadian day and reaching its lowest abundance during the early circadian night.

Knockout of PROK2 or its receptor disrupts clock-controlled behaviour and physiology (Li et al. 2006; Prosser et al. 2007; Jethwa et al. 2008) without apparent compromise of SCN function and so PROK2 has been ascribed a role in SCN output. It can, however, suppress GABAergic signalling in SCN slices (Ren et al. 2011) and through

this disinhibition increase the baseline of PER2::LUC bioluminescence without altering SCN ensemble phase (Morris et al. 2021). Conversely, PROKR2 antagonists acutely suppress the amplitude of the subsequent PER2::LUC peak (Morris et al. 2021), presumably via a disruption of signalling to CREs in the promoters of TTFL components (Colwell 2011). Pharmacological inhibition of PROKR2 also reversibly lengthens SCN period (Morris et al. 2021) with a similar small lengthening of behavioural period observed in mice lacking PROK2 or PROKR2 (Li et al. 2006; Prosser et al. 2007). Furthermore, ectopic expression of PROK2 under the control of the PROKR2 promoter disrupts the SCN TTFL and circadian behaviour (Li et al. 2018). Coherent SCN function therefore depends on where PROK2 is expressed and how it is signalling to target cells. Consistent with this, intersectional manipulations of the PROK2-PROKR2 cellular axis have revealed a pacemaking function parallel to that of the VIP-VPAC2 axis. Both PROK2 and PROKR2 populations express TTFL rhythms, with PROK2 cells in advance of the PROKR2 cells by ~ 0.5 h (Morris et al. 2021). As with VIP-VPAC2 (Patton et al. 2020), this difference between ligand- and receptor-expressing populations may contribute to the circuit-wide spatiotemporal waves of activity. Moreover, intersectional manipulations of the cell-autonomous TTFLs of PROK2 and PROKR2 alter the ensemble period of the SCN and initiate rhythms in an otherwise arrhythmic circuit (Morris et al. 2021). In contrast to the VIP-VPAC2 axis, where ensemble period is only controlled when both cell populations are targeted (Patton et al. 2020), both PROK2 cells or PROKR2 cells can individually control circuit period. To initiate rhythms, however, both PROK2 and PROKR2 populations need to have activated TTFLs, as is the case for the VIP-VPAC2 axis.

### *2.4.7 An Emerging View of the Functional Topology of the SCN Network*

The time-base over which the SCN operates, 24 h, is very different from that of other neural circuits, which process information over much shorter intervals. Various features of the SCN network architecture appear especially well adapted to this role. The first is the paracrine nature of neuropeptidergic signalling which sustains a slow but progressive flow of information through the circuit. This is reflected in the spatiotemporal waves of TTFL and neural activity, which loop across the SCN in a stereotypical pattern. The wave may comprise distinct stages, each consisting of the serial activation of ligand-releasing and receptor-expressing populations within distinct neuropeptidergic axes, themselves linked in a chain (Fig. 2.2b). Available evidence from the VIP-VPAC2 and PROK2-PROKR2 axes supports this view, and future studies may expand the network to include AVP-, GRP- and other neuropeptide-mediated signalling. The ability of distinct neuropeptidergic populations to control ensemble period, driving it at their own cell-autonomous period, further supports this view of the network as one built around serial activation through

a chain of cellular hubs. Indeed, the de novo establishment of network-wide oscillations by circadian-competent VIP-VPAC2 or PROK2-PROKR2 axes emphasises the ability of cell-derived (neuropeptidergic) circadian signals to propagate through and organise an otherwise circadian-incompetent circuit. For the purposes of entrainment to external time and to internal cues, the VIP and GRP cells are the entry point to the network. This may explain why the circadian-competent VIP-VPAC2 axis is able to establish spatiotemporal order across the SCN, whereas the PROK2-PROKR2 axis is unable to achieve this order, even though it can initiate basic rhythmicity (Patton et al. 2020; Morris et al. 2021). These results support a model whereby VIP and VPAC2 cells work together as a distributor node in the SCN network to transfer circadian timing information from the retinorecipient cells to the rest of the network, while the PROK2 and PROKR2 cells work together to integrate temporal information across the network (Fig. 2.2b).

Orthogonal to the anatomical structure of the SCN circuit, analysis of single-cell RNA sequencing data has made it possible to construct formal network topologies based on the inferred relationships between neuropeptide ligand- and receptor-expressing populations (Wen et al. 2020; Morris et al. 2021). These support both linear, recurrent and circular topologies, the latter two likely facilitating self-sustained oscillation as (again) output becomes input. Importantly, all of the neuropeptidergic axes are under circadian regulation, and from a transcriptional perspective, they are assembled during circadian day when neural electrical activity is maximal and dissolved in circadian night, when neural firing rate is minimal. The expression of VIP, AVP, GRP and PROK2, as well as their cognate receptors, all exhibit this temporal plasticity, with the PROK2-PROKR2 axis being the most dramatic as, transcriptionally speaking, it disappears at night. Assuming this is carried through at the levels of ligand release and subsequent receptor activation, it means that neuropeptidergic signals are effective during circadian daytime but exert little effect on the network at night. This implies that other, non-neuropeptidergic cues are important for nocturnal advancement and co-ordination of cell-autonomous and network-level oscillations.

## 2.5 The Role of Astrocytes in the SCN

Alongside the neurons in the SCN network sit the glial cells, of which astrocytes are the most numerous (Guldner 1983; Morris et al. 2021; Wen et al. 2020). In common with most mammalian cell types, astrocytes display clear circadian TTFL rhythms (Prolo et al. 2005; Marpegan et al. 2011; Brancaccio et al. 2017; Tso et al. 2017) alongside cytosolic circadian cycles of $[Ca^{2+}]_i$ (Brancaccio et al. 2017; Patton et al. 2022). Strikingly, however, these rhythms in the intact SCN network sit in antiphase to neuronal rhythms, i.e. they peak in circadian night when neurons are inactive (Fig. 2.3a). The overriding question, therefore, has been whether, despite being outnumbered by neurons by a factor of 3–1, astrocytes actively participate in network timekeeping?

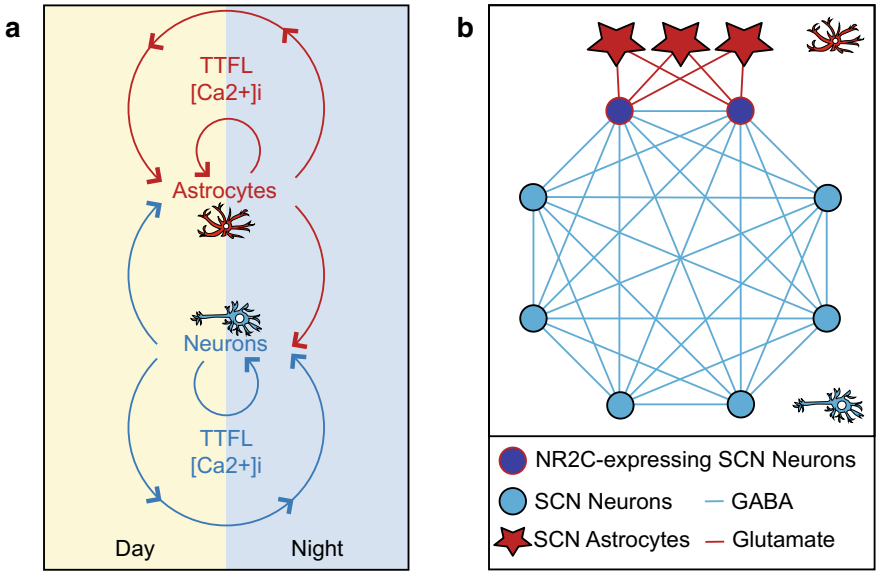


**Fig. 2.3** Neurons and astrocytes communicate within the SCN network and form a network-level re-entrant motif. **a** Astrocytes and neurons communicate with one another to correctly phase their respective TTFLs. Importantly, neurons are active during circadian day, consistent with the timing of the transcriptional presence of neuropeptidergic signalling hubs, while astrocytes are active during the night, consistent with the timing of astrocyte-derived glutamate signals. Thus, while neurons direct daytime network functions, astrocytes appear to direct night-time functions. **b** A network schematic for astrocytic control of the SCN neuronal network via nocturnal release of glutamate. This signal is sensed by a subset of SCN neurons expressing NR2C-containing NMDA receptors, which in turn utilise synaptic GABA to suppress the activity of the rest of the neuronal network

### *2.5.1 Astrocytic Control of Circadian Rhythms*

Astrocytic metabolism can be disrupted by treatment with the glial-specific metabolic toxin fluorocitrate, a suicide inhibitor of aconitase, which arrests carbon flux through the astrocytic citric acid cycle (Fonnum et al. 1997). Treatment of acute SCN explants from rats with fluorocitrate at CT6 disrupts ensemble neuronal electrical activity rhythms on the following cycles, indicating that on some level SCN astrocytes are able to direct activity within the SCN network (Prosser et al. 1994). Similarly, chronic treatment of free-running SCN explants with fluorocitrate disrupts astrocytic $[Ca^{2+}]_i$ oscillation, supresses the amplitude of PER2::LUC oscillations and compromises network synchrony (Patton et al. 2022). Furthermore, disruption of astrocytic communication through pharmacological or genetic manipulation of gap junctions or hemichannels (Shinohara et al. 2000; Brancaccio et al. 2019) and disruption of glial proliferation through antimitotic treatments (Shinohara et al. 1995) also disrupts SCN neuronal rhythms. Astrocytic competence is necessary, therefore, to maintain network-level SCN rhythmicity (Fig. 2.3a).

The sufficiency of astrocytic clocks to control the SCN circuit is demonstrated in two ways. First, if the period of the cell-autonomous TTFL specifically of astrocytes is lengthened by intersectional genetic means, then the ensemble period of the SCN is also lengthened (Brancaccio et al. 2017; Tso et al. 2017; Patton et al. 2022). In addition, deletion of BMAL1 from astrocytes lengthens ensemble period, which suggests that a BMAL1-dependent signal from astrocytes is required for timely progression of the network cycle. In contrast, shortening of the cell-autonomous period of astrocytes does not shorten ensemble period to the degree seen when the period of neurons is shortened, indicative of some differential potency between astrocytic and neuronal pacemaking (Patton et al. 2022). Second, if astrocytes are the only cells in the SCN with a functional clock, they are nevertheless able to impose rhythmicity, monitored as both TTFL and neuronal $[Ca^{2+}]_i$ oscillations, on the rest of a previously arrhythmic circuit (Brancaccio et al. 2019; Patton et al. 2022). Albeit the time-course is slower than that of neuronal initiation. SCN astrocytes are therefore not passive components within the SCN network: rather, they are effective pacemakers, and this competence is emphasised further by in vivo studies. In mice where only the cell-autonomous period of SCN astrocytes is lengthened, locomotor activity rhythms are also lengthened (Tso et al. 2017; Brancaccio et al. 2017). Furthermore, disruption of the TTFL by conditional deletion of BMAL1 only in astrocytes results in a lengthening of behavioural period (Barca-Mayo et al. 2017). Finally, CRY1-complementation into SCN astrocytes of CRY-null mice initiates behavioural rhythmicity, indicating that astrocytes are able to adequately organise neurons within the SCN network to coherently control their output (Brancaccio et al. 2019). These results therefore elevate astrocytes to active participants in SCN network timekeeping.

### *2.5.2 Astrocyte-To-Neuron-To-Astrocyte Communication Within the SCN Network*

How, then, are SCN astrocytes able to control circadian rhythmicity? The first clue to this emerged from imaging approaches that revealed rhythmic release of glutamate within isolated free-running SCN explants. This rhythmic release is striking on two fronts: first, SCN neurons are exclusively GABAergic and, second, glutamate rhythms within the SCN peak during the night, in antiphase to neuronal activity (Brancaccio et al. 2017). Glutamate release in the extracellular space is therefore co-phasic with astrocytic metabolic activity rhythms, suggesting that they are the source of this transmitter and, indeed, blockade of astrocytic glutamine synthetase, which synthesises glutamine from glutamate, increased extracellular glutamate in SCN slices. Astrocytic control was confirmed by a significant disruption in extracellular glutamate rhythms following either caspase-3 driven ablation of SCN astrocytes (but not neurons) (Brancaccio et al. 2017) or pharmacological blockade of astrocytic connexin-43 (Cx-43) hemichannels (Brancaccio et al. 2019). These manipulations in turn disrupted SCN TTFL rhythms, confirming the relevance of astrocytic control

of glutamate for clock function. So how might glutamate act on the SCN neural circuitry? Whereas NMDA receptors with NR2A and NR2B sub-units mediate retinal entrainment in the core SCN (Colwell 2011), the dorsal SCN expresses NR2C sub-units which confer different dynamics and agonist-sensitive properties to the oligomeric NMDA receptor (Fig. 2.3b). Pharmacological blockade of NR2C-containing NMDARs reduced the amplitude of SCN molecular oscillations and depolarised cells during the night (Brancaccio et al. 2017). Importantly, it also eliminated astrocyte-initiated rhythms (Brancaccio et al. 2019), indicating that signalling via the NR2C subunit is vital for linking astrocytic and neuronal clock function (Fig. 2.3b). Curiously, however, glutamate release from astrocytes appears to be inhibitory to the neuronal network, i.e. blockade of NR2C depolarised neurons, even though glutamate is conventionally an excitatory neurotransmitter. This can be reconciled by the observation that the subset of neurons that express NR2C display pre-synaptic calcium elevations during the night, and this nocturnal rise presumably facilitates the synaptic release of GABA. This will in turn suppress neuronal activity across the network, even though the action potential firing of GABAergic cells is not altered (Brancaccio et al. 2017). The effect seen in the SCN may be more general, in so far as astrocytes can manipulate extracellular levels of several neurotransmitters to mediate astrocyte-to-neuron function. The first is active release of glutamate from astrocytes, which is sensed by neuronal NR2C-containing NMDARs to control SCN GABAergic transmission (as described above) (Brancaccio et al. 2017). Second, the uptake of GABA may rebalance VIP/GABAergic signalling (Barca-Mayo et al. 2017) (and see Sect. 4.2), and, third, the active release of adenosine from astrocytes may alter neuronal GABAergic signalling (Hablitz et al. 2020). While these mechanisms are qualitatively different, they converge on a common theme: the control of GABAergic signalling.

If astrocytes are signalling to neurons to control neural activity, the neurons must also be signalling back to modulate astrocytic function. Consistent with this, endocannabinoids released by neurons induce calcium activity in SCN astrocytes following activation of astrocytic cannabinoid 1 receptors (CB1R) and thereby facilitate adenosine release (Hablitz et al. 2020). In addition, cultured cortical astrocytes can be entrained to daily VIP exposure (Marpegan et al. 2009). To understand the contributions of astrocytes to circadian timekeeping, we therefore need to understand how SCN astrocytes and neurons communicate time of day information to one another (Fig. 2.3). Such signalling adds to the emerging network model, being consistent with a necessarily slow and progressive information flow on a long timebase. This further level of paracrine cues (paracrine glutamate controlling synaptic GABA) and its nocturnally specific cellular activity will allow SCN timekeeping to "bridge" the gap when the diurnally active neuropeptidergic network is dismantled. Moreover, this adds yet another re-entrant loop motif to the SCN timekeeper.

## 2.6 SCN Outputs and Control of Circadian Behaviour and Sleep

### *2.6.1 SCN Outputs and Control of Circadian Behaviour*

The neural pathways that mediate SCN-dependent control over circadian behaviour including sleep are not well defined. This may in part reflect the diverse and highly distributed nature of circadian control. Neuropeptidergic axons from both core (VIP, GRP) and shell (AVP, PROK2) SCN neurons project out of the nucleus to the surrounding hypothalamus, where direct inputs to neuroendocrine nuclei such as the paraventricular nucleus and arcuate nucleus will mediate circadian regulation of hormonal and metabolic status (Paul et al. 2020; Mendez-Hernandez et al. 2020). SCN projections to the dorsolateral hypothalamus will impinge on wake-regulatory centres containing orexinergic and MCH-expressing neurons. A broader distribution of circadian signals arises from the sub-paraventricular zone (SPZ), which is adjacent to, and receives input from, the SCN. It projects to the medial forebrain, thalamus, hypothalamus and brainstem to provide several parallel and pathways for segregated circadian regulation of behaviour and physiology (Vujovic et al. 2015). This includes GABAergic efferents from the SPZ to the ventromedial hypothalamus that confer circadian control of aggression (Todd et al. 2018), as well as extensive input to the periaqueductal central grey, an area strongly associated with behavioural arousal. The midline paraventricular nucleus (PVT) is also interconnected, reciprocally, with the SCN and SPZ. It receives direct and indirect photic input and is densely innervated by orexinergic neurons which direct arousal-state transitions. It is, therefore, a likely route for circadian modification of affective behaviours, such as mood and motivation (Colavito et al. 2015). Notwithstanding this growing neuroanatomical knowledge, the means by which specific SCN neuronal populations control behaviour by specific pathways remains to be determined. AAV-mediated delivery of Cre-recombinase in vivo into the SCN of floxed VGAT mice decreased the quality and amplitude of circadian locomotor activity rhythms without altering period (Ono et al. 2019). Furthermore, this loss of GABAergic signalling did not disrupt timekeeping within SCN explants, suggesting that synaptically released GABA is potentially more important in the regulation of SCN outputs rather than SCN timekeeping itself (see above). Consistent with this, the same manipulation within just the AVP cells, similarly disrupted circadian behaviour without altering SCN rhythmicity (Maejima et al. 2021).

### *2.6.2 SCN in the Circadian Regulation of Sleep*

The principal overriding output of the circadian system is the control of the sleep–wake cycle. Although the exact function of sleep is not yet fully understood, its importance in relation to health is evident. Even acute sleep deprivation has known

effects on cognition and alertness (Lo et al. 2012). This is particularly relevant to the large numbers of shift workers in the population, where night shift work is associated with loss of concentration and increased workplace accidents (Budnick et al. 1994; Ryu et al. 2017). Furthermore, a breakdown of daily sleep–wake patterns is associated with many neurological and psychiatric illnesses (Leng et al. 2019; Sato and Sassone-Corsi 2021), although the extent to which these reflect cause or effect awaits clarification.

The current accepted model for sleep control involves two processes: a sleep homeostat, or Process "S" that measures sleep pressure as a function of length of time spent awake and a circadian Process "C" that allows sleep to be timed relative to the light–dark cycle (Borbely et al. 2016). In this model, the circadian clock could either behave as a passive gate on the homeostatic process, or it could actively promote sleep and/or wake at the appropriate circadian time. It is only by separating process S and C that this can be tested, and forced desynchrony protocols, which uncouple rest/activity rhythms from the internal circadian clock, have enabled this to some degree in human subjects. This has revealed that the circadian clock can modulate sleep propensity and structure and that ageing affects sleep regulation (Dijk et al. 1999). Equally, there is evidence that sleep pressure can influence circadian control of sleep [reviewed in (Deboer 2018)].

The identity of the sleep homeostat is unknown but through advanced techniques including opto- and pharmaco-genetics, viral tracing, fluorescent reporters of neuronal activity and human brain imaging, it has been possible to map individual sleep- and wake-promoting neuronal circuits within and between known sleep/wake-controlling regions in forebrain, hypothalamus and brain stem (Weber and Dan 2016; Boes et al. 2018). Within these networks, neural hubs control sleep state (REM and NREM), transitions between sleep and wakefulness and also presumably are sensitive to time spent awake. Whether homeostat(s) and wake- and sleep-promoting centres are co-located is not clear.

For Process C, early ablation studies across nocturnal and diurnal mammals demonstrated the necessity of the SCN for circadian timing of sleep, and most suggest that there is little or no effect on sleep homeostasis [reviewed in (Mistlberger 2005)]. However, SCN-ablated mice are reported to have reduced NREM compared to control animals during recovery from sleep deprivation (Easton et al. 2004), which suggests that the SCN may play a greater role in the control of sleep, beyond sleep timing. An additional level of complexity to the localisation of Process C came with the discovery of local TTFL-based clocks in various brain regions, including hippocampus, cerebellum and cerebral cortex, which can maintain autonomous rhythms in ex vivo culture for several cycles (Abe et al. 2002). These local clocks may confer circadian modulation of local functions, such as cognition (Kyriacou and Hastings 2010; Wright et al. 2012) that "chime" with central SCN-determined phases of sleep and wakefulness. As noted above, projections via the SPZ will mediate internal synchrony between SCN and local brain clocks. But what are their relative contributions to circadian control of sleep?

Genetic approaches confirm a role for the TTFL in Process C. Double deletions of either *Cry1, 2* or *Per1, 2*, or single deletion of *Bmal1* in mice abolish sleep/wake

rhythms under constant darkness (Wisor et al. 2002; Shiromani et al. 2004; Laposky et al. 2005). In contrast to the SCN neural ablations, genetic ablations variously affected total sleep time, sleep fragmentation and NREM recovery after sleep deprivation. These may arise from loss of local TTFL competence and/or the non-circadian pleiotropic effects of disrupted transcriptional programmes. Moreover, such universal genetic deletions cannot discriminate between effects originating from the SCN and those from other brain clocks. More specific conditional deletion can be achieved by intersectional means. For example, deletion of *Bmal1* in the histaminergic cells of the tuberomammillary nucleus (TMN) disrupted sleep architecture and recovery from sleep deprivation, without affecting overall circadian timing of sleep–wake cycles of mice (Yu et al. 2014). This suggests that the TTFL within local brain clocks can contribute to circadian sleep control, in this case, likely through appropriate circadian control of histamine synthesis. An alternative to such loss-of-function is genetic complementation to achieve gain-of-function. In CRY-null mice, local AAV-mediated expression of CRY1 in the SCN not only restored circadian behavioural rhythms but it also organised the previously arrhythmic sleep/wake cycle (Maywood et al. 2021a) (Fig. 2.4a–c). Moreover, deficits in NREM recovery sleep and sleep-dependent memory were reversed, as was novel object memory, a measure of sleep-dependent cognition. Thus, molecular circadian competence solely in the SCN is sufficient to effect Process C function in an otherwise "clockless" mouse. Clock competence in other brain regions is, therefore, not necessary. This does not mean, however that local clocks have no auxiliary role to play in circadian control of sleep and wakefulness. In the CRY-null mouse, local clocks are disabled, but what if time-keeping in SCN and local clocks is mismatched? Temporally chimeric mice can be created by intersectional means, such that SCN (and other) cells expressing the dopamine 1a receptor (*Drd1a*) have a cell-autonomous period of ~ 24 h, whilst all other cells and tissues have a period of ~ 20 h. This provides a suitable system where the brain has its own internal forced desynchrony, or misalignment: mutant local brain clocks running at a 20 h period, whereas the SCN provides a circadian output at ~ 24 h. The dominant pacemaking effect of the SCN *Drd1a* cells imposes a stable ~ 24 h rhythm to the SCN TTFL and also to rest/activity cycles (Smyllie et al. 2016a). In such mice, the sleep-wakefulness cycle also has a ~ 24 h period, but sleep is more fragmented compared to 24 h and 20 h, non-chimeric, control groups. Moreover, sleep-dependent novel object recognition memory is severely impaired in the temporally chimeric mice, but not in the controls, indicative of compromised sleep function (Maywood et al. 2021b) (Fig. 2.4d). This indicates that extra-SCN local brain clocks do likely play a role in circadian regulation of sleep, insofar as sleep and sleep-dependent memory are optimal when local clocks are "in tune" with the dominant circadian signal of the SCN.

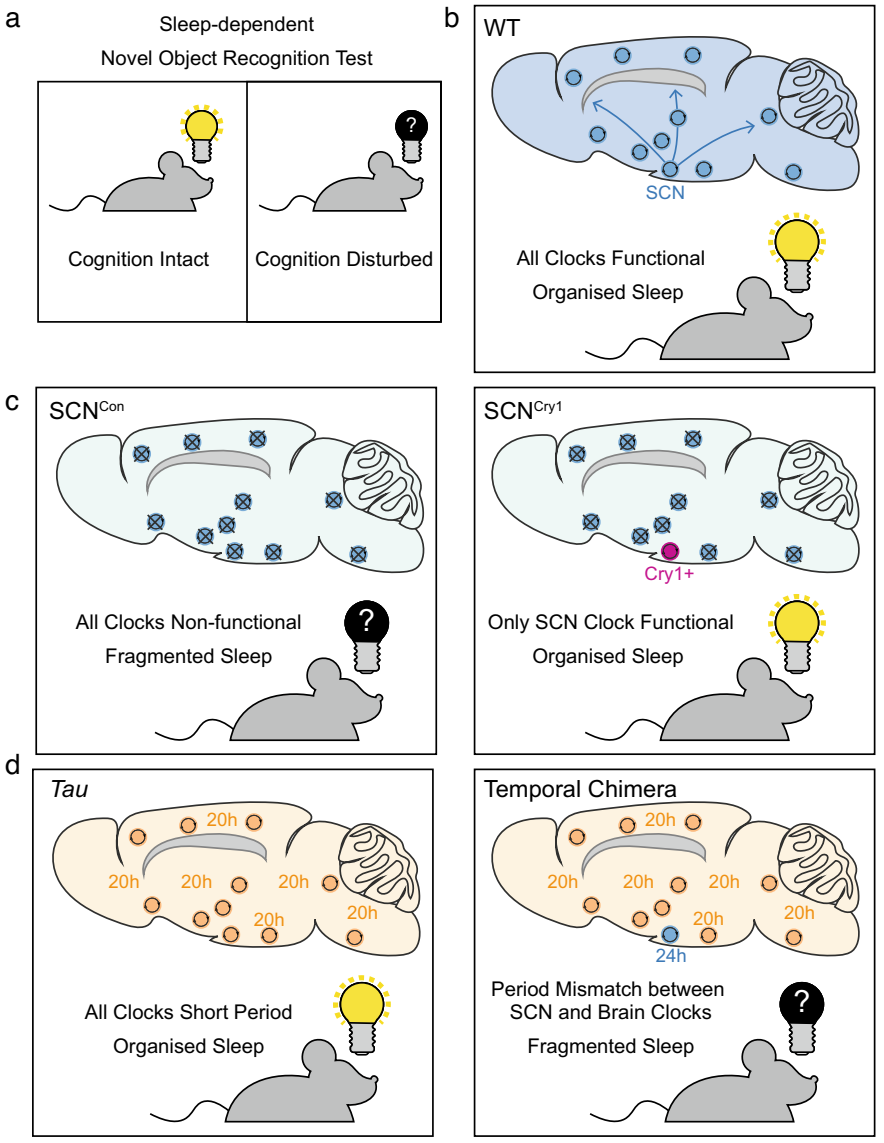


**Fig. 2.4** Circadian disruption of sleep leads to cognitive deficits. **a** Novel object recognition is a task to study sleep-dependent cognition. **b** In a wild-type mouse where all of the cell-autonomous clocks in the animal are coherent, sleep is well organised and the mouse is cognitively competent. **c** In a CRY-null mouse (left, $SCN^{Con}$) where all cell-autonomous clocks are arrhythmic, sleep is fragmented and the mouse displays cognitive deficits. If CRY1 is expressed via AAV into just the SCN of a CRY-null mouse (right, $SCN^{Cry1}$), sleep becomes organised and the mouse is cognitively competent. **d** In a short period (20 h), $CK1\varepsilon^{Tau}$ mouse (left, *Tau*) where all cell-autonomous clocks share a common, coherent period (left), sleep is well organised and the mouse is cognitively competent. In a mouse where excision of the short period $CK1\varepsilon^{Tau}$ allele is excised specifically in the SCN (right, Temporal Chimera), the SCN expresses a long period cell-autonomous oscillation (24 h) which is mismatched relative to the (20 h) cell-autonomous clocks in the rest of the mouse. This results in fragmented sleep and cognitive deficits in these mice

## 2.7 Conclusion and Future Perspectives

At all levels of organisation, the mammalian circadian timing system, focussed on the SCN, features re-entrant feedback loops that confer high-amplitude oscillation, precision and robustness (Fig. 2.5). The delineation of the cell-autonomous TTFL of mammals is a major achievement in chronobiology and neuroscience. Nevertheless, the current model is very qualitative and we have limited understanding of the exact and quantitative functions of the TTFL. This is an unmet opportunity because the operations of the SCN are ideal for formal analysis, more so than any other behaviourally relevant circuits, because its outputs are so "crystalline" and precise and the system spans seamlessly from molecules through cells and circuits to behaviour (Fig. 2.5). It is vital, therefore, to understand molecular abundances of the TTFL components through circadian time, and their affinities in the formation of complexes and how this directs their intracellular behaviour: mobility, stability and localisation. The smooth transitions between states of neural activity and quiescence reflect progressive changes in the expression of genes controlling excitability and metabolism, but how this is orchestrated at the level of the genome remains unclear.

The emerging model of the SCN circuit, based around slow and progressive paracrine signalling of time, looping around the network, highlights several critical features, such as the inter-dependence of the TTFL and neural activity, the serial activation of ligand- and receptor-expressing cell groups, and the capacity of some of these groups to impose their cell-autonomous properties on other SCN circuits. The requirement now is to understand how the assembly of these elements creates a greater whole, conferring emergent properties that are lacking from the individual cells. We also lack a clear understanding of both the topography (beyond core and shell) and topology of the circuit that could delineate the exact contribution of individual nodes (pacemaker, distributor and integrator nodes) and how they are related. Furthermore, what circuit elements actually close the loop of the spatiotemporal wave to take it full circle, and what does that wave represent for SCN output? The discovery of the central role of astrocytes in maintaining SCN timekeeping has raised a series of questions regarding neuron-to-astrocyte-to-neuron signalling, another circular motif that confers amplitude and stability. Clearly, SCN output cues are delivered by neurons but the cell-autonomous clocks of their astrocytic partners work through that neural circuitry. The transfer of information from astrocytes to neurons by paracrine means (possibly by astrocytic regulation of extracellular glutamate and thereby GABAergic tone) may be more general across the brain, and influence local changes in neural activity, such as during sleep stages and vigilance states that similarly occur over a longer time-base. The SCN is so powerful a pacemaker that if it is the only competent circadian clock in the animal, it can nevertheless impose appropriate cycles of sleep and wakefulness and in doing so maintain sleep-dependent cognitive function. The neural and neurochemical pathways by which the SCN achieves this regulation are unclear, and so we do not understand how the SCN affects the sleep–wake cycle. Is it primarily a promoter of wakefulness, and if so, does it activate wake-promoting centres and inhibit the complementary sleep-promoting circuits?

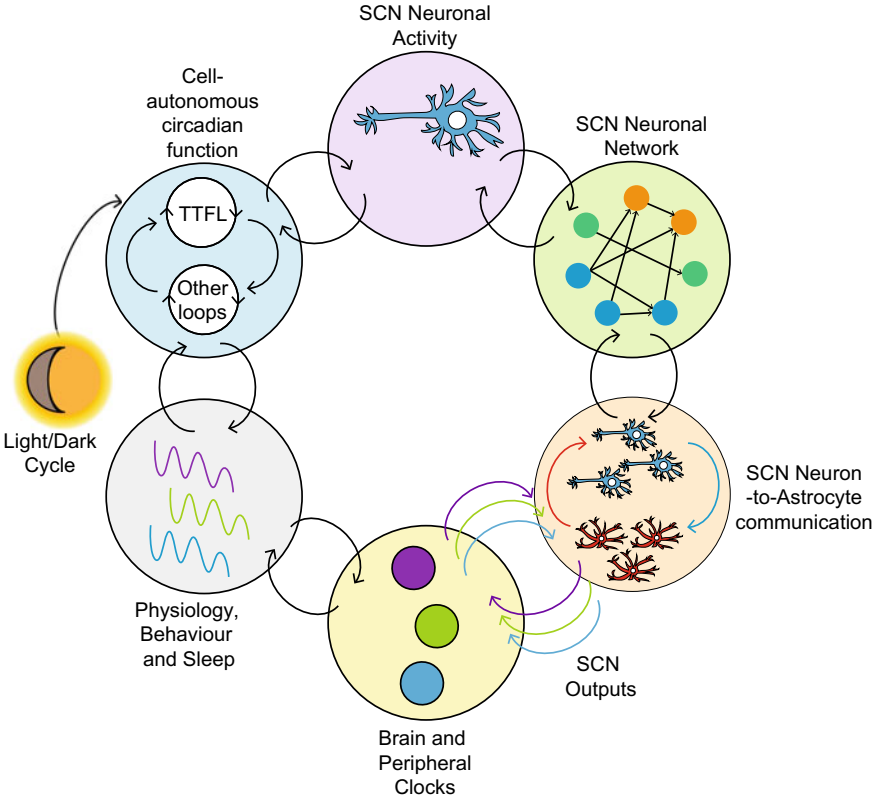


**Fig. 2.5** Different levels of circadian organisation form a re-entrant motif. Circadian organisation of physiology and behaviour extends with exquisite precision from the molecular level to the SCN neuronal and network level, to bidirectionally coupled SCN neurons and astrocytes and ultimately to SCN outputs that control behaviour and physiology on a daily timescale. Remarkably, temporal information flows seamlessly from one level to the next, with feedback between molecular and cellular elements increasing the overall precision, amplitude and robustness of the various rhythmic processes. Thus, at every level of circadian timekeeping, a re-entrant motif can be observed

Although the cell-autonomous TTFL clock is active across tissues and brain regions, the SCN is *primus inter pares*. Nevertheless, the deleterious effects of circadian chimerism, in which SCN and local brain clocks are mismatched, show that they operate in concert. Cognitive decline during ageing may therefore have some circadian origin, arising from progressive dysfunction in the SCN, in local brain clocks, and/or in their abilities to interact. This may arise in neurons and/or astrocytes in SCN and brain regions, either within their cell-autonomous clock or in cellular pathways regulated by the clock. In the context of neurodegeneration, this trajectory of decline is accelerated, particularly in diseases associated with the accumulation of toxic aggregates of misfolded proteins. Given the central role of the TTFL and its outputs in cellular homeostasis, age- or disease-dependent compromise of local TTFLs may

facilitate disease progression by reducing the capacity of the cell to prevent, process and neutralise such aggregates. These physiological defences are present in both neurons and astrocytes, the capacities of which are both compromised in neurodegenerative conditions. At the level of the whole brain, it is also clear that sleep, a dominant output of the circadian system, favours restorative functions, including cellular metabolism and brain-wide clearance of metabolites via the extracellular space and cerebrospinal fluid. Consequently, perturbed cell-autonomous clocks and sleep/wake cycles may well exacerbate the progression of aggregate-based neurodegeneration. Even though clock dysfunction may not be the primary cause of such diseases, by identifying the casual links in these processes, it should be possible to develop novel avenues to management, and possibly therapy. These translational applications of newly found circadian knowledge are in their infancy, but the pervasive roles of circadian clocks to cellular and brain health highlight the enormous range and scope of opportunities in this area.

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# Chapter 3
# Circadian Regulation of Sleep


**Zhaomin Zhong, Adeel Ahmed, and Han Wang**

## 3.1 Introduction

Sleep is a physiological and behavioral phenomenon existing in almost all animals, including humans, which is the basis of life activities (Weber and Dan 2016). Adequate sleep is fundamental to maintaining healthy physiology and bodily functions (Banks and Dinges 2007). Sleep disturbances adversely impact development, cognition, and longevity (Borges et al. 2019; Song and Zhu 2021). Approximately, one-third of a person's life is spent on sleep. The quality of sleep is one of the foundations of life quality. Numerous studies have identified the relationship between sleep and anatomical, physiological, and environmental characteristics (Trost Bobic et al. 2016; Troynikov et al. 2018; Eban-Rothschild et al. 2017). In mammals, for example, factors such as diet, social status, and BMI (body mass index) all impact the total sleep time (Binks et al. 2020; Garfield 2019).

However, the occurrence and regulation of the sleep–wake cycle are complex, involving different brain circuits, cells, and molecules (Wang et al. 2021). On the one hand, numerous neuroanatomical and neurotransmitter interactions, including acetylcholine (Ach), dopamine (DA), norepinephrine (NE), serotonin (5-HT), histamine (HA), and hypocretin (HCRT), have been shown to control wakefulness (Vanini and Torterolo 2021). On the other hand, the onset of sleep is controlled by the activity of sleep-promoting neurons located in the anterior hypothalamus, which use gamma-aminobutyric acid (GABA) to inhibit wake-promoting regions (Weber and Dan 2016), and by sleep-promoting hormone melatonin (Fatemeh et al. 2022) and substances, such as adenosine (Lazarus et al. 2019). In addition, the brainstem regions that were inhibited during wakefulness and non-rapid eye movement (NREM) sleep

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became active during rapid eye movement (REM) sleep. The accumulation of sleep-promoting molecules in the brain during prolonged wakefulness and the physiological role of gene expression during sleep add to the complexity of sleep regulation. In recent years, many groups have focused on the study of the sleep–wake cycle and have made great strides to advance the recognition of new paradigms in sleep regulation, brain-related circuits, and sleep function.

The molecular mechanisms underpinning circadian regulation of sleep have been investigated, and mutations in core circadian clock genes have been shown to lead to circadian clock sleep–wake disorders, and hormones or neuropeptides regulated by the circadian clock can also affect sleep (Gandhi et al. 2015; Ashbrook et al. 2020), while studies on homeostatic regulation investigate different brain regions or the effects of neural hormones. With the rapid development of transcriptome, metabolome, single-cell sequencing, optogenetics, chemical genetics, and other technologies, sleep-regulating genes/neurons/neurotransmitters, and neural networks underlying sleep and wake have gradually been deciphered (Weber and Dan 2016).

## 3.2 The Circadian Clock System

The circadian clock refers to the endogenous timekeeping mechanism that generates, regulates and maintains approximately 24-h rhythms, including not only the sleep–wake cycle, but also behavioral and physiological activities such as hormone secretion, body temperature, and urine production (Takahashi 2017). Almost all living things on the Earth have evolved circadian clocks. A basic feature of the circadian clock is that it can free-run without external cues such as light, indicating its endogenous nature (Sack et al. 2000). However, the circadian clock can be reset or entrained by periodic environmental factors such as light, thereby synchronizing with the external environment. In doing so, the circadian clock allows for anticipating environmental changes and better coordinating the internal machinery in advance (West et al. 2017).

In 2017, three American chronobiologists, Jeffrey Hall, Michael Rosbash and Michael Young won the Nobel Prize in Physiology and Medicine for their significant contributions to unravel the molecular mechanism of circadian rhythm regulation, which has energized the circadian field, inspired more circadian studies, and facilitated more discoveries and circadian applications in daily life and medical practices (Wang 2018). The circadian clock is characteristic of individual cells and is based on transcription-translation feedback loops. Rhythmic coordination between organ systems is achieved through signals from the suprachiasmatic nuclei (SCN), the master pacemaker in the hypothalamus (Nassan and Videnovic 2022). The SCN not only coordinates the rhythmic activity of cells and organs in the body, but also synchronizes the body's approximately 24-h rhythmic activity with the 24-h cycle of the external environment, a process known as entrainment (Stoynev et al. 1982). Functional circadian timing systems allow for organisms to anticipate and prepare for

regular changes in the environment, such as sunlight, food availability, the presence of predators, and thus provide an adaptive advantage (Abraham et al. 2013).

Most types of cells in the human body, including the nervous system, rhythmically express canonical circadian clock genes and circadian clock-controlled genes (ccgs). Circadian patterns of gene expression are driven by core clock genes that are present in SCN neurons, the nervous system, and other cell types (Takahashi 2017). In mammals, core clock genes include *Clock* and *Bmal1*, and their protein products activate transcription of *Per* and *Cry*, which are then translated into proteins that form a heterodimer and enter the nucleus, and repress the transcriptional activities of CLOCK and BMAL, thereby inhibiting their own gene transcription in the negative feedback loop (Fig. 3.1) (Takahashi 2017; Blum et al. 2018). The paralogous genes of *Per* (*Per1* and *Per2*) and *Cry* (*Cry1* and *Cry2*) have evolved non-redundant functions. Deletion of *Per1* in mice leads a free-running period 0.5–1 h shorter than wild types, whereas deletion of *Per2* results in a shortened period of 1.5 h (Zheng et al. 2001). *Per2* knockout mice are only able to maintain a circadian rhythm for nearly seven days, after which they became completely arrhythmic (Bae et al. 2001). *Cry1* knockout mice have a free-running circadian period that is one hour shorter than wild type, but *Cry2* knockout mice develop a free-running period that is one hour longer (van der Horst et al. 1999). At the molecular level, when the function of one gene in a family is lost or reduced, the expression of other paralogous genes will be up-regulated, thereby exhibiting a compensatory effect. Reduced expression of *Per1* and *Cry1* results in up-regulated expression of *Per2* and *Cry2*, respectively (Baggs et al. 2009); however, decreased or lost expression of *Per2* and *Cry2* do not lead to compensatory up-regulated expression of their paralogous genes (Baggs et al. 2009). In addition, $Per1^{-/-}$, $Per2^{-/-}$ double-knockout mice and $Cry1^{-/-}$, $Cry2^{-/-}$ double-knockout mice completely lose their intrinsic circadian rhythms (Bae et al. 2001; van der Horst et al. 1999), implicating that at least one member from each mouse *Per* and *Cry* family plays critical roles in maintaining circadian rhythm stability at the molecular and behavioral levels.

An additional negative feedback loop of nuclear receptors, such as the *Nr1d1* and *Ror* genes, is thought to further stabilize transcription-translation feedback oscillations (Guillaumond et al. 2005). Thus, disrupting the function of these core circadian genes can lead to dramatic changes in mammalian circadian rhythms. The oscillatory activity of these clock genes controls rhythmic expression of a large network of genes known as clock-controlled genes. The large-scale RNA-sequencing analyses have revealed that approximately 43% genes are rhythmically expressed in mice (Zhang et al. 2014), and more than 80% protein-coding genes display transcription rhythmicity in baboon (Mure et al. 2018), highlighting extensive roles of the circadian regulation in the body. Molecular clocks, found in nearly every cell in our body, coordinate the rhythmic expression of genes at the local level (Brown and Azzi 2013). In addition to being regulated by a transcription-translation feedback loop, the circadian clock is also regulated by post-translational modifications (Mehra et al. 2009; Rijo-Ferreira and Takahashi 2019).

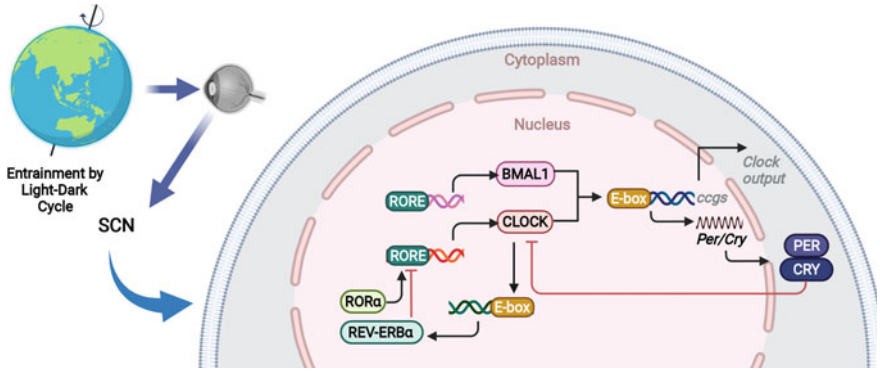


**Fig. 3.1** A mammalian circadian clock model of negative and positive transcriptional feedback loops. While the BMAL1-CLOCK heterodimer drives oscillating expression of clock-controlled genes (ccgs) with E-box containing promoters, RORs and REV-ERBs regulate *Bmal1* transcription. The clock output, including those involved in sleep, is achieved through many genes with E-box containing promoters collectively shown as ccgs (gray). See main text for further details and abbreviations

## 3.3 Regulation of Sleep—Two-Process Model

In 1982, the Hungarian-Swiss scientist Alexander Borbély proposed the "two-process model of sleep regulation" (Borbely 1982), providing a conceptual framework for dissecting the sleep–wake cycle with a better understanding of the temporality and structural properties of the sleep–wake behavior. It was postulated that a homeostatic process (S) is functionally up-regulated during sustained wakefulness and a circadian process (C) determines the 24-h temporal distribution of sleep and wakefulness (Fig. 3.2) (Borbely 1982). In humans, the phase relationship between the two processes allows arousal to be merged into a single bout, and homeostasis drives a progressively increased desire to sleep with wakefulness throughout the day (Dijk and Czeisler 1995). The interaction of the two mechanisms, one promoting sleep and the other promoting sleep and wakefulness in a time-specific manner, allows for people to stay awake at a fixed time during the day and fall asleep at a fixed time during the night, displaying an overt rhythm. Although great progress has been made in deciphering circadian rhythms, the molecular genetic and cellular mechanisms underlying sleep homeostatic regulation and its interaction with the circadian clock system are still poorly understood.

While homeostasis and the circadian clock each play an important role in sleep regulation, the two processes are also interconnected (Borbely 1982). Homeostatic regulation of sleep is discussed below.

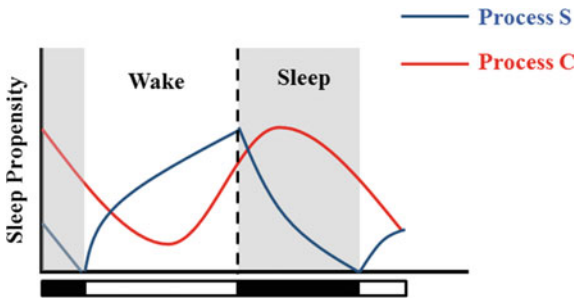


**Fig. 3.2** Borbely's two-process model of sleep regulation. Process S indicates the homeostatic built-up of sleep pressure, and Process C represents the circadian rhythm

## 3.4 Homeostatic Regulation of Sleep

Body homeostasis regulates sleep propensity, which increases exponentially at the onset of wakefulness and then tapers off during sleep (Borbely 1982). The homeostatic process is functionally distinct from the circadian clock system, as rodents with suprachiasmatic nucleus (SCN) lesions display a strong sleep compensation after complete sleep deprivation (Mistlberger et al. 1983). The correlation between sleep homeostasis and sleep intensity can be shown by the NREM EEG (Electroencephalogram) changes (delta power, 0.5–4 Hz) during NREM. In sleep homeostatic regulation, the sleep preference index increased with wake time and decreased rapidly after a typical sleep bout (Lancel et al. 1991). Furthermore, the regulation of NREM and sleep homeostasis has been shown to be associated with specific brain regions.

Sleep-promoting neurons in the ventrolateral preoptic nucleus (VLPO) and median preoptic nucleus (MnOP) underlie the neuroanatomical basis of sleep homeostatic regulation (Gong et al. 2004). These neurons are a subset of GABAergic neurons that project long distances throughout the cerebral cortex, and the number of neurons activated during sleep is proportional to NREM intensity (Gerashchenko et al. 2008). The VLPO lesions were shown to reduce sleep and alter the normal sleep architecture (Lu et al. 2000). The VLPO and MnOP neurons discharge during NREM sleep, and turn almost inactive during wake (Gompf and Anaclet 2020). In addition, some regions of the basal forebrain (BF) and lateral hypothalamus (LH) also act through GABAergic neuromodulation to generate NREM sleep (Falup-Pecurariu et al. 2021). During NREM sleep, both GABA and galanin as inhibitory signals are sent from the VLPO to the monoaminergic arousal systems including histaminergic neurons of TNM (tuberomammillary nucleus), cholinergic neurons of pedunculopontine tegmental nucleus, monoaminergic connections of LC (locus coeruleus) and DnR (dorsal nucleus of raphe), and the orexinergic neurons of the lateral hypothalamus; and these regions in turn inhibit the VLPO (Scammell et al. 2017; Gompf and Anaclet 2020; Saper et al. 2005). On the other hand, neural circuits in the pons are required for REM sleep regulation. The laterodorsal tegmental nucleus and the pedunculopontine tegmental nucleus (LDT/PPT) promote REM sleep through cholinergic excitatory projections to the pontine reticular formation (PRF), and most glutamatergic neurons and GABAergic neurons in the LDT/PPT region also contribute to REM

sleep (Fig. 3.3) (Scammell et al. 2017). The muscular atonia during REM sleep is generated by neurons in the sublaterodorsal nucleus (SLD) by suppressing the muscular tone (Scammell et al. 2017; Falup-Pecurariu et al. 2021). However, the neural circuit pathways and molecular mechanisms involved in the regulation of sleep homeostasis by these neurons have not fully been elucidated.

Neural compounds also play an important role in the regulation of sleep homeostasis, accumulating after prolonged wakefulness or sleep deprivation and gradually decreasing during sleep. For example, within 6 h of sleep deprivation, adenosine is selectively increased in the basal forebrain (Porkka-Heiskanen et al. 1997). Infusion of adenosine into the freely moving cat basal forebrain reduced both arousal and cortical excitability (Portas et al. 1997), with neuronal activation in the ventrolateral preoptic area. In an 11-h sleep deprivation experiment, nitric oxide initially accumulated in the basal forebrain, followed by adenosine accumulation, and levels of both compounds increased in the frontal cortex hours later (Kalinchuk et al. 2011). A genetically encoded adenosine sensor ($GRAB_{Ado}$) was employed to reveal that

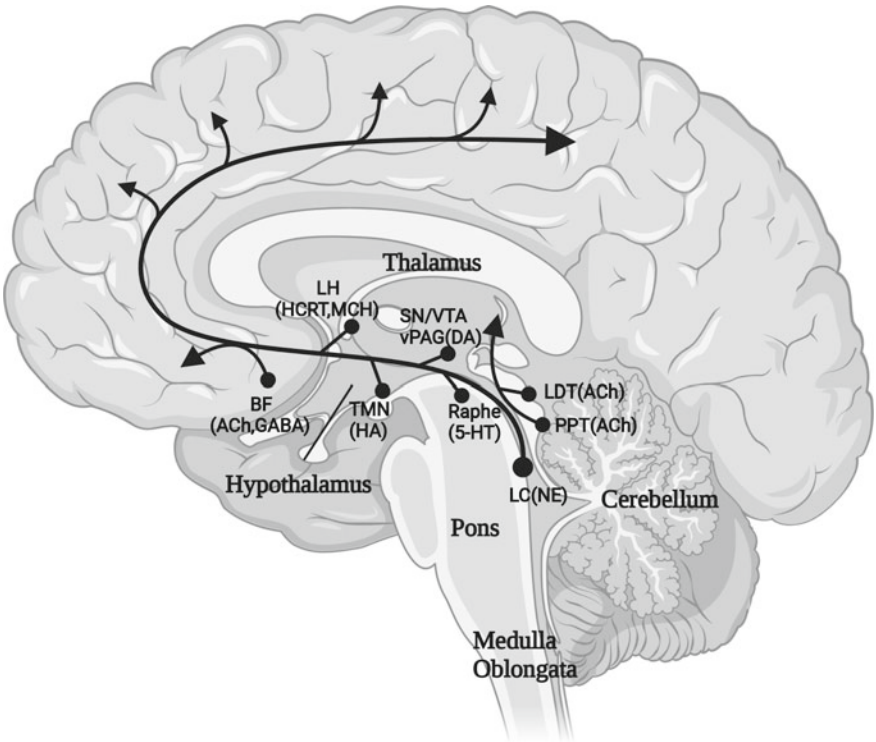


**Fig. 3.3** Sleep-promoting center in the central nervous system. VLPO promotes sleep, which is achieved by inhibiting the wake-promoting regions. VLPO projects to the LH, TMN, raphe nucleus, PPT/LDT, and LC. VLPO: ventrolateral preoptic nucleus; LH: lateral hypothalamus: TMN: tuberomammillary nucleus; PPT: pedunculopontine tegmental nucleus; LDT: laterodorsal tegmental nucleus; and LC: locus coeruleus

optogenetic activation of glutamatergic neurons, rather than cholinergic neurons, triggers adenosine release in the basal forebrain, thereby suggesting that the cell type-specific neuronal activity during wakefulness contributes to sleep propensity via releasing sleep-inducing factors such as adenosine (Peng et al. 2020). Conversely, caffeine is a potent stimulant that acts as an antagonist of adenosine by acting on the $A_1$ and $A_2$ receptors. Studies with adenosine receptor knockout mice found that caffeine can promote arousal in wild-type and A1 receptor knockout mice, but not in $A_{2A}$ receptor knockout mice (Huang et al. 2005), supporting the notion that the $A_2$ receptor mediates the wake-promoting effects of caffeine. In addition, caffeine administration in young male subjects during sleep deprivation reduced subjective sleepiness and EEG theta frequency activity and reduced NREM activity during subsequent restorative sleep (Landolt et al. 2004). Caffeine reduces sleep-prone accumulation after prolonged wakefulness, further suggesting a critical role for adenosine in sleep homeostasis. Prostaglandin D2 is also thought to be an endogenous sleep-promoting substance (Huang et al. 2007), and studies suggest that it may also trigger the sleep-promoting process through the $A_{2A}$ receptor (Satoh et al. 1996).

## 3.5 Circadian Regulation of Sleep

The sleep–wake cycle is controlled by both the circadian clock and homeostasis (Borbely 2022). However, how the circadian clock and homeostasis interact to regulate the sleep–wake behavior is far from certain. In mammals, negative feedback loops composed of a set of transcription activators and inhibitors generate a cell-autonomous oscillation of transcriptional activity (Reppert and Weaver 2002; Young and Kay 2001). The mammalian master circadian clock is located in the suprachiasmatic nucleus (SCN) of the ventral hypothalamus (Moore and Eichler 1972; Stephan and Zucker 1972), having approximately 20,000 neurons in mice (Cassone et al. 1988). Melanopsin-expressing intrinsically photosensitive retinal ganglion cells (ipRGCs) transmit light signals to the SCN (Berson et al. 2002; Hattar et al. 2002) via the retinohypothalamic tract (RHT) (Moore and Lenn 1972), synchronizing the SCN clock with the external light/dark cycle. Neurochemically heterogeneous neurons in the SCN have been subdivided into two regions: the dorsomedial "shell" region expressing high levels of arginine vasopressin (AVP), and the ventrolateral "core" region expressing high levels of vasoactive intestinal peptide (VIP) (Dierickx and Vandesande 1977; Morin 2007). In addition to these two neuropeptides, the mouse SCN also produces gastrin-releasing peptide (GRP), enkephalin, neurotensin, angiotensin II, prokineticin-2, neuromedin S (Nms), and calbindin (Abrahamson and Moore 2001; Lee et al. 2015). The SCN is able to form a coupled intercellular network for generating self-sustained circadian oscillations in both neuronal activity and gene expression (Colwell 2011; Welsh et al. 2010). VIP neurons in the core region have been shown to act as a prime coupling/synchronizing signal for the generation of SCN network synchrony (Aton et al. 2005; Maywood et al. 2006). Here, we first discuss

SCN roles in sleep regulation and then review the effects of specific circadian clock genes and clock-controlled genes on sleep.

### 3.5.1 Role of the SCN in Sleep Regulation

The SCN is known to project to other nuclei in the hypothalamus, including the subparaventricular zone (SPZ), the paraventricular nucleus (PVN), dorsomedial nucleus (DMH), ventromedial nucleus (VMH), LH, and medial preoptic area (Morin 2013; Scammell et al. 2017). Among them, the subparaventricular zone (SPZ) relays most SCN output signals, whereas the dorsomedial nucleus of the hypothalamus (DMH) regulates the timing of wakefulness via excitatory projections to the orexin neurons and locus coeruleus (LC) as well as inhibitory projections to the preoptic area (Morin 2013; Scammell et al. 2017).

In nocturnal rodents, SCN firing rates peak in the light phase, whereas SPZ firing rates peak in the dark phase when firing rates of wake-promoting neurons also peak (Kubota et al. 1981; Miyamoto et al. 2012). The rhythmicity of wake, NREM, and REM sleep was severely damaged in ventral SPZ-lesioned rats (Lu et al. 2001), implicating that the SPZ is a critical circadian relay that not only promotes arousal during the active phase but also facilitates sleep during the rest phase (Scammell et al. 2017), elegantly fulfilling the dual role of promoting wake at some times and sleep at others. While GABAergic DMH neurons innervate sleep-promoting regions including the VLPO and MnPO, glutamatergic DMH neurons strongly innervate wake-promoting brain regions, including orexin neurons, TMN, LC, ventral tegmental area (VTA), Dorsal nucleus of raphe (DNR), and LDT (Vujovic et al. 2015). Like the SPZ, the DMH is also able to act through circadian signals to promote wake at some times and facilitate sleep at others (Scammell et al. 2017).

### 3.5.2 Regulation of Sleep by Canonical Circadian Clock Genes

Identification of circadian clock genes has allowed for studying the molecular bases for temporal physiological circadian behaviors such as hormonal secretion as well as their roles in sleep regulation. Here, we review the effects of specific circadian clock genes and clock-controlled genes on the sleep duration, sleep structure and the EEG (Table 3.1).

**Table 3.1** Summary of the sleep phenotypes of circadian clock gene mutant/knockout mice

| Gene | Total sleep (24 h) | | REM/NREM | Sleep deprivation | References |
|---|---|---|---|---|---|
| | LD | DD | | | |
| *Clock*$^{-/-}$ mutant | 1.8 h ↓ in NREM | 1–2 h ↓ in NREM | NREM sleep in L ↓ | Compensatory response to sleep loss REM sleep rebound ↓ | Naylor et al. (2000) |
| *Npas2*$^{-/-}$ | ~ 40 min ↓ in NREM | | Wake time in D phase ↑ NREM/REM in D phase ↓ | Compensatory response to sleep loss ↓ (male mice only) | Dudley et al. (2003), Franken et al. (2006) |
| *Bmal1*$^{-/-}$ | 1.5 h ↑ in total sleep | 6.2% ↑ in NREM | Arrhythmic sleep/wake states in DD | REM sleep rebound ↓ | Laposky et al. (2005) |
| *Per1*$^{-/-}$,*Per2*$^{-/-}$ | No effect | No effect | Wake time in L ↑ | Compensatory response to sleep loss | Shiromani et al. (2004) |
| *Per3*$^{-/-}$ | No effect | No effect | NREM/REM after D–L transition ↑ | Accumulation of EEG delta power ↑ in recovery sleep number of NREM sleep bouts ↑ | Shiromani et al. (2004), Hasan et al. (2011) |
| *Cry1*$^{-/-}$,*Cry2*$^{-/-}$ | 1.8 h ↑ in NREM | 1.5 h ↑ in NREM | Attenuated sleep/wake rhythm across LD cycle | Compensatory response to sleep loss ↓ | Wisor et al. (2002) |
| *Dec2*$^{P385R}$ | | | NREM/REM in L phase ↓ | Compensatory response to sleep loss ↓ | He et al. (2009) |
| *Dbp*$^{-/-}$ | No effect | No effect | Sleep during L ↓ Sleep during D ↑ Circadian amplitude of the sleep distribution ↓ | Compensatory response to sleep loss REM sleep rebound absent | Franken et al. (2000) |
| *Prok2*$^{-/-}$ | 1.3 h ↓ total sleep | 1.3 h ↓ total sleep | REM sleep duration ↑ | Compensatory response to sleep loss ↓ | Hu et al. (2007) |
| *Vpac2*$^{-/-}$ | 50 min ↑ in NREM | No effect | Less defined sleep and wake phases in D and | | Sheward et al. (2010) |

#### 3.5.2.1 Regulation of Sleep by Clock, Npas2, and Bmal1

As the positive factors in the negative feedback loop, both CLOCK and BMAL1 not only play critical roles in circadian regulation, but also contribute to sleep regulation. *Clock* was the first mammalian circadian clock gene identified via a forward mutagenesis screen (Vitaterna et al. 1994). The *Clock* mutant mice, harboring an A → T nucleotide transversion in a splice donor site that results in skipping the 19th exon and deletion of 51 amino acids in the CLOCK protein (King et al. 1997), display a significantly lengthened period in the heterozygote (WT, 23.3–23.8 h; $Clock^{+/\Delta 19}$, 24.8 h) and become arrhythmic after exhibiting an extremely lengthened period ($Clock^{\Delta 19/\Delta 19}$, 26–29 h) first in the homozygote under constant dark (Vitaterna et al. 1994). Interestingly, the total sleep time of heterozygous ($Clock^{+/\Delta 19}$) and homozygous ($Clock^{\Delta 19/\Delta 19}$) mice was reduced by 1 and 2 h, respectively, in comparison to wild-type animals, and in particular, NREM sleep bout duration was also significantly reduced in $Clock^{\Delta 19/\Delta 19}$ homozygous mice, even though EEG delta power in NREM sleep was not affected (Naylor et al. 2000). In addition, $Clock^{\Delta 19/\Delta 19}$mutant mice display a normal sleep following 6-h sleep deprivation, implicating that the Clock gene plays an important role in regulating sleep duration and timing but not in homeostatic sleep regulation.

NPAS2, an analog of Clock, also forms a heterodimeric complex with BMAL1, in the forebrain nuclei, basal ganglia, limbic system and numerous peripheral organs, regulating the transcription of *Cry* and *Per* (Garcia et al. 2000). Intriguingly, NPAS2 has been shown to substitute CLOCK in the SCN, likely responsible for the different phenotypes observed in the dominant-negative $Clock^{\Delta 19/\Delta 19}$ mutant mice and $Clock^{-/-}$ knockout mice (Debruyne et al. 2006, 2007), i.e., $Clock^{-/-}$ knockout mice display robust circadian rhythms of locomotor activity instead (Debruyne et al. 2006). $Npas2^{-/-}$ mice display a shortened period but keep awake for a greater proportion of the dark period with reduced NREM and REM sleep (Dudley et al. 2003). $Npas2^{-/-}$ mice also show EEG changes during NREM sleep, characteristic of the reduced spindle frequency and the delta activity shifted toward faster frequencies (Franken et al. 2006). However, the roles of $Clock^{-/-}$ knockout or *Clock/Npas2* double knockout in sleep have not been reported to date.

On the other hand, loss of BMAL1 led to behavioral arrhythmicity with reduced locomotor activities under light/dark and constant conditions (Bunger et al. 2000). $Bmal1^{-/-}$ mice display 1.5-h increase of sleep, largely with increased NREM and REM sleep (Laposky et al. 2005). However, the number of sleep bouts in $Bmal1^{-/-}$ mice was increased during the light period, indicative of high fragmentated sleep. $Bmal1^{-/-}$ mice show the flattened distribution of EEG delta power during NREM sleep, and thus lose sleep propensity rhythm. Further, $Bmal1^{-/-}$ mice display attenuated REM sleep rebound after sleep deprivation. In other words, BMAL1 regulates both sleep amount and intensity, thereby implicating its role in the homeostatic regulation of sleep.

#### 3.5.2.2 Regulation of Sleep by Period, Cryptochrome, and Dec Genes

The CLOCK/NPAS2 and BMAL1 heterodimers regulate expression of *Cry* and *Per* genes. Similar to *Clock*, *Npas2* and *Bmal1*, studies with genetic mutations of these *Cry* and *Per* genes, show that some of them not only play roles in circadian regulation but also contribute to sleep regulation. $Per1^{-/-}$,$Per2^{-/-}$ double mutant mice display robust diurnal rhythms under the LD condition, but no alteration in sleep duration across a 24-h period under a regular LD cycle (Kopp et al. 2002) or under constant darkness (Shiromani et al. 2004), consistent with unaltered EEG recordings in single $Per1^{-/-}$ and $Per2^{-/-}$ mutant mice (Kopp et al. 2002). After sleep deprivation, these $Per1^{-/-}$ and $Per2^{-/-}$ mice display increased EEG SWA (slow-wave activity, 0.5–4 Hz) during NREM sleep, implicating their unaltered homeostatic regulation of sleep. In contrast, $Per3^{-/-}$ knockout mice display increased NREM and REM sleep immediately after the dark/light transition as well as enhanced accumulation of EEG delta power across the active period (Hasan et al. 2011). In addition, human studies have shown that *PER3* functional polymorphisms are associated with sleep homeostasis in terms of EEG SWA in NREM sleep and theta and alpha frequencies during wakefulness and REM sleep (Viola et al. 2007). Interestingly, a polymorphism in the *PER3* promoter region has been recently shown to be associated with delayed sleep phase syndrome (Archer et al. 2010). These studies suggest that mouse PER3, rather than PER1 and PER2, likely plays a role in homeostatic sleep regulation.

In contrast to $Per1^{-/-}$, $Per2^{-/-}$ double mutant mice that display unaltered sleep homeostasis, $Cry1^{-/-}$,$Cry2^{-/-}$ double-knockout mice show a 1.8-h increase in NREM sleep with increased NREM sleep bout duration. After sleep deprivation, $Cry1^{-/-}$,$Cry2^{-/-}$ double-knockout mice show compensatory rebound in NREM sleep as well as elevated EEG SWA during baseline recordings (Wisor et al. 2002). Even though these sleep phenotypes cannot be observed in single $Cry1^{-/-}$ or $Cry2^{-/-}$ knockout mice (Wisor et al. 2008), *Cry* genes appear to act together to contribute to homeostatic sleep regulation.

Mouse *Dec1* (*Sharp2*)/*Bhlhe40* (Basic helix-loop-helix family member e40) and *Dec2* (*Sharp1*/*Bhlhe41*) are rhythmically expressed in the SCN and repress the transcription activity of the CLOCK-BMALl heterodimer (Honma et al. 2002). Loss of DEC1 or DEC2 disrupted the period length, phase resetting and circadian entrainment (Rossner et al. 2008). Intriguingly, a point mutation in human *DEC2* was revealed to be associated with a short sleep phenotype as the natural short sleeper (NSS), which was recapitulated in the *DEC2 P385R*-expressiong mice that display reduced NREM and REM sleep in the light phase and elevated sleep fragmentation (He et al. 2009). Further, the *DEC2* mutation resulted in reduced NREM sleep after sleep deprivation and reduced EEG delta power, whereby implicating its role in homeostatic sleep regulation. Conversely, only minimal changes in sleep were observed in *Dec2* knockout mice (He et al. 2009).

#### 3.5.2.3 Regulation of Sleep by Circadian Clock-Related Genes

In addition to the canonical circadian clock genes, a number of clock-controlled genes have been shown to contribute to sleep regulation. Dbp as a PAR leucine zipper transcription factor is controlled by the circadian clock via E-box (Ripperger et al. 2000). *Dbp*$^{-/-}$ mice display a shortened period with reduced locomotor activity but maintain rhythmicity (Lopez-Molina et al. 1997). Even though *Dbp*$^{-/-}$ mice have the normal sleep duration, they display reduced circadian amplitudes of sleep time and sleep consolidation, and in particular, reduced REM sleep during the light period, and an elevated EEG theta frequency during exploratory behavior and REM sleep (Franken et al. 2000). These observations suggest that the direct involvement of Dpb in homeostatic sleep regulation.

Prokineticin 2 (Prok2), a humoral factor secreted by the SCN to regulate motor activity, is likely controlled by the circadian clock (Zhou and Cheng 2005). *Prok2*$^{-/-}$ mice display reduced amplitudes of activity, core body temperature and the sleep–wake cycle (Li et al. 2006). *Prok2*$^{-/-}$ mice display ~ 1.5 h reduced sleep duration compared with wild-type mice. Intriguingly, *Prok2*$^{-/-}$ mice display reduced NREM sleep during the light period but increased REM sleep occurred during both light and dark phases (Hu et al. 2007). These studies implicated that PROK2 plays roles in both in circadian regulation and sleep homeostasis.

VIP is another clock-controlled and SCN-secreted transmitter. VIP acts via its receptor VPAC2 play a critical role in sustaining circadian rhythmicity of individual SCN cells (Brown et al. 2007). *Vpac2*$^{-/-}$ mice display an altered sleep–wake rhythm, while maintaining robust activity rhythms. In addition, *Vpac2*$^{-/-}$ mice exhibit approximately 50 min longer NREM sleep time, with more sleep/wake transitions (Sheward et al. 2010). Intriguingly, the nighttime nap of mice, similar to humans' afternoon siesta, is regulated by a group of VIPergic neurons in the SCN (Collins et al. 2020).

#### 3.5.2.4 Role of Human Circadian Clock Genes in Sleep

As discussed above, dissection of knockouts and mutants for mouse circadian clock genes and circadian clock-related genes clearly showed that these genes contribute to sleep regulation, in either the circadian or homeostatic processes, or in both. In addition to *DEC2 P385R* displaying the NSS phenotype (He et al. 2009), another *DEC2/BHLHE41* variant (Y362H) has also been associated with the reduced sleep duration phenotype, likely by reducing its ability to inhibit CLOCK/BMAL1 and NPAS2/BMAL1 transactivation (Pellegrino et al. 2014). Further, mutations of several human circadian clock genes have been identified to be responsible for advanced sleep phase (ASP) or familial advanced sleep phase (FASP) displaying an extreme early-bird preference (Gentry et al. 2021). While hypophosphorylation caused by a *PER2* missense mutation (S662G) within its CKIε-binding region results in ~ 4–6 h advanced sleep phase (Toh et al. 2001), a missense mutation (T44A) in CKIδ-encoding *CSNK1D* also leads to FASP (Xu et al. 2005). Two *PER3* mutations

(P415A and H417R) both reduce its protein stability, which destabilizes PER1 and PER2 proteins and in turn cause FASP (Zhang et al. 2016), whereas a mutation (A260T) in *CRY2* results in ASP, likely due to its elevated degradation via increasing its accessibility and affinity for FBXL3 (an E3 ubiquitin ligase) (Hirano et al. 2016). Intriguingly, even though the circadian role of TIMELESS (TIM) in humans has not yet been ascertained, one *TIM* variant (R1081X) causes FASP, likely due to destabilizing the PER-CRY complex by its failure to enter the nucleus and altered affinity for CRY2 (Kurien et al. 2019). In most cases, the transgenic mice expressing the variants of these human circadian clock genes recapitulate their human variant phenotypes (Hirano et al. 2016; Kurien et al. 2019; Xu et al. 2005, 2007; Zhang et al. 2016), highlighting their conserved roles in sleep regulation.

In contrast to ASP, delayed sleep phase (DSP) exhibits an extremely late chronotype as night owls. A *CRY1* mutation was determined in a DSP proband family, which occurs in a splice donor site, resulting in skipping Exon 11 and in-frame deletion of 24 residues; this gain-of-function *CRY1* variant acts as a strong transcriptional inhibitor by increasing its binding to the CLOCK–BMAL1 heterodimer, and is responsible for the DSP phenotype (Patke et al. 2017). The prevalence of FASP and ASP was estimated to be 0.21% and 0.31% in a sleep clinic population, respectively (Curtis et al. 2019), and the frequency of *CRY1* DSP allele was estimated to be 0.1–0.6% (Patke et al. 2017), implicating that these genetic variants impact a sizeable portion of the human population. Even though these studies did not yet determine the neural and genetic links between circadian input signals to the sleep output, they indeed shed light on how the circadian clock affects human well-being.

## 3.6 Effects of Circadian Misalignment on Aging

### *3.6.1 Attenuation of Circadian Rhythms with Aging*

Aging is a multifactorial process characterized by a gradual failure of physiological functions (Harman 1981; Liochev 2015). Circadian rhythms generally weaken with aging (Hood and Amir 2017; Manoogian and Panda 2017). In rodents, while the total number of SCN neurons does not decrease significantly in aged rats and individual SCN neurons are able to maintain robust rhythmicity of canonical clock gene oscillations in aged mice (Welsh et al. 1995; Wyse and Coogan 2010), intercellular coupling and synchronization within the SCN neurons are disrupted, largely due to the reduced SCN neurons that produce coupling factors vasopressin and GABA (Mieda et al. 2015; Nygard and Palomba 2006; Roozendaal et al. 1987). This reduced intercellular SCN coupling results in their neuronal desynchronization, and in turn leads to the reduced electrical activity of the whole SCN network and a dampened SCN output (Farajnia et al. 2012; Nakamura et al. 2011; Nygard et al. 2005). Subsequently, the attenuated neuronal and humoral outputs cause malfunction of peripheral oscillators displaying weakening amplitudes of canonical clock gene oscillations

with aging, and the weakened circadian rhythms further exacerbate numerous age-related diseases. In addition, the effect of aging on the rhythmic expression of clock genes is plausible, with contradictory reports of shortening or lengthening of the SCN clock or unchanged peripheral clocks (Sato et al. 2017).

### *3.6.2 Acceleration of Aging by Circadian Misalignment/Disruption*

In modern society, irregular eating and sleep patterns, inappropriate light exposure (light at night, LAN), jet-lag, and shift work all contribute to circadian misalignment (Bass 2017; Manoogian and Panda 2017). For instance, evening use of blue light-emitting electronic readers, suppresses melatonin release, shifts the circadian clock phase, delays sleep onset and enhances morning sleepiness (Chang et al. 2015). In model organism studies, genetic knockout or mutation of canonical circadian clock genes disrupts circadian rhythms. *Bmal1*$^{-/-}$ mice also display symptoms of early onset aging, characteristic of a decrease in muscle and subcutaneous fat, cataracts, and organ shrinkage, possibly due to increased levels of reactive oxygen species (ROS) in some tissues as well as defects in stress response and impaired glucose tolerance and insulin sensitivity of these animals (Kondratov et al. 2006). Jetlag- and shiftwork-induced circadian misalignment in humans has been reported to be associated with cardiovascular diseases, metabolic disorders, and cancer (Kamdar et al. 2013; Proper et al. 2016; Vyas et al. 2012), and likely accelerates aging. In a remarkable experiment, transplantation of the SCN from a young hamster into an old hamster with dampened behavioral rhythms was able to restore robust behavioral rhythms in the older hamster and increase lifespan by 4 months (Hurd and Ralph 1998; Viswanathan and Davis 1995).

## 3.7 Effects of Sleep Disorders on Aging

Aging has been known to lead to sleep deficiency (Kondratova and Kondratov 2012), although the mechanisms are not well understood. Numerous mechanisms have been proposed from molecular, cellular, to organ levels, including deregulated autophagy, mitochondrial dysfunction, telomere shortening, oxidative stress, systemic inflammation, and metabolism dysfunction (Riera et al. 2016).

### 3.7.1 Age-Related Sleep Changes

Approximately, 40% of the elderly population have been reported to have sleep problems. In humans, the most obvious consequence of circadian disruption is an altered sleep–wake cycle (Dijk et al. 1999; Farajnia et al. 2012; Huang et al. 2002), with sleep quality and consolidation being also disrupted (Dijk et al. 2001; Farajnia et al. 2012). Many sleep-related disorders occur with increasing frequency among elderly adults (Wolkove et al. 2007). Depression and anxiety complaints, common among people over 65 years of age, frequently contribute to insomnia. Risk factors for depression in elderly people include loss of a spouse, retirement, social isolation, comorbid disease, and the onset of dementia (Tractenberg et al. 2005). It is important to know how sleep patterns change with aging and to recognize that sleep disorders are common among elderly people (Tractenberg et al. 2005).

With aging, important changes in sleep structure occur, and the most characteristic change is sleep phase advance, i.e., elderly people often go to bed and get up early. With aging, the total amount of sleep time shortens: infants and young children sleep an average of 14–20 h per day; adults, 6–8 h; and people over 60 years of age, 6.5 h daily (Rajput and Bromley 1999). Further, the elderly people display reduced slow-wave sleep, reduced rapid eye movement (REM) sleep, reduced threshold for arousal from sleep, fragmented sleep with multiple arousals and daytime napping (Wolkove et al. 2007). In a recent study, hyperexcitability of arousal-promoting hypocretin/orexin (Hcrt/OX) neurons and down-regulation of KCNQ2 was shown to be associated with fragmented sleep in aged mice, and the KCNQ-selective agonist flupirtine was able to hyperpolarize Hcrt/OX neurons and rejuvenate sleep quality in aged mice (Li et al. 2022).

### 3.7.2 Acceleration of Aging by Sleep Disorders

An around-the-clock lifestyle causes sleep deprivation (SD) Many people sacrifice their sleep to work due to psychosocial stress. Because sleep plays a crucial role in neuronal regaining and decreases the burden of plasticity, SD may have significant repercussions on the brain function (Meerlo et al. 2015). Moreover, SD has caused a community health epidemic with substantial health, economic and social impact (Hafner et al. 2017). Therefore, it is essential to investigate the neural processes of SD in order to develop effective targeted therapies. One of the pervasive sleep disorders in the elderly population is insomnia (Shochat et al. 2001; Chan et al. 2022). Further, other sleep disorders such as obstructive sleep apnea, restless leg syndrome and periodic limb movement disorder have been reported to be more prevalent in older persons (Gulia and Kumar 2018). Sleep deficiency/sleep disorders speed up aging (Carroll and Prather 2021). Indeed, chronic poor quality sleep is associated with accelerated intrinsic skin aging because lack of sleep prevents the body from properly restoring skin itself (Oyetakin-White et al. 2015). However, the accelerated aging

associated with sleep deficiency goes beyond cosmetic changes. Early postpartum sleep loss accelerates epigenetic and cellular aging, as evidenced by quicker Intrinsic Epigenetic Age Acceleration (IEAA) and Phenotypic Epigenetic Age Acceleration (PEAA) and shortened leukocyte telomere length (Carroll et al. 2021). Overall, sleep deficiency/sleep disorders are hypothesized to contribute to increasing damage accumulation, enhancing cellular senescence, shortening telomere length, disrupting telomerase activity, and accelerating epigenetic aging (Carroll and Prather 2021).

## 3.8 Concluding Remarks

It has become increasingly clear that the circadian clock plays modulatory roles in almost all the life processes and activities (Bass 2017; Rijo-Ferreira and Takahashi 2019). Sleep is hypothesized to be regulated by two processes: the homeostatic process and the circadian system (Borbely 1982). The circadian clock represents a special class of biochemical oscillators with an intrinsic period of approximately 24 h, which is regulated by negative feedback loops of the transcription-translation of circadian clock genes and proteins (Takahashi 2017). The sleep–wake cycle is the most overt rhythm controlled by the circadian clock.

Studies of knockout or mutant mice of circadian clock genes provide invaluable insights into their roles in sleep regulation. Since these canonical circadian clock genes are essential for generating and maintaining circadian rhythmicity, knocking out or mutating these genes are expected to disrupt central timekeeping mechanisms and in turn to impact sleep timing (circadian process). Surprisingly, some of the canonical circadian clock genes also contribute to homeostatic sleep regulation. For instance, BMAL1 plays a role in the homeostatic regulation of sleep by regulating both sleep amount and intensity. It appears that both the circadian process and homeostatic process of sleep are strongly coupled. One of the future efforts ought to investigate the coupling mechanisms of the circadian process and homeostatic process of sleep, and particularly, to elucidate the neural and genetic pathways that link circadian signals to the sleep output.

Aging and the circadian system are intertwined. Aging is known to affect the circadian rhythms; i.e., the amplitudes of the circadian clock are generally attenuated along with aging (Hood and Amir 2017; Manoogian and Panda 2017). On the other hand, circadian disruption and misalignment would accelerate aging. Sleep deficiency/sleep disorders represent typical circadian disruption causing a community health epidemic with substantial health impact, including accelerated aging.

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# Chapter 4
# Age-Related Decline in the Central Circadian Clock


**Shota Miyazaki, Wataru Nakamura, and Takahiro J. Nakamura**

## 4.1 Introduction

Circadian systems do not escape aging as well as other physiological functions. In humans, the sleep/wake rhythm of a newborn baby appears indistinguishable and then becomes entrained to external cues such as maternal behavior and light (Brooks and Canal 2013). In adults, the sleep/wake rhythm becomes stable, and humoral secretion rhythms also exhibit a robust circadian rhythm. However, older adults tend to have sleep disorders, such as early morning awakening and nocturnal awakening, and the amplitudes of rhythms in physiological functions also decline (Hood and Amir 2017). Age-related declines in circadian rhythms are mainly due to functional declines in the suprachiasmatic nucleus (SCN) of the hypothalamus, which is known to be the central circadian clock that regulates circadian rhythms for the whole body. This chapter addresses the regulatory mechanisms underlying the circadian rhythms in mammals and summarizes the recent literature describing the effects of aging on the circadian system, with a focus on the SCN.

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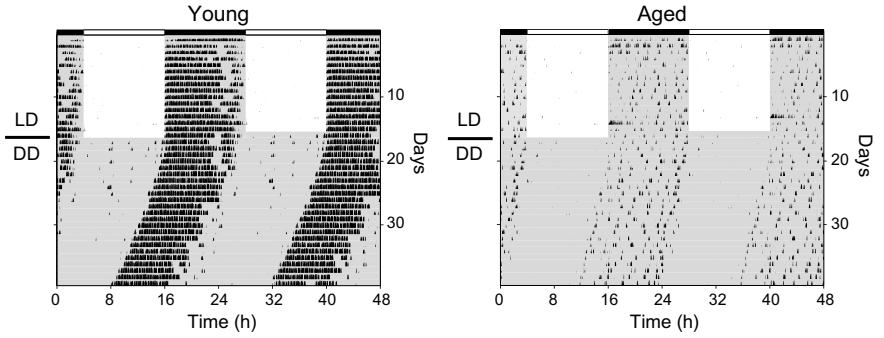


**Fig. 4.1** Effects of aging on the circadian locomotor activity rhythms in mice. Double-plotted actograms showing wheel-running activity in young (left) and aged (right) C57BL/6J mice. The vertical axis indicates the day and the horizontal axis indicates the time course (48 h). The mice were maintained under light/dark (LD = 12 h: 12 h) cycles for 2 weeks and then transferred to constant darkness (DD). In aged mice, the levels of locomotor activity are decreased, fragmented locomotor activity appears, and free-running periods are lengthened in DD. Adopted from Nakamura et al. (2016)

## 4.2 Effects of Aging on Circadian Rhythms

The effects of aging on circadian rhythms have been reported in numerous species. In aplysia, a mollusk that is commonly used as in neuroscience research, long-term recordings of neural firing activities in the retina, which has the function as a biological clock, revealed that circadian amplitudes drastically decreased in the 12-month-old organisms compared with the 3-month-old organisms (Sloan et al. 1999). Behavioral rhythms in rodents, represented by rats and mice, are also altered with age (Pittendrigh and Daan 1974). In aged mice, the levels of locomotor activity were decreased, fragmented locomotor activity appeared, and free-running periods were lengthened during constant darkness (Fig. 4.1). In particular, the balance of the activity/rest phase in aged animals is “ambiguous” (Valentinuzzi et al. 1997). Although mice used in the laboratory are nocturnal and their results cannot be applied to humans, the cause of early morning awakening in humans is interpreted as a change in the endogenous period, and a shortened/lengthened period has been verified in aged rodents. Thus, rodents can be useful models for studying neural circuits associated with aging.

## 4.3 The Central Circadian Clock in the SCN

The central circadian clock, which regulates circadian behavioral/physiological rhythms, is located in the SCN. The SCN is located above the optic chiasm where each optic nerve crosses, and the one-paired nuclei are proximal to either side of the

third ventricle. The SCN is a distinctive structure in which each nucleus, containing approximately 10,000 neurons, is densely packed with small cell bodies. Thus, we can easily identify the SCN even though it is the size of a "poppy seed" in mice (Fig. 4.2). The SCN is directly connected to the retina and is entrained to an environmental light/dark cycle. Since the 1990s, clock genes have been identified in mammals, and more recent research has revealed that the system called "cellular clock" was in each SCN cell. Several clock genes form a cellular clock that oscillates for approximately 24 h driven by a transcriptional-translational negative feedback loop. In brief, the heterodimers of clock gene products BMAL1 and CLOCK drive transcription of circadian responsive genes, including *Period* (*Per*) and *Cryptochrome* (*Cry*) via E-box elements found in their promoters. The gene products of *Per* and *Cry*, in turn, suppress the transactivation of BMAL1/CLOCK (Reviewed in Takahashi 2017). Although an individual SCN cell generates rhythmicity, the SCN generates a more robust rhythm as a nucleus by interacting with individual SCN cells (Nakamura et al. 2012). In addition, cellular clocks exist in almost all organs of the entire body (Honma 2018), and each organ can autonomously oscillate (Yoo et al. 2004). If this whole-body clock system is compared to an orchestra, the SCN plays the role of the conductor, and the clocks existing in each tissue/organ (peripheral clock) play the role of each musical instrument. Similar to a conductor, the SCN conducts and integrates each peripheral clock to adjust the time for the physiological function of the organ. Just as an incompetent conductor who misleads the harmony of music, functional decline and dysfunction of the SCN disrupt the cellular clocks in the whole body.

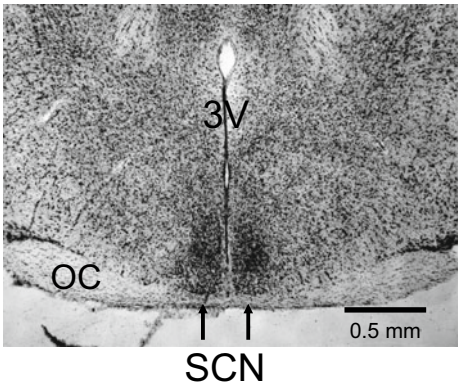


**Fig. 4.2** Central clock: suprachiasmatic nucleus (SCN) of the hypothalamus. The coronal section of the mouse brain is stained with neutral red. Many cells are highly packed within the SCN. The scale bar represents 0.5 mm. 3 V: the third ventricle

## 4.4 Age-Related Decline in Circadian Rhythms Caused by SCN Disorganization

Many studies have reported that the decline in behavioral/physiological functions is in line with dysfunction of the SCN due to aging (Reviewed in Nakamura et al. 2016). A fetal SCN transplant into the third ventricle in an aged rat with reduced circadian rhythms improved circadian rhythms in locomotor activity, body temperature, and drinking behavior (Li and Satinoff 1998). In addition, there are many reports on circadian rhythms in the SCN of aged rodents. For instance, multi-unit neural activity (MUA) recordings of extracellular potentials in SCN slices revealed that the aged SCN showed significantly smaller amplitudes than the young SCN in hamsters (Watanabe et al. 1995). In dispersal cell cultures, individual cells of the aged SCN showed neural activity rhythms with decreased amplitudes and fluctuating peak phases (Aujard et al. 2001). These results suggest that the amplitudes of the neural activity rhythm in the whole SCN are decreased due to the desynchronization of individual cells in aged animals. However, quantitative examination of *Per2* rhythms in the SCN revealed that these rhythms were not significantly influenced by aging (Asai et al. 2001). *Per1* rhythms in SCN slice cultures with a luciferase reporter also revealed that the rhythms were not significantly influenced, even though the period was slightly shortened (Yamazaki et al. 2002). These results suggest that aging does not have a large impact on the cellular clocks composed of clock genes in the SCN. Thus, there is a discrepancy in the effects of aging between the results of MUA rhythms reflecting neural outputs and clock gene expressions reflecting cellular clock generation.

## 4.5 Age-Related Dysfunction of SCN Outputs

Gene expression analyses and MUA recordings in aged mice were performed to clarify the discrepancies described in the previous section (Nakamura et al. 2011). First, experimental mice were dissected at certain intervals and examined the PER2 expressions in the SCN by immunohistochemistry. There was no difference in the rhythms of PER2 expressions between young and aged mice. Second, PER2 rhythms were recorded in SCN slice cultures using the PER2::LUCIFERASE (PER2::LUC) reporting system. The amplitude of PER2::LUC in aged SCN declined with each cycle, whereas the free-running period was indistinguishable from that of young SCN. Finally, an in vivo MUA recording system that can record neural firing subpopulations in the SCN of freely moving mice using bipolar electrodes was constructed (Nakamura et al. 2008). The method revealed that the MUA rhythms in aged wild-type SCN were essentially maintained and the counts of MUA were high during the day and low during the night, whereas the variance per recording unit (1 min) significantly increased, and the robustness of day/night activities was lost relative to

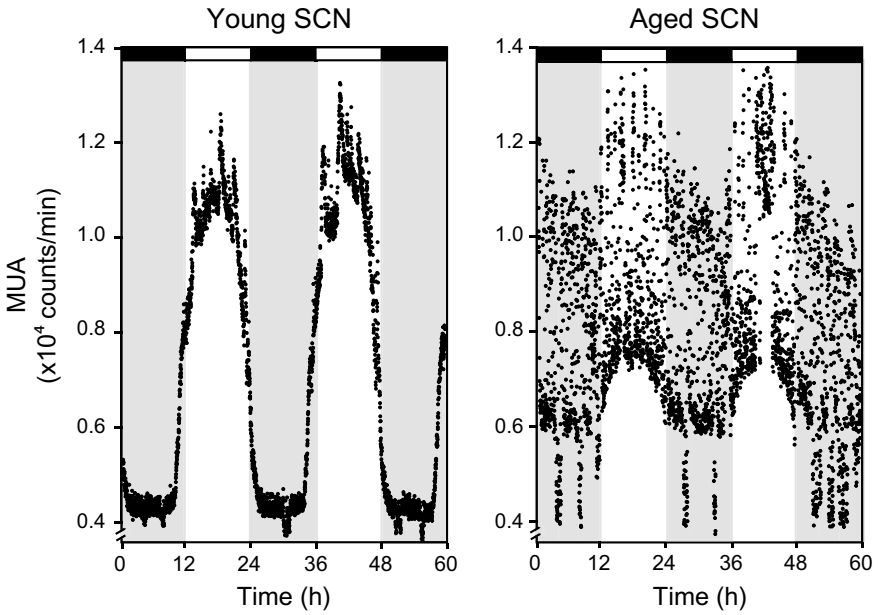


**Fig. 4.3** Effects of aging on multi-unit neural activity (MUA) rhythms in the suprachiasmatic nucleus (SCN). In vivo MUA rhythms in the SCN of mice: the MUA rhythms in the SCN were recorded by chronically inserting electrodes into the SCN of the freely moving mice. The vertical axis indicates neural activity counts per min and the horizontal axis indicates the time course. The gray square indicates the dark phase. The MUA in the SCN is high during the day and low during the night. The MUA in aged SCN shows ambiguous rhythms, even though the difference in day/night activities was maintained. Adopted from Nakamura et al. (2011)

the young wild-type SCN. The amplitudes of the MUA rhythms in aged mice were significantly lower than those in young mice (Fig. 4.3).

It is considered that the SCN projects to the dorsomedial nucleus of the hypothalamus (DMH) via the subparaventricular zone (SPZ) located just above the SCN, and that the timing signals for behavioral/physiological functions are transmitted from the DMH to some functional centers (Saper 2013). Thus, the pathway of SCN-SPZ reflects the SCN outputs. The amplitudes of the MUA rhythms in aged mice were found to decline, even in the SPZ (Nakamura et al. 2011). These results indicated that the decline in circadian rhythms at the individual level in aged mice was due to the dysfunction of SCN outputs. The age-related decline of behavioral rhythms was not observed in mice with artificial degeneration of dopaminergic neurons in the substantia nigra of the midbrain, mimicking an aged brain (Tanaka et al. 2012). This result also supports the relationship between age-related decline in behavioral rhythms and dysfunction of SCN outputs. If these results were likened to an orchestra, the conductor (SCN) became hazy and lazy.

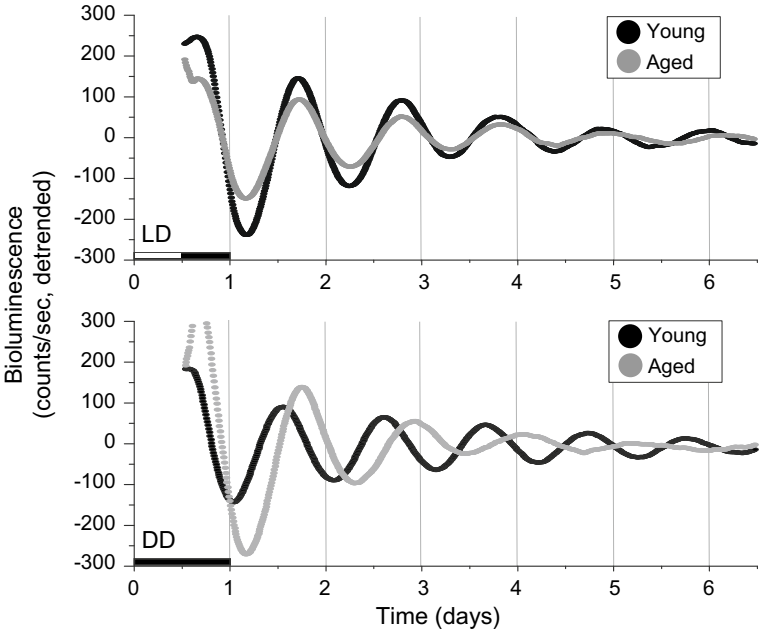


**Fig. 4.4** Constant darkness uncovers effects of aging on the cellular clock. The luminescence rhythms in the suprachiasmatic nucleus (SCN) of PER2::luciferase (PER2::LUC) mice, a luciferase is linked to PER2, were recorded in SCN slice cultures using a photomultiplier tube (PMT). PER2::LUC rhythms of mice housed in a normal light/dark (LD) cycle (upper) and constant darkness (DD) for 10 days (lower) are shown. The vertical axis indicates luminescent counts and the horizontal axis indicates the time course (days). There were no differences between the young and aged SCN during the periods and amplitudes in the LD cycle. In DD conditions, however, the aged SCN showed decreased amplitudes and fluctuated peak phases compared with the young SCN. Adopted from Nakamura et al. (2015)

## 4.6 Mechanisms Underlying SCN Output Dysfunction

Because several reports using rodents revealed that the cellular clock was almost normal even in aged SCN, and the discrepancy in the effects of aging between the neural rhythm and the cellular clock in the SCN was unsolved. In our own work, we hypothesized that rhythms in the SCN were also influenced by aging and examined the rhythms of clock gene expressions in the SCN in a non-external cues environment (constant darkness condition) (Nakamura et al. 2015). Although PER2::LUC mice have been exposed to the light/dark cycle in many experiments, in the study we cultured SCN slices from PER2::LUC mice housed in constant darkness conditions for 10 days. There were no differences in circadian periods and amplitudes between the young and aged SCN of mice housed in the light/dark environment. In contrast, the period was lengthened and the amplitude was decreased in the aged SCN of mice housed in constant darkness conditions. In brief, the effects

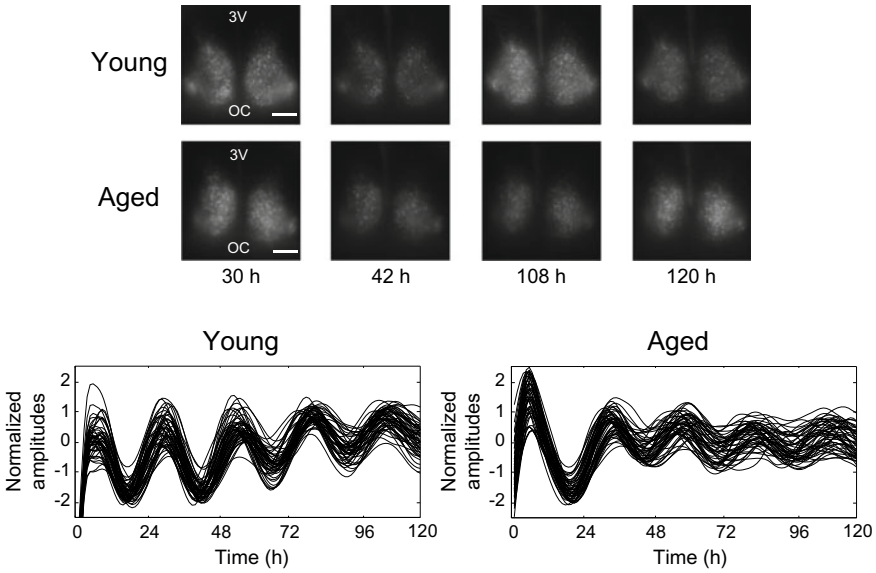


**Fig. 4.5** Effects of aging on individual suprachiasmatic nucleus (SCN) cellular clock. (Upper panel): PER2::LUC imaging of the SCN with a high-sensitivity camera. OC: optic chiasm, 3 V: the third ventricle. (Lower panel): serial plots of each PER2 rhythm picked up from individual 50 cells in the SCN for 5 days. The rhythms in the aged SCN are gradually desynchronized in culture conditions. Adopted from Nakamura et al. (2015)

of aging on the cellular clock in the SCN were remarkable under the constant darkness condition (Fig. 4.4). These results suggest that a light/dark environment masks the dysfunction of the cellular clock in aged SCN. Moreover, we performed PER2::LUC imaging of aged SCN with a high-sensitivity charge-coupled device camera under the same experimental conditions. Fifty cells were picked from each SCN, and the luminescence rhythms of each cell were recorded for 5 days. The rhythms in aged SCN were gradually desynchronized under culture conditions. Statistical analysis revealed that the variance of the peak phases significantly increased, although the amplitudes of individual cells were maintained (Fig. 4.5). These results suggest that aging disturbs the synchronization of individual SCN cells, rather than influencing individual cellular clocks.

## 4.7 Conclusion

Taken together, many experiments using rodents reveal that aging: (1) disrupts SCN internal-synchronization and (2) induces SCN output dysfunction. Therefore, the SCN cannot transmit correct timing signals to each behavioral/physiological function (Fig. 4.6). Aging does not influence the scale or number of SCN neurons. However,

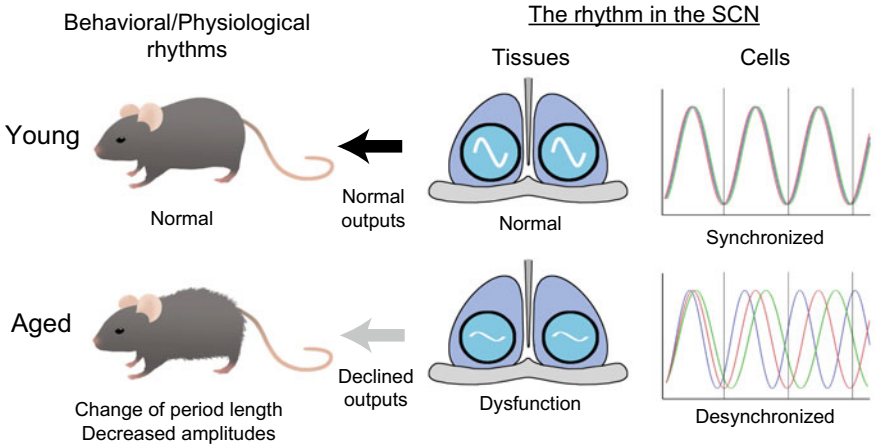


**Fig. 4.6** Summary of aging effects on the clock system. Circadian rhythm disorders of sleep arousal and physiological functions appear with age. The main cause is dysfunction of the timing signal outputs from the SCN, which is the central circadian clock. It is considered that the decline in synchronization of individual SCN cells results in the decline of functional rhythm outputs from the whole SCN in aged animals

there are more reports of a decrease in neurotransmitters in the SCN, such as gamma aminobutyric acid, with age (Hood and Amir 2017). Thus, it is considered that a functional decline in circadian rhythm due to aging is mainly due to a decline in SCN synchronization (a dysfunction of the SCN neural circuit), which may be supported by the reduction in SCN outputs caused by age-dependent alterations in specific neurotransmitter signaling (Farajnia et al. 2014). In addition, circadian rhythm disorders in neurodegenerative diseases, such as Alzheimer's disease, are considered to be a result of SCN dysfunction (Musiek and Holtzman 2016). Therapy and medicine to improve SCN neural circuits in these diseases, including aging, are expected in the future.

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# Chapter 5
# Impact of Cellular Senescence on Cellular Clocks


**Yasukazu Nakahata**

## 5.1 Introduction

Cellular senescence is a permanent cell cycle arrest caused by a variety of stressors, including genotoxic reagents, oncogene activation, mitochondrial dysfunction, and nutrient depletion, but interestingly, senescent cells are metabolically active and resistant to apoptosis (Kumari and Jat 2021). Senescent cells are also known to secrete a plethora of factors collectively referred to as senescence-associated secretory phenotype (SASP) (Kumari and Jat 2021). Cellular senescence was first observed by Hayflick and Moorhead in 1961 (Hayflick and Moorhead 1961) and was initially assumed to be simply an artifact of cell culture. It is now widely accepted that almost all cells, even post-mitotic cells such as neurons, can undergo cellular senescence and that senescent cells play both beneficial and detrimental roles in vivo in a context-dependent and temporal manner. Cellular senescence exhibits antagonistic pleiotropy, i.e., it plays beneficial roles in early life but detrimental roles in later life (Nacarelli and Sell 2017). Senescent cells in early life are associated with embryonic development (Storer et al. 2013), wound healing (Demaria et al. 2014), tissue remodeling (Munoz-Espin and Serrano 2014), and tumor suppression (Munoz-Espin and Serrano 2014). Interestingly, SASP factors secreted by senescent cells enhance immunosurveillance to eliminate themselves. On the other hand, due to immune aging in late life, the senescent cells have been accumulated at the sites of many age-related diseases and shown to contribute, at least partially, to the development of many such diseases, including contribution to tumorigenesis (He and Sharpless 2017; Herranz and Gil 2018).

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These findings have led researchers to explore how and why senescent cells accumulate and to what extent these cells have a positive or negative impact on physiological function. Given the myriad of effects of senescent cells, one system whose effects of cellular senescence are relatively unexplored is the circadian clock system, a 24-h timekeeping system found in the cells of almost every living body on earth. In this chapter, I first summarize the characteristics of cellular senescence, then describe the features of the circadian clock, and discuss the possible link between cellular senescence and the circadian clock. Finally, I discuss that senescent cells could be the underlying cause of circadian clock malfunction, which may ultimately lead to circadian rhythm dysfunction at the tissue and organism level.

## 5.2 Evidence that Cellular Senescence Is a Causative Factor for the Different Age-Related Diseases

Aging is a progressive loss of tissue and organ function over time that occurs in all multicellular organisms (Calcinotto et al. 2019). Aging is one of the greatest risk factors for non-communicable diseases such as atherosclerosis, osteoporosis, osteoarthritis, type 2 diabetes, kidney disease, neurodegenerative diseases, and cancer (Kirkland 2016). These diseases generally begin to develop in the middle of an organism's lifespan and progress with aging. Therefore, the field of aging postulates that some fundamental process must be the driving force that causes these age-related diseases to develop almost simultaneously (Lopez-Otin et al. 2013). Cellular senescence is one of the most plausible candidates that would fulfill this criterion, as the chronic presence of these cells in late life would seem to bring disarray in a tissue-specific manner. Studies to determine whether cellular senescence is a key driver of aging-related diseases have yielded compelling results for several diseases in animal models (Baker et al. 2016; Baker et al. 2011). Senescent cells have been found at the site of many aging-related pathologies and clearance of these cells using transgenic techniques can alleviate the severity of symptoms. For example, senescent cells are present in osteoarthritic joints (McCulloch et al. 2017), and transplanting senescent cells into the knee joints of mice improved osteoarthritis symptoms (Xu et al. 2017). Similarly, evidence suggests that senescent cells contribute to several chronic lung diseases (Birch et al. 2018), and the removal of these cells improved lung parameters in aging mice (Hashimoto et al. 2016).

While transgenic suicide genes that kill senescent cells have been demonstrated to ameliorate aging-related diseases and extend a healthy life span in mice, senolytic pharmacological agents that selectively kill senescent cells have also been developed for innovative therapeutic applications in aging. The first senolytics, a combination of dasatinib and quercetin, was reported to alleviate frailty symptoms and extend a healthy life span in mice (Zhu et al. 2015b). Recent studies have also highlighted the therapeutic value of senolytics-induced senescent cell elimination in natural aging and many age-related diseases (Zhu et al. 2020). Collectively, these findings indicate

that the chronic presence of senescent cells brings about havoc in many physiological systems of the body.

## 5.3 Characteristics of Senescent Cells

Irreversible growth arrest is a notable characteristic of senescent cells; however, it is not the only feature of senescent cells. To confirm the presence of senescent cells, several features are investigated (Fig. 5.1), since there is no single universal marker to confirm it (Gorgoulis et al. 2019; Sharpless and Sherr 2015). Senescent cells can be detected by an assay that stains senescent cells at suboptimal pH 6 in the presence of the substrate X-gal senescence-associated β-galactosidase (SA-β gal) (Debacq-Chainiaux et al. 2009). Senescent cells also have a greatly flattened morphology compared to proliferating cells, increased lipofuscin accumulation (Sharpless and Sherr 2015), increased expression of cyclin-dependent kinase inhibitor (CDKi) $p16^{INK4a}$ and $p21^{CIP1}$ (Hernandez-Segura et al. 2018), extensive chromatin folding called senescence-associated heterochromatic foci (SAHF) (Gorgoulis et al. 2019), and secretion of SASP as described above (Hernandez-Segura et al. 2018).

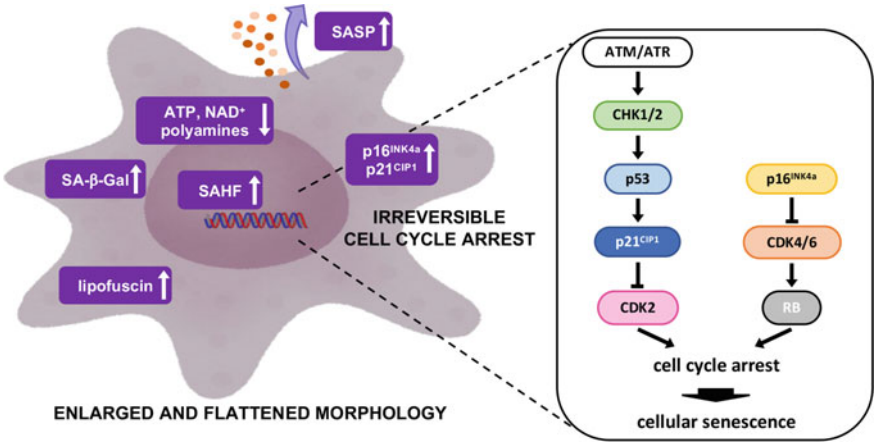


**Fig. 5.1** Characteristics of senescent cells. (Left panel) Molecular hallmarks of senescent cells. (Right panel) Molecular mechanisms modulating cell cycle arrest: DNA-damage dependent and independent mechanisms regulate the p53/$p21^{Cip1}$- and pRb/$p16^{INK4a}$-mediated mechanisms to initiate and maintain cellular senescence

## 5.4 Permanent Cell Cycle Arrest in Senescent Cells

As mentioned above, senescent cells are growth-arrested cells that do not respond to appropriate growth conditions or mitogenic stimuli. The senescent state is a cellular response to various forms of intrinsic or extrinsic stress, and the type of stressor determines whether the resulting senescent cell is replicative, oncogene-induced, stress-induced, or mitochondrial DNA damage-induced. Regardless of the type of initial stressor, cell cycle arrest is mediated primarily by two tumor suppressor pathways: the p53/$p21^{CIP1}$ and $p16^{INK4a}$/pRb pathways (Gorgoulis et al. 2019). In response to stresses such as telomere exhaustion, oxidative damage, oncogenic activation, and chemotherapeutic agents, cells initiate the DNA damage response (DDR). This involves activation of a kinase cascade that includes the serine-threonine kinases ataxia telangiectasia mutated (ATM) and ATR (ATM and Rad3-related), followed by checkpoint serine-threonine kinase CHK1 and CHK 2, ultimately leading to activation of the p53/$p21^{CIP1}$ axis. Once activated, p53 governs a complex anti-proliferative transcriptional program, inducing transcription of $p21^{CIP1}$, a CDKi, inhibiting CDK2 activity, and ultimately causing hypophosphorylation of pRB. The hypophosphorylated pRB then sequesters away the E2F transcription factor, thereby preventing the activation of genes required for DNA replication (Sharpless and Sherr 2015). However, activation of p53 and/or $p21^{CIP1}$ during senescence can also occur in a DDR-independent manner in certain situations (Storer et al. 2013). Additional or persistent stress can activate the CDKi $p16^{INK4a}$, which inhibits CDK4 and CDK6, causing hypophosphorylation of pRB and leading to a long-lasting arrest. $p21^{CIP1}$ is required for the initiation of senescence, while $p16^{INK4a}$ contributes to the maintenance of senescence (van Deursen 2014). Furthermore, an alternative reading frame protein at the $p16^{INK4a}$ locus, ARF (called $p14^{ARF}$ in humans and $p19^{ARF}$ in mice), is involved in the induction of aging. It inhibits the MDM2 E3 ubiquitin ligase, thereby preventing p53 degradation (Gorgoulis et al. 2019; Sharpless and Sherr 2015).

## 5.5 Altered Signaling Pathways in Cellular Senescence

In addition to the DDR-induced p53/$p21^{CIP1}$ and $p16^{INK4a}$/pRb pathways, various signaling pathways are altered in senescent cells. Here, I discuss signaling pathways that may be involved in the molecular clocks.

### *5.5.1 AMPK Signaling*

AMP-activated protein kinase (AMPK) functions as a bioenergetic sensor that regulates cellular responses to energy stress; AMPK is activated under conditions of increased AMP:ATP and ADP:ATP ratios (see below). To drive senescence, the

activated AMPK signaling pathway inhibits Hu antigen R (HuR)-dependent degradation of mRNAs encoding $p21^{CIP1}$ and $p16^{INK4a}$ (Wiley and Campisi 2016), or telomerase activity by phosphorylating p38 MAPK (Lanna et al. 2014). By contrast, AMPK activation has also been reported to protect cells from oxidative stress-induced senescence via autophagic flux restoration and $NAD^+$ elevation (Han et al. 2016).

### 5.5.2 P38 MAPK Signaling

p38 MAPK signaling activation following oncogenic stress, oxidative stress, or AMPK activation has been reported to induce senescence in human fibroblast (Iwasa et al. 2003), endothelial cells (Shen et al. 2013), and T cells (Lanna et al. 2014). Activated p38 MAPK represses *hTERT* mRNA expression (Lanna et al. 2014) or phosphorylates p53 directly or indirectly to induce senescence (Xu et al. 2014).

### 5.5.3 NF-κB Signaling Pathway

A remarkable diversity of stimuli, both endogenous and exogenous ligands as well as a plethora of physical and chemical stresses, leads to the activation of NF-κB signaling (Hayden and Ghosh 2008). It is composed of five different subunits, RELA (p65), RELB, c-REL, p105/p50 (NF-κB1), and p100/p52 (NF-κB2) that can homo- or hetero-dimerize to form a variety of transcriptionally active isoforms with widely different roles in the transcriptional activation or repression of genes. The major trigger for cellular senescence by NF-κB signaling activation is the DDR. Microarray analysis revealed that 65 NF-κB downstream genes are upregulated upon senescence arrest in $p53/p21^{CIP1}$ and $p16^{INK4a}$/pRb pathways-inactivated human fibroblasts (Rovillain et al. 2011). Inhibition of the NF-κB pathway by the gene ablation/silencing or pharmacological inhibition reduces senescent cells (Rovillain et al. 2011; Tilstra et al. 2012).

### 5.5.4 mTOR Signaling Pathway

The mammalian target of rapamycin (mTOR) signaling pathway integrates both intracellular and extracellular signals and serves as a central regulator of cell metabolism, growth, proliferation, and survival. The mTOR pathway is activated during various cellular processes, from protein synthesis to autophagy, and is deregulated in human diseases such as type 2 diabetes, cancer, and neurodegenerative diseases (Saxton and Sabatini 2017). mTOR is a serine/threonine protein kinase that forms the catalytic subunit of two distinct protein complexes, known as mTOR Complex 1

(mTORC1) and 2 (mTORC2) (Laplante and Sabatini 2009). mTOR pathway activation is necessary for the enlargement of the cell body of senescent cells, a key feature of cellular senescence (Bent et al. 2016). Activation of mTORC1 signaling is exhibited in senescent cells (Nacarelli and Sell 2017). mTORC1 elevation has been suggested to promote SASP by facilitating the translation of interleukin (IL) − 1α and MAPKAPK2, both of which promote the secretion of the SASP components. Furthermore, mTORC1 localizes to autolysosomes and forms a TOR-autophagy space coupling compartment that maintains the synthesis of SASP components (Narita et al. 2011). Elevated mTORC1 activity is thought to be due to defective sensing of amino acids and growth factors, as mTORC1 is constitutively activated and insensitive to serum and amino acid starvation in senescent cells (Carroll et al. 2017).

### *5.5.5 Unfolding Protein Response (UPR) Pathway*

Various factors such as oxidative stress, infections, and mutations can cause endoplasmic reticulum (ER) stress, leading to the accumulation and aggregation of unfolded and/or misfolded proteins. To eliminate these proteins and maintain ER protein homeostasis, PERK, IRE1α, and ATF6 pathways of the unfolding protein response (UPR) system in the ER are activated (Read and Schroder 2021). Interestingly, ER stressors, such as tunicamycin and thapsigargin, can induce senescence, indicating that dysfunctions of ER protein homeostasis can be a trigger for senescence. Genetic ablation of UPR genes also alters senescence levels (Pluquet et al. 2015). Notably, under non-stress conditions, PERK and IRE1α form complexes with binding immunoglobulin protein (BiP) and chaperone proteins, mainly HPS90 to inactivate and stabilize them (Wang and Kaufman 2014).

### *5.5.6 Cyto- and Nucleo-Skeletons*

One of the key features of cellular senescence is the dramatic change in cell morphology, i.e., the enlarged and flattened cell shape. The enlarged cell shape is attributed to the continued stimulation of the cell growth pathways, MAPK and mTOR (Blagosklonny 2014). Senescent cells also have an increase in the intermediate filament vimentin and a decrease in actin, tubulin, and the focal adhesion proteins, paxillin and c-Src (Nishio and Inoue 2005), resulting in decreased cytoskeletal stiffness (Dulinska-Molak et al. 2014; Ferrari and Pesce 2021; Lieber et al. 2004). Intriguingly, senescent cells also change in the shapes of the nucleus, due to the decrease in proteins composed of nuclear membranes and nuclear lamina, such as LINC (linker of nucleoskeleton and cytoskeleton) protein complex, lamin B1, and lamin B1 receptor (Pathak et al. 2021).

## 5.6 Metabolic Changes in Cellular Senescence

Levels of metabolites, such as ATP and $NAD^+$, change with aging at the cellular and organismal levels. Among them, $NAD^+$ levels which are known to decrease with aging (Khaidizar et al. 2017; Madeo et al. 2018; Yoshino et al. 2018), are one of the key metabolites that regulate the circadian clocks. Calorie restriction that increases $NAD^+$ levels restores the circadian genomic signatures of aging in the liver, epidermal, and muscle stem cells of aged mice (Sato et al. 2017; Solanas et al. 2017). Reportedly, $NAD^+$ levels are also regulated by the circadian clock (Nakahata et al. 2009; Ramsey et al. 2009).

### *5.6.1 Adenosine Triphosphate (ATP)*

Senescent cells exhibit changes in mitochondrial mass, membrane permeability, and morphology (Chapman et al. 2019). In addition, senescent cells show an increased mitochondrial accumulation (Hernandez-Segura et al. 2018), primarily due to reduced mitophagy (Korolchuk et al. 2017). A key step in triggering mitophagy is the recruitment and translocation of the E3 ubiquitin ligase, Parkin, to damaged mitochondria, ultimately leading to autophagosome-mediated degradation (Chapman et al. 2019). p53 interacts with Parkin and prevents its translocation to damaged mitochondria, thus inhibiting mitophagy. Senescent cells possess a compromised ability to generate adenosine triphosphate (ATP), although senescent cells have a higher number of mitochondria (Korolchuk et al. 2017). The membrane potential of these mitochondria is decreased, leading to the release of mitochondrial enzymes, such as endonuclease G, and intensified ROS production (Hernandez-Segura et al. 2018). Dysfunctional mitochondria may contribute to the establishment and intensification of the senescence state through excessive ROS generation (Chapman et al. 2019; Nacarelli and Sell 2017) and altered levels of other key metabolites.

Senescent cells shift to a highly glycolytic state (James et al. 2015), with elevated adenosine monophosphate (AMP) and adenosine diphosphate (ADP) levels, relative to cellular ATP levels (Wiley and Campisi 2016). Investigations have suggested that glycolysis is elevated by the upregulation of key glycolytic enzymes (James et al. 2015). Although the reason for adapting to a more glycolytic state is unclear, it is speculated that glycolysis may help provide precursors for the high demand for proteins, lipids, and other cellular macromolecules for the components of the SASP and enlarged senescent cells (Wiley and Campisi 2016).

### 5.6.2 Nicotinamide Adenine Dinucleotide ($NAD^+$)

Senescent cells have low $NAD^+$ levels (Khaidizar et al. 2017) with low $NAD^+$/NADH ratios. One of the major reasons for this is that nicotinamide phosphoribosyltransferase (NAMPT) levels, the rate-limiting enzyme in the mammalian $NAD^+$ salvage pathway, decrease in senescent cells (Khaidizar et al. 2017). Reportedly, NAMPT decreases with aging in several tissues (Lee et al. 2012). In addition, decreased levels of cytosolic malate dehydrogenase, an enzyme that mediates the conversion of oxaloacetate to malate by using NADH, in senescent cells have been suggested to contribute to the low ratio of $NAD^+$/NADH. $NAD^+$ serves as an electron carrier during oxidative phosphorylation and as an electron acceptor in the electron transport system, where it is reduced to NADH. $NAD^+$ is also important for the regulation of DNA repair signaling. Poly (ADP-ribose) polymerases (PARPs) utilize $NAD^+$ for their activation to repair genotoxic stress-induced DNA damage. The sirtuin family also utilizes $NAD^+$ for their deacetylase activity for the regulatory roles in DNA repair and metabolism (Khaidizar et al. 2017). PARP inhibition can accelerate the induction of senescence due to the accumulation of damaged DNA (Efimova et al. 2010), and low levels of $NAD^+$ can decrease the activity of sirtuins, which can aid in the senescence state (Grabowska et al. 2017).

### 5.6.3 Polyamines

Polyamines (putrescine, spermidine, and spermine) are ubiquitous polycations present in all living organisms. Polyamine concentrations in mammals are determined by their nutritional supply, synthesis by the intestinal microbiota, uptake, and cellular biosynthesis. Polyamines regulate various cellular processes such as gene regulation, protein synthesis, cell growth, and chromatin structure organization (Miller-Fleming et al. 2015; Minois 2014). Therefore, it is important to maintain optimum polyamine concentrations for the smooth functioning of various organs. Hence, reduced polyamine concentrations in organs are involved in age-related diseases and aging (Nishimura et al. 2006). Although polyamine levels in senescent cells have not been analyzed, it has been reported that reduced polyamines in human melanoma cells result in a senescent-like phenotype (Kramer et al. 2001).

## 5.7 The Circadian Clock

Circadian clocks generate 24-h rhythms in a growing body of biological, physiological, and behavioral events, ranging from bacteria to humans, and are established by cell-autonomous oscillators called cellular clocks (Takahashi 2017). Mammalian cellular clocks are part of the hierarchical multi-oscillatory network. The master

clock resides in the suprachiasmatic nuclei (SCN) of the hypothalamus. On the other hand, peripheral clocks are localized in almost all other tissues of the body (Honma 2018; Takahashi 2017). The core molecular mechanisms of cellular clocks are the same, irrespective of whether they are the master or peripheral clocks.

The mammalian cellular clocks consist of positive and negative limbs that form transcription/translation-based feedback loops (TTFLs) (Fig. 5.2). The positive limb of the clock consists of two transcriptional factors, CLOCK and BMAL1, the basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) type transcriptional activators. These two transcriptional activators form a heterodimer to bind to the E-box elements (CACGTG) present in the promoter region of the genes that form the negative limb of the clock, namely *Period* genes (*Per1*, *Per2,* and *Per3*) and *Cryptochrome* genes (*Cry1* and *Cry2*). After induction of *Per* and *Cry* genes, PER and CRY proteins form a multimeric protein complex and give feedback to repress their own transcription by binding to the activator, CLOCK-BMAL1 heterodimer (Honma 2018; Takahashi 2017). This core feedback loop is coupled with an interlocked loop, in which nuclear receptors, RORs and REV-ERBs, form an additional TTFL to the core loop. RORs and REV-ERBs recognize and compete for binding to an element, RORE (ROR/REV-ERB-response element), to serve as activators and repressors, respectively, at their target sites to form an additional TTFL that impinges on the core clock TTFL (Honma 2018; Takahashi 2017).

One of the most notable aspects of this intrinsic cellular clock is its ability to synchronize with external environmental cues, such as light–dark cycles, food, and exercise (Chaudhari et al. 2017); the components of the system can also synchronize with each other. Consequently, the circadian clock system maintains an optimal timing for nearly all physiological and behavioral activities of the organisms, such as metabolism, feeding, reproduction, and cognitive performance, thereby determining the overall health and survival of the organism. However, like other systems in the body, the circadian clock system is disrupted with aging (Davidson et al. 2008; Mattis and Sehgal 2016; Sellix et al. 2012; Valentinuzzi et al. 1997), and this has been demonstrated in the organism, tissue tissues, and at the cellular level, as evidenced. As a result, disruption of the circadian clock is associated with many age-related diseases, such as cancer, metabolic syndrome, cardiac diseases, and sleep disorders, as well as susceptibility to infectious diseases (Rijo-Ferreira and Takahashi 2019).

At the organismal level, manifestations of circadian clock disruptions with aging have been observed in both humans and other animals. For example, changes in sleep–wake cycles were observed in humans, including changes in timing, duration, and consolidation of sleep (Mattis and Sehgal 2016). Aged rodents displayed changes in activity rhythms, with an altered period, delayed phase, more days for re-entrainment to phase changes, and greater variability in activity onset time than younger animals (Davidson et al. 2008; Sellix et al. 2012; Valentinuzzi et al. 1997; Zhao et al. 2019). Although these lines of evidence suggest that circadian rhythms are affected by aging, these pieces of evidence are not sufficient to deduce that the circadian clock system is inherently affected, as the tissues or organs themselves may exhibit a functional decline independent of circadian clock changes (Zhao et al. 2019).

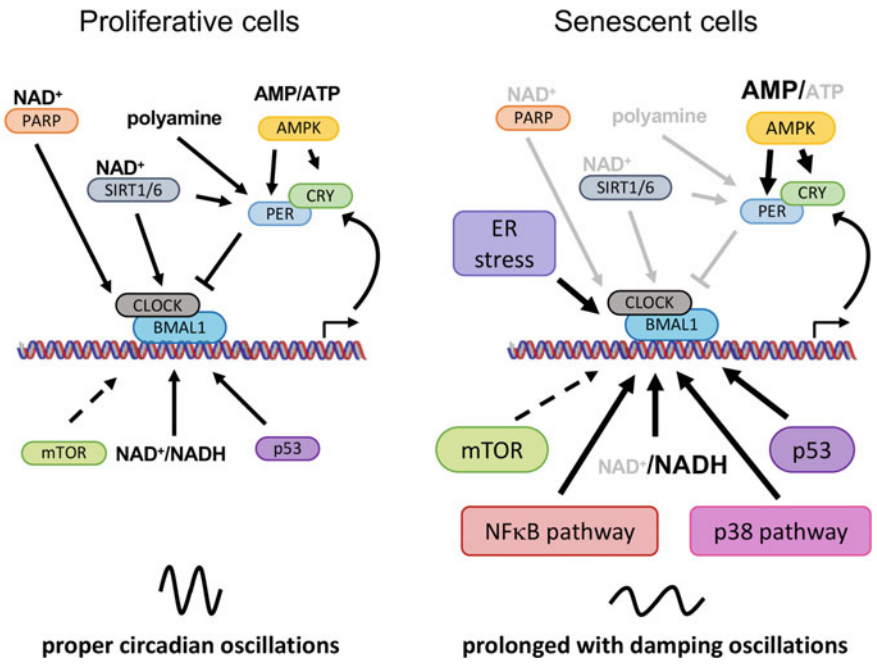


**Fig. 5.2** Molecular regulations for circadian clock. (Left panel) Proper regulations by enzymes and metabolites in proliferative cells make the 24 h rhythmicity. (Right panel) Improper regulations by altered levels of enzymes, metabolites, and signaling pathways in senescent cells lead to attenuated and prolonged rhythmicity

## 5.8 Aging of the Circadian Clock

The SCN, mammary tissues, and intervertebral disk from aged mice had longer periods, delayed phases, and attenuated amplitudes than those of young mice (Dudek et al. 2017; Nakamura et al. 2015; Yang et al. 2017). However, in other studies, changes in circadian clock properties in aged animals were tissue-specific (Davidson et al. 2008; Sellix et al. 2012; Yamazaki et al. 2002). One possibility for discrepancies between studies could be the differences in the accumulation of senescent cells. Some tissues accumulate a higher burden of senescent cells than others. Our group has recently reported that senescent cells in vitro show alterations of the cellular clocks irrespective of replicative senescent cells or stress-induced premature senescent cells (Ahmed et al. 2019, 2021). Senescent cells were found to have a prolonged period and delayed phase than proliferative cells. In the case of stress-induced premature senescent cells, these parameters intensified as senescent cells became more mature (Ahmed et al. 2021). This suggests that the dynamic nature of senescent cells may influence on the severity of clock dysfunction. Studies conducted by some groups, including ours, have elucidated that senescent cells exhibit attenuated amplitudes in several of the core circadian clock genes (Kunieda et al. 2006; Liang et al. 2021).

## 5.9 Possible Molecular Regulators of Cellular Clocks in Senescent Cells

The molecular mechanisms by which senescence affects the cellular clock remain to be elucidated, however, plenty of the pathways and factors disrupted in senescent cells are known to affect the cellular clocks (Fig. 5.2).

### *5.9.1 P53 Signaling Pathway*

Stabilization of p53 through post-translational modifications, such as phosphorylation and acetylation, is important for cell cycle arrest during senescence (Gorgoulis et al. 2019). Pharmacological stabilization of p53 suppresses *Per2* expression by which p53 directly binds to a response element that overlaps with the E-box element of the Per2 promoter, whereas $p53^{-/-}$ mice have a shorter period of locomotor activity (Miki et al. 2013).

### *5.9.2 AMPK Signaling*

AMPK is a rhythmically expressed kinase that phosphorylates and activates CK1ε to phosphorylate PER and CRY1 for degradation (Um et al. 2007; Lamia et al. 2009). Chronic AMPK activation by AMPK agonist AICAR or by glucose deprivation prolonged the circadian period and attenuated the amplitude (Lamia et al. 2009) although another AMPK agonist, metformin, shortened the circadian period (Um et al. 2007). The involvement of AMPK in cellular clocks remains controversial, however, due to an increase in the AMP/ATP ratio AMPK alters cellular clocks in senescent cells.

### *5.9.3 P38 MAPK Signaling*

p38 MAPK pathway has been shown to play a crucial role in the response to light in the SCN of rodents, the chick pineal gland, and the cultured Xenopus retina (Hasegawa and Cahill 2004; Hayashi et al. 2003; Pizzio et al. 2003). It has been reported that selective inhibitors of p38 MAPK prolong the circadian rhythm in the cultured pineal and human cells (Hayashi et al. 2003; Hirota et al. 2008) and that p38 MAPK phosphorylates CREB by TNFα stimulation, resulting in the induction of *Per1* mRNA (Petrzilka et al. 2009). However, the molecular mechanisms of how p38 MAPK signaling regulates the molecular clock remain unclear. Even if the cellular clocks are disrupted by hyperactivation of the p38 MAPK signaling pathway in

senescent cells, further studies are needed to clarify the link between the cellular clock and cellular senescence via the p38 MAPK signaling pathway.

### 5.9.4 NF-κB Signaling Pathway

Macrophages can produce proinflammatory cytokines such as IL-1β, IL-6, and TNFα in response to pathogens. Cytokine secretion by macrophages has been reported to show a circadian manner both in mice and isolated cells (Bellet et al. 2013; Curtis et al. 2015; Gibbs et al. 2012; Keller et al. 2009; Nguyen et al. 2013). NF-κB signaling is activated in response to pathogens and induces microRNA miR-155, which binds to 3'-UTR of *Bmal1* mRNA to suppress *Bmal1* mRNA and protein (Curtis et al. 2015). Interestingly, genetic ablation of miR-155 perturbs circadian function and demonstrates a shorter period (Curtis et al. 2015). RELB, one of the NF-κB components, acts as a repressor of circadian transcription. RELB forms a complex with CLOCK-BMAL1 to repress their transcriptions, while *Relb*$^{-/-}$ fibroblasts strengthen the amplitude of circadian oscillations regulated by CLOCK-BMAL1 (Bellet et al. 2012). It is noteworthy that the senescent cells in which NF-κB signaling is activated possess a prolonged period (Ahmed et al. 2019, 2021).

### 5.9.5 mTOR Signaling Pathway

mTOR is upregulated during senescence, and elevated mTOR activity is a characteristic of aging (Nacarelli and Sell 2017). mTOR perturbation by RNAi knockdown or mTOR inhibitors results in the prolonged circadian period in fibroblasts, SCN, and animal behaviors (Ramanathan et al. 2018; Zhang et al. 2009). However, molecular mechanisms of how the upregulated mTOR signaling pathway perturbs cellular clocks remain to be elucidated.

### 5.9.6 Unfolding Protein Response Pathway

ER stress inducers such as tunicamycin and thapsigargin, which can give rise to cellular senescence, reduce the amplitude of circadian oscillations with delayed phase (Bu et al. 2018; Gao et al. 2019; Pickard et al. 2019). miR-211 induced by the PERK-ATF4 pathway suppresses *Bmal1* and *Clock* mRNA and protein amounts (Bu et al. 2018; Gao et al. 2019). Overexpression of BiP or treatment of chemical chaperones, both of which inactivate the UPR pathway, strengthens the amplitude of circadian oscillations (Pickard et al. 2019).

### 5.9.7 Cyto- and Nucleo-Skeletons

Little is known about how cellular clocks sense and respond to their microenvironment. Recent studies have demonstrated the stiffness of the cellular microenvironment regulates the circadian clocks; epithelial cells have stronger circadian oscillations in soft microenvironments (Yang et al. 2017). Cell-extracellular matrix interactions transduce mechanical stress to the cellular clocks directly via integrin signaling components such as vinculin, RhoA, and ROCK (Yang et al. 2017). It is noteworthy that fibroblasts show the opposite response, exhibiting stronger oscillations in stiff microenvironments (Williams et al. 2018). Tissue stiffness alters with aging, therefore microenvironments might modify cellular clocks, although molecular mechanisms linking to the cellular clocks remain largely unclear.

### 5.9.8 $NAD^+$ and $NAD^+$/NADH Ratio

$NAD^+$ levels in cells and tissues have been demonstrated to oscillate with 24-h rhythmicity (Nakahata et al. 2009; Ramsey et al. 2009), which makes $NAD^+$-dependent deacetylase, SIRT1 and SIRT6, activity rhythmic. Thus, the acetylated histone H3 on circadian clock gene promoters exhibits circadian rhythm to fine-tune rhythmic gene expressions (Masri et al. 2014; Nakahata et al. 2008). SIRT1 also rhythmically deacetylates BMAL1 and PER2. Rhythmic acetylated BMAL1 modulates CRY recruitment to the CLOCK-BMAL1 complex (Hirayama et al. 2007; Nakahata et al. 2008). Rhythmic acetylation of PER2 regulates its protein stability and subcellular localization (Asher et al. 2008; Ashimori et al. 2021; Levine et al. 2020). PARP1, another $NAD^+$-dependent enzyme, binds and poly(ADP-ribosyl)ates CLOCK rhythmically to modulate CLOCK-BMAL1 binding affinity with PER-CRY repressors (Asher et al. 2010). Thus, low $NAD^+$ levels in senescent cells could be a possible cause of the impaired cellular clocks in senescent cells.

NADH levels also alter with aging. In contrast to the decline in $NAD^+$ levels with aging, NADH levels increase with aging, indicating a decrease in $NAD^+$/NADH ratio and the redox state of total NAD (Zhu et al. 2015a). Intriguingly, the redox state of total NAD affects the DNA-binding potential of NPAS2:BMAL1 in vitro (Rutter et al. 2001).

### 5.9.9 Polyamines

Mammals obtain polyamines by de novo synthesis and through dietary uptake. Among several enzymes in the de novo polyamine biosynthesis pathway, the rate-limiting enzyme, ornithine decarboxylase (ODC), shows circadian oscillation and

polyamines modulate the affinity of PER2 with CRY1 (Zwighaft et al. 2015). Inhibition of ODC enzymatic activity prolongs the circadian period in NIH3T3 cells, while either putrescine or spermidine treatment rescues the phenotype induced by the ODC inhibitor. Importantly, the decline in polyamines with aging in mice is associated with a longer locomotor activity period, which can be reversed by polyamine supplementation (Zwighaft et al. 2015).

#### 5.9.9.1 SASP Factors

In a variety of tissues from aging animals, senescent cells form a small fraction of the tissue, with approximately 2–13% of the tissue being senescent cells (Biran et al. 2017). However, how such a small fraction of these cells might disrupt the circadian functioning of tissues remains largely unknown. As already mentioned, senescent cells secrete SASP, a collection of cytokines, chemokines, growth factors, and proteases that exert potent biological activities on the surrounding cells and tissues (Sharpless and Sherr 2015). A plausible possibility is that senescent cells, via their SASPs, affect the cellular clocks of surrounding cells; over 300 SASP factors, some components such as IL-1β and INF-γ have been found to alter the cellular clock (Andersen et al. 2020; Dudek et al. 2017; Guo et al. 2015; Wiley et al. 2019). Thus, besides being intrinsically affected by their circadian clock mechanisms, senescent cells have the potential to spread this dysfunction to surrounding cells. Although the molecular mechanisms underlying clock dysfunction in senescent cells are not fully understood, these lines of evidence strongly suggest that senescent cells could be the underlying cause of circadian clock dysfunction, ultimately leading to circadian rhythm dysfunction at the tissue and organism level.

## 5.10 Conclusions

The above discussion highlights the need for further investigation to determine the molecular basis of cellular clock disturbances in senescent cells and to determine whether and how senescent cells can affect cellular clock function at the tissue and organismal levels. High-throughput pharmacological studies using cell-based luciferase real-time monitoring assays (Chen et al. 2012; Hirota et al. 2010; Kon et al. 2015) could be a powerful tool to address the molecular basis of the cellular clocks in senescent cells. In addition, studies of $NAD^+$ and/or polyamine supplementation could help to elucidate the molecular mechanisms of the cellular clock in senescent cells. Conditioned media from senescent cells could be used to determine whether SASP factors affect the cellular clocks in proliferating cells or vice versa. In addition, it would be worthwhile to determine whether all types of senescent cells exhibit prolonged periods and delayed phases, as seen in replicative senescent cells and stress-induced premature senescent cells, or whether there are tissue differences in these circadian characteristics of senescent cells. Ultimately, clarifying whether

different types of senescent cells have altered cellular clocks may explain the tissue-specific circadian changes observed previously (Yamazaki et al. 2002). Finally, it is possible to evaluate the effects of the removal of senescent cells on the circadian physiology of aging animals using transgenic mouse models and pharmacological compounds called senolytics, which specifically kill senescent cells. These studies will provide more insight into the relationship between senescent cells, circadian clocks, and age-related diseases, which in turn will lead to the development of new treatments to alleviate suffering in the elderly.

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# Part II
# Sleep, Ageing and Longevity

# Chapter 6
# Optimum Sleep for Healthy Ageing


**Birendra Nath Mallick and Rachna Mehta**

## 6.1 Sleep and Wakefulness

Living systems go through apparently quiescent and non-quiescent conditions in a cyclic manner. By and large, the former is associated with increased anabolic or decreased catabolic processes, while the latter, with opposite processes. These conditions may be identified grossly by the physical movement, a quantifiable parameter, of the living system. Such alternating states have been best described as the basic rest and activity cycle (BRAC), which is one of the fundamental characteristics of the living organisms. It has been proposed that the BRAC has evolved into sleep and wakefulness in higher species in evolution. In species higher in evolution, particularly where the brain has evolved, the concept of consciousness, mind, and thought processes have appeared. Without going into the philosophical or metaphysical aspects whether non-living objects and living beings without brain possess consciousness, it may be said that by and large, it is accepted that consciousness is associated with brain and its functions. To elaborate, wakefulness is associated with alertness and physical movement, rest is usually related with reduced or lack of muscle activity, while sleep, is associated with rest of the brain in addition to the voluntary muscles. Additionally, there are other phenomena, e.g. active and quiet wakefulness and rest, with or without sleep. Notwithstanding, in the absence of objectively defined characteristic parameters, although it was difficult to critically define these sleep-waking states, based on contemporary research finding it was clear that sleep serves specific purpose for the brain and is regulated by the brain.

The sleep and wakefulness are instinct behaviours. As mentioned above, although the wakefulness could be apparently identified by the expression of conscious physical activity or movement, it was difficult to objectively identify sleep. Subsequently,

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when it was possible to record the electrical activities from the brain, the electroencephalogram (EEG), the muscles, the electromyogram (EMG) and the eyes, the electrooculogram (EOG), sleep and waking could be objectively defined and characterized. Such recordings showed that sleep is not a homogenous, passive phenomenon, i.e. sleep is not merely an absence of wakefulness. It was discovered that within sleep, intermittently a stage appears when electrophysiologically the brain apparently behaves as it behaves during waking. As this stage of sleep is associated with rapid eye movement (REM), it was termed as REM sleep (REMS) (Aserinsky and Kleitman 1953). Subsequently, it was observed that this stage was associated with most of the dreams during sleep. This discovery had put death nail to the passive theory of sleep, and in other words, it was proposed that sleep is an active process. In fact, it was also confirmed that stimulation of certain brain regions may induce sleep or sleep like state (Moruzzi and Magoun 1949).

Sleep is regulated by the brain, expressed for the brain; it has been proposed that sleep serves housekeeping function of the brain. It follows a biological rhythm, which may get modulated by the circadian rhythm. Although sleep is an instinct and involuntary behaviour, it may get modulated by the voluntary and cognitive processes. It is an essential physiological process as its loss is faced with rebound recovery, while its prolonged loss may become fatal (Thakkar and McCarley 2005; Mehta et al. 2020). By and large, sleep rejuvenates the brain, as the latter directly or indirectly controls and integrates most of the other physiological processes of the body and vice versa, it is understandable that its disturbance affects most of the physiological processes. In fact, hardly any physiological process is immune to sleep disturbance, while sleep disturbance has been reported in almost all acute as well chronic diseases. Thus, sleep disturbances could be an early indicator for disturbed health. Below, we review the changes in sleep (sleep disorders) in association with dysfunctions in other systems in the body.

## 6.2 Cardiovascular and Respiratory Dysfunctions Associated with Sleep Loss

The heart rate and respiration slow down during non-REMS, while they become irregular during REMS (Lavie et al. 2000). Disturbed sleep has been reported in subjects suffering from hypertension and other heart ailments (Calhoun and Harding 2010). Cessation of respiration during sleep (sleep apnoea) is a leading cause of death (Marshall et al. 2014). Often snoring is also a symptom of disturbed respiration, when the sleep is significantly disturbed (Memon and Manganaro 2022). Deprivation of REMS alters blood parameters linked with cardiovascular disorders and thus, contributes to arthrosclerosis and arterial hypertension (Andersen et al. 2004; Martin et al. 2007). On the other hand, hypertensive patients show significantly reduced REMS (Friedman et al. 2010). Impaired noradrenaline (NA) reuptake transporter activity has been reported in hypertension and postural tachycardia syndrome (Esler

et al. 2006). Rate of respiration has been reported to be regular during NREMS, while it becomes irregular during REMS. Also, hypoxic and hypercapnic ventilatory responses fall during transition from wakefulness to NREMS, which is further reduced during REMS (Choudhary and Choudhary 2009). REMS reduces the tidal volume and ventilation efficiency, and these effects are further enhanced in patients with obstructive sleep apnoea and respiratory diseases (Millman et al. 1988; White et al. 1995). Sleep quality is impaired in patients with chronic respiratory disease; also, decreased sleep efficiency with a reduction in REMS has been reported in patients with chronic obstructive pulmonary disease (Valipour et al. 2011; McNicholas et al. 2019). Therefore, disciplined and quality sleep is essential to maintain optimum physiological processes including cardiovascular and respiratory functions.

## 6.3 Endocrine Dysfunctions Associated with Sleep Loss

Disturbed or fragmented sleep has been reported to be associated with altered levels of many hormones, e.g. thyroxine (Green et al. 2021; Gary et al. 1996), gonadotrophins (Lateef and Akintubosun 2020), corticotrophins (Hirotsu et al. 2015; Machado et al. 2010), melatonin (Davis et al. 2014), orexin (Mehta et al. 2015), etc. Elevated levels of thyroid hormones were seen to be associated with excessive daytime sleepiness and prolonged sleep latency (Sridhar et al. 2011). Hormonal imbalance caused due to sleep disturbances is also associated with metabolic dysfunctions like obesity, insulin insensitivity, diabetes and appetite dysregulation (Kim et al. 2015). Sleep deprivation is reported to be one of the important causes of infertility, which has been proposed to be due to associated changes in synthesis, release and metabolism of reproductive hormones (Lateef and Akintubosun 2020). Thus, poor sleep quality in female shift-workers or middle-aged people contributes to early pregnancy loss and decreased testosterone concentration, respectively, along with suppression of melatonin levels (Lateef and Akintubosun 2020; Alizadeh et al. 2021).

## 6.4 Metabolic Dysfunctions and Sleep Loss

Sleep is state of energy conservation in the sense that the metabolism is reduced (Schmidt et al. 2017). Accordingly, the release of metabolic enzymes, e.g. amylases, peptidases, gastric juices (Stacher et al. 1975; Pajcin et al. 2017) and hormones, e.g. insulin, gastrin (Donga et al. 2010) are affected during sleep loss. Salivary alpha-amylase has been proposed to be a peripheral measure of noradrenergic activity. Higher salivary amylases were linked with better performance while performance deficits were seen during decrease in amylases level. Sleep, particularly, REMS was found to be associated with significantly lower levels of acid secretion. Decreased sleep duration in healthy individuals has also been linked to impaired glucose homeostasis which may lead to obesity. Sleep apnoea is also linked to impaired glucose

tolerance and obesity is one of the major risk factors for the development of sleep apnoea (Mesarwi et al. 2013). Thus, the patients suffering from diabetes often complain of sleep disturbances.

## 6.5 Thermoregulatory Changes in Association with Sleep and Sleep Loss

Maintenance of body temperature is a key determinant as well as function of sleep (Okamoto-Mizuno and Mizuno 2012). The body temperature is reduced during the non-REMS and it increases during the REMS. Due to this, it has been proposed that one of the functions of REMS is "warming the CNS" (Wehr 1992). REMS appears to be more sensitive to changes in ambient temperature and thus, decreased sensitivity to hot and cold stimuli is seen during REMS as compared to other sleep stages and wakefulness (Muzet et al. 1983). NA stimulates metabolic activity elevating body temperature and triggering heat dissipation for thermoregulation (Ratheiser et al. 1998). Also, abnormalities in the body temperature rhythm are associated with insomnia and associated symptoms (Lack et al. 2008). Thus, REMS and NA (independently and dependently) play crucial roles to maintain thermoregulation and normal physiological functions.

## 6.6 Altered Immune Function in Relation to Sleep Disturbances

The immune system functions closely with the nervous system. Several studies have shown the production of immune factors by the brain and neuroendocrine mediators by the immune system (Blalock 1989; Madden and Felten 1995). Sleep is an important modulator of the immune response, and loss of sleep increases the susceptibility of an organism to infections (Krueger and Karnovsky 1987; Opp 2009). NA is also known to modulate the immune system (Kohm and Sanders 2000; Rommelfanger and Weinshenker 2007); also, it plays a significant role in controlling the susceptibility to different types of infections (Kohm and Sanders 2000). Inflammatory cytokines are known to interact with serotonin, melatonin and NA (Imeri and Opp 2009) and such interactions hint at the possible candidates linked to inflammation and sleep. REMS loss has been reported to affect several hormones (including melatonin, growth hormone and cortisol), interleukins (e.g. IL-6), several enzymes including those associated with glycolytic pathway and apoptotic markers (cytochrome C, Caspases, etc.) in the brain (Redwine et al. 2000; Somarajan et al. 2016). Several cytokines affect sleep which includes IL-1$\alpha$, IL-1$\beta$, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-15, IL-18, TNF-$\alpha$, TNF-$\beta$, IFN-$\alpha$, IFN-$\beta$, IFN-$\gamma$ and macrophage inhibitory protein (Imeri and Opp 2009). Out of these, IL-1$\alpha$, IL-1$\beta$ and TNF-$\alpha$ have been

studied more to identify their role in sleep regulation. These immune signalling molecules are present in the healthy brain, where they interact with serotoninergic, cholinergic and glutamatergic systems to regulate sleep and its loss (Imeri and Opp 2009; Grazia de Simoni et al. 1995). Receptors for IL-1$\alpha$, IL-1$\beta$ and TNF-$\alpha$ are present in the brain areas involved in sleep regulation including brain stem, hypothalamus and cerebral cortex (Imeri and Opp 2009). Some of these changes might be seen in non-neuronal tissues, however, how much they are directly associated with REMS loss, need further studies.

Sleep loss advances the onset and worsens the prognosis of many diseases. Sleep disorders like insomnia, narcolepsy, sleep-disordered breathing, etc., exacerbate existing ailments by damaging the immune system (Okun et al. 2004; Kheirandish-Gozal and Gozal 2019). Also, the role of cytokines has been strongly suggested in the development of narcolepsy. A recent meta-analysis shows that serum levels of IL-6 and TNF-α were higher in all narcoleptic patients than in control patients (Irwin and Opp 2017; Okun et al. 2004). Thus, there are enough convincing evidence that sleep and immunity have interdependencies, however, the mechanisms by which they influence each other are not completely understood.

## 6.7 Cognitive Dysfunction in Association with Sleep Disturbances

Learning and memory are among the cognitive functions that confer upon us the ability to accumulate knowledge from our experiences (Liu et al. 2009). Several studies have suggested that the quantity and quality of sleep has a profound impact on learning and memory (Stickgold and Walker 2005). REMS serves several crucial functions, and its loss affects various pathophysiological states and processes (Stickgold and Walker 2005) including loss of concentration, impairment of memory processing and memory consolidation (Stickgold 2005; Mehta et al. 2016, 2020). REMS is necessary for memory consolidation and loss of REMS has an adverse effect on memory. Indications that sleep participates in the consolidation of fresh memory traces come from a wide range of experimental observations (Maquet 2001). The cAMP signalling pathway, which regulates CREB activity, is also crucial for hippocampal synaptic plasticity and memory storage (Abel et al. 1997). It has been observed that hippocampal cAMP levels are elevated during REMS, while its levels are impaired during sleep loss (Luo et al. 2013; Vecsey et al. 2009). Although many studies have found association between changes in REMS or its loss and gene regulation, their cause-and-effect relationship cannot be correlated with reasonable confidence. Notwithstanding, analysing those studies certainly can form part of another review; however, it may be emphasized without hesitation that undertaking such studies in detail is the need of the hour.

Sleep loss and poor quality of sleep among individuals are priority issues in our society. Sleep disorders such as obstructive sleep apnoea and untreated sleep

disturbances might also lead to cognitive impairment. This may also act as an important risk factor for the development of dementia. Considering the prevalence and socioeconomic burden of several sleep loss-associated neurological disorders such as Alzheimer's and Parkinson's diseases, the age-dependent sleep loss accompanied by cognitive dysfunctions and dementia (Gagnon et al. 2008; Garcia-Alberca et al. 2013), should receive increased attention for awareness and investigations into the underlying associated causes. In support, we would like to mention that the NA from LC neurons is known to modulate the consolidation and retrieval of hippocampus-based memory (Hansen 2017). Recently, we have shown that NA level increases and GABA levels decrease in the brain during REMS loss (Mehta et al. 2017) and many of the neurodegenerative changes in the brain induced during REMS loss are mediated by increased levels of NA (Giri et al. 2021).

## 6.8 Sleep Disturbances Associated with Changes in Social Factors

Sleep is one of the most important instinct behaviours, which is indispensable for carrying out our daily activities and maintain healthy living. A quality sleep is essential for physical, cognitive and psychological well-being (Altun et al. 2012). Sleep as a universal phenomenon is represented in the sociocultural structure. The sociologist, Simon Williams, writes, "*Where we sleep, when we sleep, and with whom we sleep are important markers or indicators of social status, privilege, and prevailing power relations*". A person's genetic make-up, knowledge, beliefs, attitudes about sleep, race/ethnicity, finances, employment, overall health, etc., are few of the factors that may influence individual's sleep directly or indirectly. However, this individualistic approach is also affected at a social level which includes the home (family, bedroom, etc.), neighbourhood/environment, work, socioeconomics, religion, culture, social media, etc. These factors in turn are embedded within the social milieu, which includes factors like globalization, geography, technology, public policy, etc., affecting sleep (Grandner 2017). In a survey conducted on university students, it was found that most of the students have complained about difficulty falling asleep, sleep disturbances and excessive day time sleepiness (Wolfson 2010). The important factors that were found to affect sleep were stress, sadness, family problems, depression, anxiety and lower life satisfaction (Suen et al. 2010). Lifestyle changes have further introduced several social factors that are negatively influencing sleeping patterns. Thus, sleep hygiene affects the foundation of physical, mental and social well-being.

## 6.9 Ageing and Sleep Disturbances

Disturbed sleep is one of the symptoms common to ageing. Most of the aged people complain about reduced sleep duration, sleep fragmentation, increased sleep latency, etc. (Moraes et al. 2014; Carskadon et al. 1982). Thus, ageing impacts the ability of the brain to initiate and maintain sleep. Both sleep and ageing are natural, instinct behaviours and both, quality and quantity of sleep have been linked with ageing. Sleep duration gradually decreases from infancy to childhood and significantly decreases with old age. REMS is expressed maximum in the babies and reduces with ageing (Ohayon et al. 2004); however, it is never absent in life. Many of the REMS loss-associated symptoms have been reported upon ageing, for example, reduction in brain excitability (Oh et al. 2010), memory loss (Luszcz and Bryan 1999), loss of concentration (Park and Festini 2017) and neurodegeneration (Harada et al. 2013), while REMS is reduced in ageing-associated diseases, e.g. Alzheimer's (Gagnon et al. 2006) and Parkinson's diseases. Alterations in NA levels were also observed in different brain regions of aged rats as compared to young rats (Arivazhagan and Panneerselvam 2002). Thus, NA could be central for ageing-related sleep disturbance and pathological conditions.

## 6.10 Sleep Disturbances and Brain Maturity

Sleep is one of the fundamental and instinct behaviour expressed by the brain during early development. REMS dominates in the prenatal and neonatal periods and its quantity reduces with ageing; however, it is never absent in life (Frank and Heller 2003; Roffwarg et al. 1966). Its role in brain development has been proposed by the fact that the period spent in REMS is higher in new-born and in babies than in the adults (Dumoulin Bridi et al. 2015). REMS expression is more in babies who are born immature. Its expression decreases with maturity of the brain as well as with ageing (Roffwarg et al. 1966). It has been shown that at the end of postnatal second week in rats, a few neurons in the ventro-lateral part of the brain stem, which in adult brain corresponds to around area pontine oralis and LDT/PPT, the sites where cholinergic REM-ON neurons are located, start firing significantly faster in association with expression of REMS signs (Corner and Bour 1984). Around the same time (end of postnatal second week), the neuron in LC intermittently decreases firing. Thus, brain development and maturation correlate well with REMS quantity (Frank and Heller 2003).

Sleep disturbances during development were seen to be associated with several childhood disorders including autism spectrum disorders, attention deficit hyperactivity disorders, emotional and behavioural difficulties. In healthy children, undiagnosed sleep disturbances have also been found to significantly impact brain development (Na et al. 2021). Effects of sleep disturbances on brain maturation were obvious by the observation that children with sleep disturbances were associated with thinner

cortex in the dorsolateral prefrontal area (Kocevska et al. 2017). Also, it was observed that children with mild to severe obstructive sleep apnoea had a significant deficit in grey matter volume in the prefrontal and temporal regions (Chan et al. 2014). Thus, sleep deficiency in childhood has a profound role to play in early brain development and expression of cognitive behaviour in adult life.

## 6.11 Sleep Disorders in Association with Acute Diseases

Insomnia is one of the causes/symptoms of common cold or pneumonia while influenza has been shown to be associated with narcolepsy (Gomi 2019). A bidirectional link exists between sleep loss and obesity as obesity increases the risk for sleep disorders and reduced sleep causes fatigue reducing the ability to exercise (Cooper et al. 2018). Sleep disturbances are commonly associated with respiratory disorders like asthma (Choudhary and Choudhary 2009; Cukic et al. 2011). Also, sleep loss or poor sleep quality results into exacerbation of gastrointestinal symptoms and vice versa (Khanijow et al. 2015).

## 6.12 Sleep Disorders in Association with Chronic Diseases

Disturbed sleep or its loss is a common symptom in many of the chronic diseases like cancer, diabetes, hypertension, heart attack, stroke, lung disease, osteoporosis, arthritis, neurological diseases like Parkinson's disease, Alzheimer's disease, psychiatric ailments like schizophrenia, depression, anxiety, etc. (Fiorentino and Ancoli-Israel 2007; Cooper et al. 2018; Koo et al. 2018; Parish 2009; Gagnon et al. 2008). Therefore, we propose that accumulation of loss of sleep could be underlying cause and mother of many acute and chronic psycho-somatic disorders (Fig. 6.1).

## 6.13 Summary and Conclusion

### *6.13.1 Sleep Disruptions as Basis of Many Disorders*

Sleep is an instinct behaviour expressed by all living species, specially, higher in evolution. It has been broadly divided into NREMS and REMS. Both the stages of sleep are necessary to maintain optimum physiological processes and to lead a normal life. However, normally, as some duration of NREMS is necessary for generation of REMS, most of the experimental studies have been conducted by depriving the subjects of REMS, or total sleep. We have discussed above that sleep plays a significant role in maintaining normal physiological processes. As disturbed sleep,

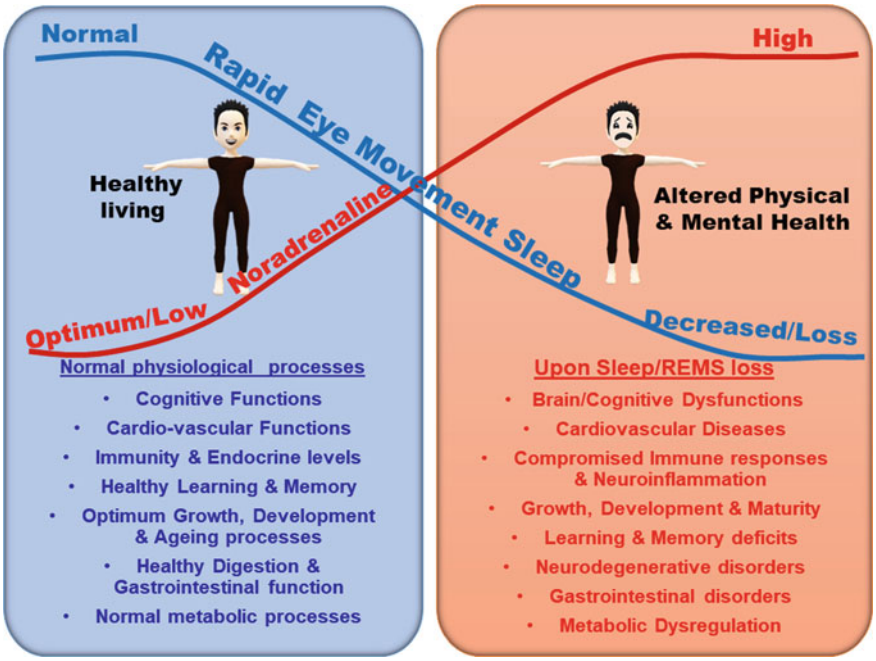


**Fig. 6.1** REMS is an instinct behaviour. An optimal level of REMS maintains the physiological processes at an optimal level leading to healthy living. On the other hand, loss of REMS affects most physiological processes. Effects of REMS loss are mediated by shift in equilibrium in the levels of several biomolecules in the body and the brain; one such molecule being NA. The effective level of NA depends on its synthesis, release and degradation. By and large, low level of NA is beneficial, while its high level is destructive to brain cells. REMS loss causes sustained release of NA and its rise in the brain at least. This elevated level of NA adversely affects the brain and directly or indirectly affects other physiological processes leading to compromised health and diseases

including REMS, affects most physiological processes, we propose that accumulation of factor(s) due to disturbed sleep could be responsible for many diseases. Thus, sleep loss could be the underlying cause of many dysregulations of physiological processes, which may lead to pathological conditions, disorders and diseases. However, expression of a disorder depends on many factors including intensity (chronicity) of sleep loss, recovery from sleep loss, associated comorbidities and pre-dispositions. As ageing is a normal physiological process, one need not necessarily become sick or diseased with ageing. However, in practice, we find most aged persons suffer from many disorders. We propose that due to lifestyle changes through childhood and adulthood living, an instinct behaviour, the sleep is affected, resulting in accumulation of one or more biomolecules, which affect(s) physiological process(es) leading to disorder(s).

### *6.13.2 NA, a Common Factor Responsible for Sleep Loss-Associated Pathophysiology*

Loss of sleep includes REMS loss as well, which is associated with elevated level of NA. Isolated studies have shown that low level of NA exerts protection to neurons and thus, is beneficial to neurons, while elevated level of NA is damaging. As the brain controls most physiological processes directly or indirectly, the sleep loss-associated modulation in NA affects physiological processes. It is also a reality that through ageing, sleep is often compromised due to various psycho-patho-physio-socio-economic related issues and lifestyle changes. We propose that through ageing, sleep loss-associated elevated NA affects neurons in the brain affecting one or more systems and physiological processes, which results in expressions of some common and some not so common symptoms. The complexity of the expression of symptoms depends on one or more of the system(s) being affected, which again depends on the vulnerability of the systems affected. However, with passage of time under chronic sleep (including REMS) loss condition, cascading effects involving many systems get affected to various degrees (Fig. 6.1). Under such condition, it becomes difficult to establish the cause-and-effect relationship of the altered state(s) or disease(s). Finally, to summarize, we propose that growing from child-to-adult-to-old is a normal physiological process, practicing healthy sleep hygiene through life (childhood and adulthood) is likely to allow one ageing gracefully with lesser health related complexities. Therefore, we propose that while diagnosing and recommending treatment for aged people, the caregivers must pay attention more closely to the present and past sleep profile of the patient and accordingly, take remedial action. Therefore, we reiterate the age-old saying in most civilizations that healthy sleep habit and hygiene throughout life is necessary for leading relatively better health and disease-free ageing.

**Compliance with Ethical Standards:** All ethical standards were followed while preparing this chapter.

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# Chapter 7
# Healthy Brain Ageing and Longevity; the Harmony of Natural Products, *APOE* Polymorphism, and Melatonin


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## 7.1 Apolipoprotein E (*APOE*) Polymorphism and Human Longevity

Life expectancy is an outcome of complicated processes that might involve thousands of genes and non-genetic factors (Christensen et al. 2006). To understand the variations in human ageing and lifespan, including exceptionally long lifespan, which is known as longevity, individual genetic differences and the role of specific genetic factors in these differences are central. Human genetic studies have shown consistently that polymorphisms in gene encoding for *APOE* influence lifespan, probably mainly thorough their association with disease (Corder et al. 1996). Several independent genome-wide association scans (GWAS) have further confirmed that *APOE* is the only gene accounted as "longevity determinant" (Garagnani et al. 2014). *APOEε* allele variants have been extensively analyzed, and the frequency of *ε4* allele has been found decreased in long-lived subjects (McKay et al. 2011; Soerensen et al. 2013) but it varies amongst different populations (Lee et al. 2001). *APOEε2* carriers have an estimated average mortality risk in adulthood that is only 4–12% less than *APOE ε3* carriers, and *APOE ε4* carriers have a risk that is only 10–14% more than for *APOE ε3* carriers throughout adulthood (Gerdes et al. 2000). Globally, *APOE* locus shows substantial allelic variation with ranges 0–20% for *ε2*, 60–90% for *ε3*,

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and 10–20% for *ε4* alleles (Corbo and Scacchi 1999; Gerdes et al. 1996) with some exceptions. At the continental level and amongst Asians, Indian populations showed the highest average value of *APOE ε3*, whilst Oceanic populations showed the lowest values.

Various clinical and epidemiological studies have highlighted the functional consequences of the phenotype–genotype relationship of *APOE* and its affiliation with diverse pathological conditions and cognitive traits (Schachter et al. 1994; Corder et al. 1993). Apolipoprotein E (ApoE) is necessary to help remove cholesterol-rich lipoprotein from the circulation. This protein not only plays an important role in lipid metabolism but also participates in other important biological functions, such as immune regulation and neurological pathway regulation (neuron repair and remodelling) (Cao et al. 2020). A search for genetic and molecular basis of ageing has directed to the identification of genes related with the maintenance of cell and of its basic metabolism as the main genetic factors affecting the individual variation of the ageing phenotype (Passarino et al. 2016). Moreover, studies on calorie restriction and on the variability of genes associated with nutrient-sensing signalling have shown that ipocaloric diet and/or a genetically efficient metabolism of nutrients can modulate lifespan by promoting an efficient maintenance of the cell and of the organism (Passarino et al. 2016).

In addition to *APOE*, understanding the contribution of human angiotensin converting enzyme (*ACE*) which is another most studied candidate genes for cardiovascular diseases (CVDs) in longevity is unclear. *ACED*-allele which predisposes to coronary artery disease (CAD) (Zintzaras et al. 2008) has been reported to be more frequent in centenarians and in nonagenarians (Seripa et al. 2006; Wufuer et al. 2004; Rahmutula et al. 2002) compared to younger ethically matched referents. However, controversies are still existed in ethically similar as well as in other population-based studies (Nacmias et al. 2007; Yang et al. 2009).

Healthy ageing is considered as one of the most complex but desirable phenotypes studied to date. Healthy ageing can be defined in various ways, generally with regard to reaching an at least moderately old age in the absence of certain diseases or disabilities, and or in the presence of desirable traits such as intact cognition or mobility (Brooks-Wilson 2013). Mounting evidence indicated that nutritional factors could have an impact on healthy ageing as diets that are rich in natural products that contain high amounts of plant bioactive including polyphenols and antioxidant vitamins which are promising dietary strategies in preventing chronic diseases and ensuring healthy ageing.

### *7.1.1 Population Ageing on Healthy Ageing*

Ageing population is a growing challenge in twenty-first century. Population ageing increases the costs in healthcare services, due to an increase in the utilization of age-related procedures and treatments that ramp-up costs for long-term care, which are expected to grow at faster pace than other healthcare needs (Cristea et al. 2020). The

global population of people aged 60 years and over was 962 million in 2017 equating to 13% of the total population, and this number is projected to 2.1 billion in 2050 and 3.1 billion in 2100 (World Population Prospects 2017). For this age range, 65% of the global increase between 2017 and 2050 will occur in Asia, 14% in Africa, 11% in Latin America and the Caribbean, and the remaining 10% in other areas. South Asia represented 24.7% of the world total population in 2015, and the people aged over 60 years was 154 million. Ageing population is often accompanied by increase in occurrence of diseases, of which dementia is the most prominent, which provide major challenges to family members, society and to the healthcare systems. Alzheimer's disease (AD) is the most common form of dementia and possibly contributes 60–70% (World Health Organization 2012). However, clinical prevalence of dementia is often underestimated in developing countries or it has not been studied extensively. Clinical prevalence of dementia was 3.98% in elderly Sri Lankans (De Silva et al. 2003), ranged from 0 to 10.6% in elderly Indians (Ravindranath and Sundarakumar 2021) and 3·6% in elderly Bangladeshians (Palmer et al. 2014), whereas it has not been reported in other South Asian countries. In our previous post-mortem brain study, we screened a total of 79 elderly brains with incomplete clinical histories obtained from two genetically and culturally related South Asian sample populations and reported that the neuropathologic changes for AD are comparable between Colombo, Sri Lanka (4.25%), and Bangalore, India (3.12%) elderly samples (Wijesinghe et al. 2016a), whereas the pathologies associated with Parkinsonism (8.5%) were found only in Colombo samples. Here, we suggested that documentation of substantial heterogeneity in dementia prevalence amongst different countries/ethnics needs to be investigated on the basis of genetic, environmental, cultural factors, and preventive approaches in reducing the burden of dementia.

### *7.1.2* APOE *Polymorphism and AD*

Genetic risk factors for AD have been studied extensively for both basic types, familial and sporadic/late onset. *APOE* is the strongest genetic risk factor associated with late onset AD, but it is far from explaining all occurrences of the disease. In late onset families, risk of AD has been increased from 20 to 90%, and the mean age of onset has been decreased with an increasing number of *APOE ε4* alleles (Corder et al. 1993). A single copy of the *APOEε4* allele triples the AD risk, whilst homozygotes have 15 times higher risk of developing AD in comparison with non-carriers (Farrer et al. 1997).

Observational studies have shown that *APOE* genotype modifies the associations between vascular risk factors and AD, so that the associations of risk factors are stronger amongst the *APOE ε4* carriers (Rönnemaa et al. 2011; Mielke et al. 2011). In general, amyloid beta (Aβ) associates with lipoproteins specifically ApoE to enable their transport and clearance (LaDu et al. 1994). LaDu and colleagues (1994) demonstrated in their study that lipoproteins ApoE2 and ApoE3 form stable complexes with Aβ (at levels 20-fold greater than that occurring with ApoE4), and so they prevent the

neurotoxic effects of Aβ by the uptake of these complexes via ApoE receptors. Moreover, Roses et al. (1996) reported that *APOE ε3* alleles (and *ε2*) prevent paired helical filaments (PHFs) formation by interacting with the microtubule binding domain of tau. However, associations reported between *APOE ε4* allele and NFTs pathologies are inconsistent (Morris et al. 2010; Kok et al. 2009). In our previous post-mortem brain study (Wijesinghe et al. 2016b), decedents with 1 or 2 *APOE ε4* allele demonstrated a significant positive association with Aβ stages and in contrast, a significant negative association with NFT stages controlling for age and sex. This observation could be related to *APOE ε4* allele frequency amongst the decedents, as it showed an age associated *APOE ε4* allelic variation that is presumed to be due to survival effect of *APOE ε4* allele carriers (Jicha et al. 2008). Frequency of the *APOE ε4* allele is an important genetic risk factor for explaining ethnic differences (Verghese et al. 2011). We recommended that *APOE* genotypes and their survival probabilities in different ethnic populations could possibly be a one of reasons for the differences observed in AD prevalence and needs to be confirmed through large-scale pathogenetic studies, across a large range of ethnicities.

Other than AD, *APOE ε4* allele is a well-known risk factor in the pathogenesis of atherosclerosis (Corder et al. 1993; Roher et al. 2003). *APOE ε4* allele has been associated repeatedly with increased risk of both cardiovascular disease and AD, whereas *APOE ε2* allele is protective (Panza et al. 2004; Bathum et al. 2006). In our previous work (Wijesinghe et al. 2020), we reported the association between posterior and anterior circulation of circle of Willis, atherosclerosis and the frequency of *APOE ε3/ε4* and *ε3/ε2* genotypes in an elderly Sri Lankan population. There we suggested that a population with predominant posterior circulation atherosclerotic stroke might be a result of increased frequency of *APOE ε3/ε4* genotypes amongst them. Similarly, a population with fewer anterior circulation atherosclerotic strokes might reflect more frequent *APOE ε3/ε2* genotypes amongst them. Although the mechanism is not clear, it provides some directions for the differences in prevalence of posterior and anterior circulation atherosclerotic stroke which have been discussed in population-based studies (Lee et al. 2006).

## 7.2 Melatonin in Circadian Rhythms and Healthy Ageing

Circadian rhythm is a natural process that occurs in approximate 24-h patterns in each day. The sleep-wake cycle is one of the most widely recognized circadian rhythms. Proper sleep allows the body to engage in circadian rhythms in the body, which initiates the build-up of energy stores for metabolic processes, neuronal remodelling for synaptic function, memory consolidation, and the assimilation of complex motor systems (Reddy et al. 2022). Studies have shown that one-third of the general population is suffering from sleep disorder (named insomnia), and there is an increasing trend because of the more stressful working conditions and the progressive ageing (Miyamoto 2009). Insomnia, characterized by poor sleep quality and in sufficient

quantity of sleep, is linked with impaired daytime functioning, physical health problems, anxiety, depression and fatigue, higher cardiovascular risk, and poor quality of life (Zeitlhofer et al. 2000; Hoevenaar-Blom et al. 2011).

Melatonin (*N*-acetyl-5-methoxy-tryptamine) is a neurohormone, and signalling molecule identified in plants in 1995 (Dubbels et al. 1995; Hattori et al. 1995). Melatonin is mainly secreted by the pineal gland in mammals, but it may also be produced by non-pineal cells like retina, bone marrow, and gut. In humans, melatonin secretion is generally decreased with increasing age. Melatonin synthesis and secretion are controlled by light/dark cycles where the production decreases during daytime and increases at night. Melatonin is an important physiological sleep regulator in diurnal species including humans where circadian melatonin rhythm is closely associated with the sleep rhythm in both normal and blind subjects (Zisapel 2001). Considering the role of sleep in memory consolidation, it is not surprising that insufficient sleep can reduce cognitive ability including attention and memory (Zisapel 2018). Mechanisms linking circadian clocks, sleep, and neurodegeneration have been demonstrated (Musiek and Holtzman 2016). Sleep disruption and or increased wakefulness may supress the function of glymphatic system that could result in decreased clearance of pathogenic proteins such as Aβ, which may lead to Aβ accumulation and the development of the symptoms of AD (Musiek and Holtzman 2016).

### *7.2.1 Melatonin in AD*

Melatonin has various physiological functions in the brain, including regulating circadian rhythms, clearing free radicals, inhibiting biomolecular oxidation, and suppressing neuroinflammation and has a wide range of neuroprotective roles by regulating pathophysiological mechanisms and signalling pathways (Chen et al. 2020). Melatonin levels in the serum and cerebrospinal fluid (CSF) are lower in AD patients than those in age-matched control subjects (Wu and Swaab 2005) which could be resulted via the defects in melatonin receptor expressions in AD patients (Savaskan et al. 2007). Intracellularly, melatonin is targeted to mitochondria where it provides potent antioxidant protection, and its deficiency may result in reduced antioxidant protection in elderly individuals in which mitochondrial dysfunction may contribute to the incidence or severity of neurodegenerative diseases, such as AD (Paradies et al. 2017; Wongprayoon and Govitrapong 2017). Increased brain melatonin concentrations could lead to a reduction in Aβ, which may retard neurogenerative changes in AD (Lahiri et al. 2004a, b). Melatonin has been reported to inhibit Aβ production and aggregation both in vivo and in vitro (Wang et al. 2008; Chinchalongporn et al. 2018). In addition, combinations of Aβ and ApoE4 synergistically aggravate Aβ neurotoxicity, which can be prevented by melatonin through interactions with ApoE4 (Poeggeler et al. 2001). Melatonin supplementation is suggested to reverse the synaptic dysfunction and cognitive impairment via epigenetic regulations (Lahiri et al. 2004a, b; Wang et al. 2013). In the Aβ1-42-treated mouse model of AD, melatonin treatment ameliorated the Aβ1-42-induced neurotoxicity,

attenuated memory impairment and tau hyperphosphorylation, reversed synaptic disorder, and reduced the apoptosis and neurodegeneration via PI3K/Akt/GSK3β signalling pathway (Ali and Kim 2015). Above studies highlight that melatonin could be an effective, promising, and safe neuroprotective candidate for the treatment of progressive neurodegenerative disorders, such as AD.

### *7.2.2 Melatonin Via Plant-Based Diet*

Melatonin is now well recognized as a universal amphiphilic antioxidant molecule that, due to its small size and strong solubility in both water and lipids, can infiltrate all compartments of a cell. In lieu of synthetic melatonin, which may contain various by-products, the idea of extracting this bioactive chemical from natural plants for use in dietary supplements is intriguing (Arnao et al. 2018). Melatonin was found in concentrations ranging from a few to several thousand nanograms per gram of tissue in 108 herbs species typically used in Chinese medicine (Chen et al. 2003), indicating that they are good natural sources of this molecule. Melatonin has been found in roots, shoots, leaves, flowers, fruits, and seeds, but the highest concentrations have been found particularly in seeds. This abundance is likely due to the importance of reproductive organs in plant life, as well as their requirement to adequately defend them against numerous environmental challenges, such as secondary oxidative stress (Kołodziejczyk et al. 2015). Moreover, the melatonin concentration in the plant food products is also related with the environment, in which the plants are cultivated, including the temperature, duration of sunlight exposure, ripening process, agrochemical treatment, etc. (Wang et al. 2016).

Exogenous melatonin is well absorbed, broadly distributed, and nearly totally metabolized in humans after oral treatment. Melatonin receptors are abundant in the brain, and easily crosses the blood–brain barrier. Melatonin is easily absorbed into circulation when ingested as a drink or as a galenic tablet (Lee et al. 1996). Meng et al. (2017) suggest that the intake of melatonin containing foods could significantly increase the melatonin concentration in human serum, indicating melatonin could provide beneficial effects on health through foods. In addition, Meng et al. (2017) recommend more clinical trials that are necessary to clarify the effects of food-based melatonin on human beings.

## 7.3 Antioxidant Properties of Tea

Black tea represents approximately 72% of total consumed tea in the world, whereas green tea accounts for approximately 26% (Katiyar and Mukhtar 1996). Black tea mostly comes from plantations in Africa, India, Sri Lanka, and Indonesia, whilst green tea comes from countries in the far East such as China and Japan. The nutrition

**Table 7.1** Mean nutrient composition in percentage in green and black tea (Belitz and Grosh 1997)

| Compound | Green tea | Black tea |
|---|---|---|
| Proteins | 15 | 15 |
| Amino acids | 4 | 4 |
| Fibre | 26 | 26 |
| Other carbohydrates | 7 | 7 |
| Lipids | 7 | 7 |
| Pigments | 2 | 2 |
| Minerals | 5 | 5 |
| Phenolic compounds | 30 | 5 |
| Oxidized phenolic compounds | 0 | 25 |

composition between green and black tea is same, except phenolic compounds (Table 7.1), which depend on the processing methods.

The production of green tea is characterized by an initial heating process, which kills the enzyme polyphenol oxidase, which is responsible for the conversion of flavonoids in the leaf into the dark polyphenolic compounds found in black tea. The polyphenols constitute the most interesting group of green tea leaf components, and in consequence, green tea can be considered as an important dietary source of polyphenols, particularly flavonoids. It includes the four major catechins (flavan-3-ols) are epigallocatechin-3-gallate (EGCG) 59%, epigallocatechin (EGC) 19%, epicatechin-3-gallate (ECG) 13.6%, and epicatechin (EC) 6.4% (McKay and Blumberg 2002). In black tea, the polymerized catechins such as theaflavins and thearubigins predominate. Black and green teas both contain similar amount of flavonoids, however, they differ in their chemical structure; green tea contains more catechins (simple flavonoids), whilst the oxidation undergone by the leaves in order to make black tea converts these simple flavonoids into the aflavins and thearubigins (Fig. 7.1) (McKay and Blumberg 2002).

A graphical representation of the total catechins between green and black teas estimated from commercially available teas of different geographical regions; China, Japan, Kenya, Sri Lanka, and India are given in Fig. 7.2 (Cabrera et al. 2003).

Green tea is considered as a major dietary source of antioxidants; rich in polyphenols (catechins particularly) but it also contains carotenoids, tocopherols, ascorbic acid (vitamin C), minerals such as Cr, Mn, Se, or Zn, and certain phytochemical compounds. These compounds could increase the green tea polyphenol's antioxidant potential. It is antioxidant activity exhibited by scavenging reactive oxygen and nitrogen species and chelating redox active transition metal ions (McKay and Blumberg 2002; Kim et al. 2003 and Skrzydlewska et al. 2002).

The majority of the research demonstrating the antioxidant activity of tea flavonoids was either on animal models or laboratory cellular studies (Warden et al. 2001; Shahrzad et al. 2001; Leenen et al. 2000; Yang et al. 1999; van het Hof et al. 1998). Increasing numbers of human studies are now concluding that the body does in fact absorb some of these antioxidants. As well as being absorbed these flavonoids

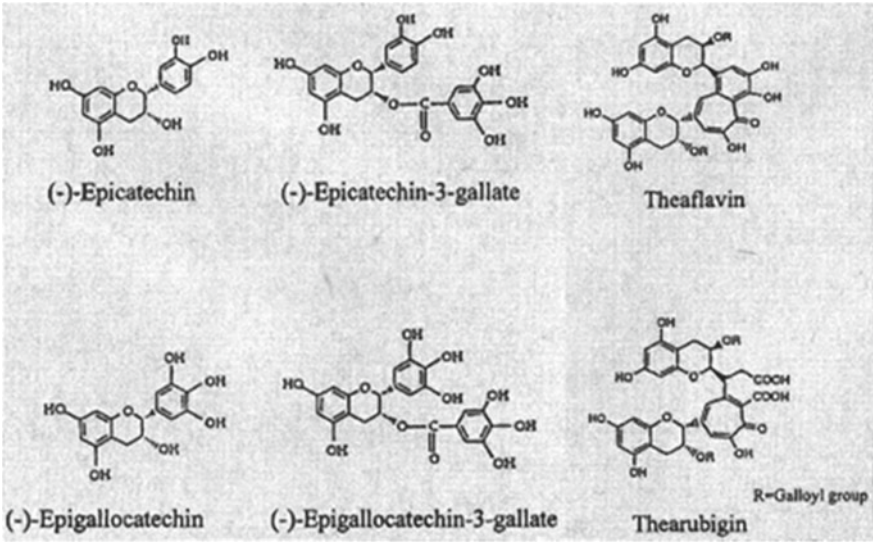


**Fig. 7.1** Major flavonoids in tea

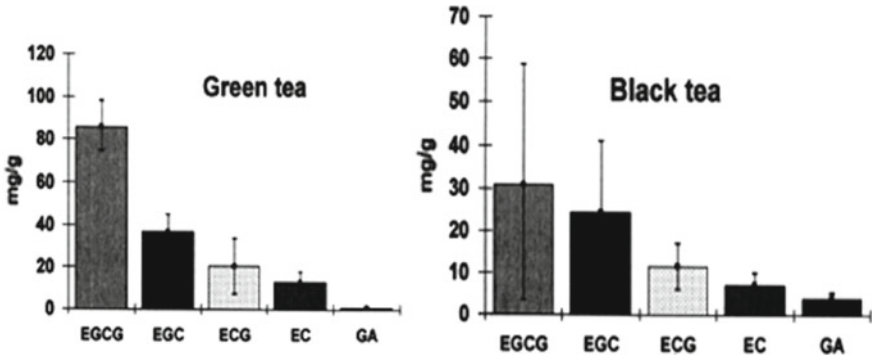


**Fig. 7.2** Mean content of total catechins and gallic acid of green and black teas (dry weight data; Cabrera et al. 2003)

demonstrate antioxidative potential in vivo. A number of studies have shown that plasma antioxidant activity peaks 30–60 min after moderate tea consumption (1–6 cups). There is some controversy about which tea has higher antioxidant potential. Although the oxidization process modifies the type of flavonoids present, the total level and their overall antioxidant activity are similar in both teas. It was proved in some studies where they found that whilst green tea was 6 times more potent in inhibiting lipid peroxidation in vitro, when healthy human subjects ingested the same amount of either black or green tea, the plasma antioxidant capacity (expressed as TRAP, or total radical-trapping antioxidant parameter) was similar in both groups

(Łuczaj and Skrzydlewska 2005; Leung et al. 2001). This finding has led to the suggestion that the theaflavins and thearubigens in black tea also have antioxidative potential (Stewart et al. 2005). The addition of milk to tea, as enjoyed by the majority, does not appear to affect the bioavailability or antioxidant activity of the tea flavonoids (Reddy et al. 2005; Hollman et al. 2001; Leenen et al. 2000; van het Hof et al. 1998).

## 7.4 Protective Effect of Tea in AD Leading Towards Healthy Ageing

Accumulating evidence suggests that oxidative stress resulting from reactive oxygen species generation and inflammation play a pivotal role in neurodegenerative diseases, supporting the implementation of radical scavengers, chelators, and nonvitamin natural antioxidant polyphenols in the clinics. Oxidative damage is a hallmark of the aged brain and is especially elevated in the brains of AD patients. Oxidative stress and/or metabolic problems adversely affect neuronal function in AD patients. Some studies also found that people who develop AD consume fewer fruits and vegetables and fewer antioxidant nutrients (Singh et al. 2008). As a consequence, tea and tea polyphenols (which include catechins and their derivatives) particularly those from green tea are now being considered as therapeutic agents in well controlled epidemiological studies, aimed to alter brain ageing processes and to serve as possible neuroprotective agents that can help to ameliorate neurodegenerative diseases such as AD and Parkinson's disease (PD) (Avramovich-Tirosh et al. 2007; Weinreb et al. 2004; Mandel and Youdim 2004). Green tea catechins, formerly thought to be simple radical scavengers, are now considered to invoke a spectrum of cellular mechanisms related to neuroprotective as well as neurorescue activities (Reznichenko et al. 2005). As the secondary and tertiary structure determined the function of the protein, a misfolding caused by metabolic glycation, amination following oxidative stress and release of free radicals, phytochemicals in the beverages and food can modulate this detrimental biochemical event. Amyloid plaque containing misfolded Aβ protein is commonly seen in the brains of AD and appears to disrupt the function of cells. Some of the fragments of Aβ protein is found to be cytotoxic. Strategies to prevent the development of amyloid plaque are one of the avenues being explored in the prevention and treatment of AD (Ringman et al. 2005; Yang et al. 2005). Furthermore, Rezai-Zadeh et al. (2008) demonstrated that the tea antioxidant EGCG has potent anti-plaque ability and seems to change potentially harmful proteins into proteins that are not detrimental to brain cells. Both green tea and black tea have potent antioxidant properties and play a pivotal role in retarding age-related changes (Okello et al. 2004). Okello et al. (2004) observed in their study that both green and black tea inhibited the activity of enzymes associated with the development of AD, but coffee had no significant effect. Both teas retard the activity of the enzyme

*acetylcholinesterase*, which breaks down the neurotransmitter, acetylcholine, essential for cognition and memory. AD is characterized by cholinergic depletion in the critical areas in the brain, leading to clinical manifestation. Green tea and black tea also hinder the activity of the enzyme *butyrylcholinesterase*, which has been found in protein deposits in the brain of patients with AD. Furthermore, green tea has been shown to counter the activity of *β-secretase*, which also plays a role in the production of amyloid protein in AD pathogenesis. The major caveat is the very poor absorption and delivery of EGCG seen in some studies (Cabrera et al. 2003).

Countries like India and Sri Lanka tend to retain traditional herbal medical practices in their daily life and thus offer a valuable resource for new anti-dementia therapies (Perry 2007). Several species of medicinal plants have activities *in vitro* or *in vivo* those are relevant to dementia, e.g.: anticholinesterase, anti-amyloid, antioxidant, anti-inflammatory, neuroprotective, and memory enhancing (Burgener et al. 2008; Ramassamy 2006; Martin et al. 2002a, b). However, the usefulness of such a resource relies on documented evidence of the effects.

## 7.5 Therapeutic Potential of Cinnamon on AD

Cinnamon (*Cinnamomum zeylanicum* and Cinnamon cassia) is well-known for its nutritional and pharmacological benefits, which are mostly attributable to the polyphenolic content and volatile essential oils extracted from various portions of the plant (bark, leaves, flowers, or buds) (Ooi et al. 2006). Cinnamon is made up primarily of essential oils and compounds including cinnamaldehyde, cinnamic acid, and cinnamate (Rao and Gan 2014). Cinnamon, in addition to its anti-inflammatory, anti-diabetic, and anti-cancer characteristics, has considerable brain protective and pro-cognitive effects in multiple neurodegenerative models including Alzheimer's disease (Rao and Gan 2014). In vitro research suggests that the essential oils of Cinnamomum species, particularly cinnamaldehyde and sodium benzoate, may protect against oxidative stress-induced cell death, reactive oxygen species production, and autophagy dysregulation, implying that they may have neuroprotective properties (Rao and Gan 2014). In AD, oxidative impairment is generally derived from mitochondrial dysfunction and microglial activation (the formation of ROS and NOS), thereby provoking calcium overload and excitotoxicity and eventually leading to neuronal apoptosis (Cenini et al. 2019). Cinnamon exerts its neuroprotective effects by interfering with oxidative stress, calcium overload, proinflammatory pathways, and tau aggregation and has been shown to ameliorate Alzheimer's disease progression in in vitro and in vivo models (Momtaz et al. 2018). Cinnamon inhibited the formation, accumulation, and toxic effects of A$\beta$ plaques in PC12 neuronal cells. PC12 cell viability was reported about 100% after administration of cinnamon extract. Cinnamon has shown to improve those factors which are associated with AD and cognitive impairment through blocking tau formation and inhibiting aggregation of amyloid precursor protein (Frydman-Marom et al. 2011). extract of *C. zeylanicum* inhibited human tau accumulation induced dissociation of tau

tangles and unravelled paired helical filaments in the AD mouse brain. A$\beta$-type doubly linked procyanidin oligomers and cinnamaldehyde were responsible for such inhibitory activity (Peterson et al. 2009).

## 7.6 *APOE* and *ACE* Polymorphism in Human Longevity and the Protective Effect of Black Tea on AD-Related Neuropathologic Changes: A Proof of Concept

Total content of antioxidants in Ceylon green and black teas was 190.0 mg/g and 186.6 mg/g, respectively (Yashin et al. 2010). Notably, tea catechins showed potent anti-plaque activity and altered potentially harmful proteins into proteins that are not detrimental to brain cells (Yashin et al. 2011). Interestingly, Yashin et al. (2011) reported that Ceylon black tea demonstrates greatest antioxidant activity compared with other world black tea products. As tea is main beverage in Sri Lanka, it is worthy investigating the association between AD-related neuropathologic changes and black tea consumption pattern in elderly Sri Lankan brains as a proof of concept.

### *7.6.1 Sample Collection*

Consecutive human brain samples were obtained from 76 elders (age range = 50–89 yrs, mean age ±S.D. = 67 · 3 yrs ±0 · 0, median age = 65 · 5 yrs, male: female = 52:24, mean post-mortem interval ±S.D. = 17 · 3 h ±14 · 2) between May 2009 and March 2010, in the Department of Judicial Medical Office, Colombo South Teaching Hospital following approval by the Institutional Scientific Ethics Committee to carry out the study and informed consent from the kin to utilize the material for research. An ante-mortem questionnaire was given to kin who were familiar with intellectual and motor functional status of the subjects before death. The purpose of this questionnaire was to obtain information on demographic data, past medical history, family history, health habits, and consumption pattern of the deceased. This information was held strictly confidential. All the recruited cases had incomplete clinical history except three cases which were clinically diagnosed of Parkinson's disease.

In addition, cadaver blood/clotted blood was collected at autopsy from all the recruited cases. Cadaver blood clots were then sliced into small pieces and washed in 1ml of saline (9g/L NaCl) and suspended. The mixture was subjected to series of centrifugations and digestions of nucleated leukocytes with lysis buffers. Finally, genomic DNA was extracted and purified using phenol/chiasm method. Genes encoding for *APOE* and *ACE* was genotyped using standard protocols with polymerase chain reaction (PCR)-based restriction fragment length polymorphisms (RFLP).

### 7.6.2 Screening of AD-Related Neuropathologic Changes Using Histopathological and Immunohistochemical Techniques

For this purpose, brain samples from 50 out of 76 elderly decedents ($\geq 60$ years; mean age $72 \cdot 1$ years $\pm 7 \cdot 8$, mean $\pm$S.D., male: female = 29:21) from both hemispheres including hippocampus along with parahippocampal gyrus, superior frontal gyrus, middle temporal gyrus, superior parietal lobule, and midbrain at superior colliculus level were used for paraffin embedding and sectioning. Following routine histological evaluation [Haematoxylin and Eosin (H&E) staining], brain sections (4 μm thick) were immunostained blindly to the case histories by standard immunoperoxidase technique following antigen retrieval by heat and DAB/$H_2O_2$ as the chromogen to visualize the immunolabelling (DAKO Envision Detection System). For this screening, the following three antibodies were used.

(a) Beta amyloid–monoclonal antibody (1:200 dilution) from Novacastra™
(b) Ubiquitin–monoclonal antibody (1:150 dilution) from Novacastra™
(c) Phosphorylated tau–PHF−1 monoclonal antibody (1:50 dilution) (Gift)

The diagnostic criteria for AD neuropathologic changes and Lewy body diseases were based on National Institute on Ageing-Alzheimer's Association guidelines—a practical approach (NIA-AA) (Montine et al. 2012). Actual burden of AD related-changes [neurofibrillary tangles (NFTs), NPs, and SPs] was counted in specific brain regions such as hippocampus and parahippocampus, superior frontal gyrus, and midbrain based on the methods described by Purohit and colleagues (2011). For this purpose, a medium high power (20×) objective lens producing a visual field of 0.785 $mm^2$ (field diameter = 2.0 mm) were used. Lesions were counted in medium high (200× magnification, Olympus U-CTR30-2 Trinocular objective tubes and 10× eye piece) power fields and then converted into average per 200× as follows: for superior frontal gyrus, areas with high NFTs/NPs/SPs were selected, and the visual counts were carried out in five non overlapping fields. For other regions, areas with high NFTs/ NPs/ SPs were identified in each sub fields, and then, visual counts were carried out in non-overlapping fields (wherever possible five non-overlapping fields were selected). In addition, β-amyloid positive extent of cerebral amyloid angiopathy (CAA) in leptomeningeal and cortical arteries of the specific neuroanatomical regions was also assessed based on Greenberg and Vonsattel (1997) specifications, and the average CAA grade was recorded for each case.

### 7.6.3 Statistical Analysis

All the analyzes were carried out using statistical software SPSS version 25.0 (IBM Corp. Armonk, New York). Fisher's exact test (2 × 2 contingency table) was used to determine the degree of association between *APOE* and *ACE* allelic frequency

and life expectancy. Relationship between black tea consumption pattern (≤ 2–3 cups/day and > 2–3 cups/day) and AD-related pathologies was assessed for square root value of NFTs, SPs, and CAA scores using two sample independent *t*-test.

### 7.6.4 Major Findings

Our study consisted of 76 elderly decedents aged between 50 and 89 years (mean age 67 · 3 years ± 10 · 0, mean ± S.D., male: female = 52:24). Polymorphisms in genes encoding for *APOE* and *ACE* are summarized in Table 7.2. Frequency of *APOE* ε4 allele was high in young decedents who died between 50 and 69 years (57.9%) compared with old decedents who died at the age of 70 and above (42.1%), however, it was not statistically significant ($p = 0.792$). Whereas frequency of *ACE DD* genotype was high in old decedents (65.2%) compared with young decedents (34.8%), and it was statistically significant with Fisher's exact test ($p = 0.041$). Notably, frequency of *ACE ID* genotype was significantly low ($p = 0.004$) in old decedents (24.1%) compared with young decedents (75.9%). Figure 7.3 illustrates the frequency of *APOE ε4* allele, *ACE ID,* and *DD* genotypes amongst the elderly decedents representing a semi urban Sri Lanka population. In this study, gender differences did not show significant associations with life expectancy ($p = 0.623$).

Table 7.3 summarizes the association between AD-related neuropathologic changes and black tea consumption pattern (≤ 2–3 cups/day referred as "light tea drinkers" and > 2–3 cups/day referred as "frequent tea drinkers"). Mean counts of both NFTs and SPs were relatively high in light tea drinkers compared with frequent tea drinkers. Particularly, mean SPs counts were significantly low in the brain regions of entorhinal cortex ($p = 0.009$) and superior frontal gyrus ($p = 0.041$)

**Table 7.2** Polymorphisms of longevity associated genes *APOE* and *ACE* in an elderly Sri Lankan population

| Gene | Genotype | Number of individuals | Allele frequency |
|---|---|---|---|
| *APOE* | *ε3/ε3* | 47 | *ε3*−0.799<br>*ε4*−0.146<br>*ε2*−0.055 |
| | *ε3/ε4* | 16 | |
| | *ε3/ε2* | 05 | |
| | *ε2/ε2* | 01 | |
| | *ε2/ε4* | 01 | |
| | *ε4/ε4* | 02 | |
| *ACE* | Insertion (II) | 20 | *I*−0.479<br>*D*−0.521 |
| | Insertion/deletion (ID) | 29 | |
| | Deletion (DD) | 23 | |

(Missing data $n = 4$)
*APOE* apolipoprotein E; *ACE* angiotensin converting enzyme

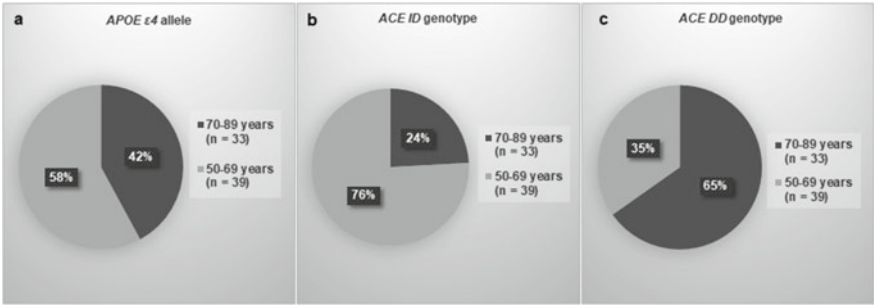


**Fig. 7.3** Frequency of **a** *APOE ε4* allele, **b** *ACE ID*, and **c** *DD* genotypes amongst the decedents who died at the age ranges of 50–69 years and 70–89 years. *APOE*, apolipoprotein E; *ACE*, angiotensin converting enzyme; *n*, sample size

in frequent tea drinkers. In addition, the average CAA grade obtained for cortical and leptomeningeal arteries was also significantly low in frequent tea drinkers ($p = 0.037$) compared with light tea drinkers. As these pathologies are age dependent, mean age at death between light and frequent tea drinkers was also analyzed, and it was statistically nonsignificant ($p = 0.173$) between the groups.

## 7.7 Future Perspectives

Population ageing is having a profound impact on the emergence of the dementia epidemic. Recent reviews estimate that globally nearly 9.9 million people develop dementia each year; this figure translates into one new cases every three seconds. About 60% of people with dementia currently live in low- and middle-income countries, and most new cases (71%) are expected to occur in those countries (Prince et al. 2013). The estimated worldwide annual cost for the society of dementia was US$818 billion in 2015, an increase of 35% since 2010, and the 86% of the total cost, incurred in high income countries and the rest in low- and middle-income countries (Wimo et al. 2017).

Frequency of *APOE ε4* allele is high in Sri Lankans compared to the general frequency in Asians (14.6 vs. 9.0%) (Wijesinghe et al. 2016b; Singh et al. 2006). A recent meta-analysis study (Sebastiani et al. 2019) shows that *ε4* is associated with a substantially decreased odds for extreme longevity and increased risk for death that persists even beyond ages reached by less than 1% of the population. Sebastiani et al. (2019) also show that carrying *ε2ε2* or *ε2ε3* genotype is associated with significantly increased odds to reach extreme longevity, with decreased risk for death compared with carrying the genotype *ε3ε3* but with only a modest reduction in risk for death beyond an age reached by less than 1% of the population. Though it is statistically not sought, in our study, 42% of the old decedents aged 70 years and above had at least one *APOE ε4* allele which shows the general tendency that decreased frequency

**Table 7.3** Relationship between Alzheimer's disease-related neuropathologic changes and black tea consumption pattern

| Regions | Lesion | Light tea drinkers (≤ 2–3 cups/day) mean counts/mm$^2$ (S.E.) | Frequent tea drinkers (> 2–3 cups/day) mean counts/mm$^2$ (S.E.) | Two sample independent *t*-test (*P* value) |
|---|---|---|---|---|
| Hippocampus | NFTs | 4.69 (0.85) | 3.22 (0.75) | 0.279 |
| | SPs | 0.44 (0.18) | 0.10 (0.10) | 0.119 |
| Entorhinal cortex | NFTs | 2.78 (0.45) | 2.66 (0.81) | 0.887 |
| | SPs | 0.64 (0.21) | 0.04 (0.04) | 0.009** |
| Superior frontal gyrus | NFTs | 0.24 (0.08) | 0.10 (0.07) | 0.303 |
| | SPs | 0.60 (0.20) | 0.11 (0.11) | 0.041* |
| Midbrain | NFTs | 1.71 (0.31) | 1.30 (0.36) | 0.431 |
| | SPs | 0.14 (0.12) | 0.05 (0.05) | 0.588 |
| All regions | NFTs | 6.27 (0.88) | 5.02 (0.92) | 0.383 |
| | SPs | 1.17 (0.35) | 0.16 (0.16) | 0.014* |
| Cortical and leptomeningeal arteries | CAA score | 0.45 (0.15) | 0.08 (0.08) | 0.037* |

Both NFTs and SPs counts and CAA score were converted into square root values and then analyzed using two sample independent *t*-test. Significant levels were set at $^{**}P \leq 0.01$ and $^{*}P \leq 0.05$ (≤ 2−3 cups/day referred as light tea drinkers and > 2−3 cups/day referred as frequent tea drinkers) SE, standard error; NFTs, neurofibrillary tangles; SPs, senile plaques; CAA, cerebral amyloid angiopathy

of *APOE ε4* allele in long-lived elderly Sri Lankans. Whereas 58% of the young decedents aged below 70 years had at least one *APOE ε4* allele which might confer their short life expectancy. On the other hand, a meta-analysis study of Garatachea et al. (2013) reported that the *ACE D*-allele and the *DD* genotype might confer a modest, albeit significant advantage to reach exceptional longevity. CVD is the leading cause of morbidity and mortality amongst the Asian populations. *ACE* has received much attention in the recent years as a candidate gene for hypertension, CVD, and type 2 diabetes. Bhatti et al. (2017) recently investigated the association of *ACE I/D* polymorphism with CVD in north Indian population, and no significant difference was observed in the distribution of *ID* genotypes between CAD patients and control subjects, whereas *DD* genotypes were significantly distributed in CAD patients. As of most literatures, in our study, *ACE* polymorphism showed a consistent association with longevity where 65% of *ACE DD* genotypes were identified in old decedents aged 70 years and above, and 76% of *ACE ID* genotypes was found in young decedents aged below 70 years where most of the death causes were associated with CVD. Thus, *ACE ID* polymorphism shows discrepancy between the South Asian study populations with respect to incidence of CAD. Therefore, we recommend investigating the association between *ACE* polymorphism and the incidence of CVD via a large-scale Sri Lankan population-based studies.

Observational studies have suggested that lower risk of dementia in some developing countries can be attributed to their type of diet (Luchsinger et al. 2007). There have been several studies highlighted that oxidative stress via generating free radicals plays a pivotal role in neurodegenerative diseases and that can be reduced by diets that are rich in antioxidants such as fruits, vegetables, and tea (Martin et al. 2002a, b; Mandel and Youdim 2004; Rezai-Zadeh et al. 2008; Okello et al. 2004). Studies confirming the high antioxidant potential of tea beverages claim that it originates from the considerable content of catechins, a type of phenolic compound with beneficial effects on human health (Kochman et al. 2020). Its health-promoting properties are attributed to the high content of antioxidant and anti-inflammatory substances. Although polyphenol content profiles vary between tea types due to the extent of oxidation, similar potential antioxidant activity is observed. For example, green tea is minimally oxidized and contains high levels of catechins (flavanols and flavanol gallates). In comparison, black tea is fully oxidized, and through enzymatic processes, catechin content declines and complex flavanols such as theaflavins and thearubgins are formed. Catechins and theaflavins have been shown to have similar antioxidant potential (Łuczaj and Skrzydlewska 2005; Cleverdon et al. 2018). Moreover, India and Sri Lanka are the two largest black tea exporters in the world, and their annual per capita tea consumption, respectively, is 0.64–0.66 kg and 0.9–1.29 kg (Pandy and Chadha 1993), respectively. In this proof of concept, a significant reduction in Aβ accumulations in the regions of entorhinal cortex, frontal gyrus, and in the cortical and leptomeningeal arteries was noted in decedents who had consumed black tea frequently (> 2–3 cups/day) compared to the decedents who had consumed lightly ($\leq 2-3$ cups/day). This observation supports our concept that protective effect of black tea consumption against neuropathologic change associated with AD. In sum, our findings open a new biologic mean to investigate in-depth scientific and large-scale observational studies in relation to neuroprotective role of Ceylon black tea in the future.

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# Chapter 8
# Role of Sleep in Imprinting Healthy Aging


**Kamalesh K. Gulia and Velayudhan Mohan Kumar**

## 8.1 Introduction

In this article, the role of sleep in imprinting the healthy aging is discussed, based on the recent evidences both from the pre-clinical studies and well as the epidemiological data. The literal dictionary meaning of imprinting is a rapid learning that occurs during a brief receptive period, typically soon after birth, and establishes a long-lasting behavioral response. But in the animal kingdom where gestation window is fairly long like in humans, the prenatal imprinting is important. Recent studies have shown that in the current lifestyle of 24 × 7, sleep during conception is emerging as a robust prenatal factor affecting the pregnancy outcomes (Okun et al. 2009; Chang et al. 2010; Palagini et al. 2014; Gulia et al. 2014, 2015; Zhao et al. 2014; Radhakrishnan et al. 2015; Peng et al. 2016; Gulia and Kumar 2018a, b; Aswathy et al. 2018a, b; Gulia et al. 2021; Pires et al. 2021). Prenatal stress was documented as one of the reasons for altered sexual orientation, based on the studies of births that occurred during World War II (Ellis et al. 1988; Ellis and Cole-Harding 2001; Gulia and Mallick 2010). Extreme stresses during the war would have also contributed to sleep loss during pregnancy, but this aspect was not studied in these reports. Recent reports that LGBT community experience longer sleep latency and shorter sleep (Galinsky et al. 2018; Caceres et al. 2019; Butler et al. 2020) also need further investigation. It is essential to understand the process of sleep, in order to focus the studies on healthy aging (Gulia 2012; Gulia and Kumar 2018c).

The altered physiology of the cardiovascular, respiratory, and gastrointestinal systems during the various components of sleep–wake cycle forms the basis of good

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health (Douglas et al. 1982; Nagai et al. 2010; Xie 2012; Buchanan 2013; Khanijow et al. 2015; Grandner et al. 2016; Makarem et al. 2019; Taillard et al. 2021; Singh et al. 2022). Several functions are performed during sleep like adaptive immobilization, brain maturation (early development), synaptic plasticity, memory consolidation, discharge of emotions, cognition, learning, restoration of glymphatic system, energy balance, etc., (Stickgold 2005; Tononi and Cirelli 2006; Alhola and Polo-Kantola 2007; Rasch and Born 2013; St-Onge 2013; Hauglund et al. 2020; Gulia et al. 2021; Vanek et al. 2020). Chronic sleep disturbances impair brain-body functioning and overall health due to failure in the above-mentioned functions (Lack et al. 2008; Leproult and Van Cauter 2009; Besedovsky et al. 2012, 2019; Kim et al. 2015). Moreover, sleep is disturbed in almost every neurological disorder including Autism spectral disorder, Parkinson disease, Alzheimer disease, ADHD, headache, anxiety, depression, and infection (Blau 1990; Tsuno et al. 2005; Nutt et al. 2008; Menza et al. 2010; Konofal et al. 2010; Devnani and Hegde 2015; Bubu et al. 2017; Gulia and Kumar 2020). This article highlights the effects of prenatal sleep loss on the health outcomes in the offspring.

## 8.2 Maternal Sleep Loss During Pregnancy and Poor Health Consequences in F1 Generation

In the twenty-first century, there are growing concerns on poor sleep quality during pregnancy and adverse pregnancy outcomes. Even though several factors including malnutrition, substance abuse (cocaine, marijuana, etc.), alcohol consumption, HIV/AIDS, infection, stress, and smoking during pregnancy were identified as risks for the development of the fetus (Garcia-Rill et al. 2007; Richardson et al. 2009; Hunt et al. 2008; Schetter and Tanner 2012; Hambleton et al. 2013; Zeskind et al. 2014; Forray 2016; Stephan-Blanchard et al. 2016; O'Donnell and Meaney 2017; Stringer et al. 2018; Haugland et al. 2020), sleep loss during pregnancy still remains as an under-investigated factor. World Health Organization had reported that about 10% of women during pregnancy, and 13% of women who have just given birth to babies have experienced mental disorders including anxiety and depression (WHO, mental health action plan 2013–2020). In developing nations, this percentage escalates by another 5–6%. This has to be viewed with the fact that globally 10–20% of children and adolescents also had experienced mental disorders. These neuropsychiatric conditions were the leading causes of disabilities in youngsters, and they severely influenced their development, educational attainments, and abilities to live fulfilling and productive lives. It is always a difficult task to conduct controlled sleep deprivation experimental studies during pregnancy in human subjects due to ethical and practical reasons. However, controlled studies in the rodent model provided evidences of the effects of sleep deprivation during pregnancy on the cognitive development of the offspring (Fig. 8.1).

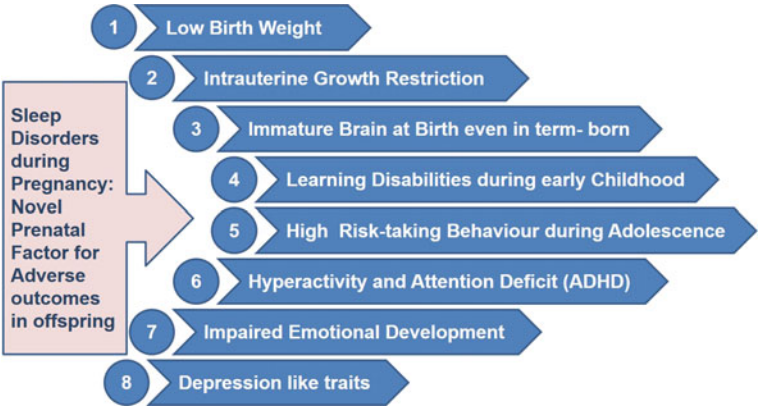


**Fig. 8.1** Effects of sleep disorders during pregnancy on adverse outcomes in offspring

Rodents are also altricial in nature similar to humans. So, it is easy to simulate human pregnancy states with three trimesters of 3 weeks of the total gestation period in this species. The developmental profile of sleep-wakefulness in normal rat dams was assessed by recording EEG and EMG for 24 h, starting from pre-pregnancy days, during pregnancy, post-partum (lactation), and post-weaning days, along with the monitoring of their anxiety (Sivadas et al. 2017). Sleep fragmentation was observed during the third trimester of pregnancy and post-partum days with a concomitant rise in the NREM sleep delta power (Fig. 8.2). Delta power in the NREM sleep, a measure of the homeostatic drive for sleep, was calculated from EEG traces, using fast Fourier transformation. Increased NREM sleep delta power during late pregnancy—lactation continuum was evident as shown in Fig. 8.2. During normal pregnancy, the post-partum sleep and anxiety decreased compared to the ante-partum levels (Sivadas et al. 2017).

Poor sleep quality in dams due to reduced REM sleep and sleep fragmentation is common observations during the last trimester even in a normal pregnancy (Hertz et al. 1992; Bourjeily 2009; Wilson et al. 2013; Mindell et al. 2015). However, further sleep loss during late pregnancy is a growing concern. The effects of REM sleep restriction (REMSR) and total sleep restriction (TSR) during last trimester (gestational days 15 to 20), on the pregnancy outcomes, were studied in separate groups of rats. REMSR involved restriction of REM sleep of 22 h per day during 9 am to 11 am (next day) using classical platform method, whereas in TSR, restriction of total sleep of 5 h per day during 9 am to 2 pm was carried out by gentle handling procedure.

Pups born to the TSR dams showed low birth weight in spite of having a longer gestational period (one day longer) than the control dams (Aswathy et al. 2018b; Gulia et al. 2021). Similarly, pups born to the REMSR dams during the last trimester showed lower birth weight compared to the control dams, but no change in the gestational period was observed (Aswathy et al. 2018a; Gulia et al. 2021). There were no cases of preterm births. The pups in TSR group gained their body weights

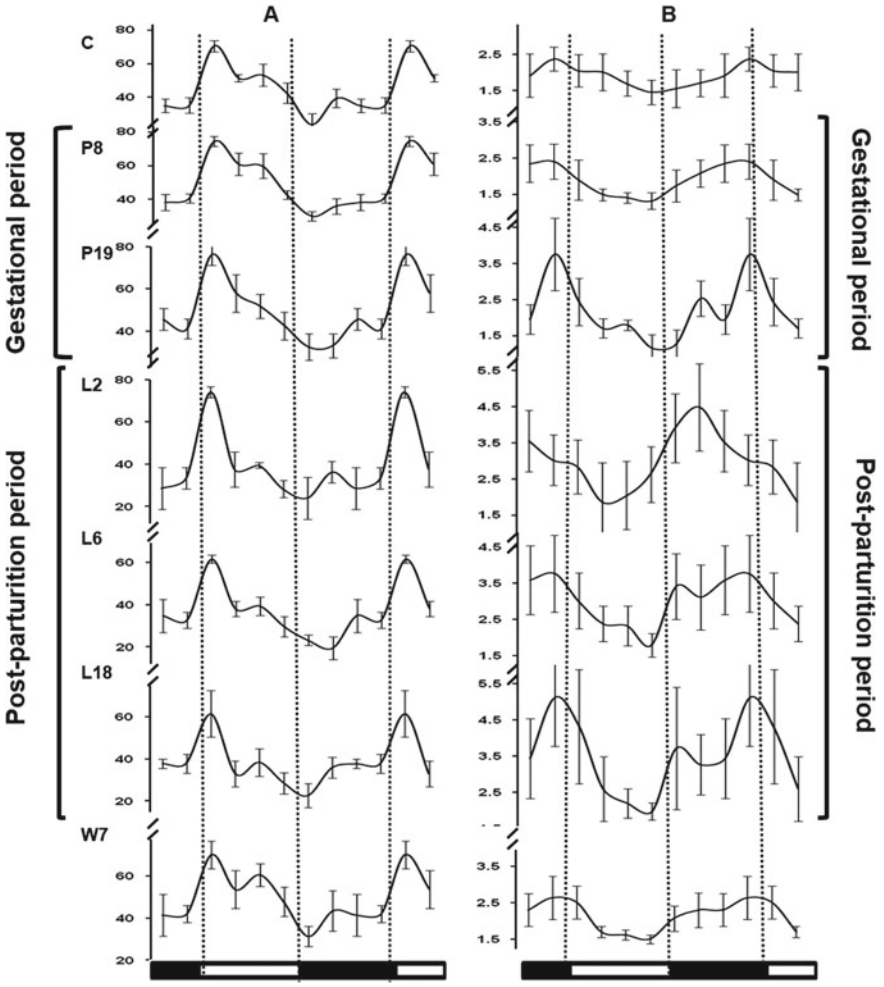


**Fig. 8.2** Changes in delta power during NREM sleep during pregnancy, post-partum and post-weaning during normal rats. **a** NREM sleep (%) in 3 hourly bins across day and night for control (C), gestational day 8 (P8), 19 (P19), post-delivery days 2 (L2), 6 (L6), 18 (L18), and post-weaning day 7 (W7). In horizontal axis, the dark bar denotes dark period beginning at 6 pm, and light bar shows light period beginning at 6 am. **b** Normalized delta power is shown for respective bins ($n = 4$)

within 2–3 days of birth, compared to the growth of the control group; however, the pups in the REMSR group had lower weights throughout the study period compared to the control group. This probably indicated fetal growth restriction that continued post birth also in this condition of sleep restriction.

Sleep loss during pregnancy is reflected in a complex way in sleep-wakefulness and the cognitive development of the offspring (Aswathy et al. 2018a, b; Gulia et al.

2021). Distinct differences were observed between TSR and REMSR group pups in the neonatal calls, i.e., ultrasonic vocalizations (USVs), recorded using isolation paradigm to measure their affective state. The pups in the TSR group displayed higher rate of USVs after birth until peak calling range, i.e., postnatal day 9, while the REMSR group pups made fewer calls on postnatal days 1–9 and also showed delay in calling pattern (Gulia et al. 2014, 2015; Gulia and Kumar 2018a, b, c). Recent studies in human have also shown that the maternal sleep quality influences neonatal auditory event-related potentials (ERP) as happy and angry stimuli induced different ERPs (Lavonius et al. 2020). Observation of altered sleep-wakefulness patterns with higher percentage of active sleep (AS) is the precursor of adult REM sleep, post birth in pups from both the groups of maternal sleep restriction during pregnancy (TSR and REMSR). These indicated that they had immature brain in spite of having born at term or a day later than the control pups (Aswathy et al. 2018a, b; Gulia et al. 2021). Furthermore, sleep restriction produced distinctly different phenotypes in the two groups, as TSR group pups showed symptoms of hyperactivity, and increased risk-taking behavior during peri-adolescence, while the pups in the REMSR group showed signs of depression-like traits in neonates, which persisted until middle age (Radhakrishnan et al. 2015; Gulia et al. 2021). These outcomes indicated the risk in the emotional development, in the offspring, if the mothers had chronic sleep restriction during late pregnancy. These are also evidences to support the relationship between early development of sleep and brain functional connectivity in the preterm and term born babies (Uchitel et al. 2022). Moreover, children born to mothers having anxiety disorders during pregnancy and had more sleep problems (Harskamp-van Ginkel et al. 2020). A thorough understanding of sleep in relation to age and state (pregnancy) is essential for preventing the above-mentioned conditions of prenatal origin. Though sleep is essential for all the wake time behaviors, it still remains as a poorly recognized health concern.

## 8.3 Dynamic Role of Sleep and Healthy Aging

There are escalating evidences of prenatal origin of many heart and brain malfunctions (Barker et al. 2002; O'Donnell et al. 2009; Calkins and Devaskar 2011; Radhakrishnan et al. 2015; Haugland et al. 2020; Porges and Furman 2011; Fyfe et al. 2015; Faa et al. 2016; Nijland et al. 2008; O'Donnell and Meaney 2017; Aris et al. 2018; Aswathy et al. 2018a; Monk et al. 2019; Amgalan et al. 2021; Jia et al. 2021; Su et al. 2021). A few systematic reviews and meta-analysis in human cohorts identified sleep during pregnancy as a significant risk factor for gestational diabetes and insulin resistance. Though the trends are evident, it cannot be confirmed due to the subjective nature of sleep and outcomes of several other parameters that cannot be normalized (Du et al. 2021; Wang et al. 2022). In humans, factors like maternal malnutrition, alcohol or substance abuse, smoking during pregnancy are comparatively well worked out for their outcomes. But the chronic sleep loss, hypoxia,

restless leg syndrome, sleep disordered breathing, preterm birth, etc., during the critical period of both pre and postnatal window, are emerging as novel factors that are detrimental for the overall growth and neurocognitive development of the offspring (Badran et al. 2019; Morrakotkhiew et al. 2021; Visser et al. 2021). The role of sleep in developmental programming requires attention to understand the mechanism of neurocognitive disorders. It will help to reduce the disease burden and in meeting the healthy longevity challenge.

## 8.4 Conclusion

As sleep is the foundation of health even during the fetal life, if given due care, one can achieve healthy aging and prevent disease burden. It is emphasized that the UN decade of Healthy Aging 2021–2030 is a timely global collaborative goal amidst the fast-changing proportion of world's population over 60 years, which will rise up from 12 to 22% within 35 years, counting from 2015 to 2050 (WHO estimation). Sleep is for the whole body including brain. If due importance is given to sleep during the fetal life, it can help in optimizing the functional abilities during later life. If one can achieve healthy aging, he/she can continue to learn and make decisions, to be mobile, to build and maintain relationships, and to contribute to society. Moreover, considering that high percentage of older people (80%) will belong to the low- and middle-income countries in 2050, it is time to seriously look for simple viable strategies for sleep management, which is one of the pillars of health (Gulia et al. 2017). This will also help in achieving the good health and well-being, the Sustainable Developmental Goal 3 (SDG3). To prevent early aging and disease burden, awareness of sleep is required in the general population, along with nutrition and exercise regimen.

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# Chapter 9
# Sleep, Ageing, and Cognitive Decline


**Krishna Melnattur**

## 9.1 Introduction

This chapter focuses on changes in sleep architecture, physiology, and function in healthy ageing. We begin with a description of the phenomenology of age-dependent changes in sleep distribution and oscillations and discussion of potential underlying neurobiological mechanisms. We next consider two candidate consequences of sleep—glymphatic brain clearance and learning and memory. This chapter focuses largely on work on humans and rodents. However, in each section, we will also briefly consider parallels with invertebrate models. Specifically, we will draw comparisons with sleep in the fly *Drosophila* as a canonical example of an invertebrate. In the last 20 years, *Drosophila* has emerged as powerful model to study sleep regulation and function and is certainly the best studied invertebrate sleep model.

## 9.2 Age-Dependent Changes in Sleep Distribution and Oscillations

Sleep in humans undergoes characteristic ontogenic changes, with the prototypical young adult pattern of sleep distribution and oscillatory activity only emerging by late adolescence.

Newborn infants spend a large proportion of their day asleep (16–18 h). Infant sleep is, however, not consolidated into a single sleep bout. Instead, bouts of sleep alternate with bouts of feeding. Further, each sleep cycle lasts ~ 50 min and consists of equal amounts of rapid eye movement (REM) sleep and non-rapid eye movement

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(NREM) sleep. Sleep onset is also frequently into REM sleep (Daftary et al. 2019; Grigg-Damberger et al. 2007; Grigg-Damberger 2016; Ohayon et al. 2004). Sleep stages in newborns and infants do not exhibit all of the characteristics seen in young adults. Muscle atonia is thus incomplete in infant REM sleep, and the slow waves that are an important characteristic of NREM sleep in adults (see below) are not present in every cycle (Grigg-Damberger 2016; Bes et al. 1991). REM and NREM sleep in infants are classified as active sleep and quiet sleep to reflect this fact (Grigg-Damberger 2016).

By one to four years of age, total sleep time decreases to about 11–12 h a day. Sleep is also more consolidated, consisting of one primary sleep bout at night, and one to two naps during the day (Ohayon et al. 2004). Important differences remain in characteristics of different sleep stages in young children versus young adults. NREM is typically much deeper in young children versus young adults (Busby and Pivik 1983).

As children get older sleep duration further decreases. Teenage sleep shares many traits with sleep in young adults discussed below, with the important exception that the timing of sleep is delayed.

In young adults, sleep is characterised by a single consolidated bout at night and a regular cyclical pattern of alternation between sleep stages. Each sleep cycle lasts ~ 90 min, with a regular alternation between NREM and REM sleep (Fig. 9.1a). The sleep stages are defined by characteristic signatures in the electro encephalogram (Carskadon and Dement 2016).

### *9.2.1 Age-Dependent Changes in Sleep*

Healthy normal ageing is associated with characteristic changes in sleep duration, quality, and timing. Overall sleep duration decreases in older adults. Sleep is more fragmented and associated with more awakenings and arousals. Further, the timing of sleep onset and offset is advanced, and sleep latency is increased. In addition, ageing is also associated with changes in sleep stage architecture. Thus, ageing is associated with lower amounts of deep slow wave sleep, more time in lighter NREM stages 1 and 2, and fewer NREM-REM cycles (Fig. 9.1) (Landolt et al. 1996; Zepelin et al. 1984; Feinberg and Carlson 1968; Kales et al. 1967; Klerman and Dijk 2008; Van Cauter et al. 2000).

Further, it is not just sleep at night that is altered with age. Daytime sleep is also altered with age. Older adults report increased frequency of daytime naps, and daytime sleepiness severe enough to impair normal functioning (Foley et al. 2007).

#### 9.2.1.1 Anatomical Basis of Age-Dependent Sleep Changes

What might be the neurobiological basis for these phenomena? We begin our discussion of this question, with a little historical background.

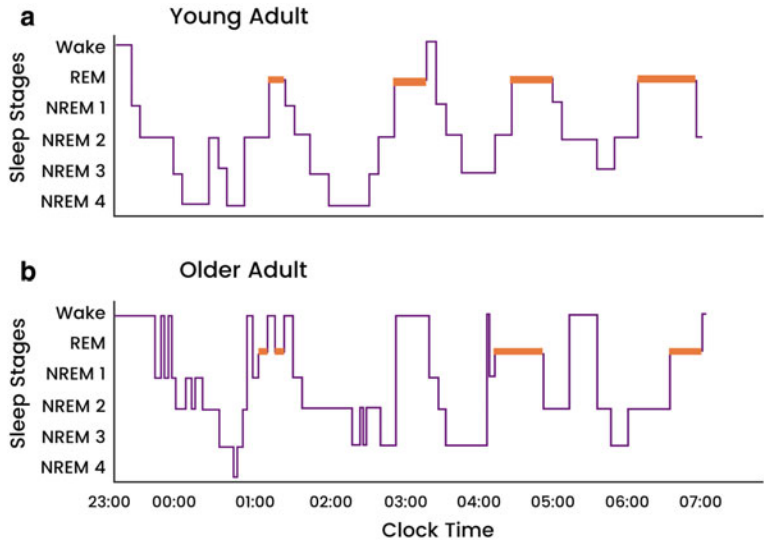


**Fig. 9.1** **a** Sleep hypnogram of a young adult. Sleep consists of a single consolidated bout at night and is characterised by a ~ 90 min cycle of NREM and REM sleep (orange). The relative time spent in REM sleep increases through the night, concomitant with a decrease in time spent in deeper NREM stages. **b** Sleep hypnogram of an older adult. Sleep of older adults is characterised by longer sleep latency, more fragmented sleep with greater awakenings from sleep, and less time in deeper slow wave sleep stages. Figure adapted from Mander et al. (2017)

The study of the anatomical basis of sleep and wakefulness in mammals owes a lot to an unfortunate epidemic of *encephalitis lethargica* almost a 100 years ago. Upon examining encephalitis patients who presented with insomnia, von Economo observed inflammatory lesions in the preoptic area (POA). Patients with hypersomnia presented with lesions in the posterior hypothalamus (PH) (von Economo 1930). Based on these results, von Economo postulated a sleep-promoting area in the POA and a wake promoting region in the PH. Subsequent lesion studies in animal models supported this idea and suggested a model, whereby sleep-promoting POA neurons inhibit arousal promoting PH neurons (Nauta 1946). Around the same time, electrical stimulation of the reticular formation was shown to induce a wake like state in anaesthetised cats (Moruzzi and Magoun 1949).

Since these classic studies, application of more modern circuit dissection techniques has led to a more nuanced understanding of the circuitry for sleep and wakefulness (Scammell et al. 2017; Szymusiak and McGinty 2016) (Fig. 9.2). The sleep-promoting area in the POA was shown to comprise of GABA and galaninergic neurons in the ventrolateral preoptic area (VLPO) (Kroeger et al. 2018; Sherin et al. 1996). The idea of an undifferentiated reticular formation has been replaced by the identification of multiple arousal promoting systems distributed along the neuraxis. These include serotonergic neurons from the Dorsal Raphae, noradrenergic neurons in the Locus Coeruleus, dopaminergic neurons from Ventro Tegmental Area,

histaminergic neurons from the Tubero Mammillary Nucleus, orexinergic neurons in the hypothalamus, and cholinergic neurons in the basal forebrain. These arousal promoting systems innervate broadly in the cortex enabling the brain to transition to a wake state. Interestingly, wake and sleep-promoting systems inhibit each other, resulting in what has been termed a flip-flop switch, that enables rapid transitions between sleep and wake with little time spent in an in-between state (Saper et al. 2010).

Perhaps unsurprisingly, ageing affects both sleep and arousal promoting centres. The number of galanin expressing neurons in the POA was shown to decline with age in humans, with the severity of loss correlating with extent of sleep fragmentation (Lim et al. 2014). Further, the number of orexinergic neurons in the lateral hypothalamus was also reduced in both aged rodents and older humans (Kessler et al. 2011; Hunt et al. 2015). A recent study in rodents found that neuronal excitability of orexinergic neurons was causally linked to age-dependent sleep disruptions (Li et al. 2022). Oxerinergic neurons in aged mice were found to have a lower resting membrane potential. They were also found to express lower levels of the voltage gated potassium channel subfamily Q member 2 subunit (KCNQ2) and a lower basal M current ($I_m$). Disrupting KCNQ2 in young mice fragmented sleep, conversely increasing KCNQ2 activity increased sleep stability in aged mice (Li et al. 2022). These results provide an interesting and detailed mechanistic explanation for sleep

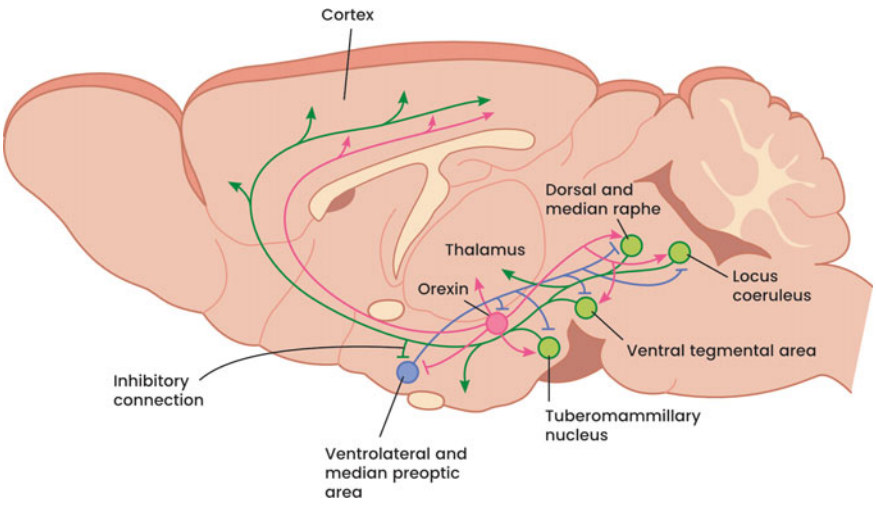


**Fig. 9.2** Schematic highlighting select sleep and arousal promoting nuclei in the mouse brain. Green is arousal promoting monoaminergic nuclei, including norepinephrine secreting nuclei in the Locus Coeruleus, serotonergic neurons in the Dorsal Raphae, dopaminergic neurons in the Ventro Tegmental Area, and histaminergic neurons in the TuberoMammalary Nucleus. Pink is arousal promoting orexinergic neurons of the Lateral Hypothalamus. Blue is the sleep-promoting GABA and galanin positive neurons of the ventrolateral preoptic area. Sleep and arousal promoting neurons have mutually inhibitory connections that result in a sleep–wake 'flip-flop' switch that enables rapid transitions between sleep and wake. Figure adapted from Scammell et al. (2017)

deficits in ageing and suggest potential therapeutic avenues for improving sleep in older human subjects.

Another, non-exclusive possibility to explain some of the age related sleep deficits is changes in neurogenesis. Neurogenesis has been reported in the hypothalamus of rodents (Lee and Blackshaw 2012; Haan et al. 2013; Robins et al. 2013). Ageing affects hypothalamic neurogenesis (Zhang et al. 2017; Matsuzaki et al. 2015). Chronic suppression of neurogenesis and gliogenesis by administration of an antimitotic agent disrupted sleep in young animals (Kostin et al. 2019). These animals exhibited reduced NREM and REM sleep amount, sleep fragmentation, and altered sleep homeostasis—all sleep deficits also associated with ageing (Kostin et al. 2019). The molecular processes that underlie the effects of ageing on neurogenesis are not very well understood. However, there are some hints that changes in neuroinflammatory products might explain some of these effects (Rosano et al. 2012; Ekdahl et al. 2003, 2009; Vallières et al. 2002). Further, systemic changes, such as in exercise and calorie restriction, that reduce inflammation, can improve neurogenesis and mitigate sleep deficits (Varrasse et al. 2015; Stangl and Thuret 2009; Blanco-Centurion and Shiromani 2006; Salin-Pascual et al. 2002). These manipulations, however, can affect multiple systems. The field will likely thus benefit from a more targeted means of enhancing neurogenesis, which would be expected to help better establish a causal link between neurogenesis and ageing-related deficits.

### *9.2.2 Age-Dependent Changes in Sleep Oscillations*

In addition to changes in overall sleep amounts, substantial changes are observed in the electrical oscillations of sleep–slow wave activity and sleep spindles.

#### 9.2.2.1 Changes in Slow Waves with Age

One important measure of slow waves is the spectral power in the slow and delta frequency range (0.5–4 Hz) that has been termed slow wave activity (SWA). SWA is most associated with drive to sleep and the phenomenon of homeostatic rebound sleep. Sleep pressure or the drive to sleep is classically modelled as increasing in proportion to time spent awake and dissipating during subsequent sleep (Borbély 1982; Borbely and Tobler 2011). SWA is highest in the early part of the sleep period and reduces over the length of the sleep period as sleep pressure dissipates.

Substantial SWA reductions are seen in baseline sleep of older adults. Further, the process of homeostatic SWA increase and decrease is also altered in older adults. Specifically, homeostatic increases in SWA in response to time awake are blunted (Landolt and Borbély 2001; Münch et al. 2004), and the slope of SWA dissipation across the night is also shallower (Landolt and Borbély 2001; Landolt et al. 1996).

SWA changes are accompanied by changes in slow wave amplitude and density. Both amplitude and density of slow waves are reduced in older adults (Carrier et al.

2011; Dubé et al. 2015). These changes suggest that ageing might diminish synchronised firing—the switching between a depolarised up state and a hyperpolarised down state that might underlie slow wave changes.

#### 9.2.2.2 Neurobiological Basis of Slow Wave Impairments

In mammals, adenosine in the basal forebrain plays an important role in sleep homeostasis (Porkka-Heiskanen et al. 1997). Prolonged wakefulness increases adenosine levels in the basal forebrain. Adenosine levels in the basal forebrain, however, appeared higher in older rodents versus younger siblings (Mackiewicz et al. 2006; Murillo-Rodriguez et al. 2004). This finding is surprising given the age-dependent impairments in homeostasis discussed above. However, there is also age-dependent loss of adenosine A1 receptors and A1 receptor gene expression (Ekonomou et al. 2000; Pagonopoulou and Angelatou 1992; Cheng et al. 2000). This receptor loss may decrease sensitivity to adenosine and thus may form the basis for the observed age-dependent defects in homeostasis. Interestingly, age-dependent impairments in slow wave features correlated with structural atrophy in prefrontal cortex (PFC) areas in older adult humans (Mander et al. 2013; Varga et al. 2016). These structural changes thus might also at least partially explain the observed defects in slow wave features discussed above.

#### 9.2.2.3 Changes in Sleep Spindles with Age

Sleep spindles are oscillatory activity in the 12–15 Hz range, thought to be generated by thalamocortical activity (Huguenard and McCormick 2007; De Gennaro and Ferrara 2003). Power in this 12–15 Hz range is decreased in older versus younger adults (Dijk et al. 1989; Landolt et al. 1996). This power reduction could be explained in part by a reduction in the number of generated spindles (Mander et al. 2014; Martin et al. 2013). Other features of the spindle waveform, e.g. duration and peak amplitude are also decreased in older versus younger adults (Mander et al. 2014; Martin et al. 2013).

#### 9.2.2.4 Neurobiological Basis of Spindle Impairments

What might be the neurobiological basis for age-dependent spindle defects? This is less clear. Reductions in hippocampal grey matter predict spindle defects in older adult humans (Fogel et al. 2017). Although spindles are classically thought to result from thalamocortical activity, they are also linked to burst firing of sharp wave ripples in hippocampus so these structural defects in the hippocampus could plausibly underlie the observed defects in spindles (Fell et al. 2001).

### *9.2.3 Connection to Invertebrates*

*Drosophila* was also shown to exhibit age-dependent changes in sleep amount, quality, and homeostasis (Shaw et al. 2000; Vienne et al. 2016; Melnattur et al. 2021). The anatomical basis of these age-dependent sleep changes in the fly has not been systematically investigated. The extrinsic fan-shaped lateral (ExFl2) neurons of the dorsal fan shaped body are a particularly interesting candidate in this regard (Donlea et al. 2011). These sleep-promoting neurons secrete GABA and allatostatin (Ni et al. 2019; Donlea et al. 2018). Allatostatin is the invertebrate analogue of mammalian galanin. Further, they have been proposed to form the output arm of the fly homeostat and are thought to be analogous to mammalian VLPO neurons (Liu et al. 2012, 2016; Donlea et al. 2011, 2014, 2018; Pimentel et al. 2016). It would thus be interesting to investigate whether there is age-dependent loss of these ExFl2 neurons in flies as has been reported for VLPO neurons in mammals.

## 9.3 Consequences of Age-Dependent Sleep Loss

The previous sections clearly demonstrate that ageing leads to sleep deficits. But are these defects of any consequence? To get at this question, we need to examine some functional outcome of sleep (Dissel et al. 2015).

### *9.3.1 Glymphatic Clearance*

One interesting idea about the function of sleep comes from a flurry of papers over the last 10 years that describe a system for fluid flow in the brain that has been termed the glymphatic system (Nedergaard and Goldman 2020). To appreciate the significance of these discoveries, we first have to take a brief detour into anatomy. Brain neuropil lacks lymphatic capillaries that enable fluid flow as is common in other organ systems. Directional flow is instead achieved by means of astrocytic processes that constitute a glia—lymphatic or 'glymphatic' conduit for cerebrospinal fluid (CSF) flow (Iliff et al. 2012; Xie et al. 2013). CSF flows into periarterial spaces in the brain driven by arterial pulsations that result from pulse waves along arteries driven by heart beats (Mestre et al. 2018; Iliff et al. 2013). Perivascular spaces are channels that run along the vasculature enclosed by endfeet of astrocytes (Wardlaw et al. 2020). Astrocytic endfeet expresses the water channel Aquaporin 4 (AQP4) (Hasegawa et al. 1994; Jung et al. 1994; Nielsen et al. 1997; Rash et al. 1998). Glymphatic flow consists of CSF entering periarterial space, mixing with interstitial fluid (ISF), carrying solutes and exiting the brain via perivenous spaces, cranial nerves, etc. Importantly, for the purposes of this review, glymphatic flow was dramatically higher (up to a fold higher) in sleep versus wake (Xie et al. 2013). This increase in flow also correlated

with increased AQP4 at astrocytic endfeet. Further, the flow was AQP4 dependent as deletion of AQP4 dramatically reduced flow (Iliff et al. 2012).

In parallel, recent studies reported the discovery of lymphatic vessels in the meningeal dura and clearance of injected tracers via lymphatic vessels (Aspelund et al. 2015; Louveau et al. 2015). Glymphatic clearance along perivenous spaces could drain into sinus lymphatics as veins merge (Fig. 9.3a) (Wardlaw et al. 2020; Ma et al. 2017), suggesting an anatomical connection between glymphatic and lymphatic systems.

#### 9.3.1.1 Impairment of Glymphatic Flow with Age

Glymphatic flow is reduced with sleep deprivation (Plog et al. 2015; Eide et al. 2021) and with ageing (Da Mesquita et al. 2018; Kress et al. 2014; Zhou et al. 2020). Ageing was also associated with mislocalisation of AQP4 away from endfeet towards soma and perisynaptic processes (Kress et al. 2014). Tortuosity of the vasculature was also increased in aged animals, providing another mechanism by which CSF flow could be reduced with age (Fig. 9.3e). Further, brain lymphatic vessels also degenerate with age (Ma et al. 2017; Ahn et al. 2019), thereby possibly providing another mechanism for reduction of flow. These age-dependent reductions in flow could have important consequences as glymphatic clearance has been implicated in clearance of toxic metabolites such Amyloidβ (Aβ)—the toxic fragment associated with Alzheimer's disease (Iliff et al. 2012; Xie et al. 2013). Decreased glymphatic flow increased Aβ (Iliff et al. 2012; Xie et al. 2013), conversely increased Aβ decreased flow (Da Mesquita et al. 2018; Peng et al. 2016), suggesting a vicious cycle. Indeed, polymorphisms in AQP4 are also linked to Alzheimer's disease (Zeppenfeld et al. 2017; Burfeind et al. 2017).

#### 9.3.1.2 Connection to Invertebrates

A sleep stage associated with brain clearance was recently reported in *Drosophila* (van Alphen et al. 2021). This sleep stage was defined by characteristic proboscis extension and retraction movements and elevated arousal thresholds. The proboscis extensions appear to causally drive haemolymph flow facilitating clearance and supported recovery from brain injury, suggesting parallels with mammalian glymphatic clearance (van Alphen et al. 2021).

### *9.3.2 Learning and Memory*

Brain clearance is clearly one important function of sleep. Another very influential theory of sleep function is that sleep is critical for learning and memory (Diekelmann and Born 2010; Walker and Stickgold 2004).

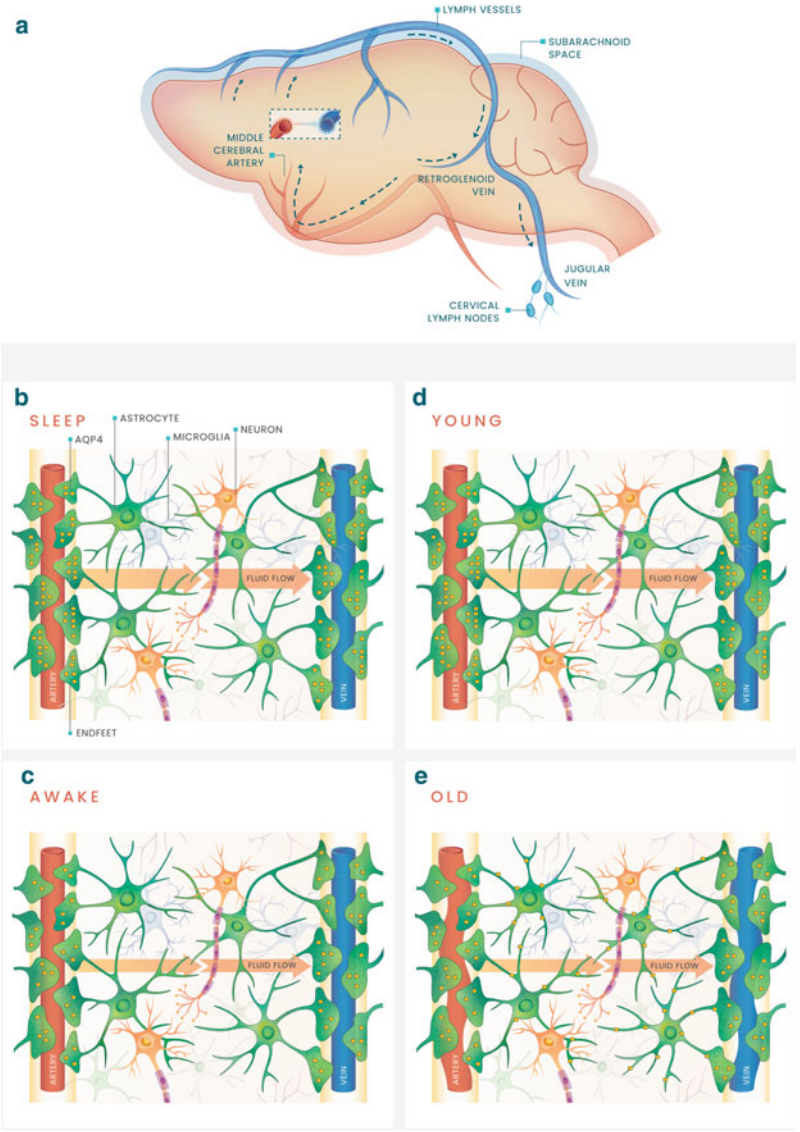


**Fig. 9.3** **a** Anatomy of glymphatic and meningeal lymphatic systems. Arrows depict direction of CSF flow. Arteries are in red, veins in dark blue, and lymph vessels in light blue. Figure adapted from (Iliff et al. 2015). **b–e** Insets highlighting the glymphatic system that facilitates fluid flow in the neuropil. Astrocytic endfeet tile the vasculature, astrocytic processes create a conduit for CSF + ISF flow across the neuropil. Increased AQP4 at astrocytic endfeet during sleep (**b**) versus wake (**c**) facilitates increased fluid flow. AQP4 is mislocalised away from endfeet towards the soma in old (**e**) versus young (**d**) animals. AQP4 mislocalisation combined with arterial tortuosity decreases fluid flow in old versus young animals. **b–e** Artery—red, Veins—Blue, astrocytes—green, AQP4—gold. Figure adapted from Nedergaard and Goldman (2020)

#### 9.3.2.1 Learning

In humans, although sleep supports many kinds of memories, hippocampus-dependent declarative memories appear to particularly benefit from sleep (Diekelmann and Born 2010). Thus, sleep loss in young adults was shown to impair learning of new episodic memories and verbal memories (Yoo et al. 2007; Drummond et al. 2000). Ageing also similarly disrupted encoding of hippocampus-dependent declarative memories and spatial memories (Jennings and Jacoby 1997; Toth and Parks 2006; Newman and Kaszniak 2000).

#### 9.3.2.2 Age-Dependent Learning Defects

In older adults, the extent of overnight sleep impairments correlated with the extent of next day encoding impairments (Lo et al. 2016; Cavuoto et al. 2016). Consistent with these findings, in rodents, ageing disrupted hippocampus-dependent spatial learning but not hippocampus independent non-spatial learning (Rapp et al. 1987; Barnes 1979; Bach et al. 1999).

#### 9.3.2.3 Memory Consolidation

Sleep is clearly important for learning new information, but the idea that sleep is critical for memory and plasticity perhaps only really took flight after the discovery of hippocampal place cell replay in rodents by Wilson, McNaughton, and colleagues (Wilson and McNaughton 1993, 1994). In these classic experiments, rats were trained to run along a linear track. The trajectory of the rat along the track was shown to be represented as a sequence of activation of place cells in the rat's hippocampus (Wilson and McNaughton 1993). This sequence was shown to be replayed during subsequent sleep in a kind of 'fast-forward' replay (Wilson and McNaughton 1994; Lee and Wilson 2002; Nadasdy et al. 1999). Hippocampal replay was accompanied by sequence reactivations in the cortex, and this dialogue between hippocampus and cortex consolidated the experience into a memory (Siapas and Wilson 1998; Sirota et al. 2003; Ji and Wilson 2007; Rothschild et al. 2017). Further, disrupting sleep-dependent replay impaired memory, thus establishing causality (Girardeau et al. 2009; Ego-Stengel and Wilson 2010).

#### 9.3.2.4 Age-Dependent Memory Consolidation Defects

Ageing impaired sleep-dependent sequence reactivation in rodents and resulted in lower memory scores (Gerrard et al. 2008). Ageing was also shown to impair long-term potentiation and $Ca^{2+}$ signalling in hippocampal neurons (Barnes 1988; de Souza et al. 2012). Further, in older adult humans, impairment in SWA was associated

with a continued reliance on hippocampal storage rather than cortical representations (Mander et al. 2013) indicating that ageing might disrupt replay in humans as well.

#### 9.3.2.5 Age-Dependent Declines in Hippocampal Neurogenesis

In small mammal systems, adult neurogenesis has been reported in the dentate gyrus (DG) of the hippocampus (Altman and Das 1965, 1967; Caviness 1973, Guéneau et al. 1982). Neurogenesis in the DG has been associated with context encoding and memory, including REM sleep-dependent memory consolidation (Shors et al. 2001; Danielson et al. 2016; Kumar et al. 2020). Neurogenesis in rodents is impaired with sleep deprivation and fragmentation (Guzman-Marin et al. 2007; 2003). Ageing also impairs rate of neurogenesis in the DG at least in rodents (Seki and Arai 1995; Kuhn et al. 1996). Impairments in neurogenesis might thus explain some of the age-related cognitive deficits. That said, clearly not all hippocampal-dependent memories require neurogenesis (Shors et al. 2002). Further, the extent of neurogenesis in the adult human hippocampus remains somewhat unclear (Sorrells et al. 2018; Boldrini et al. 2018). Additional experiments might help clarify the roles of neurogenesis in age-related impairments in cognition and sleep-dependent processes.

#### 9.3.2.6 Enhancing Sleep to Restore Learning

Ageing clearly impairs sleep, learning, and sleep-dependent memories. This suggests that enhancing sleep could potentially be a viable strategy to restore functioning to aged brains. Indeed enhancing sleep of older adults was shown to improve memory (Papalambros et al. 2017; Westerberg et al. 2015).

#### 9.3.2.7 Connection to Invertebrates

Sleep is critical for learning and memory in *Drosophila* as well (Dissel et al. 2015). Flies also exhibit age-dependent declines in learning, including spatial learning (Tamura et al. 2003; Rieche et al. 2018; Melnattur et al. 2021). Interestingly, enhancing sleep of aged flies was sufficient to ameliorate age-dependent spatial learning defects (Melnattur et al. 2021), indicating that in flies as in mammals, sleep can restore functioning to impaired brains. Enhancing sleep might thus be widely applicable as a viable therapeutic strategy in a range of different contexts.

## 9.4 Conclusions

Age-dependent changes in sleep architecture and physiology are fairly well characterised. The precise neurobiological mechanisms underlying these changes in sleep

and sleep outcomes are still being worked out. The emergence of powerful invertebrate models of sleep such as the fly *Drosophila* holds promise as vehicles to solve some of these problems.

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**Compliance with Ethical Standards:** This article does not contain any studies with human participants performed by any of the authors.

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# Part III
# Clock, Ageing and Longevity

# Chapter 10
# How Non-photic Cues for the Circadian Time System Matter in Healthy Aging

**Maristela Oliveira Poletini, Paola Fernandes, and Nayara Abreu Coelho Horta**

## 10.1 Introduction

Cannon could not have been more precise when he coined the term homeostasis to define the equilibrium of the internal *milieu*, because while *stasis* means a condition, *homeo* indicates similar (but not same), i.e., the internal *milieu* is in constant variation within a range (Cannon 1929). Homeostasis is achieved by the action of several integrative mechanisms, which operate autonomously or in a reactive manner. In anticipation of periodic changes into the environment as the light and dark (LD) cycle, the autonomous mechanisms are a consequence of the circadian time system. This system is composed of a set of endogenous oscillators named circadian clocks that determine circadian rhythms (Fig. 10.1). The *circa-Diem* (from Latin: about a day) nature of the temporal system is most likely because it has evolved under LD cycle imposed by the earth's rotation, which has made windows of opportunities and challenges for all living things, which lead to compartmentalization to optimize physiology and behaviors (Pittendrigh 1993).

In mammals, for example, the rest/activity cycle compartmentalizes the other meaningful inputs to the system. In diurnal species, activity occurs in the light (from the sun or artificial) phase, whereas resting in the dark phase. While in activity (awake), feeding behavior, and social interactions take place: while resting, these behaviors are not observed (Fig. 10.1). Hence, there is an organization of these temporal cues according to the external light input.

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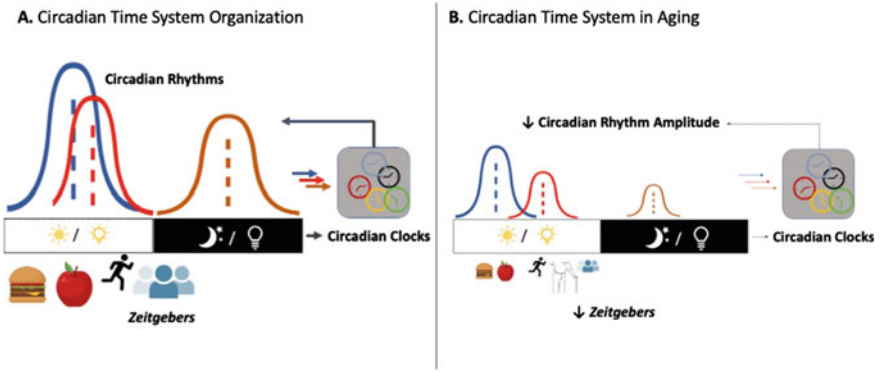


**Fig. 10.1** **Panel A**, Circadian time system organization is presented. The *zeitgebers* are compartmentalized according to the light and dark (LD) cycle that is illustrated by the white and black rectangles. Sunlight or artificial light determines the photoperiod. Circadian rhythms in behavior and physiology are represented by the blue, red, and brown lines. These rhythms are a consequence of the endogenous oscillators or circadian clocks that are illustrated by colored clocks. Each clock represents peripheral oscillation in the clock molecular machinery expressed throughout the body. LD cycles, time of feeding, social interactions, and activity are all *zeitgebers* (or temporal cues) that entrain the circadian rhythms through circadian clocks. These rhythms also function as internal *zeitgebers* to the circadian clocks. **Panel B**, Changes in lifestyle such as alterations in eating pattern, locomotion issues, and impairment in social interactions are observed in the elderly. These changes weaken the input to the circadian clocks, which may be the consequence of reduced circadian rhythm amplitude observed in aging

Remarkably, feeding and social interaction are clock-driven temporal cues, which means they occur also in the absence of LD cycles in synchrony with the rest/activity phase. In nocturnal species, the phase relationship between LD cycles and circadian behaviors is opposite to what was shown in Fig. 10.1. Temporal cues that can entrain circadian clocks are commonly named *Zeitgebers*, a German word that means time givers (Fig. 10.1). To fulfill the mission of homeostasis predicting changes in the environment, the circadian clock can respond to these changes, which entrains the endogenous rhythms to them. Therefore, circadian rhythms such as in glucocorticoid and melatonin secretion, in the core body temperature, among others are all entrained to the external temporal cues following the compartmentalization described above. Importantly, these rhythms may also be considered internal *zeitgebers* that maintain phase and amplitude of the multiple endogenous oscillators throughout the body (Fig. 10.1). These internal *zeitgebers* act by means of reinforcing the internal synchrony at the systemic level (West and Bechtold 2015).

Aging is correlated with gradual changes in internal physiological parameters as well as in the lifestyle. For example, there is an age-related reduction in the core body temperature (Hernandes Júnior and Sardeli 2021), and alterations in the pattern of feeding, activity, and in social interactions (Manoogian and Panda 2017). Thus, age-related decline in organism fitness may also be a consequence of a desynchronization and/or decline of the circadian time system by weakening *zeitgebers*. Given this

proposition, what are the impacts of aging in the circadian clocks when there is a reduction and/or misalignment of *zeitgebers*? (Fig. 10.1). To answer this question, the chapter gives an overview of the function of the circadian time system. Firstly, there will be a characterization of central and peripheral clocks, followed by a definition of entrainment and pathways currently described that is used by the light and by the main non-photic *zeitgebers* (mealtime and social interaction) in the entraining of the system, and, then by possible impacts of aging in this process. Lastly, some remarks on how re-alignment of the circadian time system matters for healthy aging.

### *10.1.1 Central and Peripheral Clocks: Categories of a Hierarchical System Model*

Circadian clocks are codified in the DNA. This statement is based on the first observations made by Konopka and Benzer (1971) in their groundbreaking paper at the time showing that *Drosophila melanogaster* mutants can display cycles of activity and rest spread randomly across 24 h, or a shorter daily cycle of around 19 h, or a longer daily cycle of around 28 h (Konopka and Benzer 1971). From then on, the molecular chronobiology field has advanced a great deal, so much that in 2017 Jeffrey C. Hall (contemporary of Konopka in Dr. Benzer's lab), Michael Rosbach, and Michael W. Young received the Nobel Prize in Physiology or Medicine for their discoveries of the molecular mechanism controlling the circadian time system (https://www.nobelprize.org/prizes/medicine/2017/press-release/).

The circadian molecular mechanism operates at the cellular level through a set of transcription factors regulated by a self-sustained feedback loop consisting of positive and negative elements, generally named clock proteins. Several well-written reviews describe the functioning of circadian molecular clocks [see Takahashi 2016]. Briefly, in mammals, the positive elements are the proteins Circadian Locomotor Output Cycles Kaput (CLOCK) and BMAL1 (aryl hydrocarbon receptor nuclear translocator-like protein 1, also known as ARNTL), they form a heterodimer that binds to the E-box enhancer of specific genes, driving transcription. The negative elements are *Period* (isoforms: *Per1*, *Per2* and *Per3*) and *Cryptochrome* (isoforms *Cry1* and *Cry2*). In the cytoplasm PER and CRY proteins form heterodimers (PER/CRY) and are subsequently phosphorylated by casein kinase δ or ε, which results in PER/CRY migration to the nucleus. At the nucleus, these heterodimers exert negative feedback on the activity of CLOCK/BMAL1. In another loop of regulation, CLOCK/BMAL1 stimulates the transcription of the nuclear receptor subfamily 1, group D, member 1/2 (Rev-Erbα/β also known as Nr1d1/2) and RAR-related orphan receptor alpha/beta (RORα/β also known as Nr1f1/2) genes: Rev-Erbα/β stimulates while RORα/β inhibits *Bmal1* transcription. The interlocked feedback loops of activators and repressors described above form the canonical positive- negative- feedback loop. CLOCK/BMAL1 heterodimer also modulates other genes known as clock-controlled genes (CCGs). In a very simplistic view, the expression of the positive

elements (for example, *Bmal1*) is an anti-phase of the negative element (for example, *Per*) in each tissue/organ of the body. This is important to keep in mind for the understanding of how different *zeitgebers* entrain circadian clocks. Mostly, they alter the phase of clock gene expression but keep the phase-relationship among them, as will be explained later in this chapter in the section on the effects of time-restricted feeding (TRF) on peripheral clocks. An interplay between this molecular loop with transcriptional programs within different cell types is responsible to keep the physiology of these cells time-coordinated and aligned with the geophysical time.

Circadian clocks are categorized as central clock located in the suprachiasmatic nucleus of the hypothalamus (SCN) and in peripheral clocks located in tissue/organ in the periphery or in non-SCN areas of the brain (Mohawk et al. 2012). Cells from both the central and peripheral clocks can generate an autonomous circadian rhythm of clock gene expression. However, there are important properties that make the SCN necessary and sufficient for regulating circadian physiology and behavior. The first property is that SCN neurons receive direct photic input from the retina allowing direct synchronization to the LD cycles (Morin et al. 2006). Secondly, SCN neurons display circadian rhythm of a spontaneous firing rate (meaning in the membrane excitability or action potential) (Herzog et al.1998; Honma et al. 1998; Welsh et al. 1995). Third, SCN neurons act as a network, which is responsible for the synchronization among them in constant conditions, such as in darkness (Aton and Herzog 2005). This network favors the strength of cellular rhythmicity (Webb et al. 2009) by communicating SCN neurons via synapses, diffuse messengers, and gap junctions (Colwell 2000; Maywood et al. 2011; Yamaguchi et al. 2003). In addition, the SCN network modulates the intracellular calcium concentration [$Ca^{2+}$] in dispersed SCN cells (Noguchi et al. 2017). Importantly, these dispersed SCN cells have the intrinsic property of displaying circadian rhythm in spontaneous electrical activity (Welsh et al. 1995). As a result of these proprieties, SCN synchronizes peripheral clocks via several direct and indirect output pathways, either by regulating hormone secretion, as glucocorticoids and melatonin, or by regulating autonomic nervous system-driven physiological parameters, as changes in core body temperature, and behaviors. As previously discussed in this chapter, these parameters work as second-order or reinforcing *zeitgebers*, which ensure synchronization of all peripheral clocks with each other and with the external environment.

Central and peripheral clocks function through a hierarchical system or an "orchestra model", where the SCN-instructs all subordinate peripheral clocks regarding the time of the day. This working model arose from classical experiments in which several biological rhythms were lost when the SCN was lesioned (Stephan and Zucker 1972). In addition, based on the experiments where SCN-lesioned mice and hamsters assume the circadian period of the donor animal SCN transplants, it is attributed to the central clock the ability to generate rhythms (Lehman et al. 1995; Ralph et al. 1990; Sujino et al. 2003).

#### 10.1.1.1 The Central Clock

The central clock is in the SCN. SCN neurons are heterogeneous in terms of neurotransmitter/neuropeptide synthesis. Two distinct portions are identified in the medial region of the nucleus: the ventral portion (v-SCN), characterized by neurons that synthesize vaso-intestinal polypeptide (VIP), and the dorsal portion (d-SCN) characterized by neurons that synthesize arginine-vasopressin (AVP). These regions are also named, respectively, core and shell (Fig. 10.2). They communicate with each other through neurochemical signals and gap junctions that maintain the SCN neurons synchronized and working as a pacemaker (Fig. 10.2). VIP is necessary to synchronize the pace among individual neurons, as inhibition of VIP-mediated coupling leads to loss of synchrony in clock expression throughout the SCN tissue *(*Aton et al. 2005; Hastings et al. 2019*).* VIP actions on SCN networks modulate GABA. In most SCN neurons, neuropeptides are colocalized with GABA (Moore and Speh 1993). In addition to AVP and VIP, SCN expresses other peptides such as calbindin, calretinin, gastrin-releasing peptide, neurotensin, and prokinectin 2 (Abrahamson and Moore 2001; Schwartz 2002). The v-SCN receives light information from the retinal-hypothalamic tract, while the main projections from the SCN depart from the d-SCN to other brain areas in the hypothalamus and brainstem (Abrahamson and Moore 2001) that regulates circadian rhythms (Fig. 10.2).

#### 10.1.1.2 Peripheral Clocks

Peripheral clocks are endogenous oscillators, which can be found in virtually all mammalian cells. Their importance for homeostasis can be appreciated by the growing body of evidence linking them with the circadian rhythms in physiology. Interestingly, human and mouse adipose tissue display circadian oscillation in several physiological parameters, including lipogenesis, lipolysis, adipokine expression, and thermogenesis, which is due to the presence of local clock components that are in line with the central clock. Metabolic tissues/organs such as white adipose tissue (WAT), brown adipose tissue (BAT), and liver show rhythmic expression of several transcripts, many of which encode important lipid metabolic regulators and clock genes. In addition, it was demonstrated that the molecular clock in the pancreatic beta cells regulates circadian insulin secretion (Marcheva et al. 2011; Dibner and Schibler 2015; Suter and Schibler 2009).

The communication between the SCN with peripheral clocks regulates the phase coherence and the period of the peripheral clock at a systemic level. This was demonstrated by recording the transcriptional activity of the *Per2* promoter. As shown in Fig. 10.3, both the acrophase and the period of *Per2* circadian rhythm are altered in explants of several peripheral tissues from SCN-lesioned mice (Yoo et al. 2004).

More recently, it has been proposed that under LD, SCN clock is dispensable for the synchronization of peripheral clocks, while in the absence of *zeitgebers*, SCN clock is required to sustain the circadian organization and integration of the whole system. This claim was based on studies with mice bearing SCN clock disruption;

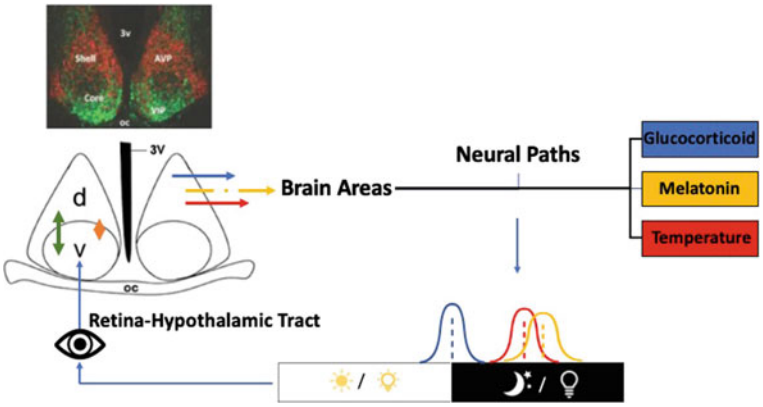


**Fig. 10.2** The suprachiasmatic nucleus of the hypothalamus (SCN) is anatomically and morphologically divided in ventral (v) and dorsal (d) portions. A diagram of these portions is presented showing the third ventricle (3 V), and a drawing of the SCN located bilateral to the 3 V above the optic chiasm (oc). The vSCN and dSCN communicate with each other by means of gap junctions (green arrow) and neurotransmitters (orange arrow) which form the SCN network. Fibers depart mainly from the dSCN to brain areas that regulate several circadian rhythms, such as glucocorticoid blood levels (blue line), melatonin blood levels (orange line), and core body temperature (red line). In this illustration a phase-relationship between these rhythms expressed in a nocturnal animal was used. Light from the LD cycles entrain the SCN through vSCN, which is also named core and is rich in vasointestinal polypeptide (VIP), this light signal is communicated to the dSCN, also named shell, that mainly contains neurons expressing arginine-vasopressin (AVP). A coronal section of a mouse SCN showing dSCN and vSCN delimited by the expression of tdTomate fluorescent protein in AVP neurons (red) and the core region containing VIP neurons labeled (Mieda 2019)

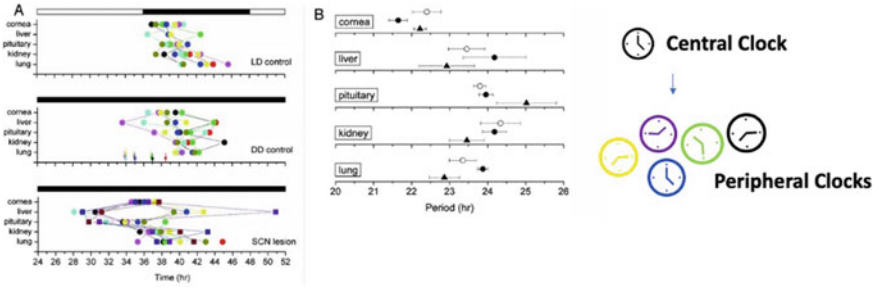


**Fig. 10.3** **Panel A** shows results from Yoo et al. (2004) demonstrating the acrophase of the circadian rhythm in the *Per2* transcriptional activity that were measured from bioluminescence recording from explants of cornea, liver, pituitary, kidney, and lung of control PER2: LUC mice under light–dark (LD) cycles or darkness (DD). The third graph in this panel shows results obtained from DD mice bearing lesion of the suprachiasmatic nucleus (SCN lesion). **Panel B** shows a graph from the same study showing a phase-map of those organs regarding *Per2* expression circadian rhythm. From these results, the main function of the central clock is shown, i.e. it determines the phase-relationship among oscillators or peripheral clocks (colored clocks)

these animals still display oscillatory profiles under LD cycles but not under darkness (Husse et al. 2015).

### *10.1.2 Are Age-Altering Circadian Rhythms Consequences of Impaired Oscillators?*

The functionality of the circadian time system blunts with aging. This decline is characterized by a reduced amplitude and increased scatter in circadian acrophase of circadian rhythms, and an increased tendency toward internal desynchronization. The endogenous circadian period of locomotor activity shortens in old hamsters (Pittendrigh and Daan 1974), in primates (Aujard et al. 2006), and in rats (van Gool et al. 1987). However the period lengthens with age in inbred mice (Farajnia et al. 2012). In humans, there is not a significant change in the intrinsic period of circadian rhythm of activity, which averages 24.18 h in young and old subjects (Duffy and Czeisler 2002). However, the daily decrease in core body temperature and the onset of sleep occurs approximately 2 h earlier in old compared to younger subjects (Dijk et al. 2000). Also, there is a gradual increase of early chronotype with age, which may predict phase-advancement of circadian rhythms, since chronotypes and phase are correlated (Roenneberg and Merrow 2007). Because similar features are observed in experimental animals after SCN lesion, this nucleus has been implicated in aging. Indeed, all SCN properties recognized as fundamental in regulating circadian rhythms are altered by aging.

As such, studies in aging rodents have shown that there is a reduction in the amplitude of neuronal SCN electrical activity rhythm (Satinoff et al. 1993; Watanabe et al. 1995). Although the total number of neurons within the SCN remains the same in aged rats, the number of neurons expressing AVP is decreased within the SCN (Mieda et al. 2016). Similarly, VIP mRNA and content are decreased in older male rodents (Harper et al. 2008; Duncan et al. 1995). On the other hand, in female rats, AVP mRNA in SCN neurons increases in the dark phase (Nicola et al. 2021). In addition, there are alterations in the density of GABAergic axon terminals and in the alpha-3 subunit of the GABA receptor in aging mice (Palomba et al. 2008), which may impair the SCN network. Farajnia et al. (2012) thoroughly compares alterations at all levels of the circadian time system (organismal, electrical, neuronal network, single cellular, and molecular) between young and old mice. This complete study shows that old mice display fragmentation in the sleep/wake cycle, reduction in the amount of activity, reduced amplitude of the electrical activity rhythm within SCN neurons, larger distribution of the neuronal activity in dispersed SCN, and reduction in *Bmal1* SCN expression whereas there is an increase in *Per2* SCN expression (Farajnia et al. 2015, 2012). Despite these alterations, analysis of *Per2:Luc* bioluminescence imaging in female mice show no changes in the amplitude of SCN *Per2* rhythm comparing old and young mice (Polidarová et al. 2016). The definitive proof that links the central clock with aging comes from transplantation studies. Implantation

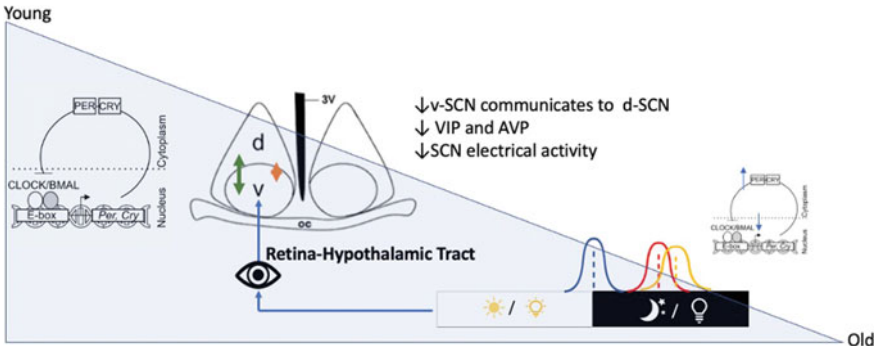


**Fig. 10.4** Aging promotes alterations in the central clocks that might be responsible for the decline in the amplitude of circadian rhythms as consequence of aging

of fetal SCN tissue in old animals rescues circadian rhythms, such as daily activity in hamsters (Viswanathan and Davis 1995) and the diurnal rhythm of corticotropin-releasing hormone in rats (Cai et al. 1997). Of note, animals with restored-clock increased longevity (Hurd and Ralph 1998). The main alterations observed in the central clock during aging are illustrated in Fig. 10.4.

Regarding peripheral clocks, several studies have shown that there are no changes in clock gene profiles within different tissues. *Per1*, *Per2*, or *Cry1* expression is similar when comparing young and aged rats in the paraventricular nucleus of the hypothalamus and pineal gland (Asai et al. 2001). Likewise, the expression of *Per2* and *Bmal1* does not differ between young and aged brain, heart, liver, and kidney submandibular gland mice (Oishi et al. 2011; Takahashi et al. 2017). Also, no differences were found in the in vitro transcriptional activity of clock genes measured in different tissues (Yamazaki et al. 2002; Novosadová et al. 2018; Yang et al. 2016; Yamaguchi et al. 2018).

Although there is no clear evidence that associates aging with alteration in peripheral clocks, it is not possible thus far to exclude age-associated changes in transcriptional cellular programming. Supporting this idea, analyzing the whole transcriptome in aged mice revealed epidermal and muscle stem cells though still showing rhythmicity in clock genes, the downstream oscillating transcriptome is pronounced reprogrammed, switching from genes related to homeostasis to those involved in stress, inflammation, DNA damage, and cellular autophagy (Solanas et al. 2017). In addition, comparison of tissue-specific transcriptional profiles of mature, aged, and old-age *Mus musculus*, *Danio rerio*, and *Nothobranchius furzeri* show conserved aging-related expression patterns. This emphasizes how the circadian system and aging might influence each other over a long lifespan (Barth et al. 2021). This opens a new avenue to correlate aging with circadian oscillations.

## 10.2 Light Versus Non-photic *Zeitgebers*

*Zeitgebers* possess the ability to alter the period ($\tau$, the amount of time it takes for a full cycle in constant conditions) and the phase ($\varphi$, the relative timing of a given circadian event within the external 24-h day) of the circadian rhythms (Roenneberg et al. 2003). Light is the most powerful *zeitgeber* for the circadian time system. Colin Pittendrigh was a pioneer in exploring the effect of short discrete pulses of light on the free-running rhythms of animals kept in constant darkness. His classical study shows that light-pulse delivered at different times of the circadian cycle have different phase-shifting effects on the observed free-running rhythm activity (Pittendrigh and Daan 1976). As such, a light-pulse delivered on the subjective day has little effect on the rhythm, whereas a light-pulse delivered during the first half of the subjective night causes a delay in the animal's activity during the following day. Light exposure during the second half of the subjective night advances the clock, as observed in the activity rhythm (Fig. 10.5). The effect of the light of advancing an animal's activity earlier the next day meets the animal's necessity to finish its activity before dawn arrives. This brought an advantage for the animal in nature which illustrates the main function of the circadian time system which is to predict cyclical changes in the environment.

The phase response curve shown in Fig. 10.5 is remarkably similar among different organisms. Differences in the shape of the curve, as well as species-specific interactions between light intensity, the phase of light exposure, the length of the free-running period, and the size of the delay, and advanced portions of the phase response curve may occur. Light activates the intrinsic photosensitive retinal ganglionar cells (ipRGCs) that possesses the photopigment melanopsin. Melanopsin is a G-protein coupled receptor covalently attached to a chromophore. In mammals, the chromophore is 11-cis-retinal that absorb light resulting in its isomerization to all-trans-retinal and activation of the phototransduction cascade. This eventually depolarizing ipRGCs, and, as consequence, the retinal-hypothalamic tract is activated. This leads to a release of glutamate and/or adenil-cyclic activator peptide (PACAP) into the retino-recipient portion of the SCN, stimulating its neurons. Through this path, higher SCN neuronal activity is found in the light phase or in response to a light-pulse in the dark phase. This route activates early immediate-factor AP-1 c-Fos, and cAMPC, increasing the expression of *Per1* within SCN neurons. Therefore, resetting the central clock occurs, leading to the photoentrainment (Do 2019).

It is well-known that non-photic cues provide meaningful information to track time. Like what has been described in the photoentrainment, non-photic *zeitgebers* produce a curve-phase response in the circadian rhythms. But their shape is different because it is based on the premise that the increased arousal state induced by an external stimulus will delay or advance the oscillation, as shown in Fig. 10.5. Therefore, higher phase-shift will happen in the resting phase, i.e., and in the dark phase for nocturnal animals (Golombek and Rosenstein 2010). The best-known non-photic *zeitgebers* are feeding time, activity, and social interactions. Different from light,

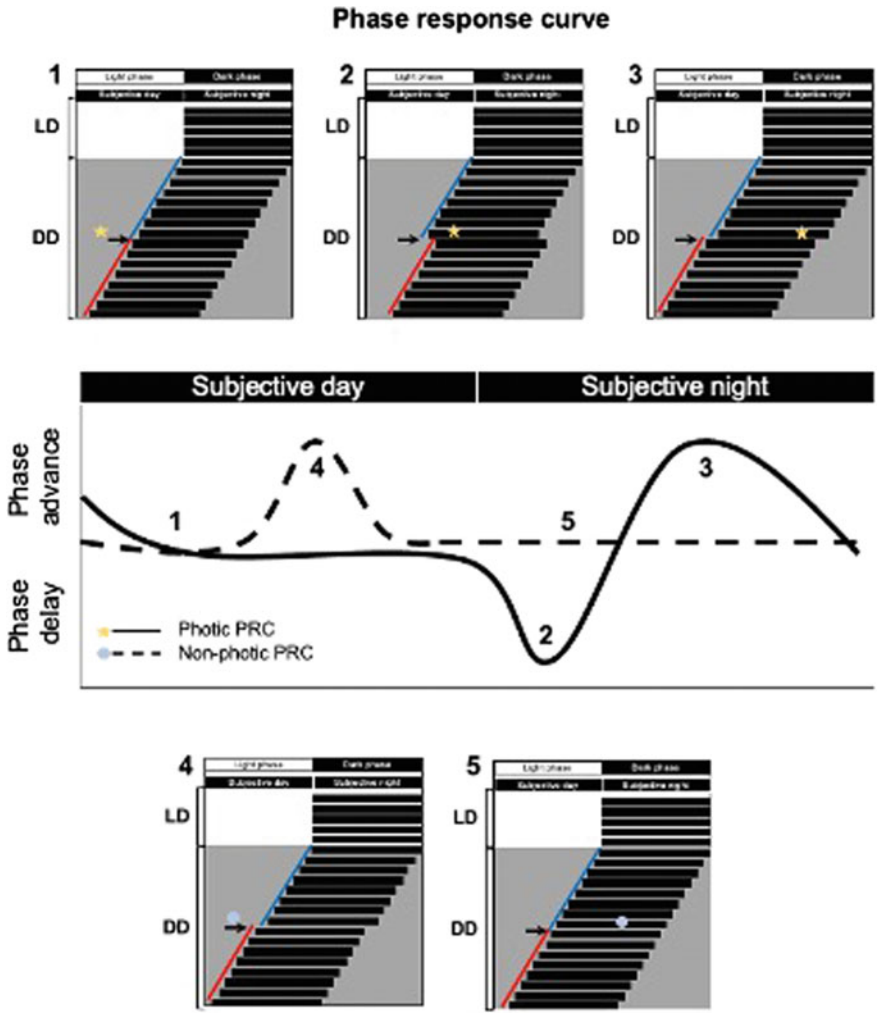


**Fig. 10.5** Representation of photic and non-photic phase response curves. In the upper panels, actograms of nocturnal rodents that received a pulse on the subjective day (1), at the beginning of the subjective night (2) and at the end of the subjective night (3) are represented. In the lower panels, actograms of nocturnal rodents that were exposed to the activity wheel on the subjective day (4) and on the subjective night (5) are represented. The response to light pulse exposure or the activity wheel is represented on the phase-response curve (middle panel) where the solid line represents the photic phase-response curve, and the dotted line represents the non-photic phase-response curve. This illustration was modified from Golombek and Rosenstein (2010)

they seem not to depend on the central clock to entrain the circadian time system (Moran-Ramos et al. 2016).

### *10.2.1 Photoentrainment and Aging*

Aging is associated with yellowing and thickened lenses in humans. This may impair light transmission from the eyes to the SCN (Brainard and Maloney 2004; Kessel et al. 2010; Najjar et al. 2014) and rodents (Zhang et al. 1998). Also, the retinal function is declined in humans (Freund et al. 2011; Gerth et al. 2002), as well as in rodents, the number of ipRGCs is reduced as aging progresses (Semo et al. 2003). In addition, aging reduces light responsiveness of the SCN, but it does not affect the retinal hypothalamic tract (Lupi et al. 2012; Zhang et al. 1998). Therefore, uncoupling among oscillators within the SCN may be a consequence of the aging-related decline of light entrainment resulting in impairment of the central clock maintaining amplitude of the circadian rhythms (Fig. 10.4).

### *10.2.2 Feeding Time Entrains the Circadian Time Systems: Impact on Aging*

Food is largely recognized as a temporal clue to the circadian time system. A misalignment between feeding time and the active phase can be as dangerous as a high-fat diet to impairing health. Thus, misalignment of feeding time within the circadian activity rhythm contributes to several metabolic disorders, such as hepatic steatosis, hyperglycemia, insulin resistance, dyslipidemia, and metabolic syndrome (Bass and Takahashi 2010; Huang et al. 2011; Turek et al. 2005).

Nocturnal rodents show feeding behavior in the dark phase when they are active. Hence, the fasting period of nocturnal animals is through the light phase, when food consumption is very low (Santoso et al. 2018). Notably, clock-mutant mice lack the circadian variation of food consumption; they eat similar amounts of food in the light and dark phase, although they still show circadian rhythm in the locomotor activity. These results imply that feeding behavior and locomotor activity are two independent circadian rhythms (Turek et al. 2005), although they are compartmentalized in the same phase of circadian cycle. Also, regardless of a higher-fat diet, clock-mutant mice display higher body weight gain compared to wild-type mice, indicating that the alignment of feeding and activity rhythms may contribute to energetic balance (Turek et al. 2005).

Feeding time is the dominant *zeitgeber* for peripheral clocks (Pickel and Sung 2020). This is largely demonstrated in animal studies showing the effect of time-restricted feeding (TRF) on circadian clock gene expression (Fig. 10.6). Rodents with food access exclusively at the light phase for several days show a complete inversion

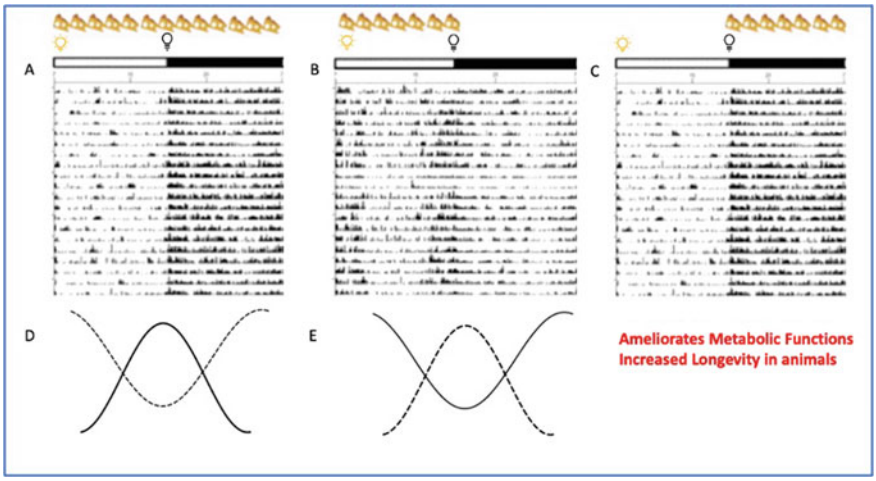


**Fig. 10.6** Time-restricted feeding (TRF) effects on locomotor activity circadian rhythm are represented by actograms obtained from rats (results from the Dr Poletini' lab, author of this chapter). Rats were fed ad libitum (**Panel A**) or at the light phase (**Panel B**) for 21 days. Lines in panel D and E illustrates the expression of *Per* genes (solid line) and *Clock* or *Bmal1* (dashed line), note that TRF inverts the phase of the circadian expression of those genes, mainly in the liver (see text for details). In addition, note that TRF reduces the locomotor activity at the end of the dark phase (panel B). Panel C duplicates the actogram of panel A to illustrate the design experiment that has shown to improve metabolic function and association with increased longevity in rodents

of the acrophase of the circadian rhythm in clock gene expression, when compared to those with food access at the night phase or *ad libitium* (Fig. 10.6). This has been demonstrated in various tissues, such as the liver, heart, and kidney, (Damiola et al. 2000; Schibler et al. 2003; Stokkan et al. 2001; Vollmers et al. 2009). Amazingly, the alignment of feeding schedule to active period has been associated with ameliorating of metabolic function and increased longevity in animals (Fig. 10.6).

It is recognized that each tissue differently responds to cycles of nutrients, hormonal secretion, and other demands imposed by the fast/feeding cycles. Accordingly, it is conceivable that their responses to feeding time were different. Indeed, TRF at the light phase strongly phase-shifts clock genes expression in the liver and white adipose tissue, while it partially affects the kidney, and it has no effects on the lung (Manella et al. 2021). Thus, feeding at the resting phase in nocturnal animals not only entrains peripheral clocks but also promotes an uncoupling among them.

The liver is entrained earlier by food when compared to other organs (Damiola et al. 2000). In addition, it is known that the liver exerts a crucial role in processing nutrients. This largely accepted view relies on studies showing that the liver participates in the maintaining of glycemia by increasing glucose production in the fasting (kalsbeek et al. 2014). And it regulates lipid flow throughout fast-feeding cycles through the molecular clock (Doi et al. 2010; Han et al. 2016, Jones 2016). Given that, the liver plays a critical role in the entrainment promoted by food, aligning glucose and lipid metabolism to the fast-feeding cycles (Chaix et al. 2019; Mukherji

et al. 2015). Nevertheless, it seems that the liver clock does not regulate the clock itself in other tissues, but rather it modulates peripheral tissue transcriptional rhythmicity, mainly upon feeding at the light phase schedule (Manella et al. 2021). This, once more, emphasizes the importance of the liver to metabolic homeostasis under conditions of nutrient challenges.

Aside from phase-shifts in the clock gene expression, TRF increases the locomotor activity in anticipation of food availability, leading to a bimodal rhythm of activity (Fig. 10.6). This is known as food anticipatory behavior, which causes alterations in the activity depending on the time of food exposure (Kessler and Pivovarova-Ramich 2019). For example, chow or high-fat diet offered for 8 h in the dark phase to a nocturnal rodent does not alter the locomotor activity circadian rhythm, although the animals display an increase in activity at the end of dark phase (Hatori et al. 2012). Whereas chow or high-fat diet offered throughout the light phase reduces activity at the end of the dark phase (Reznick et al. 2013; Yasumoto et al. 2016). The food anticipatory behavior is accompanied by an increased neuronal activity and *Per1* expression in the dorso-medial hypothalamus and has no effects on the SCN, showing that it is a behavioral output separated from the known light-entrained oscillator located in the SCN (Carneiro and Araujo 2012).

There are many theories about aging that will not be the scope of this chapter. However, the aspects of two long-standing hypotheses will be provided to recognize of the significance of calorie restriction time (not only the calorie restriction per se) in improving health throughout aging. Briefly, the theories of aging are based on the rate of living and oxidative damage. The former proposed by Pearl (1928) stated that mammalian longevity is inversely related to their metabolic rate per unit of tissue mass (= rate of living). The latter was proposed by Harman (1956) and stated that reactive oxygen species (ROS)—by-products of oxidative phosphorylation in mitochondria—impair DNA, lipids, and proteins, leading to accelerated biological aging (Redman et al. 2018). The combination of these theories brings us to the general idea that calorie restriction reduces body weight and ameliorates aging hallmarks, which leads to longevity. As such, calorie restriction beginning early or in mid-life and sustained for a substantial portion of the lifespan, increases longevity in a wide variety of species (Speakman and Mitchell 2011; Balasubramanian et al. 2017). On the other hand, calorie restriction imposed chronic cycles of feeding—fasting which raises the question of whether calories, fasting, or time of day are the contributors to increase lifespan. Remarkably, mice submitted to daily fasting intervals and circadian alignment of feeding show extended lifespan, independent of reducing body weight gain. Calorie restriction at night (active phase in mice) also ameliorates aging-related gene expression encoding components of metabolic genes in mice liver (Acosta-Rodríguez et al. 2022).

This striking animal study linking TRF protocols and longevity provides strong evidence in favor of using circadian-aligned calorie restriction or TRF as a strategy for a healthier metabolic life in aging. Several studies in humans have shown clinical benefits of TRF, such as a reduction in insulin levels; improved insulin signaling; a reduction in oxidative stress; an increase in antioxidant defenses and autophagy; and a reprogramming of aging-related pathways (Manoogian and Panda 2017). A

randomized crossover study in humans showed that early TRF (eating between 8 am and 2 pm) reduces the 24-h glucose levels, the fasting glucose, and the insulin levels in overweight individuals compared to feeding time between 8 am and 8 pm (control schedule). In the morning, before the eating time, early-TRF increases the ketone beta-hydroxybutyrate, cholesterol, and the expression of the stress response and aging gene coding the SIRT1 (silencing information regulator 2 related enzyme - sirtuin 1) and the autophagy gene LC3A, while in the evening, it increases the expression of mTOR (mammalian/mechanistic target of rapamycin), a major nutrient-sensing protein that regulates cell growth (Jamshed et al. 2019). Furthermore, a trial pilot study with sedentary older adults ($\geq$ 65 years) reported improvements in quality of life and meaningful changes in walking speed with few reported adverse events after TRF (Anton et al. 2019).

The whole picture of the clinical effects of TRF or calorie restriction in humans is still not known, therefore applying TRF or calorie restriction as therapeutic tools to overcome aging-related deleterious effects on health should be carefully considered.

### *10.2.3 Social Interactions Entrain the Circadian Time Systems: Impacts on Aging*

Social interaction either entrains or masks the circadian time system. In nature, individuals need to adapt to the rhythms of activity and rest of their sexual partners, prey, predators, and family members, allowing the formation of groups, success in the search for food and reproduction, which may have contributed to the evolution of the species (Mistlberger et al. 2011). Therefore, the maintenance of social bonds is extremely important for the survival of social species. This also applies to humans, since maintaining social contact is of utmost importance for physical and mental health. Proof of this is that social interactions and positive social stimuli are considered social reward stimuli, since they can activate circuits and brain regions involved in reward processing, both in humans and rodents (Gunaydin et al. 2014; Rademacher et al. 2015). On the other hand, social isolation (or social rejection) which leads to the feeling of loneliness, can be an aversive emotional state in humans and rodents, being considered a challenge for physiological homeostasis like hunger and thirst (Lee et al. 2021a, b; Tomova et al. 2020).

The role of social interaction as *a zeitgeber* has already been investigated by evaluating its phase response curve in several rodents (Fig. 10.5). For example, isolated nocturnal hamsters (*Mesocricetus auratus*) exposed to 30 min of social interaction with a conspecific of the same sex or changing housing-cages at different times over 24 h show a typical response curve (Mrosovsky 1988). Cohabitation of an arrhythmic rat (showing unstable rhythm) with a rhythmic rat (more stable rhythm) under constant light leads to stability in the locomotor activity and core body temperature rhythms. This might be explained by the social interactions among the arrhythmic and rhythmic rats (Cambras et al. 2012).

More recently, it has been shown that social interactions with conspecifics of the opposite-sex increase c-Fos (a marker of neuronal activity) in the olfactory bulb, as well as the PER1/*Per1* in the SCN, and the c-Fos and PER-1 in the piriform cortex of both male and female, pointing to participation of odor-related neuronal structures in the resetting of the central clock by social stimulus (Sonker and Singaravel 2021). The social interaction as well as other non-photic *zeitgebers* have their occurrence in the active phase leading to an increase in locomotor activity and the wake/arousal state. On the other hand, the circadian time system is prone to respond to them in the resting phase. This implies that social interactions may affect circuits regulating the sleep/wake cycle (Mrosovsky 1988; Mistlberger and Antle 2011).

Studies using dual tract-tracing have shown that the medial preoptic area (MPA), the subparaventricular zone (SPVZ), and the dorsomedial hypothalamic nucleus (DMH) are SCN relaying output to two key sleep-promoting nuclei, namely, the ventrolateral and median preoptic nuclei. The MPA, SPVZ, and DMH are believed to link the SCN with wake-regulatory neuronal groups, such as the tuberomammillary nucleus, the locus coeruleus (LC), the ventral tegmental area (VTA), the dorsal raphe nucleus (DRN), and the substantia innominata (Deurveilher and Semba 2005). Vipergic and vasopressinergic fibers from the SCN reach LC via SPVZ and DMH. The neuronal circuit SCN—DMH—noradrenergic LC controls the sleep/wake cycle (Aston-Jones et al. 2001; González and Aston-Jones 2006). Noradrenergic LC neurons display higher activity in the wake phase compared to the resting phase in mice and rats (Aston-Jones and Bloom 1981; Poletini et al. 2007). Inhibition of noradrenergic LC neurons reduces the alertness and enhances the sleep state during the active phase, and the opposite occurs when these neurons are stimulated (Carter et al. 2010; González and Aston-Jones 2006). In addition, LC participates in the stress-regulated circuits that includes paraventricular nucleus of the hypothalamus (PVN) projections (Valentino and Van Bockstaele 2008).

On the other hand, lack of social interaction is considered mild stress. There have been reported mixed results measuring the effect of social isolation on the hypothalamus–pituitary–adrenal (HPA) axis activity (Greco et al. 1992; Ieraci et al. 2016; Lopez and Laber 2015; Perelló et al. 2006). This is mainly because the mild stress effect of social isolation varies according to species, age, and time of isolation. Aside from the classical physiological functions of PVN regulating HPA activity, this nucleus plays a role in regulating the waking state in mice (Liu et al. 2020; Ono et al. 2020) and it is a target of SCN projections (Abrahamson et al. 2001). PVN can be part of the social interaction entrainment of the circadian time system. Social stimulus inactivates GABAergic neurons of the SCN in the subjective night leading to an increase in the neuronal activity of corticotropin-releasing factor (CRF) neurons in the PVN, which in turn activates orexinergic neurons in the lateral hypothalamus. The activation of these neurons increases the alertness state (Ono et al. 2020). In addition, social stimulus increases the oxytocin-producing PVN neurons (Resendez et al. 2020).

Social interaction is also a reward stimulus to social species (Krach 2010; Rademacher et al. 2015). It is well established that the mesolimbic pathway composed by dopaminergic VTA projections to the nucleus accumbens (NAc) participates in the

control of motivational behavior of searching for reward (Rademacher et al. 2015). In addition, optogenetic activation of the dopaminergic VTA-NAc pathway, as well as from the serotonergic pathway from the NDR to the NAc increases the search for social interaction with a conspecific (Gunaydin et al. 2014; Walsh et al. 2018).

Serotonin (5-hydroxytryptophan, 5-HT) from medial raphe nucleus (MRN) and DRN plays a role in the non-photic entrainment of the circadian time system. Stimulus inducing alertness applied during the rest phase increases the 5-HT levels in the SCN. In addition, both electrical stimulation of MRN/DRN and the serotonergic-receptor agonist treatment cause phase-shift in the circadian rhythm of activity (Mistlberger and Antle 2011). The retino-recipient portion of the SCN receives direct projections from MRN and indirect projections from DRN (Abrahamson et al. 2001; Bang et al. 2011; Muzerelle et al. 2014). The increase in the number of c-Fos positive SCN neurons induced by a light pulse is drastically reduced after previous electrical stimulation of the MRN or DRN (Meyer-Bernstein and Morin 1999).

Dopamine signaling also modulates light responses of the SCN. Dopamine-positive-β-hydroxylase and tyrosine-hydroxylase (TH) fibers are found in the d-SCN portion of mice (Abrahamson et al. 2001). Chemogenetic activation of the dopamine receptor type 1 (D1) in the SCN causes a phase-shift like those induced by light-pulse (Grippo et al. 2017). And D1 receptor knockout mice (Drd1-KO) subjected to a jet-lag protocol show a slower entrainment rate compared to wild-type mice, while the restoration of this receptor in the SCN normalizes this behavior. On the other hand, the activation of dopaminergic neurons in VTA accelerates the entrainment rate (Grippo et al. 2017). Finally, dopaminergic signaling via the D1 receptor also seems to be important for appropriate activity of SCN neurons, since incubation of brain slices with D1 receptor agonist reduces the firing rate of SCN neurons (Grippo et al. 2020). Therefore, 5-HT and dopamine neurotransmitter systems can modulate SCN light responses.

Importantly, social isolation significantly changes the circadian rhythm of 5-HT in the rat hypothalamus (Greco et al. 1992), as well as reducing and increasing the activity of serotonergic DRN neurons and VTA dopaminergic neurons, respectively (Fabricius et al. 2010; Sargin et al. 2016). In addition, social isolation leads to a reduction in the activity of the SCN neurons at the beginning of the light phase. This is associated with a reduced amplitude of the daily rhythm of core temperature and uncoupling between this rhythm the locomotor activity (Fernandes et al. 2021). On the other hand, both serotonergic and dopaminergic pathways are associated with the reward caused by social interaction (Dölen et al. 2013; Eban-Rothschild et al. 2016). Therefore, social interaction ensures proper activation of SCN neurons in the light phase and maintains the proper phase relationship between the daily rhythms of activity and core body temperature, as well as 5-HT and dopamine signaling to the central clock.

Aging is associated with decreased circadian rhythmicity of sleep, as well as sleep timing, duration, and consolidation (Wei et al. 1999). Accordingly, in the elderly, sleep tends to be more fragmented with poorer quality, which is correlated with worsened cognitive performance (Miyata et al. 2013). In addition, aging induces

significant changes in social relationships for reasons that include a decline in physical or cognitive abilities and decreased interactions with friends and relatives (Cudjoe et al. 2018). Subjective social isolation from both family and friends is associated with depressive symptoms, and psychological distress (Taylor et al. 2016). Therefore, the circuits regulating sleep–wake cycle, rewarding, and social stimulus can be diminished as age progresses leading to an impairment of circadian time system.

Indeed, in older rats, the PVN activity in response to acute and chronic stress reduces during aging (Kovács et al. 2019). Also, decreasing PVN inputs to hypothalamus–pituitary–adrenal axis results in the age-related circulating cortisol fall in non-human primates (Yang et al. 2017). Age-related decline in the LC neuron number by ~ 20–40% was reported in humans, although in this study individuals presenting neurodegenerative diseases were not excluded (Mather and Harley 2016).

The dopaminergic function declines with normal aging resulting in impairment of cognition, reward processing, and motor function, which is proposed to influence the development of a depressive phenotype in the elderly (Taylor et al. 2021). A decrease in the circadian amplitude of dopamine, noradrenaline, and 5-HT occurs as age progresses (Cornelissen and Otsuka 2016). The 5-HT SCN levels in 24-month rats are low, and a disruption in the daily 5-HT rhythm is observed at this age (Jagota et al. 2010). In general, 5-HT signaling impairment has been implicated in changes in mood behavior, such as depression and anxiety and neurological diseases across the lifespan, including age-related diseases like Alzheimer's disease (Daut and Fonken 2019).

Figure 10.7 summarizes the brain areas affected by social interaction and how they are connected to the central clock in rodents. As described, there are strong intersections between these circuits with the regulation of sleep/wake cycles, reward, and arousal state. There are growing evidence showing aging affecting brain areas and neurotransmitter systems, which, in turn might be involved in the social stimulus entrainment.

Importantly, depressive symptoms and cognitive problems are all social-isolation health issues that lead to increased risk of mortality in the elderly (Domènech-abella et al. 2017). Currently, due to the isolation and social distancing as an alternative to containing the spread of the new coronavirus (SARS-Cov2), it has become evident how important and vital the establishment of social bonds are. Recent studies show that, in addition to social isolation per se, the lockdown has the consequence of limiting and disorganizing exposure to temporal cues of entrainment in the circadian system, such as exposure to sunlight and social routines, resulting in an increase of reports of depressive symptoms and feelings of loneliness, increased use of electronic devices at night, changes in the sleep–wake cycle, and reduced sleep quality (Cellini et al. 2020; Leone et al. 2020; Majumdar et al. 2020). Therefore, keeping social interactions as aging progress may delay symptoms of mental health impairment because it provides a reinforcing stimulus to track time.

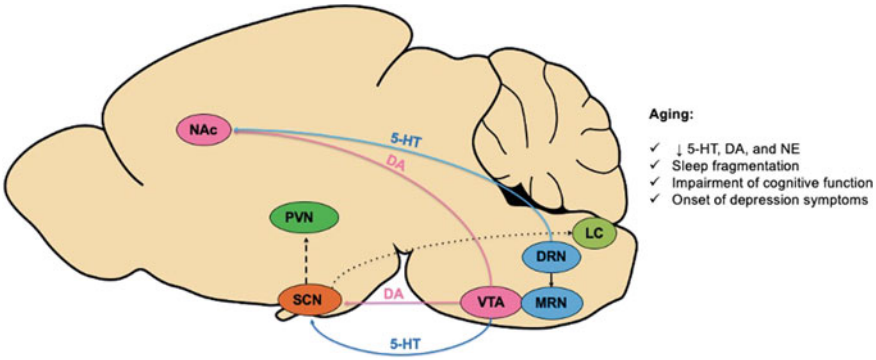


**Fig. 10.7** Proposal of neural pathways involved in the social interaction entrainment of the circadian time system based on rodent studies. Suprachiasmatic nucleus (SCN), paraventricular nucleus of the hypothalamus (PVN), ventral tegmental area (VTA), dorsal raphe nucleus (DRN), medial raphe nucleus (MRN), locus coeruleus (LC), nucleus accumbens (NAc), serotonin (5-HT), dopamine (DA), norepinephrine (NE)

## 10.3 Conclusion

In conclusion, given that aging is associated with a decline of the circadian time system and changes in lifestyle, increasing the quality of social activities and monitoring feeding time in elderly may contribute to improve health and/or decrease the hallmarks of aging. Remarkably, adopting modifiable healthy lifestyles was associated with lifetime gain, even in individuals aged 80 years or more (Sakaniwa et al. 2022). In other words, it is never too late to adopt habits that improve the quality of aging. Attentions to mealtime and social interactions may help getting healthier along the way because they function as reinforcing *zeitgebers* in the circadian time system.

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**Compliance with Ethical Standards:** This article does not contain any studies with human participants performed by any of the authors. An animal study performed by the authors is displayed in Figure 6. This study was approved by the Ethics Committee for Animal Use (CEUA ICB/UFMG, protocol number 289/2016).

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# Chapter 11
# Pineal Gland Physiology and Aging-Related Alterations in the Circadian Timing System

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## 11.1 Introduction

The pineal gland (PG) is part of the epithalamus and is situated in the midline of the 3rd ventricle (i.e., the geometric center, hence "Seat of the Soul" by René Descartes) of the human brain. The circadian timing system (CTS), sleep/wake control, immunity, reproduction, cell protection, and neuroprotection are some of the examples of important functions of the pineal gland. The physiologically active proteins, peptides, and enzymes produced by the mammalian pineal body have several physiological activities in the pineal gland and help maintain the biological clock and circadian timing (Blask et al. 1983; Benson 1989; Bharti et al. 2009; Jagota and Mattam 2017). It constitutes active peptides, serotonin (5-HT), melatonin (MLT), and several other pineal indoles, which have recently been discovered to be strong regulators of a variety of physiological functions, including aging and longevity. Many researchers revealed that the blood concentrations of melatonin turn down with increasing age and are documented to be negatively associated with quite a lot of diseases together with neurodegenerative diseases (Cheng et al. 2021). As melatonin is implicated in autophagic flux, quenching of free radicals, suppressing the discharge of pro-inflammatory protein factors, and jamming apoptotic pathways, the amplitude of its rhythm during aging is crucial. Melatonin rhythm, in general, deteriorates in aged mammals and humans, the above-mentioned processes are weakened causing

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them more vulnerable to numerous disorders and diseases. The pineal gland exhibits clear age-associated deteriorations (Cheng et al. 2021). The pineal gland in humans frequently becomes calcified with age, making it a suitable imaging marker. Several studies have linked pineal calcification to a disrupted 24 h rhythm of sleep control and a reduction of melatonin synthesis during the aging process (Yoon et al. 2003).

The deficiency of melatonin is linked not just to age but also to the severity of the deficiency's impact on mental health. Extensive investigations document that nocturnal melatonin concentrations are diminished explicitly in Alzheimer's disease (AD) and that diurnal melatonin concentrations are elevated in AD patients, representing that the neurodegenerative development influences the circadian-pineal organization. Patients often report distressing sleep patterns. The absence of a daily melatonin rhythm in AD patients is inextricably linked to clinical circadian rhythm disease. These patients exhibit agitation, agitation, insomnia, and sleep dysregulation (Wu et al. 2003).

Melatonin is available without a prescription in practically every country on the planet and is available in the form of tablets, capsules, syrup, and transdermal patches (Wu et al. 2003). Nonetheless, despite its modest side effects profile and stumpy potential for misuse, there are concerns associated with the continuous usage of melatonin in the elderly. These concerns also emerge from improper administration and use in specific therapeutic situations, such as an adjuvant in benzodiazepine dose-decreasing protocols, where clinical studies are insufficient to maintain the drug's efficacy.

## 11.2 Neuroendocrine Perspective of Circadian Rhythm and Aging

There are various metabolic activity and cellular secretions of the hypothalamus, pituitary, pineal (SCN), adrenal, thyroid, thymus, and gonads which are associated with circadian rhythm and aging and controlled through neuroendocrine secretions. Among others, aging progressions in mammalian systems lead to major changes in the circadian clock's output rhythms, neuroendocrine disruption, compromised immunity, and loss of collagen fiber and tissue elasticity. The changes include phase shifts (usually a phase advance) and amplitude reduction. In rodents, aging causes a change in the circadian timing system and several other parameters such as regulation of body temperature, locomotor rhythm, sleep/wakefulness, drinking, and feeding rhythms (Weinert 2000). A shift in melatonin synthesis and changes in body temperature rhythms are also noted. Unlike young adults, elderly people have an earlier usual time of sleeping and awakening and sleep disturbances (Yoon et al. 2003). This has been linked to inadequate pineal secretion rhythmicities in the aged pineal gland in older adults. In older animals, this is also linked to a reduction in thyroxin, thyroxin-releasing hormone (TRH), and thymus secretion (Rezzani et al. 2020). There is a shred of evidence that pineal calcification with aging inhibits melatonin secretion

affecting immunomodulation and metabolic balance (Tan et al. 2018). Hence, it is imperative to rejuvenate the pineal gland for its normal endocrine function to control the aging process initiated due to poor melatonin and pineal secretion in the calcified gland. Melatonin regulates other cellular functions that control cell death, thereby aging, viz., mitochondrial function, free radical generation, apoptosis, anti-inflammatory function, etc. (Mattam and Jagota 2014; Hardeland 2017; Subramanian et al. 2021; Xie et al. 2021). Pineal secretions up-regulate aging suppressor sirtuin-1, which resulting better mitochondrial metabolic function and circadian rhythm.

Hence, re-normalizing the circadian clock may improve health and longevity, while disrupting the clock may cause associated medical and mental dysfunctions. So, resetting circadian clocks would help synchronization in physiology and metabolism and then increase longevity and overall health. Some studies reported resetting of circadian clocks through changing feeding regimes (Froy 2011). As a result, maintaining pineal endocrine secretions is beneficial to homeostasis and longevity.

Recently, it is stated that the pineal gland secretes neurosteroids, e.g., 7α-hydroxypregnenolone, estradiol-17β, testosterone, etc., in circadian rhythm and controls age related physiological activities (Tsutsui et al. 2018).

## 11.3 Changes in Sleep Pattern with Aging

Sleep is a vital physiological process that contains important curative activities necessary for optimal daytime functioning. Inadequate or poor-quality sleep has also been linked to chronic health problems and end-organ dysfunction, including an increase in mortality rates and aging (Verstraeten 2007; Punjabi et al. 2009; BaHammam and Pandi-Perumal 2010). Several physiological changes occur during normal aging. This includes sleep quality (subjective as per self reports and objective, as per polysomnographic or other diagnostic devices findings), sleep quantity, and sleep intensity. Age-associated alterations in sleep include, but are not limited to, duration of sleep and waking, the timing of sleep onset, the overall efficiency of sleep maintenance, alteration in sleep staging (a polysomnographic finding), and daytime sleep behaviors (Pandi-Perumal et al., 2010). Aging is associated with increased light (NREM Stage N1 and N2 sleep) and decreased deep (NREM Stage N3 sleep) (refer, Table 11.1). Increased frequency of unprompted arousals is also reported (Edwards et al. 2010).

The process of aging is often associated with qualitative and quantitative changes in terms of sleep/wake patterns and their robustness. The sleep period in infancy, for example, is at an all-time high, with newborn children napping for about 16 h almost every day. This need for sleep decreases throughout development eventually resulting in 7–8 h in adults. Though less widely studied, there is evidence that sleep duration decreases from young adulthood through the later years of life in humans. Other studies, however, show that sleep quantity does not change with age; rather, sleep in aging is highly fragmented and is frequently consolidated during daytime naps. Various factors, vision-related issues, including inadequate natural light exposure,

**Table 11.1** Sleep changes that occur during normal aging

| Sleep-related changes that occur as a result of normal aging | |
|---|---|
| i | Circadian changes, e.g., amplitude reduction, acrophase becomes labile |
| ii | Advanced sleep timing, i.e., early bedtime, early morning awakening |
| iii | Circadian dysregulation, e.g., Advanced sleep phase syndrome (ASPS) |
| iv | Decrease in the ability to sleep:<br>a. Sleep fragmentation, i.e., increased a number of nocturnal awakenings<br>b. Prolonged nocturnal awakenings (lack of consolidation) |
| v | Increased sleep onset latency (SOL) |
| vi | Increased sleep fragmentation, i.e., less consolidation, more awakening, increased arousals, and increased transition to lighter sleep stages N1 and N2 |
| vii | Increased time spent in lighter and fragile sleep (NREM sleep stage R1 and stage R2; easily woken by external stimuli) |
| viii | Decreased slow-wave sleep (SWS; deep sleep or NREM Stage N3)<br>Advanced sleep timing (going to bed too early) |
| ix | Reduction in overnight sleep |
| x | Increased daytime nap frequency |
| xi | Increased wake after sleep onset (time spent awake throughout the night) |
| xii | Decreased overall nocturnal sleep duration |
| xiii | Reduced and fewer NREM-REM sleep cycles and other related changes |

rising health concerns, and an alteration in circadian zeitgebers, have been proposed as processes causing poor sleep quality in the aged individuals (Pandi-Perumal et al. 2010; Kun et al. 2018). Additionally, the incidence of sleep-related problems, which are becoming more common among the aged society, is a significant contributor to poor sleep quality (Figs. 11.1 and 11.2).

## 11.4 The Relationship Between Aging Physiology and Circadian Rhythm

Aging is typically linked with dwindling or disorganization of the circadian system. The circadian acrophase becomes highly displaced, tending to happen in advance with progressing age. The participation of clock genes in the aging physiology as they are involved in an assortment of disease processes is also noted. Current work has been giving insights into the underlying molecular pathways associated with aging physiology, with the assurance of involvement(s) to augment healthy life spans. Caloric constraint, which is constantly and recurrently connected with lengthening life in diverse animal models, is linked with amplified circadian amplitude. These data suggest the decisive significance of circadian biology in comprehending aging problems, from the circadian clock machinery coordinating metabolism to the progress up to geroprotectors (Arul and Subramanian 2014; Duffy et al. 2015). The quantitative

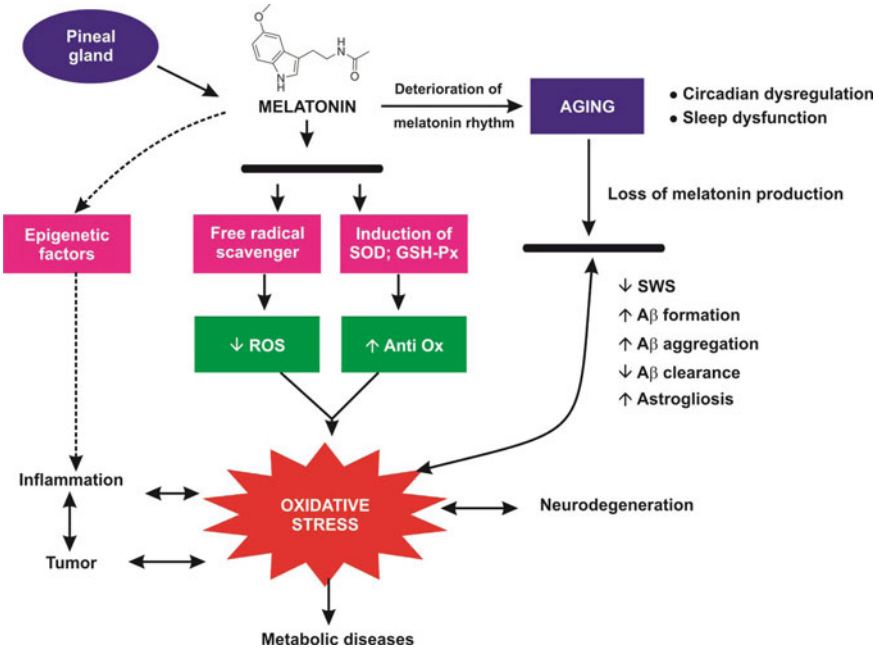


**Fig. 11.1** Neuroendocrine mechanism of aging in mammals

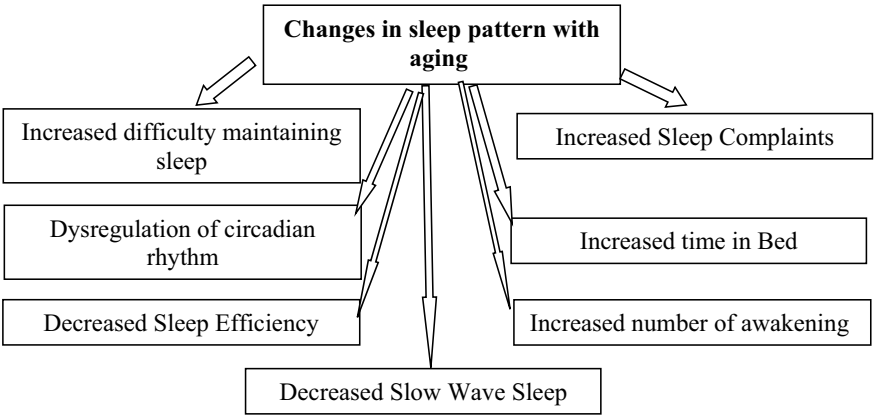


**Fig. 11.2** Incidents of sleep-related problems with aging

inference of circadian rhythm hallmarks construed in light of time-dependent reference values aids in (i) distinguishing influences of normal healthy aging from those associated with disease, disorder, and disease/disorder prognosis; (ii) in identifying changes in rhythm characteristics as indicators of increased risk before the appearance of disease; and (iii) in optimizing prophylactic and/or curative intercessions

aimed at disease/disorder separately. Comprehending the alterations in amplitude and/or acrophase that may outshine any modification in normal value also shuns away depicting counterfeit interpretations resultant from data collected at a fixed clock hour. Appropriate risk recognition, along with the management optimization of the disease by timing (chronotherapy), is the objective of several ongoing widespread population-based investigations focusing on the health of the aged people, with the intention that long life is not accomplished at the outlay of an abridged quality of healthy life (Cornelissen and Otsuka 2017; Martín Giménez et al. 2022).

## 11.5 Aging and Circadian Rhythms

Aging has been shown to disrupt circadian rhythmicity at multiple levels of biological organization (Tabibzadeh 2021; Nathan et al. 2021; Kim et al. 2022). There is a substantial body of literature on age-related changes in the 24 h periodicity in animals. These studies documented age-associated variations in the circadian behavioral rhythms (e.g. amplitude of numerous neuronal, endocrine, and metabolic rhythms) have been documented in the literature, and discuss how the circadian clock drives these rhythms (Acosta-Rodríguez et al. 2021). However, there are still some inconsistencies (Pohl 1993). Age-associated alterations in circadian pacemakers have been investigated in both humans and other animals. Such studies include, but are not limited to morphological, behavioral, as well as electrophysiological investigations. This points to the fact that older individuals may be due to a lack of *zeitgebers* (time clues or time givers), especially if they are housebound or institutionalized.

As outlined above, the circadian clocks become weaker and often damped or phase advanced during aging. This is further evident with regular or routine adjustments in routine bedtimes and arousing times in the older individuals. These changes denote, the phase progression of the sleep–wake cycle, and these alterations could be either by a slowing or a quickening of the circadian pacemaker (suprachiasmatic nucleus, SCN). This phase moves forward phase could be linked to amplitude attenuation and phase advance in the core body temperature (cBT) rhythm. Furthermore, a reduction in endogenous period length for waking and paradoxical sleep (PS) is one of the most significant age-related changes in a temporal structure, and phase advancement or dampening of hormonal and other overt rhythms may lead to a disease state or altered physiology (Morris et al. 2016).

The phase-reversal mechanism in older animals also varies. For example, aged animals respond to a phase-reversal of the LD cycle slower than younger animals. During the day, there is a common tendency for sleep loss. Because older animals sleep less during the light phase of the LD cycle, the majority of the age-related decrease in total sleep time (TST) is due to selective sleep loss).

## 11.6 Modifications in Circadian Rhythms with Age

As organisms age, rhythmic processes also undergo a variety of systematic changes. While these changes may be regarded as generic representations of normal aging, it is clear that within a species, individual variations in the way aging occurs exist. As a result, many natural, distinct progressions toward disorder within circadian systems manifest themselves as increases in the standard deviations (SD) of their measured values. Several changes in overt rhythmicity appear to be linked to aging. Some of these have been linked to a loss of SCN function, while others may be the result of a decline in either entrainment mechanisms or clock-controlled systemic activities.

(a) Changes in overt circadian patterns include amplitude reduction, rhythm fragmentation, and temporal order disruption
(b) Loss of entrainment stability and sensitivity to zeitgebers. Besides, the clock-controlled process itself is likely to change. For example, changes in the volume or intensity of specific activities, the distribution of different behaviors, the amounts of circulating hormones, and the density of specific peptides, neurotransmitters, and receptors; and.
(c) Changes in period or period stability.

## 11.7 Amplitude and Circadian Organization

Losses in "stability" and level of rhythmic function are reflected in amplitude reductions. Numerous studies have looked at the link between rhythm abnormalities and aging. Earlier research has discovered changes in circadian hormonal rhythms. As rodents get older, many studies have found that their wheel-running activity deteriorates. Humans have also been reported to have age-related changes in locomotor activity. A decline in the amplitude of other behavioral rhythms such as feeding, drinking, and sleep/wake is also connected with aging (Hennion and Etain 2022). Aging has an impact on other physiologic rhythms in a similar way, e.g., body temperature rhythms (mice and rats), audiogenic convulsions (mice), oxygen consumption (mice), potassium excretion (humans), growth hormone (GH), testosterone, and luteinizing hormone (LH) (humans). There is a report on altered diurnal rhythms of blood cortisol, aldosterone, prolactin, and GH in older humans. The sex difference was also noted. The circadian amplitude and mesor of epinephrine and norepinephrine are decreased with age while the acrophase remained constant (Halberg 1982). There has been a decrease in the rhythm of pineal N-acetyl transferase in hamsters (Reiter et al. 1980). One of the difficult questions to answer is whether the decline in the amplitude of overt rhythms reflects a shift in circadian pacemaker activity or an age-related loss of peripheral function. When compared to young rats, Satinoff and co-workers (1993) found that the pacemaker of older rats had disrupted patterns and lower amplitude of neuronal activity, without disturbing behavioral rhythms. In response to LD transitions, Wise et al. (1987, 1988) found a reduction in glucose utilization in suprachiasmatic nucleus (SCN) tissues in aging rats.

Furthermore, there have been numerous reports of age-associated morphological and neurochemical alterations in the SCN, including alterations in cells producing vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) (Roozendaal et al. 1987). Although these changes do not always correspond to explicit changes in behavior and physiology. There are adequate variations in the SCN of younger and older animals which advocate an association between dysregulation of SCN and changes in circadian patterns. Furthermore, the re-consolidation of host-driven locomotor rhythmicity in aged hamsters following transplantation of SCN demonstrates its vital role in maintaining organization and rhythmicity during the aging process (Murd and Ralph 1998).

A lack of synchronization or incorrect phase connections among rhythms is a predictable result of reduced rhythm amplitude. The major purposes of biological clocks, according to popular belief, are to elicit a time-oriented structure within rhythmic processes and to synchronize them to the geophysical environment. As a result, it's safe to assume that this organization will be jeopardized if the clock or its control mechanisms fail. Alteration in time-oriented structure (e.g., biochemical, physiology, and behavior) is a common sign of disorganization. Rhythms remain in sync with one another, yet they may have incongruent or changeable phase relationships. In humans, such a form of the disorder has been thoroughly established.

In summary, several rhythms have shown age-related changes in amplitude, including the rest/activity cycle, core body temperature (cBT), feeding, drinking, eating, and response to zeitgeber (e.g., LD cycle non-photic zeitgeber) (Mohawk et al. 2019). However, differences in the amplitude of circadian cycles could not be explained only by age-related changes in visual sensitivity. Similar to the diminished LD disparities in sleep/wake rhythms, behavioral rhythms have lower amplitudes.

## 11.8 Entrainment and Responsiveness to *Zeitgebers*: Influence of Aging

When compared to young adults, old persons' sleep/wake patterns become disordered and varied. A lack of organization in a light cycle could be caused by either a malfunctioning clock or a drop in sensitivity or response to *zeitgebers*. For most organisms, the environmental LD cycle serves as a pervasive and prominent zeitgeber. Other rhythmic features of the geophysical environment may also serve in this capacity (Amir and Stewart 1998). Additionally, non-photic zeitgebers will indirectly alter rhythms and serve as a potential zeitgeber (Mrosovsky and Biello 1994; Mrosovsky 1996).

Entrainment to light cycles, which is the ultimate measure of overall circadian activity, is affected by changes in the period, photoreceptor sensitivity, and circadian function. Entrainment, on the other hand, is regulated by the organism's acute light reactions, which may mask circadian gating. The most basic experiments that look at circadian reactions to external stimuli are re-entrainment and phase-shifting

paradigms. Unfortunately, while these reactions change with age, they are not consistent among species or even within experiments. Peng et al. (1980) and Peng and Kang (1984) showed no difference in the rate of re-entrainment between young and old rats. Rosenberg et al. (1979) found that older rats took longer to respond to a phase-reversal of the LD cycle than younger rats. In 1992, Zee and co-workers observed that young hamsters take longer to re-entrain to a phase advanced light cycle but take less time when the cycle is delayed, whereas Valentinuzzi et al. (1997) reported that in old mice, re-entrainment is accelerated when the cycle is advanced but unchanged when the cycle is delayed. Finally, light-induced phase delays rise in old rats but decrease in mice, and light-induced phase shifts decrease in old hamsters, but this change can be reversed by powerful light pulses (Zhang et al. 1996). The reasons for these inconsistencies are unknown, given the variety of species used and the fact that experimental conditions differ from lab to lab, this mismatch may not be surprising. Variations are more than likely related to individual differences in how animals age. Some hamsters lose their highly consolidated pattern of wheel-running activity as they get older, while others keep it (Antoniadis et al. 2000). Because activity influences circadian responsiveness to light, as well as the phase and duration of rhythms, aging may have varying effects on rhythmicity as a result of changes in wheel-running patterns. Age has an impact on non-photic cue reactions as well. Phase shifts induced by a serotonin (5-HT) agonist or the benzodiazepine (BZD), triazolam, are reduced in aged hamsters, and a prenatal SCN transplant and a melatonin agonist can restore the latter effect. Further, melatonin can also assist you in readjusting to a new light cycle (Weibel et al. 2000).

## 11.9 Age-Associated Changes in Circadian Dysregulation

There are numerous differences in age-related pineal secretions and physiological changes in period length to be noticed (Reiter et al. 1981). The most significant age-related changes in circadian behavioral rhythms are seen in the free-running and entrained rhythms. The amplitude of many intrinsic rhythms of metabolic and physiological indices decreases as people get older, with an apparent decrease in the rhythm's maxima, viz., body temperature cycles (mouse and rat), audiogenic convulsions (mouse), cellular oxygen utilization (mouse), excretion of potassium (humans), and secretion of growth hormone (human), testosterone (human), and luteinizing hormone (human) (Davis 1981; Sehirli et al. 2021).

Age-related defects in the circadian organization are linked to changes in the association between endogenous and ambient rhythms. Circadian rhythms are "free-run" in permanent darkness, with an intrinsic period (Tau) slightly longer or shorter than 24 h, and in humans, it is between 24.2 and 24.4 h. As the age advances, Tau gets shorter slightly. Similarly, the free-running duration in rats decreases from adolescence to old age. There are a variety of viewpoints on these changes. Several investigations found shorter periods, while others found more extended periods. On

the other hand, other researchers have claimed that no alterations have occurred (Sharma and Chandrashekaran 1998).

However, experimental, technical, and methodological variations such as age differences, exposure to earlier entrainment, and potential feedback response could skew the results. Further, experimental setup, time of the experiment, or observation time (e.g. LL vs DD) can also influence the results. Therefore, due to such a wide range of confounding factors, making any clear judgments about age-related alterations in circadian systems is not that easy. Proper caution should be exercised during the interpretation of the findings.

## 11.10 Conclusions

Throughout animals' and humans' lives, the pineal gland plays a critical part in the circadian timing system and maintaining homeostasis and biological clock. Melatonin levels decline with age, and in neurodegeneration, there is a marked reduction in this hormone. Melatonin has both chronobiotic and cytoprotective (antioxidant and neuroprotector) effects (Cardinali 2019). Melatonin, as a chronobiotic, can alter the phase and amplitude of biological cycles. Melatonin, as a cytoprotective molecule, prevents the low-level inflammatory damage found in aging and neurodegeneration. Administration of melatonin reset the circadian dysregulation, promotes sleep, reduces sundowning, and delays the course of cognitive deterioration in neurodegenerative disorders. Recent evidence suggests that melatonin effectively protects neuronal cells against Aβ-mediated toxicity through antioxidative defense and antiamyloid properties. Melatonin not only suppresses Aβ production, but also stops the development of amyloid fibrils through a structure-dependent interaction with Aβ. More research on the use of melatonin in the treatment of various disorders is needed, particularly at an early stage of neurodegenerative diseases.

This chapter provides and overview of some of the aspects of circadian rhythms, and its relevance to aging and neurodegeneration (Fig. 11.2. Interaction between pineal gland, aging, and sleep).

Over the years, several theories have been proposed, and recent research on pineal secretions revealed that they slow aging by reducing mitochondrial processes and physiological body defense mechanisms. For clear understanding, readers are encouraged to refer the accompanying chapters in this volume and related publications. In summary, a significant need for the study on the disruptors of the physiology of pineal, its immunomodulatory functions, and aging in variety of species should be conducted to determine their translational potential in gerontology and enhancing longevity.

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# Chapter 12
# Circadian Rhythmicity in Aging and Parkinson's Disease


**H. J. Jyothi, Bidisha Bhaduri, Maithily Hingmire, Priyansha Verma, T. C. Yasha, and Phalguni Anand Alladi**

## 12.1 Basal Ganglia

The group of nuclei that are encased within or considered a part of the basal ganglia (BG) extend from the forebrain to midbrain and comprise of striatum, globus pallidus (GP), subthalamic nuclei (STN), and substantia nigra (SN). The striatum is the neuroanatomical cooperative of the sub-cortical regions known as caudate and putamen, and they harbor 90–95% of medium spiny neurons (MSN), which are their functional principal neurons. SN on the other hand is the hub for the dopaminergic neurons, wherein the soma is localized primarily in the pars compacta (SNpc) region, while the pars reticulata is the output nucleus. The age-related neurobiological changes in the substantia nigra pars compacta have been studied (Alladi et al. 2009; Alladi et al. 2010a, b; Jyothi et al. 2015; Naskar et al. 2019) and reviewed extensively elsewhere (Reeves et al. 2002; Stark and Pakkenberg 2004).

Classical studies on post-mortem brains and experimental animal models divulge the heterogeneous nature of BG disorders, although the same anatomical loci within the BG are involved (Albin et al. 1989). The manifestations can be of either hyperkinetic nature, characterized by the excess, rapid, and uncontrollable movements

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seen in Huntington's disease (HD), tics, ballism (Carpenter et al. 1950), or hypokinetic, which involves akinesia, bradykinesia, rigidity, mostly noted in Parkinson's disease (PD), and dystonia (Jankovic and Rohaidy 1987). These hyper or hypokinetic movements are incumbent to the alterations in the striatal projection neurons and/or destruction of cellular repertoire of the subthalamic nucleus. Most clinical therapies available currently for PD, HD, etc., partly alleviate the symptoms and offer no cure to prevent or curtail the disease progression. The next section deals with PD, a debilitating age-associated disorder.

### *12.1.1 Parkinson's Disease: A Major Basal Ganglia Disorder*

Among the basal ganglia diseases, Parkinson's disease (PD) is the most common disorder. Understanding etiopathology of PD also gains relevance, since it is the second most common neurodegenerative disorder, after Alzheimer's disease (AD) with an incidence of 8–18 per 1,00,000 people/year. Its incidence increases manifolds after the age of 60, affecting nearly 1% of the population above 60 (Alves et al. 2008; Poewe et al. 2017).

PD is characterized by the loss of dopaminergic neurons in the SNpc. By the time of manifestation of initial motor symptoms, nearly 30% of the dopaminergic neurons are lost (Fearnley and Lees 1991). A 50–60% loss of SNpc neurons brings about a loss of 80–90% of tyrosine hydroxylase (TH) immunoreactive (IR), i.e., dopaminergic terminals in the striatum, which dominoes into specific manifestations like shuffling gait, tremor, and rigidity—the critical features of the disease. These were described in the original "An essay on the shaking palsy", wherein James Parkinson identified the cardinal motor triad of bradykinesia, rigidity, and tremor. Thus, the symptoms of PD are classified into motor and non-motor symptoms; the latter involve sleep and cognition and often arise during the earlier stages of the disease. Some of these also found a mention in the original essay. Recovery in PD is currently improbable and unyielding to even sustained treatment for decades.

### *12.1.2 The Neuroanatomical Basis of Parkinson's Disease*

The neuroanatomical aspects of PD are very complex, since they involve several parts of the brain and not just the basal ganglia. It is equally pertinent that multiple cellular factors contribute to the dopaminergic neuron degeneration, viz. the formation of Lewy bodies, oxidative stress, microglial activation, release of pro-inflammatory cytokines, mitochondrial dysfunction, etc. Braak and colleagues developed a six-leveled staging method to propose the origin and spread of the disease by assessing the magnitude of α-synuclein (α-syn) containing Lewy neurites and bodies to propose the seminal "Braak hypothesis". As per the hypothesis, Stage 1 was associated with α-syn lesions in the dorsal motor nucleus of the vagus (DMV), olfactory bulb, and anterior

olfactory nucleus, which manifest as loss of olfaction and autonomic dysfunction (Braak et al. 2003a, b). As per their evidence, α-syn deposits follow a rostro-caudal pattern of accumulation in the digestive tract, exhibiting maximum concentration in submandibular gland, and is much less in the colon (Fasano et al. 2015). DMV is the crucial junction for the passage of phosphorylated α-syn deposits from the digestive tract to the CNS (Braak et al. 2003a, b; Fasano et al. 2015). By stage 2, the disease spreads to lower brain stem nuclei including raphe nucleus, locus coeruleus, and pedunculopontine nucleus resulting in abnormal sleep-associated patterns and sleep disturbances. Thus, the stages 1 and 2 are mainly related to non-motor concerns.

By stage 3, the basal ganglia and other midbrain nuclei are affected; consequently, the motor symptoms begin to appear, owing to the dopaminergic deficits that deregulate the direct and indirect components of the nigrostriatal pathways. The involvement of mesocortex is considered as stage 4, leading to worsening of motor symptoms, and the disease becomes "clinically evident". By the stages 5 and 6, the higher-function areas of the neocortex are involved, causing cognitive deficits (Braak et al. 2003a, b).

## 12.2 Circadian Rhythmicity in Aging and PD

### *12.2.1 Role of BG and Clock Genes*

Circadian rhythm is facilitated by transcriptional and translational regulation of "CLOCK" (circadian locomotor output cycles kaput) genes and their proteins. Vitaterna et al. (1994) discovered mutations in "CLOCK" gene that disrupted the mammalian circadian rhythm. Those belonging to the molecular CLOCK gene family are termed as "circadian locomotor output cycles kaput", i.e., (Clock), brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (Bmal1, Arntl), cryptochrome 1 and 2 (Cry1, Cry2) and period 1, 2, 3 (Per1, Per2, Per3), rev-erbα (Reppert and Weaver 2002), etc., and their proteins encode time via interlocked transcriptional-translational feedback loops (TTFLs). The biological clock is not controlled exclusively by the suprachiasmatic nucleus (SCN). Complex neuronal and non-neuronal extra-SCN oscillators exist in hippocampus, amygdala, paraventricular thalamus, arcuate, and dorsomedial nuclei of the hypothalamus that contribute to the clock control (Guilding and Piggins 2007, a review). The extra-SCN oscillators are also known as "slave oscillators".

Age-associated sleep changes support the possibility of a link between behavior and circadian disruption (Hood and Amir 2017). Aged individuals exhibit difficulty falling and staying asleep (Foley et al. 1995) in addition to increased sleep fragmentation (Yoon et al. 2003; Duffy et al. 2002), resulting in increased daytime drowsiness and napping (Carskadon et al. 1982). This circadian rhythm misalignment in older people affects the "homeostatic drive for sleep" and disrupts sleep promoting signals like melatonin and body temperature (Dijk et al. 1999). A 37% loss of melatonin in

elderly men during sleep shows a significant effect of age and melatonin on circadian homeostasis (Zeitzer et al. 2007).

Using an aging mice model, Wyse and Coogan (2010) performed 4 hourly gene expression studies for clock and Bmal1 for a period of 24 h in non-SCN brain areas. In their observation, age affected the amplitude and patterns of expression of Bmal1 and clock significantly in the non-SCN regions, but spared the CA3. On the other hand, the "clock expression pattern" turned rhythmic in older animals, as opposed to being constitutively expressed in the young. The acrophase, i.e., the phase of peak expression, also shifted toward the late evening (Wyse and Coogan 2010). Interestingly, the slave oscillators showed diurnal pattern, and with the onset of aging, these areas lost their ability to receive signals from the SCN. The most serious consequence of circadian dysfunction was gathered in view of increased mortality of aged animals (Davidson et al. 2008). It is independent of stress and explains why aged population adapts poorly to sudden time zone shifts. Potentially with age, the "slave oscillators", controlling the circadian rhythm in peripheral tissues, lose their ability to receive entrainment from SCN.

Wynchank et al. (2019) reported that a dysfunctional biological clock caused age reminiscing effects and preceded neurodegenerative states. They earmarked reduction in telomere length in aged individuals afflicted by delayed sleep phase syndrome, as deterioration of cellular health. Earlier findings equated aligned circadian rhythm with the quality of life (Hurd and Ralph 1998). Implantation of SCN cells of young animals improved the lifespan and behavioral patterns of the aged recipient (Li and Satinoff 1998).

Zhang and colleagues studied the age-related changes in DNA methylation frequency. They reported that Bmal1 promoter in the stomach was methylated in the majority of animals, while its expression was suppressed in the aged rodent striatum (Zhang et al. 2013). Deletion of Bmal1 was also responsible for age-related astrogliosis in the cortex and hippocampus. Synaptic terminals were damaged with loss of cortical connectivity and oxidative damage to cells. Upon treatment with a mitochondrial complex 3 inhibitor, Bmal1-deficient hemizygous mice developed striatal degeneration (Musiek et al. 2013). Thus, Bmal1 dysfunction leads to circadian arrhythmicity (Kondratov et al. 2006; Duncan et al. 2013), and DNA methylation disrupts the clock gene expression (Taniguchi et al. 2009). Rev-erbα is circadian modulator having a direct role in the transcription of both cyclic Bmal1 and CLOCK (Crumbley and Burris 2011). The clock gene rev-erbα loses its daily fluctuations in MPTP mice model of PD. The diurnal variations in microglial immunoreactivity are also affected. This signifies the contribution of rev-erbα toward microglial activation and elevated neuroinflammation. Use of a rev-erbα agonist small molecule improved the dopaminergic terminals in the striatum of MPTP mice (Kou et al. 2022). These observations are very similar to those by Griffin et al. (2019) and Kim et al. (2018).

Esquifino et al. (2002) employed multiple time points and demonstrated a fall in dopaminergic activity, DA turnover, and GABA expression at middle age, in aged rodents. They suggested that age-related changes in striatal function are dependent on an intact internal clock, incongruence with earlier reports on serotonergic sprouting during damage to the dopaminergic system (Bédard et al. 2011). They also showed an

increase in serotonin turnover with age. Detailed investigations showed a reduction in the duration of slow-wave sleep, which is otherwise crucial for memory consolidation, emphasizing the role of circadian clock in dementia (Dijk et al. 2010). Studies thus far suggest a close involvement of basal ganglia in sleep.

### *12.2.2 Sleep and PD: The Intriguing Prelude*

The rodent striatum is divisible into the caudate-putamen and ventral striatum. The latter also comprises the NAc. Ibotenic acid-induced lesioning of the striatum indicated a significant association of the dorsal striatum in controlling wakefulness (Qiu et al. 2010). Further studies using modafinil showed that NAc participated in inducing and sustaining sleep (Qiu et al. 2012). In view of the prominent association of BG in sleep induction and sustenance, deficits in this region may well be seen in PD, as well as other BG disorders. Relevantly so, sleep-associated non-motor deficits, like insomnia, frequent nighttime awakening and sleep fragmentation, nocturia, restless legs syndrome, sleep breathing disorders, drug-induced symptoms, parasomnias associated with REM sleep, sleep attacks, and excessive daytime sleepiness (Raggi et al. 2013), and even vocalizations, (Chaudhuri et al. 2006) are common in the patients.

### *12.2.3 Rapid Eye Movement (REM) Sleep Behavior Disorder (RBD)*

RBD is denoted by abnormal behaviors and loss of muscle atonia during the REM phase of sleep (Postuma et al. 2015). It is commonly observed in synucleinopathy patients such as PD, dementia with Lewy bodies (DLB), and multiple system atrophy (MSA). Although a tight association was earlier proposed with synucleinopathies, emerging evidence points at occurrence of RBD, even in the non-synucleinopathic cluster of neurodegenerative diseases such as AD, HD, and amyotrophic lateral sclerosis (ALS). Experimental evidence suggested the presence of a reciprocal connectivity between SNpc, VTA (ventral tegmental area), pedunculopontine nucleus, and reticular formation which influence the REM sleep behavior (Lima 2013). Polysomnography-based findings suggest that oculomotor abnormalities seen in RBD including insomnia, movements during sleep, and daytime sleepiness (Arnulf et al. 2008) were common, but poorly studied aspects of HD (Annapureddy et al. 2021).

In PD, the RBD symptoms do precede the onset of motor symptoms by years. RBD patients show reduced DAT density and reduced dopamine uptake in the striatum, suggestive of early stages of PD pathology (Eisensehr et al. 2000). In an earlier study, almost all PD patients were plagued by sleep disturbances, which reflected in the

involvement of dopaminergic system in REM sleep-related structures (Steinfels et al. 1983). In a 10-year follow-up study on 89 patients, 30% of the subjects developed PD and other neuropathologies after 3 years, up to 66% after 7.5 years, whereby the authors proposed it to be a potential biomarker for preclinical diagnosis of PD (Postuma et al. 2006).

It is theorized that Lewy body deposition in certain areas of the lower brainstem, possibly pedunculopontine nucleus and sub-coeruleus nucleus, is the underlying pathology in RBD (Braak et al. 2003a, b). In a study on PD patients with and without RBD, a significant reduction in gray matter volume was observed in thalamus of PD-RBD patients indicating thalamic involvement. A positive correlation between RBD, cognitive impairment in PD, and greater α-syn load has also been established (Vendette et al. 2007; Postuma et al. 2015; Gong et al. 2014). These patients also show greater incidence of hallucinations (Vibha et al. 2011; Gong et al. 2014).

Optogenetics and fiber-photometry-based studies in animal models revealed that orexin-enhanced sublaterodorsal tegmental nucleus (SLD) output prolongs REM sleep episodes by consolidating brain state activation or muscle tone inhibition. This is disrupted upon chemogenetic silencing of SLD orexin signaling. Thus, orexin is a stabilizer in REM sleep (Feng et al. 2020). Studies on MPTP-treated marmoset opened up new avenues for quantitative research involving mechanisms and treatment strategies for RBD and the premotor phase of Parkinson's disease. Unlike mice and rats, which have nocturnal preferences and fragmented sleep patterns, marmosets are diurnal and their night sleep architecture matches well with humans. It comprises a cyclical pattern of light, deep, and REM sleep (Verhave et al. 2011). Mizrahi-Kliger et al. (2020) suggested that the synchronous cortico-BG β oscillations modulate destabilization of slow oscillation and insomnia during sleep, analogous to the creation of hypokinesia during wakefulness, thereby governing two apparently separate manifestations of PD.

In humans, positron emission tomography (PET) and magnetic resonance imaging (MRI) studies have shown reductions in cerebral blood flow in the striatum, while transiting to NREM sleep, as well as an increase in REM compared to NREM sleep, especially in the posterior part of putamen and caudate (Kaufmann et al. 2006; Braun et al. 1997). Electroencephalogram (EEG) and functional MRI (fMRI) studies in young and older individuals reported decrease in network connectivity during. Though in the older age group, a lower decrease in connectivity was observed while in some cases, increase was also noted in thalamo/basal ganglia connectivity. This could indicate a suppression in the older ages to disconnect causing lighter and more fragmented sleep, and leading to deleterious effects of age on brain plasticity (Daneault et al. 2021).

### *12.2.4 Stress*

One of the several ways that the neurons respond to cellular stress is by induction of heat shock protein (HSP) expression. These are chaperons that help degrade or

re-fold misfolded proteins as also help in apoptosis. In aging female rats, striatal heme oxygenase 1 (HO1) and Hsp40 were higher at middle age than at the oldest age studied, whereas Hsp60 was higher in the older animals. Hsp25 was elevated with advancing age in both nigra and striatum. Hsp25 also co-localized with tyrosine hydroxylase in nigral neurons. Age-related increase in Hsp25 may well indicate an endogenous adaptation to combat cellular stress (Gleixner et al. 2014). Their functional role toward cellular stress was reinforced in a finding of significant positive correlation between decrease in glutathione and an increase in Hsp72, during aging (Calabrese et al. 2004). HSP72 was significantly reduced with age in a rodent model (Gupte et al. 2010).

One of the most debilitating manifestations of stress is that of perinatal stress (PRS). PRS mice demonstrated a longer LTP during postnatal day (PND) 12–60 unlike in control which lasted from PND 12 to 14. Recovery of LTD was noted with dopamine 2 receptor (D2R) agonist quinpirole. In adult PRS mice, it also improved the behavior. Untreated adult PRS mice show downregulation of D2R, excess DNA methyltransferase 1 (DNMT1), increased binding of DNMT1 to D2R promoter, and hypermethylation at D2R promoter in the striatum. A DNMT1 inhibitor could successfully restore striatal synaptic plasticity via D2R-mediated dopamine signaling. This study clearly suggests that the effects of early stressors on striatal health run well into adulthood and senescence (Li et al. 2021).

Reduced dopamine release, along with loss of TH-positive neurons and DAT, was noted at adulthood, when rats were exposed to stress during perinatal period. Further, a reduction in the D2R signaling indicates that the indirect pathway is affected, manifesting in poor motor function. "Perinatal stress" rats had poor striatal and synaptic vesicle function. This study highlights the effect stress has on increasing the vulnerability of the striatum to age effects (Marrocco et al. 2020). Early life stress as modeled by maternal separation rats shows behavioral, olfactory, motor, and gait disturbances (Ren et al. 2022). Enriched environment retarded and eliminated the depletion of 3,4-dihydroxyphenylacetic acid (DOPAC) and HVA, respectively. Enriched environment (EE) also restored D1R and choline acetyltransferase expression to baseline in the nigrostriatal pathway in MPTP model of PD, thus providing direct evidence of the positive outcomes of EE in BG diseases (Hilario et al. 2016).

## 12.3 Factors Ascribing Longevity and Symptom Alleviation in PD

Both aging and PD often affect several aspects of the lives of the subjects and the patients (Fig. 12.1). Realizing the need for addressing these deficits, many studies thus far have applied different modalities to decrease or alleviate specific symptoms or deficits (Fig. 12.2).

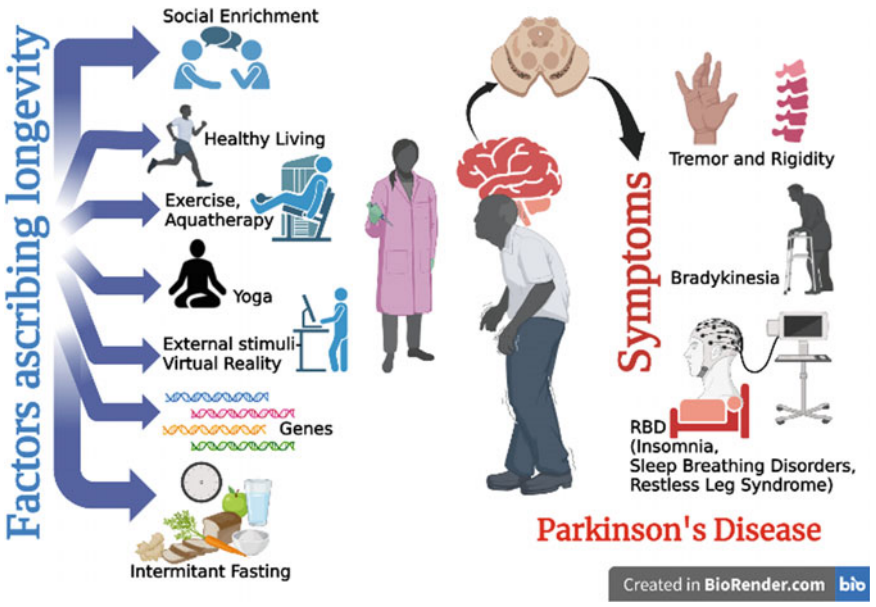


**Fig. 12.1** Pictorial representation of factors responsible for alleviating symptoms and ascribing longevity in Parkinson's disease. Recent researches have shown social enrichment, yoga, virtual reality, genes, exercise training, and food habits to be beneficial in improving PD symptoms (picture created on BioRender.com; concept and creation Bidisha Bhaduri and PA Alladi)

### *12.3.1 Relevance of Social Enrichment*

Social enrichment popularly termed as EE is a common neuroprotective strategy both during development and in the mature nervous system. 6-OHDA brought about a significant loss of the rat dopaminergic neurons; however, postnatal enrichment restricted the cell loss and hypokinesia. In a first of its kind study, postnatally enriched environment prevented PD later in life (Jungling et al. 2017). The aged rats exposed to EE during childhood were better protected against 6-OHDA injections. In mice overexpressing human SNCA gene, EE reduced the pro-inflammatory cytokines in the feces and inflammation inducing genes in the colon indicating a positive effect on intestine by gut microbiota (Singh et al. 2019). EE accelerated motor recovery, prevented short-term memory impairment, and avoided a decrease in striatal brain-derived neurotrophic factor (BDNF) levels in mice (Campêlo et al. 2017). In combination with exercise, EE altered the behavior and cellular morphology in normal and injured CNS (Döbrössy and Dunnett 2006).

MPTP-injected young and old mice exhibited locomotor recovery upon exposure to EE (Goldberg et al. 2011). Despite rapid disease progression, even limited EE improved the rotarod performance of R6/2 mice, whereas maximal enrichment was required to induce any improvement in the behavior of normal littermates. In R6/2 brains, enrichment also delayed the loss of peristriatal cerebral volume, which

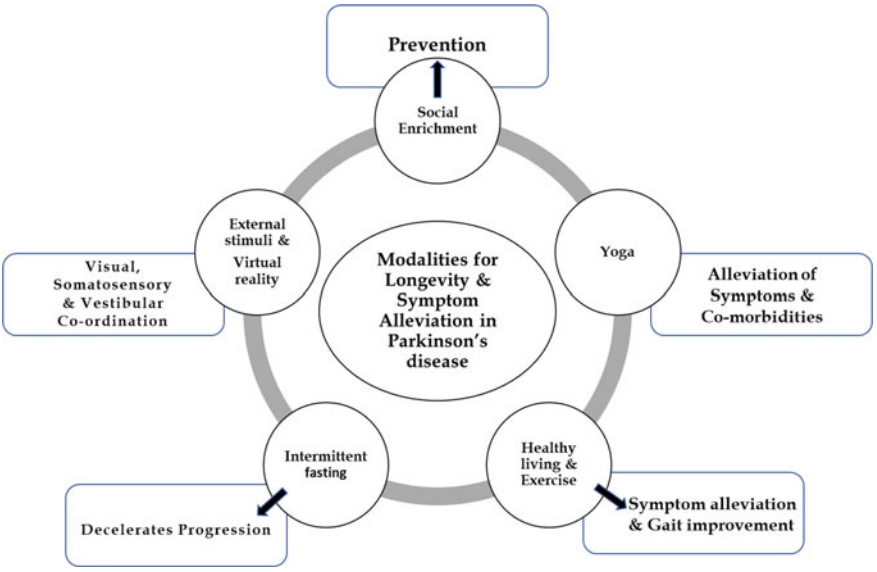


**Fig. 12.2** Proposed interventional modalities for Parkinson's disease and the different aspects that they target: environmental/social enrichment reduced neurotoxin-induced neuronal death in mice model, thus promising prevention. Exercises improved gait and other factors contributing to healthy living. Yoga and intermittent fasting reduced other geriatric co-morbidities like diabetes, hypertension, etc., and increased longevity. Virtual reality improves co-ordination between different somatosensory systems. A holistic approach may need to be tailored for each patient (concept and creation PA Alladi)

suggests that EE ameliorates the effects of HD (Hockly et al. 2002). Few animal studies demonstrated that exposing transgenic HD mice to EE prevented cerebral volume loss and delayed the onset of motor disorders. Similarly, a more stimulating environment or remotivation improved physical, mental, and social functioning in people with HD (Sullivan et al. 2001).

### *12.3.2 Yoga*

This is an ancient Indian practice applying mental and physical faculties along with spirituality. It includes mindfulness protocols applying breathing maneuvers as also physical activities. Yoga involves stretching exercises which activate stretch receptors in muscles, ligaments, and joints thus helping with flexibility and posture (Tran et al. 2001). Many studies demonstrated the usefulness of yoga in maintaining or restoring balance, posture, flexibility, relaxation, agility, physical alignment, strength, and overall physical and mental well-being (Schmid et al. 2010). Although not many studies have applied Yoga in PD, it is held to be therapeutic (Kwok et al. 2017). Tolahunase et al. (2017) studied the effect of yoga and meditation-based

lifestyle intervention (YMLI) on cellular aging in healthy individuals by assessing the biomarkers of cellular aging in blood from baseline to week 12, which isncluded DNA damage marker 8-hydroxy-2′-deoxyguanosine (8-OH2dG), oxidative stress markers reactive oxygen species (ROS), and total antioxidant capacity (TAC), as well as telomere attrition markers like telomere length and telomerase activity, and metabotropic markers cortisol, endorphin, IL-6, BDNF, and sirtuin-1. After 12 weeks of YMLI, significant improvements were noted in both the markers of cellular aging compared to baseline values. Intervention with yoga significantly reduced the rate of cellular aging in the healthy. The effect of age on fluid intelligence and resting state brain functional network architecture in middle-aged yoga and meditation practitioners, and matched controls was studied by Gard and colleagues (Gard et al. 2014). In yoga, practitioners and meditators' fluid intelligence depleted at a slower rate than in controls. Resting state functional networks in practitioners and meditators were more integrated and resistant to damage. Therefore, mindfulness positively correlates with fluid intelligence, resilience, and global network efficiency. In a landmark preliminary study on meditation, mindfulness, and cellular aging, Epel et al. (2009), proposed "that some forms of meditation may have salutary effects on telomere length by reducing cognitive stress and stress arousal and increasing positive states of mind and hormonal factors that may promote telomere maintenance".

### *12.3.3 Healthy Living and Exercise*

Collective evidences suggest that exercise reduces the risk of PD by inhibiting oxidative stress, repairing mitochondrial damage, and promoting the production of growth factors as well as controlling other geriatric co-morbidities such as diabetes, hypertension, and cardiovascular disease. It positively influences both motor and non-motor symptoms in PD. It is proposed that gait velocity decreases during forward and backward walking, and that otherwise persists even with the best medications. Furthermore, in the early phase of the disease, reduced gait, balance, and mobility can be identified, and a further deterioration in gait signals the start of impairment. These considerations emphasize the need for combining medications with modalities like exercise, in PD (Rawson et al. 2019).

Exercise training is feasible in HD patients, specifically in the early-to-middle stages of disease, although one study proposed its practicability even in the late stages (Quinn et al. 2016). A subsequent case series in late-stage HD supports the feasibility and benefits of exercise training as well (Fritz et al. 2017). The depressive-like behaviors, in premotor symptomatic female HD mice, were due to a serotonin (5-$HT_{1A}$) autoreceptor dysfunction (Héry et al. 2000), which was corrected with chronic sertraline treatment and physical activity. Other findings suggest that running wields an antidepressant effect on HD mice, independent of hippocampal cell proliferation (Renoir et al. 2012). In a rodent model of HD, improvements in mitochondrial

function were linked to better motor performance on the rotarod test, thus inferring that exercise improves motor behavior by reversing deficits pertaining to the mitochondrial functions (Caldwell et al. 2020).

Contrarily and surprisingly, a rare study on a mouse model of HD suggested that physical activity had no beneficial effects on weight, lifespan, hyperglycemia, Morris water maze learning deficits, hippocampal neurogenesis, neuronal morphology, intranuclear inclusions, or dentate gyrus volume—concluding that exercise is not only "not beneficial" but may even harm the vulnerable nervous system (Potter et al. 2010).

Aquatherapy or hydrotherapy or pool exercise is suitable for rehabilitation and exercise as water provides a challenging, yet safe exercise environment for PD, multiple sclerosis, ALS, and HD patients; however, further evidence is required to make specific recommendations (Plecash and Leavitt 2014). The sweat output (SSwR; sympathetic sweat response) and the cutaneous blood flow (SVR; skin vasomotor reflex) in the hand are various parameters to evaluate the autonomic dysfunction in MSA patients. SSwR results correlated with the cardiovascular autonomic dysfunctions and were absent in about half of the MSA patients. However, the SVR was relatively preserved (Asahina et al. 2003). In patients with MSA, a short duration of physiotherapy was reported feasible and safe and assisted the improvement of the gait performance, vis-à-vis the often ineffective pharmacotherapy (Raccagni et al. 2019).

Tang et al. (2020) compared older adults with 10 years of mindfulness meditation (integrative body-mind training, IBMT) with those who had physical exercise (PE) experience. IBMT group fared significantly higher on dimensions of life quality. They also show better parasympathetic activity, as indicated by skin conductance response and high-frequency heart rate variability. Interestingly, the PE group had lower basal heart rate and greater chest respiratory amplitude. Cortisol concentration, an indicator for stress, was lower in the meditation group. They also have a stronger connection between the dorsal anterior cingulate cortex (dACC) and the striatum at resting state, as well as greater volume of gray matter in the striatum. This study emphasizes on combining exercise and meditation to achieve better health and quality of life in an aging population.

### *12.3.4 External Stimuli and the Imaginary World*

Adaptation to different environments necessitates postural control, in addition to visual, somatosensory, and vestibular responses. The invention of virtual reality (VR) opened up new vistas in the treatment of PD by integrating these aspects. VR prescribes execution of precise goal-oriented tasks, while being used in a completely immersive environment via simple simulations. It primarily builds an image realistic model on the computer, which results in the formation of a simulation environment, and can immerse the patients in this environment via sensing devices (Badarny et al. 2014). This modality is an extension of the external stimuli, provided using

different visual means. External stimuli improve gait in PD patients, without a need to modify their medication regimens. The patients registered an additional increase in speed when supplemented with visual cues, i.e., VR (Suteerawattananon et al. 2004; Nieuwboer et al. 2007), while also providing auditory and somatosensory stimuli. PD patients' brains habitually lag in interactions between the vestibular, visual, and proprioceptive systems, resulting in changes in body biomechanics, which is rebooted and boosted by VR.

When compared to the traditional rehabilitation methods, VR-assisted rehabilitation technology improved the BBS, TUGT, UPDRS3, and FGA scores of PD patients, an important aspect for future research. Furthermore, it is a highly sophisticated intelligent treatment modality, but needs more maneuverability, and easier application in community settings, although it adds fun and enjoyment to patients' recovery.

### *12.3.5 Intermittent Fasting*

Intermittent fasting (IF) is a popular type of dietary pattern, based upon timed periods of fasting with two different regimens, i.e., alternative day fasting (ADF) and time-restricted fasting (TRF; Dong et al. 2020). This dietary pattern is beneficial in slowing down the progression of neurodegenerative diseases like AD and PD (Martin et al. 2006) and also is likely to improve cardiovascular health. A number of hypotheses are proposed to explain the basis for the efficacy of intermittent fasting. For example, calorie restriction increased the dopamine levels in the striatum in aged rats (Portero-Tresserra et al. 2020).

The first one relates to oxidative stress theory (Merry 2004) proposing that IF reduces the energy consumption causing mitochondria to produce lesser free radicals, and this helped obese patients with asthma. IF reduced the levels of inflammatory factors like tumor necrosis factor-alpha as well as oxidative stress including nitrotyrosine, 8-isoprostane, protein carbonyls, and 4-hydroxynoneal adducts. However, ADR raised the levels of the antioxidant uric acid (Johnson et al. 2007).

The second one, i.e., the circadian rhythm hypothesis, proposes that IF, when timed properly, may synchronize with the circadian rhythm to improve cardiac health. Among different TRF regimens, subjects allowed to eat in the middle of the day lost more weight and had better glucose control, lipid levels, and inflammatory responses (Moro et al. 2016), compared to those allowed to have late afternoon or evening intake. They suggested that "beyond 16:00" had no improvement, rather worsening of glucose control, blood pressure, and lipid levels (Carlson et al. 2007; Stote et al. 2007).

The third suggests that a ketogenic state is induced by IF, as evidenced by the rise in β-hydroxybutyrate levels after 6–8 h of fasting in overweight individuals. When ketone levels were detectable, it indicates a switch from fat storage to fat utilization

with decrease in low-density lipoproteins (LDL) and increase in high-density lipoproteins (HDL) levels (Dashti et al. 2006). PD is strongly linked to lifestyle, particularly dietary habits, which have gained attention as disease modifiers. In a study on a Parkinsonian mice model, MPTP depleted propionic acid and isobutyric acid, while it increased butyric acid, valeric acid, and other metabolites. Insulin-sensitizing hormone glucagon-like peptide 1 GLP1 (glucagon-like peptide 1) and metformin, a small molecule drug, are neuroprotective in animal models of PD (Athauda et al. 2017). GLP-1 receptors are involved in the production of cyclic AMP and the activation of the cAMP response element-binding protein (CREB); therefore, GLP-1 receptor agonists improve insulin sensitivity. The amount and frequency of energy intake can impact brain health and vulnerability to diseases like, AD, PD, and stroke. Only a few studies reported the role of the ketogenic diet in the prevention of PD and AD as it reduces appetite and is also unappealing from an organoleptic standpoint, which may further be associated with gastrointestinal side effects and reduction in food intake by elderly people with neurodegenerative diseases, resulting in reduction in the supply of nutrients provided by the diet (Włodarek 2019). Contrary to the above studies, a dramatic quantitative reduction in dopaminergic neurons along with an increase in α-syn accumulation was reported in intermittent fasting in a rotenone model of PD (Tatulli et al. 2018). This was accompanied by elevated excitatory amino acids, inflammatory lysophospholipids, and sphingolipids, indicating more pronounced neuronal damage.

## 12.4 Conclusion

PD being the second most common neurodegenerative disease is a major cause for economic burden on the exchequers of several countries. The disease shows a complex involvement of the basal ganglia and several other cortical and non-cortical structures, leading to motor symptoms. Nevertheless, it also has a long prodromal phase where several non-motor symptoms manifest. In-depth studies reveal that although normal aging and PD share some common pathogenic pathways, the PD pathology is quite unique. The age-related alterations in the striatal medium spiny neurons result in several biochemical and morphological deficits, viz. the loss of spines, neurotransmitter dopamine, dopamine receptors and transporters, post-synaptic density protein 95, and pre-synaptic dopamine markers as well as leading to loss of neurons. It is often noticed that PD patients show loss of circadian rhythmicity affecting their sleep pattern. Sleep disturbance, e.g., the REM behavioral sleep disorder, is the commonest and longest prodrome in the PD patients. Certain modalities for symptom alleviation have been established in recent times through engagements like social enrichment, healthy living, and exercise as well as by use of external stimuli like virtual reality. Despite rapid strides in the research in the field, several aspects still remain elusive.

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# Part IV
# Melatonin, Sleep and Clock

# Chapter 13
# Sleep Hormone Melatonin, Inflammation and Aging


**Yaoyao Xia, Xiaoyan Wu, Zhangzheng Yin, Yikun Li, and Fang He**

## 13.1 Introduction

Aging is a progressive irreversible degenerative change in the structure and function of various tissues and organs with the growth of age, under the influences of many factors such as heredity, mental stress, and environmental pollution (Soto-Gamez and Demaria 2017; Bektas et al. 2018). Aging can be divided into physiological aging and pathological aging. The former refers to the state of natural aging of body function and metabolism over time, for example, protein degradation, tissue atrophy, decreased metabolic rate, and abnormal calcium metabolism (López-Otín et al. 2013; Verkhratsky 2019). The latter refers to the aging state caused by various diseases with the passage of age, like Alzheimer's disease (AD), Parkinson's disease (PD), cardiovascular and cerebrovascular diseases, infection-related diseases, and even cancers (Correa et al. 2018).

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It should be noted that aging is mainly characterized by a weakened immune system accompanied by persistent inflammation, oxidative damage, and accumulation of advanced glycation end products (AGEs) (Basta 2008; Lian et al. 2020). Melatonin is primary secreted by the pineal gland in vertebrates, which is also destined to have a rhythmic secretion that can coordinate adaptive physiology (Cipolla-Neto and Amaral 2018). Moreover, melatonin has anti-inflammatory, antioxidant, and other activities (He et al. 2021, 2022; Bocheva et al. 2022; Xia et al. 2022), thus melatonin is thought to be a potential anti-aging substance. This chapter will mainly describe the roles of melatonin in inflammation and aging, as well as the involved mechanisms.

## 13.2 Aging and Inflammation

Actually, after birth, the body constantly carries out life activities such as nutrient metabolism, resists the threat of exogenous pathogens, removes own damaged components, and keeps the body in a state of balance (Cullum et al. 2020; Helman et al. 2020). The "free radical theory of aging" proposed in the mid-twentieth century (Harman 1956), that is, intracellular metabolism produces oxygen free radicals and leads to accumulated cell damage, which accelerates cell aging and supports the longevity hypothesis (Balaban et al. 2005). In addition to intracellular metabolism, chemical stimuli, heat sources, and ultraviolet radiation in the environment cause oxidative stress in cells (Finkel and Holbrook 2000). However, with the deepening of research, the telomere hypothesis that was proposed later causing aging has gradually been recognized by the public (Aubert and Lansdorp 2008), which links cellular aging with genomic changes and provides new research ideas for human aging and cancer (Aubert and Lansdorp 2008). Of not, as described in earlier, aging is formed by a variety of complex factors, and there are differences in various organisms; thus, it is hard to explain all aging phenomena with one theory currently.

The trend of global aging and the associated diseases during aging have become a challenge that cannot be ignored in the current human society (Partridge et al. 2018). Indeed, as the body becoming aging, the immune system undergoes a corresponding remodeling known as immunosenescence, resulting in long-standing chronic inflammation and weakened immune responses (Lian et al. 2020). Immunosenescence makes the elderly more vulnerable and more susceptible to various diseases. For instance, the coronavirus disease 2019 (COVID-19) pandemic predisposes the elderly, especially those older than 40 s, prone to adverse outcomes such as intensive care unit (ICU) admission or death (Chen et al. 2021a). Specifically, by assessing epigenetic aging in the blood of healthy people, non-severe and severe COVID-19 patients, researchers found that infection with COVID-19 might accelerate epigenetic clock and telomere attrition, promote epigenetic aging, and lead to post-COVID-19 syndrome (Cao et al. 2022).

In addition to changes in the immune status, metabolic disorders in the elderly also drive the progression of inflammation. For example, glycemic disorders in the elderly are prone to type 2 diabetes (T2D), and the imbalance of fat metabolism predisposes

the elderly to obesity and hyperlipidemia (Barbé-Tuana et al. 2020). With altered metabolism, aging organisms are accompanied by accumulation of AGEs, which bind to receptors and exacerbate tissue damage through the nuclear factor kappa-B (NF-κB) signaling pathway (Basta 2008).

Furthermore, aging causes an imbalance in the gut microbiota, which is interestingly gender specific, for example, decreased *Bifidobacterium* and increased *Blautia* and *Roseburia* in aged males, but the opposite was detected in aged females (Ma et al. 2020). The gut microbiome may act through inflammatory signaling, for instance, inhibition of caspase-1 shapes the fecal microbiome, with the increased relative abundances of *Akkermansia* spp. and *Blautia* spp., thereby favoring to lessen inflammation and rebalance the gut microbiota to protect the host (Wong et al. 2016). Studies also found that intestinal *Bifidobacterium* and *Roseburia* were negatively correlated with T2D, and *Blautia* was positively correlated with T2D (Gurung et al. 2020). These similar changes in gut microbiota provide us with interesting speculations about whether the imbalances in gut microbiome of the elderly interact with inflammatory diseases and metabolic disorders associated with immunosenescence. Of course, the relevant conclusions need to be experimentally confirmed.

On the other side, the persistent inflammatory responses in the aging population also exacerbate the aging of the body, and this process involves complex changes in a variety of immune cells. For example, $CD8^+$ T cells play an important role in controlling chronic infections, but studies have shown that persistent antigenic stimulation of inflammation leads to T cell exhaustion and that overexpression of programmed cell death (PD)-1 reduces the proliferative capacity of $CD8^+$ T cells (Hashimoto et al. 2018). In chronic persistent infection caused by Hepatitis B Virus (HBV), monocytes express high levels of PD-L1 and interleukin (IL)-10, and the suppressive monocytes induce natural killer (NK) cells to produce IL-10 and suppress T cell activation, including $CD4^+$ and $CD8^+$ T cells (Li et al. 2018). As mentioned earlier, senescence increases susceptibility to viruses, $CD4^+$ T cells are essential for antiviral infection, and enhance the lethality of $CD8^+$ T cells in the context of chronic infection by secreting IL-12 (Zander et al. 2019). Therefore, immunosenescence is further promoted by multiple cellular immune blunting due to chronic inflammation. The presence of leukocytes in the chronic inflammatory microenvironment continues to stimulate the body, locally induces fibrosis, and eventually leads to irreversible tissue damage and organ failure (Sebastiani et al. 2014; Eming et al. 2017; George et al. 2020).

More importantly, the immunosenescence of the elderly is accompanied by an impaired immune response to vaccination (Pawelec 2018), that is, due to changes in the degeneration of the thymus and the lack of naive T cells in the older adults, vaccination may not have the desired effect when the elderly disease occurs. However, persistent inflammation is not harmful absolutely in the aging population, and research suggests that a new balance of pro- and anti-inflammatory responses in some older adults, especially centenarians, contributes to longevity (Santoro et al. 2021). Considering that aging is a systemic event involving changes in the immunity, nutrient metabolism, and intestinal microecology, and also is related to the accumulation of aging markers in non-invasive biological fluids such as plasma and urine (Adav

and Wang 2021); therefore, the treatment of senile diseases requires consideration of multiple factors. Actually, the suboptimal responses of older people to vaccines have prompted scientists to develop more comprehensive treatments and interventions, for example, the use of autophagy enhancers, stem cell therapy, Chinese herbal medicine treatment, enhanced exercise, and other multi-faceted means (Shetty et al. 2018).

## 13.3 Melatonin and Aging

Melatonin is an indole hormone mainly secreted by the pineal gland in mammals with the multiple functions, including anti-oxidation, regulating sleep, modulating circadian rhythm, enhancing immunity, and suppressing tumor progression (Xia et al. 2019). Indeed, melatonin also plays an important role in the complex aging process of mammals (Boga et al. 2019). Interestingly, aging is accompanied by metabolic and physiological decline and has the characteristics of circadian rhythm disorder (Roenneberg et al. 2013; Nohara et al. 2019). Moreover, in most vertebrates, the link between aging and melatonin is that melatonin level decreases with age (Bubenik and Konturek 2011; Hardeland et al. 2012). There are two possible reasons for this: 1) The decreased density of β-adrenergic receptors leads to the weakening of melatonin synthesis in pineal gland during aging through downregulating the gene expression or phosphorylation of aralkylamine *N*-acetyltransferase (AANAT) (Suwazono et al. 2000; Jiang et al. 2017; Hohl et al. 2018); 2) the depletion of melatonin increases due to the metabolic events, resulting in changes in the overall content (Obayashi et al. 2014).

Importantly, it has been demonstrated that when the pineal gland of rats was excised to lower the production of melatonin, the accumulation of oxidative damage products accelerated their aging process (Kumazaki and Yoshida 1984). In contrast, when young pineal glands were transplanted into older animals or supplemented with exogenous melatonin, both significantly increased the life span of experimental animals (Kumazaki and Yoshida 1984; Doron et al. 2019). The study by Jauhari et al. also pointed out that AANAT knockout mice are an accelerated aging model (Jauhari et al. 2020), which further suggests that melatonin has an anti-aging effect. Moreover, age differences in the infection rate and severity of COVID-19 have been shown higher in the elder than in youth, and studies have linked this difference to melatonin (Zimmermann and Curtis 2020). Also, experiments have shown that children have higher levels of melatonin, which may be related to lower rates of COVID-19 infection. Therefore, these aforementioned findings suggest that low melatonin level could be considered as a biomarker of aging (Huffnagle and Noverr 2013; Obayashi et al. 2014; Brazao et al. 2017); and more importantly, melatonin might modulate aging process.

## 13.4 Regulatory Effects of Melatonin on Aging

### *13.4.1 Melatonin Slows Aging Through Antioxidant Function*

The oxidative damage of aging originates from the "aging free radical theory." The vast majority of intracellular ROS comes from nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) and mitochondrial oxidative phosphorylation (OXPHOS) (Balaban et al. 2005; Dan Dunn et al. 2015). Nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH) generate superoxide anion ($O_2 \cdot^-$) in the process of passing through the I and III sites of the mitochondrial electron transport chain (ETC) (Balaban et al. 2005). When the OXPHOS is disturbed by excessive cell inflammation, autophagy, and/or apoptosis, the balance is inclined to the production of ROS, which also called mitochondrial ROS (mtROS) (Dan Dunn et al. 2015). Likewise, oxidative damage causes changes in the morphology and physicochemical properties of senescent cells (Muñoz-Espín and Serrano 2014). Mitochondrial metabolism is also implicated in aging, for example, alterations in mitochondrial pyruvate dehydrogenase (PDH) activity lead to increased pyruvate, which in turn leads to mtROS production and promotes mitochondrial aging (Kaplon et al. 2013; Sun et al. 2016). In conclusion, mitochondria may play an important role in aging-induced oxidative damage.

Melatonin has anti-oxidative activity and its anti-oxidation efficacy dependently of direct and/or indirect means. As for the direct action, melatonin and its metabolites directly scavenge free radicals and ROS. For example, melatonin scavenges hydroxyl radicals (·OH) by tautomerization (Purushothaman et al. 2020). Therefore, melatonin has the potential to inhibit cancer by regulating angiogenesis by inhibiting the hypoxia-inducible factor 1α (HIF-1α)/ROS/vascular endothelial growth factor (VEGF) pathway (Cheng et al. 2019). The melatonin metabolite *N*(1)-acetyl-5-methoxykynuramine (AMK) also exhibits excellent scavenging efficiency for ·OH and $\cdot OOCCl_3$ (Galano et al. 2013). And for the indirect effects: (i) Melatonin upregulates glutathione (GSH) synthesis by stimulating antioxidant enzymes [e.g., glutathione reductase (GR)], which in turn carry out antioxidant activities (NaveenKumar et al. 2020). (ii) Melatonin restores immune cell functions, such as enhancing neutrophil phagocytosis and NETosis function and defending against infections (NaveenKumar et al. 2020). (iii) Melatonin neutralizes nitrogen-based poisons such as nitric oxide (NO) and chelates transition metals to resist oxidation (Reiter et al. 2016).

In view of the aforementioned role of mitochondria in oxidative damage, melatonin exerts an antioxidant effect through the melatonin–mitochondrial axis in the process of resisting infection, reducing tissue damage, thereby inhibiting or delaying tissue aging (Reiter et al. 2018). Actually, the oligopeptide transporter peptide transporter 1 and 2 (PEPT1/2) promotes the transport of melatonin to mitochondria (Fig. 13.1) (Huo et al. 2017). In addition, mitochondria express AANAT and *N*-acetylserotonin-*O*-methyltransferase (ASMT), which are involved in the production

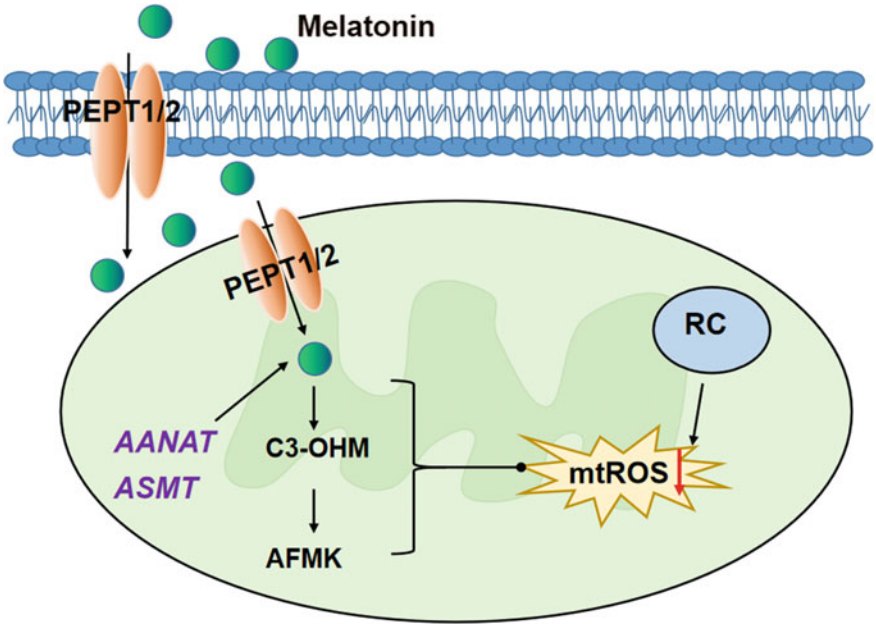


**Fig. 13.1** Melatonin reduces oxidative damage and delays aging by inhibiting mtROS. Melatonin in mitochondria may rely on oligopeptide transporter PEPT1/2 transport or AANAT/ASMT-mediated synthesis. Melatonin and its metabolites C3-OHM and AFMK can reduce mtROS generated by mitochondrial RC, thereby reducing mitochondrial oxidative damage and inhibiting aging. *("↓" arrows represent decreases.) mtROS: mitochondrial ROS; AANAT: aralkylamine *N*-acetyltransferase; ASMT: *N*-acetylserotonin-*O*-methyltransferase; C3-OHM: Cyclic 3-hydroxymelatonin; AFMK: *N1*-acetyl-*N2*-formyl-5-methoxykynuramine; and RC: respiratory chain

of melatonin (Fig. 13.1) (Tan et al. 2013; Reiter et al. 2018). Melatonin reduces mtROS after entering mitochondria (Chen et al. 2020); its metabolite prevents mitochondrial permeability transition (MPT) and limits mitochondria-related apoptosis (Fig. 13.1) (Jou et al. 2019). Moreover, melatonin activates adenosine 5′-monophosphate-activated protein kinase α (AMPKα), attenuates dynamin-related protein 1 (Drp1)-dependent mitochondrial fission, restores the interaction of voltage-dependent anion channel 1 (VDAC1) and hexokinase 2 (HK2), prevents MPT pore (MPTP) opening and activation of PINK1/Parkin pathway, and ultimately blocks mitophagy-mediated cell death (Fig. 13.2) (Zhou et al. 2017). In addition, mitochondrial DNA (mtDNA) released into the cytoplasm activates the cyclic guanosine monophosphate–adenosine monophosphate synthase (cGAS)/stimulator of interferon genes (STING)/interferonregulatory factor 3 (IRF3) pathway, which mediates the inflammatory response in aging, and melatonin could reduce mtDNA to delay aging (Fig. 13.3) (Jauhari et al. 2020). Sirtuin 3 (Sirt3) is a mitochondrial nicotinic adenine dinucleotide (NAD) dependent deacetylase (Wang et al. 2019b), and melatonin reduces the acetylation of superoxide dismutase 2 (SOD2) through Sirt3–SOD2 signal and inhibits the generation of mitochondrial $O_2 \cdot^-$ (Fig. 13.3) (Pi et al. 2015).

Excessive oxidative stress will damage oocytes and thus affect reproductive function, which is also called ovarian aging (Tamura et al. 2020). Melatonin reduces the ROS level of oocytes through melatonin receptor 1 (MT1)/AMPK pathway, maintains mitochondrial membrane potential, and ultimately delays ovarian aging and improves fertility (Zhang et al. 2019a).

In conclusion, melatonin signaling, such as melatonin receptor MT1, melatonin transporter PEPT1/2, melatonin synthases AANAT and ASMT, and melatonin metabolite AMK, acts as the target of mitochondrial antioxidant through AMPK/Drp1, HIF-1α/ROS, cGAS/STING, SIRT3-SOD2, and other signals to reduce cellular and/or tissue senescence in the process of alleviating oxidative damage.

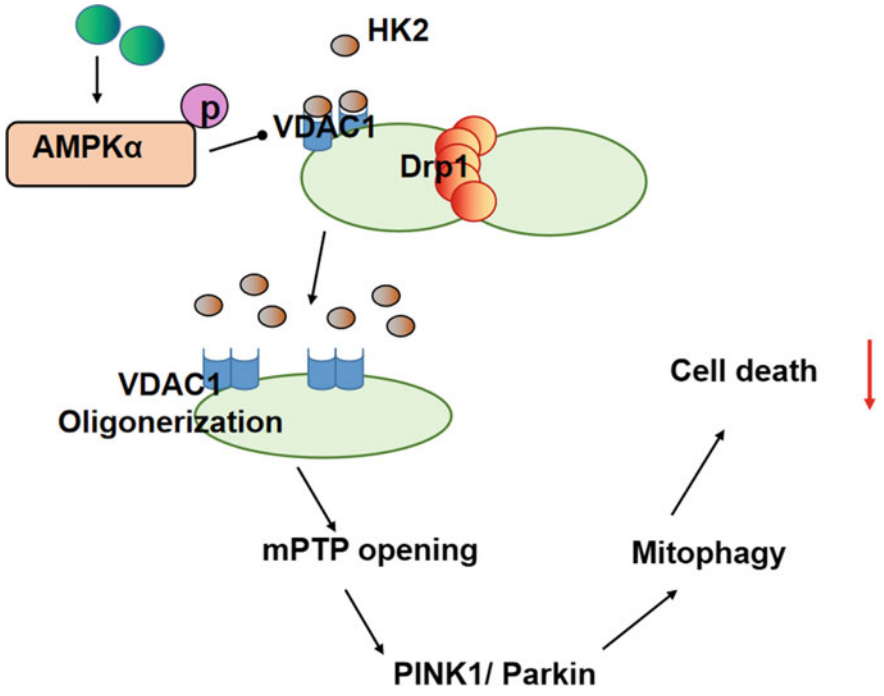


**Fig. 13.2** Activation of AMPKα by melatonin inhibits mitochondrial fission and autophagy-induced cell death. Melatonin inhibits Drp1-induced mitochondrial fission by activating AMPKα, reverses VDAC1 oligomerization, promotes VDAC1-HK2 interaction, prevents mPTP opening and PINK1/Parkin activation, and ultimately prevents mitophagy-induced cell death. *("↓" arrows represent decreases.) Drp1: dynamin-related protein 1; VDAC1: voltage-dependent anion channel 1; HK2: hexokinase 2; and mPTP: mitochondrial permeability transition pore

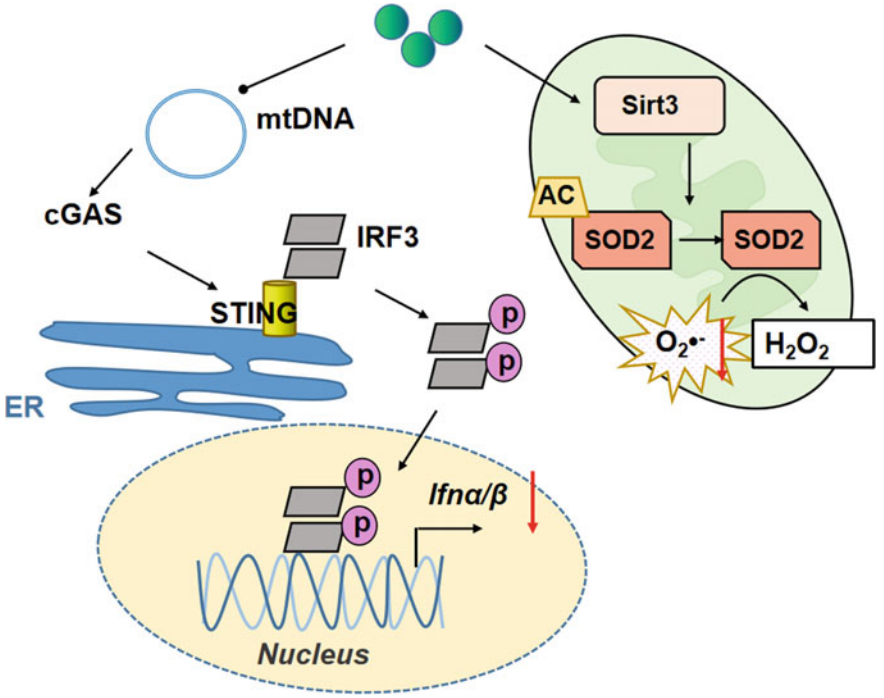


**Fig. 13.3** Melatonin reduces mtDNA to suppress inflammation or promotes Sirt3 to reduce oxidative damage. The mtDNA released into the cytoplasm activates the cGAS/STING/IRF3 pathway and promotes Ifnα/β expression to mediate inflammatory responses during aging. Melatonin can reduce mtDNA, thereby inhibiting its cascade reaction and delaying aging. Melatonin promotes the deacetylation of SOD2 by enhancing the activity of Sirt3, thereby reducing mitochondria-derived $O_2\cdot^-$, reducing cellular oxidative damage, and delaying aging. *("↓" arrows represent decreases.) mtDNA: mitochondrial DNA; Sirt3: sirtuin 3; cGAS: cyclic guanosine monophosphate–adenosine monophosphate synthase; and SOD2: superoxide dismutase 2

### *13.4.2 Melatonin Delays Aging by Repairing DNA Damage*

Another theory about aging is the "telomere theory." As cells becoming aging, the telomeres at the ends of eukaryotic chromosomes shorten or change structurally, leading to replication aging and chromosome instability, and consequently, universal cell aging causes qualitative changes in body aging (Aguado et al. 2020). Melatonin may target human cytochrome P450 1A1 (CYP1A1) gene-mediated 15-hydroxyeicosatetraenoic acid (15-HETE)/telomerase reverse transcriptase (TERT) pathway to regulate telomerase activity, improve telomerase activity, reduce DNA damage, and inhibit cell senescence (Xie et al. 2021). Melatonin also participates in epigenetic modification of genes, inhibiting gene silencing such as DNA methylation and lysine 9 trimethylation of histone H3 (H3K9me3), promoting transcription of activation genes such as acetylation of histone H3, promoting gene reprogramming,

further facilitating gene rejuvenation, and inhibiting cell aging (Yang et al. 2019). The prolongation of telomere and the stimulation of ribosome function by melatonin save the aging of endothelial tissue (Xie et al. 2021), retinal pigment epithelium (RPE) (Blasiak et al. 2016), ovary, and other tissues (Tamura et al. 2017). Therefore, the ability of melatonin in repairing DNA damage gives it the potential of delaying aging.

### *13.4.3 Melatonin Promotes Autophagy and Reduces the Accumulation of Harmful Substances During Aging*

Autophagy is induced by the internal environment or external stress, which can guide the degradation of various substances and avoid the accumulation of damaged substances in the aging process (Glick et al. 2010). Therefore, impairing autophagy would promote the aging. In addition, lysosomal function declines with age, and the accumulation of damaged proteins or organelles induces senescence (Levine and Kroemer 2008; Lawrence and Zoncu 2019). Wong et al. concluded that autophagy defects, involving changes such as impaired nucleocytoplasmic transport and abnormal phase separation, are major risk factors for aging and some neurodegenerative diseases (NDDs) (Wong et al. 2020).

Melatonin prevents aging by regulating autophagy through inflammation-related signal pathways (Fig. 13.4). Melatonin blocks toll-like receptor 4 (TLR4)/protein kinase B (PKB or AKT)/mammalian target of rapamycin (mTOR) pathway to activate autophagy, thereby inhibiting neuro-inflammation and microglial apoptosis in T2D mice (Cui et al. 2021), or to promote mitophagy, reducing tumor cell viability and inhibiting tumors' growth (Shen et al. 2018). Melatonin also promotes mitophagy through SIRT3-SOD2 or AMPK/optic atrophy 1 (OPA1) signaling, which blocks caspase-9-induced mitochondrial apoptosis and maintains cellular homeostasis against cardiac injury (Pi et al. 2015; Zhang et al. 2019b). Melatonin can also activate the AMPK/Forkhead box O 3 (Foxo3) pathway to maintain mitochondrial redox homeostasis or inhibit NF-κB to promote autophagy, respectively, protecting chondrocytes and alleviating intervertebral disk degeneration (IVDD) (Chen et al. 2020, 2021b). Besides, melatonin attenuates AD, PD, Huntington's disease (HD), organophosphate-induced delayed neuropathy (OPIDN), amyotrophic lateral sclerosis (ALS), and other age-related NDDs. *Luo* and colleagues summarized the association between melatonin and aging-related NDDs via autophagy (Luo et al. 2020), which we would not discuss here again.

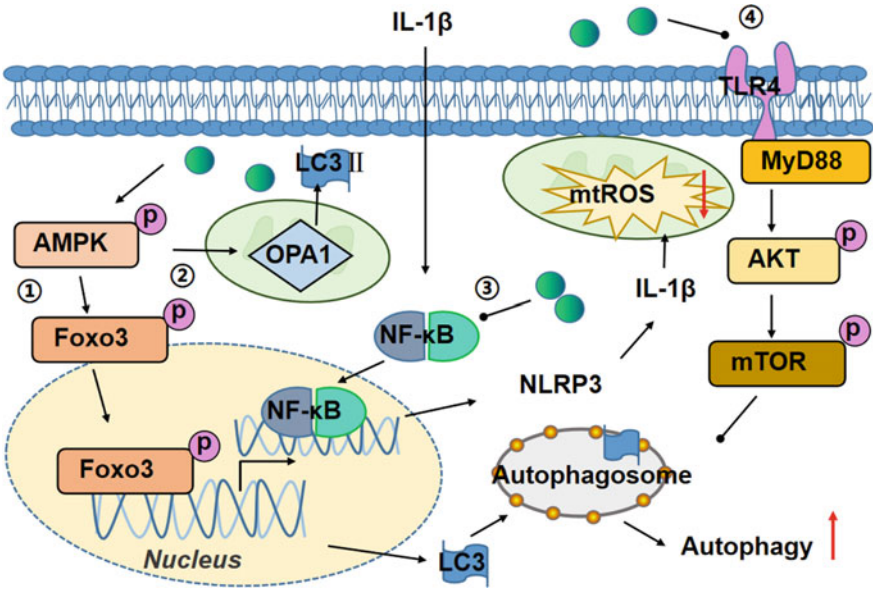


**Fig. 13.4** Melatonin delays aging by promoting autophagy through inflammatory signaling. ① Activation of the AMPK/Foxo3 pathway by melatonin increases the expression of autophagy genes and promotes the expression of LC3, which promotes autophagy. ② Melatonin also promotes mitophagy by increasing the levels of LC3II and mito-LC3II through AMPK/OPA1 signaling. ③ IL-1β/NF-κB/NLRP3 activates a positive feedback loop to promote mtROS production, and melatonin mediates the disruption of the IL-1β positive feedback loop and inhibits mtROS production. ④ Melatonin inhibits the TLR4/AKT/mTOR pathway and promotes autophagosome generation to activate autophagy. *("↑" arrows represent increases, and "↓" arrows represent decreases.) Foxo3: Forkhead box O 3; LC3: microtubule-associated protein 1 light chain 3; OPA1: optic atrophy 1; and mtROS: mitochondrial ROS

### *13.4.4 Melatonin May Rescue Aging by Inhibiting Hyperactive Sympathetic Nerve Activity*

In addition to facing changes in cellular aging and tissue dysfunction, both healthy aging and disease aging are accompanied by autonomic dysfunction, especially the overactive sympathetic nerve activity (SNA), which is innervated by projections from the paraventricular nucleus (PVN) of the hypothalamus and the rostral ventrolateral medulla (RVLM) of the brainstem (Balasubramanian et al. 2019).

Oral melatonin (30 mg/kg/day) for 15 days was shown to effectively inhibit sympathetic excitation, reduce baseline mean arterial pressure (MAP) and ROS levels in RVLM, and alleviate neurogenic hypertension, but the specific mechanism remains to be explored (Nishi et al. 2019). Study indicated that topical application of melatonin or its analog *N*-butanoyl-2-(2-methoxy-6H-isoindolo[2,1-a]indol-11-yl) ethanamine (IIK7) could reduce the intraocular pressure through MT2, and β-adrenergic agonists contribute to this effect; however, the co-localization of MT2 with the sympathetic

nervous system (SNS) was not observed in this article (Alarma-Estrany et al. 2008), so there may be other mechanisms involving in the aforementioned progress. At the end of the 20th, some studies focused on the effect of melatonin on the SNA and found that the SNS directly regulates the synthesis of melatonin in the pineal gland (Wurtman et al. 1964), and melatonin can be used as an endogenous mediator to participate in short photoperiods to inhibit peripheral SNA (Viswanathan et al. 1986). However, whether the role of melatonin in regulating the SNS is related to aging and the mechanism of how melatonin regulates the SNS remains an open question.

### *13.4.5 Melatonin Regulates Infection and Delays Aging by Modulating Gut Microbiota*

Aging is accompanied by changes in host-microorganism homeostasis. This process is affected by factors such as diet, living environment, and lifestyle, as well as by the health status of the host. Ghosh et al. concluded that healthy aging and disease-related aging have both similar and distinct gut microbial changes (Ghosh et al. 2022). Among them, *Akkermansia*, *Butyricimonas*, *Christensenellaceae*, *Oscillospira*, and *Roseburia* increased in normal aging, but decreased in disease aging; *Ruminococcus* decreased in normal aging but increased in disease aging; Pathobionts, *Parabacteroides* are elevated in both normal and diseased aging, while short chain fat acid (SCFA) producers and *Bifidobacterium*, *Prevotella*, and *Eubacterium* are decreased in both normal and diseased aging (Ghosh et al. 2022). In addition, butyrate-producing *Faecalibacterium* and *Coprococcus* (Valles-Colomer et al. 2019) and *Lachnospiraceae* involved in the conversion of primary bile acids to secondary bile acids (Sorbara et al. 2020) are decreased in disease aging; *Anaerotruncus* associated with high cholesterol (Zhang et al. 2021) and *Coprobacillus* associated with the severity of COVID-19 (Zuo et al. 2020) are increased in disease aging (Ghosh et al. 2022). These above findings suggest that alterations in the gut microbiota during disease aging might affect host metabolism and make the host susceptible to infection. And the microbiota intervention with the same change trend in disease aging and healthy aging will become a new idea for regulating aging signals.

Melatonin modulates inflammatory responses in a microbe-dependent manner. For example, melatonin increases the abundance of probiotic *Bifidobacterium* and reduces the abundance of harmful bacteria such as *Desulfovibrio*, and has a relieving effect on oxazolone (Oxa)-induced colitis (Zhao et al. 2021). Melatonin blocks *Prevotella* lipopolysaccharide-induced nitric oxide and interleukin-6-induced host damage by inhibiting NF-κB and signal transducer and activator of transcription 1 (STAT1) activity (Choi et al. 2011). Furthermore, the whole metagenomic sequencing of gut microbiota in children with autism spectrum disorder (ASD) found decreased *Parabacteroides* in gut microbiota and decreased abundance of genes associated with melatonin and SCFAs in the ASD metagenome (Averina et al. 2020). Importantly,

**Table 13.1** Melatonin affects microbiota with the same trends in healthy aging and diseased aging

| Microbiota | Changes in healthy aging | Changes in disease aging | Melatonin effects | Reference |
|---|---|---|---|---|
| *Bifidobacterium* | ↓ | ↓ | ↑ | Zhao et al. (2021) |
| *Prevotella* | ↓ | ↓ | Inhibits *Prevotella*-LPS induced inflammation | Choi et al. (2011) |
| SCFA producers | ↓ | ↓ | ↑ | Lv et al. (2020) |
| *Eubacterium* | ↓ | ↓ | ▲ | Averina et al. (2020) |
| *Faecalibacterium* | ↓ | ↓ | ▲ | Averina et al. (2020) |
| *Parabacteroides* | ↑ | ↑ | ▲ | Averina et al. (2020) |
| Pathobionts | ↑ | ↑ | ↓ | Zhao et al. (2021) |

*"↓" indicate decrease, "↑" indicate increase, "▲" indicate association

the metabolite genes of *Eubacterium*, *Faecalibacterium*, and *Roseburia* are mainly related to melatonin (Averina et al. 2020). Gut-derived plasma SCFAs showed significant circadian oscillations, possibly related to plasma melatonin (Swanson et al. 2020). Gut microbiota that produces SCFAs may promote melatonin receptor expression (Wang et al. 2019a), and melatonin supplementation can promote the abundance of SCFAs production-related flora and increase the production of SCFAs, finally alleviating neuro-inflammation (Lv et al. 2020). Therefore, melatonin may improve inflammation and ultimately affect the aging process by regulating the microbiota that commonly changes in healthy aging and disease aging (Table 13.1). However, whether there is a direct link between melatonin-gut microbiota-aging needs to be experimentally verified.

## 13.5 Conclusion

In conclusion, melatonin plays an important role in the complex aging process of mammals. For example, melatonergic signaling, including melatonin receptor MT1, melatonin transporter PEPT1/2, melatonin synthases AANAT and ASMT, and melatonin metabolite AMK might reduce cellular and/or tissue senescence through alleviating oxidative damage, promoting autophagy, and reducing the accumulation of harmful substances during aging. Notably, melatonin may curtail excessive inflammation and ultimately affect the aging process by regulating the microbiota that commonly changes in healthy aging and disease aging. Moreover, numerous reports

indicate that melatonin possesses anti-infection capability (He et al. 2021, 2022); and the susceptibility of pathogens increases during aging which may also be related to the decreased secretion of melatonin. Therefore, the melatonin level functions as a biomarker of aging and exogenous melatonin could be a potential age regulator.

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# Chapter 14
# Melatonin as a Chronobiotic and Cytoprotector in Healthy Aging

**Daniel P. Cardinali, Seithikurippu R. Pandi-Perumal, and Gregory M. Brown**

## 14.1 Introduction

The impending aging of human population is an undeniably, remarkable event. According to the World Health Organization (WHO), the number of individuals aged 60 and up will double from 1 billion in 2019 to 2 billion in 2050 (WHO, Aging and health), with 80% of all older people living in low- and middle-income countries (WHO, Non-communicable diseases). As individuals live longer, they will encounter a variety of health and quality-of-life concerns, including an increase in the prevalence of non-communicable diseases (NCDs). According to the WHO, NCDs kill 41 million people each year, accounting for 71% of all deaths worldwide (Khan 2019). Over 80% of NCD deaths are caused by cardiovascular illnesses, malignancies, respiratory disorders, diabetes, and neurological diseases. NCDs are highly associated with impairment, dependency, and the need for long-term care.

NCDs are characterized by a chronic low-grade pro-inflammatory condition termed as "inflammaging" (Barbé-Tuana et al. 2020; Franceschi et al. 2018; Fulop et al. 2018). Indeed, as the aging process progresses, the human body's ability to resolve inflammation decreases, resulting in an imbalance of pro- and anti-inflammatory events. Circadian disturbance, as evidenced by interrupted sleep, is

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another critical process in the aging organism. Sleep cycle disruption, as a comorbidity of inflammaging, results in a slew of pathophysiological alterations that hasten the aging process.

Melatonin is a methoxyindole having several features that make it useful for dealing with circadian disturbance and inflammation. It functions as a circadian synchronizer and amplitude enhancer, a direct and indirect antioxidant, an immunological modulator, and a protector and modulator of mitochondrial activity. Melatonin levels tend to drop as people age, and they are even lower in people with NCDs.

This chapter examines melatonin's many functions as a chronobiotic and cytoprotector in relation to age-related NCDs (Cardinali 2019a, b, c). Melatonin attenuates inflammatory responses and progression of inflammation (Cardinali and Hardeland 2017). Furthermore, the late afternoon/night surge in melatonin synchronizes both the central circadian pacemaker found in the hypothalamic suprachiasmatic nuclei (SCN) and a slew of peripheral cellular clocks (melatonin's "chronobiotic action") (Cardinali et al. 2021). The link of melatonin with sirtuins, known by their relevant qualities as aging suppressors and accessory components or downstream elements of circadian oscillators, will be dealt with in depth (Hardeland 2019). SIRT1 and SIRT3 appear to be at the heart of melatonin's chronobiotic and cytoprotective activities in healthy aging.

## 14.2 Inflammaging

Claudio Franceschi and colleagues coined the word "inflammaging" to describe the imbalance between inflammatory and anti-inflammatory signals that occurs as people age (Fulop et al. 2021). This imbalance contributes to the onset of age-related diseases such as cardiovascular disease, metabolic syndrome, and diabetes, as well as neurodegenerative, renal, lung, and skin diseases. Increased inflammatory indexes such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1, IL-6, IL-8, IL-12, IL-17 and IL-22, chemokines, and inflammatory factors like monocyte chemoattractant protein-1 (MCP-1) and C-reactive protein characterize inflammatory aging (Xia et al. 2016).

Macrophages are important players in the delicate balance of pro- and anti-inflammatory reactions. They carry out critical innate immunological tasks, including the clearing of dying cells through phagocytosis (Lu et al. 2021). Macrophages can be divided into two types of polarization states: conventionally activated (M1) and alternatively activated (M2). Genes associated to pro-inflammatory cytokines or oxidative stress, such as TNF-α, IL-6, MCP-1, and inducible nitric oxide synthase (iNOS), are substantially expressed in M1 macrophages, whereas anti-inflammatory cytokine IL-10 is highly expressed in M2 macrophages (Lu et al. 2021).

It is worth noting that, in the absence of acute infection or physiological stress, the levels of inflammatory mediators tend to rise with age. When stress occurs, however, it causes inflammatory damage to cellular components such as proteins, lipids, and DNA, as well as contributing to the age-related decline in physiological functions,

particularly in cells regulating homeostasis, such as neural, immune, and endocrine cells (Bulut et al. 2021). As a result, the functional losses seen with aging include a slow-moving, long-lasting type of oxidative stress caused by increased production of reactive oxygen and nitrogen species (ROS and RNS), which is exacerbated by mitochondrial damage (Bader and Winklhofer 2020; García et al. 2020).

Because of thymic involution and extended germ exposure, which both lead to the depletion of numerous subtypes in developmental stages of leukocytes, an age-related pro-inflammatory propensity is almost unavoidable (Hardeland 2019). However, considerable interindividual differences exist in the velocity of these changes and in the balance between pro-inflammatory and anti-inflammatory cytokines. This could be attributable to a genetic predisposition as well as previous viral load histories, both of which contribute to an immunological risk profile. In centenarians, protective phenotypes include an inverted immunological risk profile (Pawelec 2018; Wikby et al. 2008). A higher proclivity for inflammatory responses could shorten life expectancy. It is likely, then, that a sound immune system is the most reliable predictor of human longevity and healthy aging (Bulut et al. 2021; Fulop et al. 2021; Santoro et al. 2021).

As previously stated, inflammaging is a symptom of oxidative stress, which is defined as an increase in the generation of ROS and RNS compared to the quantity of antioxidants present in the body's natural defensive systems. Melatonin stands out among antioxidants for its anti-inflammatory and antioxidant effects, as well as its role as a metabolic regulator (Cardinali 2019a, b, c; García et al. 2020; Hardeland et al. 2015; Majidinia et al. 2018). Melatonin may have a therapeutic value in promoting healthy aging because it controls several inflammaging-related pathways.

## 14.3 The Circadian Apparatus

The daily and seasonal changes caused by the planet's rotation and orbit around the sun have a consistent impact on the organisms that live on it. The light–dark cycle is the most visible manifestation of this periodic pattern, which has led to the development of endogenous circadian timing systems that synchronize biological functions with the environment (Foster 2020). This is the basis of predictive homeostasis evolving as an adaptation to anticipate predictable changes in the environment, such as light and darkness, temperature, food availability, or predator activity (Burdakov 2019). Therefore, the circadian clock is one of the most indispensable biological functions for living organisms and acts like a multifunctional timer to adjust the homeostatic system, including sleep and wakefulness, hormonal secretions, immune function, and most other bodily functions, to the 24-h cycle.

The circadian system in mammals is made up of numerous distinct tissue-specific cellular clocks. The phases of this plethora of cellular clocks are controlled by a master circadian pacemaker found in the hypothalamic suprachiasmatic nuclei (SCN) to generate coherent physiological and behavioral responses (Hastings et al. 2018).

Among the environmental photic (natural/artificial light) and non-photic (food, behavioral arousal, etc.) cues, natural light is the pervasive and prominent synchronizer ("zeitgeber"). The retinohypothalamic tract entrains the SCN via neurotransmitters that act as messengers, controlling the differential expression of clock genes and clock-controlled genes inside SCN cells and influencing the observable output in the form of physiology and behavior (Hastings et al. 2020).

Circadian clocks are based on clock genes, some of which encode proteins that can feedback and repress their transcription on a molecular level. These cellular oscillators are made up of interlocked transcriptional and post-translational feedback loops that are controlled by a small number of core clock genes (Welz and Benitah 2020). Transgenic gene deletion technology was used to characterize the negative and positive transcriptional/translational feedback loops that make up the core clockwork in rats. The delay in the feedback loops, which is regulated in part by phosphorylation of the clock proteins that affect their stability, nuclear re-entry, and transcription complex formation, causes clock gene expression to oscillate (Takahashi 2017).

The circadian clock's complicated molecular mechanisms are conserved across animals. The transcription factors CLOCK and BMAL1, which form dimers through basic helix-loop-helix domains, are formed when the genes *Clock* and *Bmal1* are transcribed in mammals. The dimer then promotes transcription of two more genes, *Per* and *Cry*, resulting in the creation of the proteins PER and CRY, which dimerize and are then inhibited by CLOCK and BMAL1 expression. As PER and CRY deteriorate with time, the loop must be restarted (Takahashi 2017).

In both nocturnal and diurnal mammals, the levels of *Per* and *Cry* mRNAs in the SCN peak in the middle to late afternoon (Hastings et al. 2020). *Bmal1* mRNA increases around midnight, but *Clock* is expressed in the SCN throughout the whole time (Lee et al. 2001). Through binding to the CLOCK/BMAL1 complex, PER and CRY bind to the E-box element of the promoter regions of *Bmal1*, *Clock*, *Rev-Erb*, and other clock-controlled genes to limit their production (Takahashi 2017). After casein kinase 1 ε/δ phosphorylates PER and CRY, they are translocated to the nucleus (Lee et al. 2001). The master oscillation is modulated further by a secondary regulatory loop comprised of the nuclear receptors REV-ERB and ROR (retinoid-related orphan receptor). REV-ERB inhibits *Bmal1* and ROR promotes it through attaching to the RORE (response element-binding site) sequence in the promoter region of Bmal1 (Fontaine and Staels 2007; Preitner et al. 2003). Phosphorylation and ubiquitylation via the E3 ligase complex govern the stability of PER and CRY, culminating in their proteasomal destruction (Takahashi 2017).

Physiological and behavioral processes are visibly manifested in the complex interaction between the core clock genes and other clock-controlled genes. Interruptions of the circadian rhythm are harmful to one's health (Welz and Benitah 2020). Chronic jet lag and shift work have been linked to heart disease (Crnko et al. 2019), memory loss (Snider and Obrietan 2018), disruptions in hormone timing (Maierova et al. 2016), diabetes (Oosterman et al. 2020; Stenvers et al. 2019; Tsereteli et al. 2021), cancer (Asadi et al. 2021; Stangherlin et al. 2021; Wang et al. 2019), impaired reproductive health (Caba et al. 2018; Pan et al. 2020), and metabolic disorders (Che

et al. 2021; Reinke and Asher 2019; Spiegel et al. 1999). The use of chronotherapies, such as melatonin, to modulate the molecular elements of circadian rhythms to alleviate the ill-effects of circadian rhythm disorders and diseases with a circadian correlate is an area receiving growing attention in the scientific literature (Cardinali et al. 2021).

## 14.4 Melatonin as a Chronobiotic

Borbély et al. (2016) propose that the physiological regulation of the circadian rhythm of sleep/wakefulness (the body's main circadian rhythm) is divided into two parts: a circadian (24-h) component and a homeostatic component. Melatonin is an important component of the circadian clock, which controls the timing of sleep. In both normal and blind patients, the circadian rhythm in the synthesis and secretion of pineal melatonin is intimately linked to the sleep rhythm (Emens and Eastman 2017). The initiation of nocturnal melatonin secretion occurs roughly 2 h before a person's usual bedtime and has been linked to the onset of evening tiredness. Endogenous melatonin has been implicated in the physiological regulation of the circadian systems that govern sleep propensity in several studies (Auld et al. 2017; Gobbi and Comai 2019).

Aging has been linked to a decrease in sleep efficiency and consistency, as well as a decrease in the amplitude of the melatonin cycle and thus many other circadian rhythms in the body (Duffy et al. 2015; Kim and Duffy 2018). Early morning awakenings and trouble falling asleep have been noted regularly among the elderly. Sleep issues that affect senior insomniacs can be linked to melatonin secretion problems. Indeed, melatonin insufficiency causes a relative circadian desynchrony, which can lead to aging. As a result of its well-known chronobiotic capacity, melatonin supplementation can help to improve the quality of life of the aged.

Melatonin is a key player in circadian rhythmicity's coordination. Melatonin secretion is an "arm" of the biologic clock in the sense that it responds to signals from the SCN and that the timing of the melatonin rhythm reveals the status of the clock in terms of phase (i.e., internal clock time relative to external clock time) and amplitude (Pevet et al. 2021). Melatonin is also a chemical code of night in another sense: the longer the night, the longer the length of its secretion. This pattern of secretion serves as a temporal cue for seasonal rhythms in most mammalian species (Clarke and Caraty 2013; Wahab et al. 2018).

Pineal melatonin production is controlled by a complex neural system originating in the hypothalamic paraventricular nucleus (PVN) and ending in the highest levels of the thoracic spinal cord—the superior cervical ganglion sympathetic system (Pevet et al. 2021). The superior cervical ganglion's postganglionic sympathetic nerve terminals release norepinephrine into the pineal gland, which activates melatonin synthesis by interacting with β- (primarily) and α-adrenoceptors on pineal cell membranes. Melatonin is not kept in the pineal because of its high diffusibility, and it is expelled as soon as it is created (Tan et al. 2018). The SCN-melatonin loop is a group of components that govern circadian rhythms. Melanopsin-containing retinal ganglion cells,

the retinohypothalamic tract, SCN, PVN, intermediolateral cell column, sympathetic cervical ganglia, pineal gland, and the melatonin rhythm, all of which have feedback effects on the SCN, make up this loop (Tan et al. 2018).

In fact, all mammalian species' circadian pineal melatonin production is confined to the dark phase of the light/dark cycle. Melatonin is always synthesized throughout the night, regardless of the species' daily cycle of activity/rest, demonstrating its close link with the external photoperiod. If there is no light in the surroundings, melatonin is created at night (Pevet et al. 2021).

When present at night, blue light activates melanopsin-containing retinal ganglion cells, a specific retinal mechanism that suppresses pineal sympathetic norepinephrine release, reducing or eliminating melatonin generation. Melatonin can synchronize the circadian cycles of various organs and their functions due to the regularity of daily melatonin production, which is associated with high and low blood concentrations during the night and day, respectively. In vitro studies have revealed that a synthetic day and night melatonin profile can act as a pacemaker for most cells' daily rhythmic processes (Hardeland et al. 2011).

The effects of the internal zeitgeber melatonin on the circadian clock are time-dependent, just like the effects of the external zeitgeber light. Melatonin given to rats daily modifies the phase of the circadian clock, which could explain how melatonin affects sleep in humans (Pevet et al. 2021). Clinical trials in blind subjects (who have free running of circadian rhythms) treated with melatonin provide indirect support for such a physiological involvement (Skene and Arendt 2007). The revelation that the phase response curve for melatonin was opposite (i.e., around 180° out of phase) to that of light offered more concrete evidence for this notion (Lewy 2010).

Melatonin receptors have been discovered both in the CNS and in the periphery (Dubocovich et al. 2010). The MT1 and MT2 receptors, which belong to the G-protein coupled receptors (GPCR) families of membrane receptors, have all been cloned. GPR50, a new member of the melatonin receptor subfamily, was recently added (Cecon et al. 2018). GPR50 has a lot of similarities to MT1 and MT2, but it does not bind to melatonin or any other known ligand. The ability of these receptors to form homo- and heteromers with each other and with other GPCRs, such as the serotonin 5-HT2C receptor, is an intriguing property (Cecon et al. 2018).

Although melatonin's major physiological function is to regulate circadian and seasonal rhythmicity, the methoxyindole's activities are not limited to receptor-rich locations. Melatonin influences mitochondrial electron flux, the mitochondrial permeability transition pore, and mitochondrial biogenesis, as well as anti-excitatory activities, immunomodulation, including pro- and anti-inflammatory qualities, antioxidant actions, and energy metabolism (Tan and Reiter 2019). Many of these actions are independent of receptors.

Melatonin in the blood is loosely linked to albumin, and it is hydroxylated in the liver before being conjugated with sulfate or glucuronide (Claustrat and Leston 2015). The primary metabolite in human urine is 6-sulfatoxymelatonin. Melatonin is converted to kynurenine derivatives in the brain. Some of melatonin's metabolites, such as cyclic 3-hydroxymelatonin, $N^1$-acetyl-$N^2$-formyl-5-methoxykynuramine (AFMK), and, with the highest efficacy, $N^1$-acetyl-5-methoxykynuramine, share its

well-documented antioxidant effects (AMK). Melatonin administration to experimental animals and people therefore initiates an "antioxidant cascade" (Reiter et al. 2017).

As already stated, circulatory melatonin in mammals is virtually entirely produced from the pineal gland. However, melatonin is also generated locally in most cells, tissues, and organs, including lymphocytes, bone marrow, thymus, gastrointestinal tract, skin, and eyes, where it can have an autocrine or paracrine role (Acuña-Castroviejo et al. 2014). There is now strong evidence that melatonin is synthesized in every animal cell with mitochondria.

Although it is usually assumed that the endogenous melatonin's chronobiotic impact is mediated by MT receptors, a chronobiotic effect can also be detected when pharmaceutical quantities of fast-release melatonin (that saturate receptors) are used. Even at a high dose, melatonin employed as a fast-release preparation administered at a single time point in the day (bedtime) keeps the chronobiotic effects (Fig. 14.1). Hence, the rationale for using melatonin as a preventive medication in NCDs caused by aging is based not only on the amelioration of the immunoinflammatory disorder, but also on the general improvement and prevention of potential complications caused by maintaining optimal circadian rhythmicity.

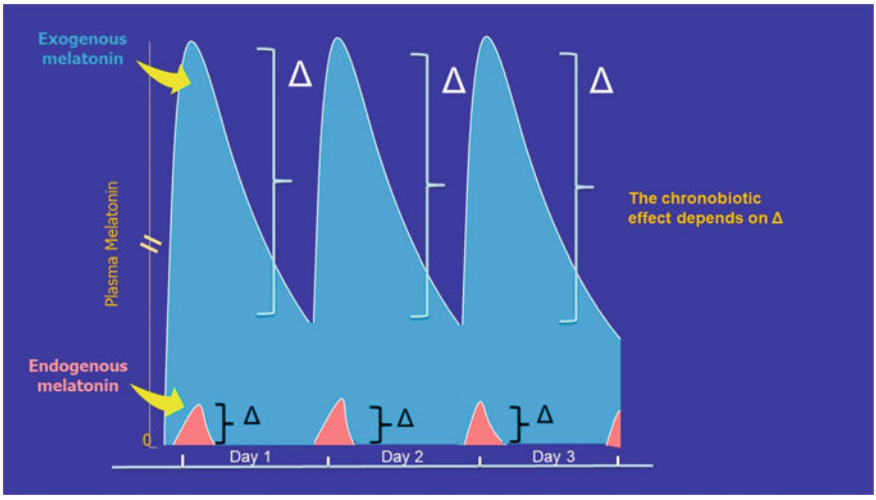


**Fig. 14.1** Because of its pharmacokinetic properties, i.e., a very short half-life in the blood, when melatonin is given orally as a fast release preparation at bedtime, melatonin gives rise to a chronobiotic signal regardless of the amounts given (reproduced with permission from Cardinali et al. (2020a, b)

## 14.5 Use of Melatonin in Aged Sleep

Melatonin is a potent chronobiotic with a mild hypnotic potential. In sighted people who live in environments that are likely to produce a free-running rhythm, daily melatonin doses of 2–10 mg, timed to advance the phase of the internal clock in the SCN, preserve circadian rhythms synchronized to a 24-h cycle (Lewy 2010). After a brief period of free running, melatonin synchronizes the rhythm in people. Giving melatonin to blind subjects with free-running rhythms has been shown to stabilize, or entrain, the sleep/wake cycle to a 24-h period, resulting in improved sleep and mood (Arendt 2019). Melatonin administration helps to minimize the variation in the onset time of sleep in normal aged adults and demented patients with disrupted sleep/wake cycle synchronization. Melatonin's phase-shifting properties also account for its usefulness as a treatment for circadian-related sleep disorders such jet lag and delayed phase sleep syndrome (Burgess and Emens 2018).

The sleep/wake cycle has a bidirectional association with aging. Inadequate sleep, both in terms of duration and quality, can have a negative impact on health and consequently hasten the aging process. Sleep/wake cycle problems, on the other hand, tend to worsen with age due to the flattening and misalignment of circadian rhythms such as melatonin secretion, as well as the sleep-disturbing effects of aging-related ailments and diseases (Hardeland 2015). The most striking examples are immunosenescence, which also affects the brain (Cardinali et al. 2008; Hardeland 2018), and the nearly exponential increase in hydroxyl radical generation reported in the senescent brain (Poeggeler et al. 1993; Reiter 1995).

There is a considerable literature that suggests that the sleep/wake issues become more common as people get older. According to epidemiological studies, more than half of all persons over the age of 65 suffer from a persistent sleep-related ailment (Foley et al. 1995). Several meta-analyses support the view that the chronobiotic/hypnotic properties of melatonin are useful in aged patients with primary sleep disorders to decrease sleep onset latency and to increase total sleep time, with fewer effects on sleep efficiency (Auld et al. 2017; Ferracioli-Oda et al. 2013; Zhang et al. 2019). A role for melatonin in adult insomnia is also supported by several expert consensus reports (Geoffroy et al. 2019; Palagini et al. 2021; Wilson et al. 2019).

Sleep/wake disturbance has been linked to a variety of neuropathologies in numerous studies. In healthy participants, sleep loss or slow wave sleep disruption raised amyloid β (Aβ) levels in the CSF (Olsson et al. 2018; Ooms et al. 2014). A single night of total sleep deprivation was said to prevent the normal decline in CSF Aβ. The brain "glymphatic" hypothesis states that perivascular astrocytes, which are highly enriched in aquaporin-4, and changes in the vascular lumen generate active, lymphatic-like motions in the extracellular space of the brain (Boespflug and Iliff 2018; Braun and Iliff 2020). The exchange of solutes between the CSF and the interstitial fluid takes place mostly during slow wave sleep, when the cortical interstitial space expands by more than 60% and provides a low-resistance conduit for CSF and interstitial fluid movement in the brain parenchyma. The aging human brain has an impact on this. Various neurological disease states, such as stroke, traumatic brain

injury, and AD, have been understood in terms of glymphatic dysfunction's impact (Boespflug and Iliff 2018). It is worth noting that giving melatonin to AD transgenic mice improves their glymphatic clearance of Aβ (Pappolla et al. 2018).

Primary insomnia affects up to ten percent of the general population and up to 25–30% of the elderly, for whom insomnia therapy is an obvious medical necessity. Insomnia's direct and indirect costs add up to a significant socioeconomic burden. The most recommended medicines for the treatment of insomnia in the elderly are benzodiazepines (BZD) and other BZD receptor agonists (Z-drugs such as zolpidem, zaleplon, and zopiclone). Several meta-analyses that investigated the risks and advantages of these therapy choices in older patients found statistically significant improvements in sleep, but also a statistically significant risk of life-threatening side events (Schroeck et al. 2016; Winkler et al. 2014). Due to safety concerns, regulatory agencies have only approved these medications for treatment of older persons for no more than a few weeks. More than 40% of users of both BZD and Z medicines have had negative side effects.

European health authorities are implementing rules and making recommendations to decrease the use of BZD and Z-drug medicines. Despite national guidelines and recommendations, however, the campaigns have been largely unsuccessful, and the usage of these medications has continued to rise (Clay et al. 2013). The more obvious method for reducing chronic BZD use is to discontinue the medicine gradually; abrupt discontinuation can only be justified if a major side effect arises during therapy. There is no clear data about the best way to proceed with BZD withdrawal, and times range from four weeks to several months (Edinoff et al. 2021).

The interaction of melatonin with central BZD receptors was initially reported in 1986 (Acuña-Castroviejo et al. 1986) and the first study on the reduction of BZD use in melatonin-treated elderly people was published in 1997 (Fainstein et al. 1997). Melatonin's anxiolytic, antihyperalgesic, and antinociceptive actions are explained by its facilitation of γ-aminobutyric acid neurotransmission (Cardinali et al. 2016). Several clinical investigations have now confirmed melatonin's usefulness in reducing BZD use in chronically treated patients (Morera-Fumero et al. 2020). The results of a pharmaco-epidemiologic study aimed at assessing the impact of anti-BZD/Z-drug campaigns and the availability of alternative pharmacotherapy (melatonin) on BZD and Z-drug consumption in several European countries revealed that campaigns failed unless they were linked to the availability of melatonin on the market (Clay et al. 2013). Melatonin has therefore proven to be an excellent medication for maintaining healthy sleep patterns in the elderly.

## 14.6 Melatonin and Inflammaging

The significance of melatonin in reducing inflammation and its progression has gotten a lot of attention, especially when it comes to therapy options for people who have low endogenous melatonin levels. Melatonin is one of the hormones that is known to drop with age and, more importantly, in various age-related NCDs (Hardeland 2012; Vasey

et al. 2021). In coronary heart disease, metabolic syndrome, and type 2 diabetes, melatonin levels were found to be lower (Altun et al. 2002; Girotti et al. 2000; Hernández et al. 2007; Nagtegaal et al. 1995; Yaprak et al. 2003). Additional evidence from polymorphisms of human melatonin receptor genes indicates that deviations in melatonergic signaling may favor the development of prediabetic states, diabetes type 2, elevated cholesterol, and coronary heart disease. Furthermore, knocking down the melatonin receptor MT1 in mice resulted in insulin resistance (Contreras-Alcantara et al. 2010).

Melatonin acts as an anti-inflammatory at different levels. One of these is metabolic dysregulation repair, which includes preventing insulin resistance, an inflammation-promoting alteration that is a characteristic of the metabolic syndrome (Cuesta et al. 2013; Lee et al. 2020). Melatonin was found to be efficient in decreasing insulin resistance in a variety of animals, tissues, and induction approaches. Reduced serine phosphorylation of insulin receptor substrate 1 (IRS-1) is the key effect at which the relevant pathways converge in this regard, which is frequently followed by an increase of IRS-1 expression (Du and Wei 2014). Melatonin and the melatonergic agonist piromelatine have been found to reverse insulin signal transduction inhibition (She et al. 2009). Insulin resistance has been found to be an early indicator of low-grade neuroinflammation in neurodegenerative illnesses such as AD and Parkinson's disease (Sun et al. 2020a, b; Verdile et al. 2015).

The avoidance of processes that encourage or lead to inflammation is another level of action. Calcium overload, excessive nitric oxide (NO) release, which leads to the creation of peroxynitrite, peroxynitrite-derived free radicals, and eventually, tyrosine nitration, as well as mitochondrial malfunction because of oxidative stress, are all examples of it (Cardinali and Hardeland 2017; Hardeland et al. 2015). All these alterations are known to generate low-grade inflammation in numerous organs, which is linked to aging. In the central nervous system, this includes microglia activation and vicious cycles caused by overexcitation and oxidant damage, which result in reduced neuronal and astrocytic activities. Melatonin has been proven in animal models to prevent these harmful processes by acting as an anti-excitatory agent, protecting mitochondria, reducing peroxynitrite-related damage, and reducing microglia activation.

Melatonin's immunological effects are a third aspect of inflammaging to consider. Melatonin's many functions as an immunomodulatory drug include both pro-inflammatory and anti-inflammatory effects, resulting in either pro-oxidant or antioxidant equilibrium (Carrillo-Vico et al. 2013; Hardeland 2019; Markus et al. 2021). Melatonin is generally pro-inflammatory in immunosuppressive circumstances. The exact reasons for when melatonin acts pro- or anti-inflammatory are yet unknown, while the severity of inflammation and the chronological sequence of initiation and healing processes are certain to play a part.

Melatonin's anti-inflammatory properties take precedence as people age. Melatonin reduced pro-inflammatory cytokines including TNF-α, IL-1, and IL-6 in the livers of elderly, ovariectomized female rats while increasing the anti-inflammatory cytokine IL-10 (Kireev et al. 2008). Corresponding findings were verified in the

dentate gyrus, along with an increase of sirtuin 1 (SIRT1), a protein with strong anti-inflammatory characteristics. TNF-α and IL-1 levels were reduced, whereas IL-10 levels were elevated, in the liver, pancreas, and heart of the senescence-accelerated mouse strain SAMP8 (Cuesta et al. 2011, 2010; Forman et al. 2011).

Other studies have found that melatonin has anti-inflammatory effects in brain damage, ischemia/reperfusion (I/R) lesions, hemorrhagic shock, and various forms of high-grade inflammation, such as endotoxemia and sepsis. Remarkably, the use of melatonin as a countermeasure to a SARS-CoV-2 infection has been advocated (Reiter et al. 2020a; Zhang et al. 2020). Melatonin has pan-antiviral effects, and it diminishes the severity of viral infections and reduces the death of animals infected with numerous different viruses, including three different coronaviruses. Network analyses, which compared drugs used to treat SARS-CoV-2 in humans, also predicted that melatonin would be a most effective agent for preventing/treating COVID-19 (Cardinali et al. 2020a, b). Finally, when badly infected COVID-19 patients were treated with melatonin alone or in conjunction with other drugs, the severity of infection was reduced, the death rate was lowered, and the length of hospitalization was shortened (Farnoosh et al. 2021; ZT et al. 2021).

From a molecular standpoint, distinguishing between direct and indirect anti-inflammatory effects of melatonin via changes in phase or amplitude of local circadian oscillators is not always attainable (Boivin et al. 2003; Bollinger et al. 2011; Hardeland et al. 2012). Melatonin has been shown to affect metabolic sensing factors such as peroxisome proliferator-activated -activated receptor-γ coactivator-1α (PGC-1α), peroxisome proliferator-activated receptor-γ (PPARγ), phosphoinositide 3-kinase, protein kinase B, including the accessory oscillator components AMP kinase, nicotinamide phosphoribosyl transferase (NAMPT), and SIRT1.

The induction of antioxidant enzymes in the rat liver and pancreas under inflammatory conditions, where melatonin promotes the expression and nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) that mediates the upregulation of the protective enzymes, is an example of a direct melatonin effect not mediated by oscillators (Jung et al. 2010). Melatonin suppresses the expression of nuclear factor-κB (NF-κB) by recruiting a histone deacetylase (HDAC) to its promoter, which decreases pro-inflammatory factors like TNF-α, IL-1, and iNOS.

Various other effects of melatonin on gene expression are mediated by the circadian system. In particular, the role of SIRT1 must be considered, which is not only believed to be an aging suppressor, but acts as a protein deacetylase and, moreover, as a component of circadian oscillators that interacts with the BMAL1/CLOCK dimer and is required for high rhythm amplitudes (Bellet et al. 2011). SIRT1 was activated by melatonin in multiple aging scenarios, including senescence-accelerated animals, and induced increased deacetylation of various of its substrates, including PGC-1α, Forkhead box protein O1 (FOXO1), NF-κB, and p53 (Hardeland et al. 2015). Notably, these effects strongly contrast with the opposite effects in epigenetically dysregulated oscillators of cancer cells (Hardeland 2014). SIRT3 is another sirtuin associated with melatonin effects at the mitochondrial level (Mayo et al. 2017).

## 14.7 Melatonin, Sirtuins, and the Anti-inflammatory Network

Sirtuins, a family of seven proteins encoded by Silent Information Regulator (*Sir*) genes, play a key role in senescence control and survival under adverse conditions (Watroba and Szukiewicz 2021). Sirtuins are nicotinamide adenine dinucleotide (NAD)-dependent HDAC type-III enzymes that control a variety of cellular and molecular processes through deacetylation (Mayo et al. 2017). According to the targeting sequences they include, sirtuins can be cytoplasmic or nuclear (SIRT 1,6,7) and can even be directed to migrate to the mitochondria (SIRT 3,4,5). While nuclear/cytosolic sirtuins regulate cellular processes by deacetylating histone and non-histone targets (Yamamoto et al. 2007), mitochondrial sirtuins regulate energy metabolism by acting as either NAD+ dependent class III histone deacetylase enzymes (e.g., SIRT3), auto-ADP-ribosyltransferases (SIRT6) or as mono-ADP-ribosyltransferases (particularly SIRT4) to control energy metabolism (Watroba and Szukiewicz 2021). They could influence mitochondrial biogenesis, insulin sensitivity, glucose and lipid metabolism (Poulose and Raju 2015), urea cycle, cell cycle, DNA repair, and rDNA transcription due to their deacetylating and ADP-ribosylation properties (Elkhwanky and Hakkola 2018; Singh et al. 2018).

SIRT1 and SIRT3 are two sirtuins that are particularly critical for melatonin's anti-inflammatory properties (Mayo et al. 2017). SIRT1 is a versatile protein that deacetylates both histone and non-histone sites to control gene transcription. P53, FOXO transcription factor, PGC1α and NF-κB are examples of non-histone targets that regulate stress responses, inflammation, cellular senescence, and apoptosis (Watroba and Szukiewicz, 2021) (Fig. 14.2). Because SIRT1's activity is reliant on the co-factor NAD+, it was first thought to be a NAD+-dependent histone deacetylase. Overexpression of *Sirt1* enhances insulin sensitivity by deacetylating PGC-1α, a transcriptional coactivator that regulates glucose homeostasis at the transcriptional level, which influences glucose tolerance (Milne et al. 2007). *Sirt1* overexpression in the progeny of mice fed a high-fat diet decreases insulin resistance, improves glucose tolerance, avoids hepatic steatosis, and lowers ROS generation (Nguyen et al. 2019). SIRT1 also plays several functions in signaling pathways involved in development, cognition impairment, heart disease, aging, cancer, and energy homeostasis, including lipid and glucose homeostasis.

SIRT1 has been linked to a longer lifespan and the prevention of neurodegenerative diseases. The overexpression of SIRT1 in AD reduces the increase in Aβ deposition (Fernando and Wijayasinghe 2021). Overexpression of SIRT1 is also advantageous in Parkinson's disease, as it reduces acetylation of SIRT1 substrate (FOXO3a) and inhibits α-synuclein aggregation by preventing misfolding of α-synuclein protein (Jęśko et al. 2017).

SIRT1 has been shown in numerous studies to have antioxidant and anti-inflammatory properties like melatonin (Mayo et al. 2017). This includes suppressing NF-κB activation, upregulating Nrf2, suppressing NLRP3 inflammasome activation, and inhibiting TLR4 (toll-like receptor 4) signaling. High mobility group

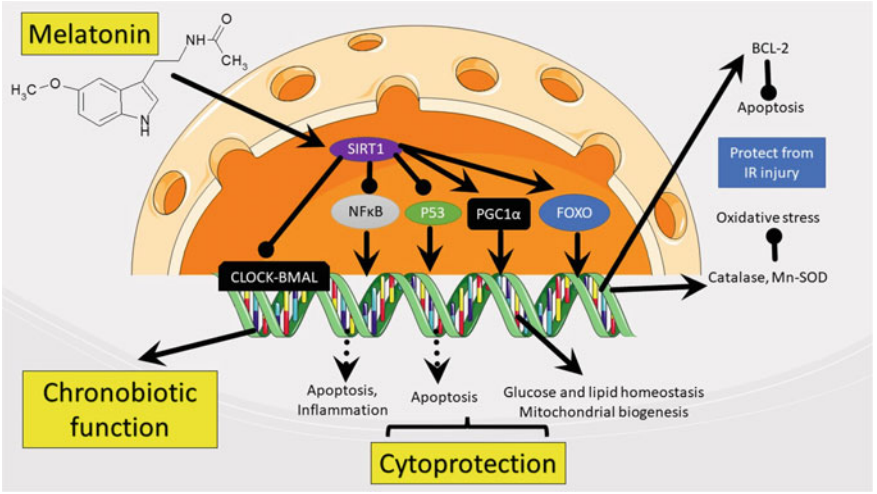


**Fig. 14.2** SIRT1 it is a multifunctional protein that controls gene transcription by deacetylating both histone and non-histone targets. Non-histone targets include P53, forkhead homeobox type O (FOXO) transcription factor, peroxisome proliferator-activated receptor γ coactivator 1-α (PGC1α) and nuclear factor (NF)-κB, thus regulating stress responses, inflammation, cellular senescence and apoptosis. In addition, SIRT1 and the circadian clock interact. SIRT1-deficient mice exhibit alterations in the expression patterns of Per1, Per2, Cry1, and Cry2 circadian genes. Melatonin modulates SIRT1 activity, and this modulation may be in the core of the cytoprotective and chronobiotic properties of the methoxyindole

box-1 (HMGB1), an inflammatory signaling protein secreted by monocytes and macrophages, is a key role in TLR4 activation (Hardeland 2019). SIRT1 has been shown to deacetylate HMGB1, preventing its nucleocytoplasmic transfer and release. Importantly, HMGB1 promotes macrophage and microglia polarization toward the pro-inflammatory M1 type (Hardeland 2019). Melatonin has also been shown to have anti-inflammatory properties via inhibition of HMGB1 (Mayo et al. 2017). Under more severe inflammation, several different findings on sirtuin-mediated suppression by melatonin were discovered. This was seen in normal and diabetic rats with cardiac ischemia/reperfusion, in H9C2 cardiomyocytes with endoplasmic reticulum stress, in LPS-treated microglial cell lines, and in mice with brain injury caused by cecal ligation/puncture (Hardeland 2019).

SIRT3 is a key factor in mitochondrial function, as it regulates the pyruvate dehydrogenase complex (PDH) and participates in ATP synthesis. Several investigations have found that melatonin operates at the mitochondrial level via SIRT3 (Mayo et al. 2017) (Fig. 14.3). Higher ATP generation, an elevated ATP production-coupled oxygen consumption rate, and reduced lactic acid secretion resulted from a switch from cytosolic aerobic glycolysis to oxidative phosphorylation (OXPHOS). Melatonin activated SIRT3 and PDH, which increased the mitochondrial membrane potential and the activity of complexes I and IV in the electron transport chain. Melatonin

greatly improved mitochondrial energy metabolism by reversing the Warburg effect via raising PDH activity and stimulating SIRT3.

Melatonin changes pro-inflammatory glycolytic M1 macrophages into anti-inflammatory OXPHOS-using M2 macrophages (Reiter et al. 2020b). Melatonin causes the mitochondrial metabolism of pyruvate, stimulation of the tricarboxylic acid cycle, improved OXPHOS, and reduced ROS by down-regulating hypoxia-inducible factor 1, which leads to PDH disinhibition. Melatonin and its metabolites are particularly effective direct scavengers of partially reduced derivatives of oxygen under these conditions, in addition to lowering mitochondrial ROS production.

Because macrophages and associated cells are key participants in inflammation, their differentiation into pro-inflammatory M1 or anti-inflammatory M2 phenotypes is critical for maintaining the pro-/anti-inflammatory balance (Fujisaka 2021). By promoting M2 polarization and disfavoring M1 polarization, melatonin can move this balance toward the anti-inflammatory side (Reiter et al. 2020b). One of the major anti-inflammatory effects in the inhibition of M1 function consists in the MT1 receptor-mediated activation of NF-κB degradation. Additionally, suppression of NF-κB actions has been reported for RORα. Because ROR is unable to bind melatonin,

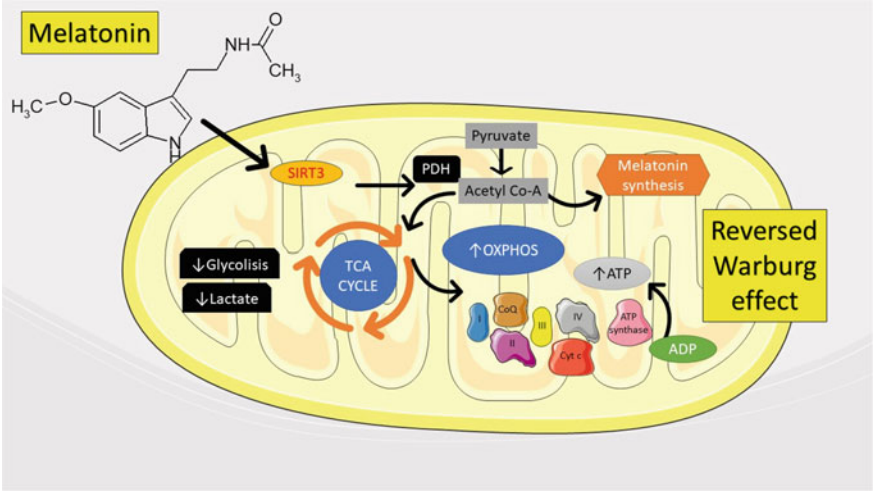


**Fig. 14.3** In resting macrophages (M2), the glucose metabolite pyruvate enters the mitochondria where it is enzymatically converted to acetyl-coenzyme A by the enzyme pyruvate dehydrogenase complex (PDH). Acetyl-CoA feeds the tricarboxylic acid cycle (TCA) and supports oxidative phosphorylation (OXPHOS). Additionally, acetyl-CoA is an essential co-factor/substrate for the rate-limiting enzyme in melatonin synthesis, arylalkylamine *N*-acetyltransferase. Mitochondrial melatonin functions intracellularly and is released into the cellular microenvironment, but not into the blood. Melatonin scavenges ROS generated during OXPHOS and improves mitochondrial membrane potential and the activities of complexes I and IV in the electron transport chain. Additionally, melatonin stimulates SIRT3 allowing PDH stimulation and the activation of superoxide dismutase 2. As a result of these changes, melatonin significantly enhanced mitochondrial energy metabolism to reverse the Warburg effect

the methoxyindole's effect on the transcription factor must be indirect (Hardeland 2019). A possibility of particular interest concerns the effect of SIRT1 on RORα, in its function as a partial mediator of melatonin effects. Upregulation of SIRT1 deacetylates PGC-1α and facilitates the binding of RORα to its response elements.

SIRT1 and the circadian clock interact (Fig. 14.2). SIRT1 influences the circadian clock in both the brain and in peripheral tissues (Masri, 2015; Soni et al. 2021). The expression patterns of *Per1*, *Per2*, *Cry1*, and *Cry2* circadian genes are altered in Sirt1-deficient mice. *Sirt1* and *Per2* work together to suppress each other (Wang et al. 2016). SIRT1 deacetylates and degrades PER2 in the liver. SIRT1 also regulates circadian rhythms by binding to the CLOCK-BMAL1 complex in a rhythmic way. As a result, the acetylation and deacetylation of its components affect the molecular circadian clock.

Sirtuins and circadian clock proteins work cooperatively to regulate oxidative metabolism via NAD+ and NADH responses (Anderson et al. 2017; Griffiths et al. 2020). Apart from activating the clock genes *Per* and *Cry* and other clock-controlled genes, the heterodimer CLOCK-BMAL1 also regulates the activity of the gene *Nampt*, which encodes the rate-limiting enzyme nicotinamide phosphoribosyltransferase, whose metabolite is NAD+ . Because of oscillations in NAMPT levels, NAD + synthesis has a specific circadian cycle. The cellular redox status is maintained by the distribution of NAD+ in the cytosol, nucleus, and mitochondria, which is necessary for the normal functioning of the bioenergetic enzymatic machinery (Anderson et al. 2017; Griffiths et al. 2020). These findings suggest that a complex system of regulators, of which SIRT1 is a key component, controls the molecular circadian clock's stability via various pathways (Griffiths et al. 2020; Xu et al. 2021).

Melatonin regulates SIRT1 activity, which may be at the heart of the methoxyindole's cytoprotective and chronobiotic effects (Bonomini et al. 2018; Emamgholipour et al. 2016; Favero et al. 2020; Stacchiotti et al. 2019) (Fig. 14.2). Melatonin's cardioprotective action during I/R is mediated by SIRT1 signaling in antioxidative response pathways. SIRT1 deacetylation activates FOXO1, which in turn produces the antioxidant enzymes manganese superoxide dismutase (MnSOD) and catalase. Apoptosis is aided by the presence of acetylated FOXO1 (Ac-FOXO1). SIRT1 and Ac-FOXO1 expression were dramatically increased and lowered in melatonin-treated myocardial I/R rats, respectively. In I/R plus vehicle group, SIRT1 expression was reduced and Ac-FOXO1 expression was significantly boosted (Yu et al. 2014). Melatonin therapy boosted the expression of the antiapoptotic gene *Bcl-2* via upregulating SIRT1 and thereby lowering Ac-FOXO1. Hence, melatonin works with SIRT1 to alleviate oxidative stress and to prevent apoptosis (Fig. 14.2).

SIRT1 is also the effector responsible for melatonin's protective role in kidney function in badly burned rats, since it reduces oxidative stress, regulates inflammatory responses, and inhibits apoptotic pathways (Bai et al. 2016; Owczarek et al. 2020). In a C57BL/6 J mouse model of sepsis, SIRT1 contributes to the protective role of melatonin following cecal ligation and puncture (Zhao et al. 2015). Melatonin reduces the load of neuroinflammatory and oxidative stress caused by septic encephalopathy (Hu et al. 2017). This benefit was reduced by a SIRT1 inhibitor,

implying that melatonin's beneficial effect was mediated through SIRT1 (Zhao et al. 2015).

Activation of the NLRP3 inflammasome in various systems, under different conditions and counteractions by melatonin, has been recently reviewed (Sayed et al. 2021; Volt et al. 2016; Zheng et al. 2021). Melatonin's regulation of NF-κB signaling, which is also critical in the prevention of oxidative damage, was linked to these findings. In addition, NF-κB has been shown to cause pyroptosis in adipose tissue, which is suppressed by melatonin.

TLR4 activation, for example, via the IFN-γ-adaptor protein, a toll-receptor-associated activator of interferon (TRIF), is another pro-inflammatory mechanism (Feng et al. 2022; Lwin et al. 2021). By inhibiting TRIF and TLR4, melatonin has been found to reduce the release of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8. Because TLR4 also causes pro-oxidant actions via NF-κB, melatonin's impacts on this pathway are likely to be more widespread.

## 14.8 Therapeutic Value of Melatonin in Animal and Clinical Models of Age-Related NCDs

As already mentioned, circulating melatonin levels in humans are consistently reduced in age-related NCDs. In a limited number of clinical trials employing melatonin in the 2–5 mg/day range, partial beneficial effects were obtained. However, in animal model studies of NCDs, melatonin was highly effective in curtailing symptomatology. Allometric calculations derived from animal studies indicate projected cytoprotective melatonin doses for humans in the 40–100 mg/day range, doses that are rarely employed clinically.

Melatonin treatment reduces obesity, type 2 diabetes, and hepatic steatosis in rats (Martínez Soriano et al. 2020; Pan et al. 2006) Melatonin injections normalized most of the identified changes and corrected the altered biochemical pro-inflammatory profile in many animal models of hyperadiposity (Cardinali 2019a, b, c). Melatonin treatment of streptozotocin-induced type 1 diabetic mice results in the regeneration and proliferation of β-cells in the pancreas, lowering blood glucose levels (Hajam et al. 2021; Kanter et al. 2006). The loss of melatonin in the circulation following pinealectomy causes hyperinsulinemia and lipid buildup in the rat liver (Nishida et al. 2003). Melatonin improves lipid metabolism in type 2 diabetic mice by improving insulin sensitivity after long-term treatment (Nishida et al. 2003). Melatonin administration increased glycogen content in the liver of rats, while intraperitoneal injection of 10 mg/kg melatonin improved glucose consumption, insulin sensitivity, and alleviated hepatic steatosis in high-fat diet-induced diabetic mice (Shieh et al. 2009).

The causes for the drop in body weight after taking melatonin in the absence of major changes in food intake should be investigated further. The fact that melatonin plays a role in seasonal changes in adiposity by increasing the activity of the sympathetic nervous system, which innervates white and brown fat, is an important piece of

evidence in this respect (Bartness et al. 2002; Ryu et al. 2018). Melatonin influences not only white adipose tissue, but also brown adipocyte recruitment and metabolic activity in mammals (de Souza et al. 2019; Fernández Vázquez et al. 2018; Halpern et al. 2019, 2020; Tan et al. 2011). Melatonin's hypertrophic impact and functional activation of brown adipose tissue have been suggested as potential treatments for obesity in humans.

The human equivalent dose (HED) of melatonin for a 75 kg adult was estimated by normalizing body surface area from the doses of melatonin used in animals (Blanchard and Smoliga 2015; Nair et al. 2018; Reagan-Shaw et al. 2008). Body surface area has been advocated as a factor to use when converting a dose for translation from animals to humans because it correlates well with several biological parameters such as oxygen utilization, caloric expenditure, basal metabolism, blood volume, circulating plasma proteins, and renal function across several mammalian species. It is worth noting that theoretical HED of melatonin derived from various research studies are 2–3 orders of magnitude higher than those used in people.

For a summary of the effect of melatonin in animal models of age-related NCDs, see Cardinali (2019a, b, c). Melatonin reduced 87% of the area of injury and 80% of the number of injured myocardium regions in a rat model of myocardial infarction (caused by closure of the left anterior descending coronary artery 3 h earlier) (Castagnino et al. 2002). Several investigations in rats and mice have shown that melatonin can lower heart damage signs, boost cardiac antioxidant defenses, and normalize lipid profiles (see for ref. Cardinali 2019a, b, c). The same was observed in cardiomyopathy induced by streptozotocin (Kandemir et al. 2019) or doxorubicin (Kandemir et al. 2019). Melatonin boosts the therapeutic efficacy of cardiac progenitor cells for myocardial infarction in a mouse model of myocardial infarction treated with cardiac progenitor cells (Ma et al. 2018). A study of the subcellular distribution of melatonin in the heart of rats found that at a dose of 40 mg/kg b.w., the nucleus and mitochondrion attained their maximum concentration of melatonin. The authors calculated a HED of melatonin $\geq$ 112 mg/day for therapeutic purposes in a 70 kg human adult (Acuña-Castroviejo et al. 2018).

Cell line studies regarding AD and melatonin have delineated important melatonin mediated mechanisms in AD prevention. For comprehensive reviews on melatonin activity to reverse disrupted signaling mechanisms in neurodegeneration, including proteostasis dysfunction, disruption of autophagic integrity, and anomalies in the insulin, Notch, and Wnt/β-catenin signaling pathways, see (Melhuish Beaupre et al. 2021; Shukla et al. 2019).

The results obtained in transgenic models of AD are consistent with the hypothesis that melatonin affects Aß metabolism mostly during the early stages of the pathogenic process (Corpas et al. 2018; Jürgenson et al. 2019; Sun et al. 2020a, b). From the doses of melatonin used in the different transgenic models employed, the HED of melatonin for a 75 kg adult ranged from 2- to 3-orders of magnitude greater than those employed in humans.

The mechanism through which melatonin inhibits the production of Aβ is unknown. Melatonin inhibits progressive-sheet and/or amyloid fibrils via interacting with Aß40 and Aß42 (Pappolla et al. 1998), an interaction which appears to depend

on structural melatonin characteristics rather than on its antioxidant properties. Melatonin may help peptide clearance by enhancing proteolytic breakdown by blocking the production of secondary sheets. Oxidative stress is involved in Aß-induced neurotoxicity and cell death, and melatonin efficiently protects cells in vitro and in vivo. Melatonin was found to protect against Aβ toxicity, particularly at the mitochondrial level (Cardinali 2019a, b, c).

Melatonin effectively reduces tau hyperphosphorylation in neuroblastoma cells by influencing protein kinases and phosphatases (Solís-Chagoyán et al. 2020). Melatonin increases the clearance of Aß in the glymphatic system in AD transgenic mice (Pappolla et al. 2018). As a result, sleep disturbance as a comorbidity in AD may contribute to the disease's development and progression through a failure of Aß clearance (Bitar et al. 2021).

The activation of microglia, which results in increased expression of pro-inflammatory cytokines, is another element in the pathophysiology of AD. Melatonin reduced pro-inflammatory cytokine production in microglia triggered by Aß, NF-kB, and NO (Baeeri et al. 2021; Rosales-Corral et al. 2003; Zhang et al. 2021a, b). In addition, the DNA binding activity of NF-kB was inhibited by melatonin (Hardeland 2019).

As far as clinical studies on melatonin therapeutic value in age-related NCDs, type 2 diabetic patients have low circulating levels of melatonin with a simultaneous and expected regulation of mRNA expression of the melatonin membrane receptors (el Aghoury et al. 2020; Otamas et al. 2020; Tanaka et al. 2021; Tütüncü et al. 2005). In addition, allelic variants for melatonin receptors were associated with an increase in fasting blood glucose levels and/or an increased risk of type 2 diabetes (Bai et al. 2020; Bonnefond and Froguel 2017; Bouatia-Naji et al. 2009; Prokopenko et al. 2009; Tam et al. 2010) and with the polycystic ovarian syndrome (PCOS) (Song et al. 2015; Yi et al. 2020).

Melatonin secretion is reduced in patients with coronary artery disease (Brugger et al. 1995; Domínguez-Rodríguez et al. 2002; Girotti et al. 2003, 2000; Misaka et al. 2019; Sakotnik et al. 1999; Yaprak et al. 2003), and among the elderly hypertensive patients, nocturnal urinary melatonin excretion was inversely associated with the non-dipper pattern of hypertensive disease (Jonas et al. 2003; Obayashi et al. 2013). Melatonin therapy ($\leq$ 5 mg/day) reduced nocturnal blood pressure in hypertensives and mitigated age-related cardiovascular rhythm abnormalities (Cagnacci et al. 2005; Campos et al. 2020; Gubin et al. 2016; Grossman et al. 2006; Imenshahidi et al. 2020; Scheer 2005).

Melatonin (5 mg/day) treatment improves metabolic syndrome in obese and PCOS patients (Alizadeh et al. 2021; Koziróg et al. 2011; Mohammadi et al. 2021; Tagliaferri et al. 2018), and in bipolar and schizophrenic patients receiving second generation antipsychotics (Agahi et al. 2018; Duan et al. 2021; Modabbernia et al. 2014; Romo-Nava et al. 2014). Melatonin treatment improves the enzyme profile in alcoholic hepatic steatosis patients (Abdi et al. 2021; Gonciarz et al. 2010). In several studies melatonin therapy improves glycemic control in type 2 diabetes patients (Anton et al. 2021; Bazyar et al. 2021; Kadhim et al. 2006; Ostadmohammadi et al. 2020; Pourhanifeh et al. 2020; Raygan et al. 2019; Satari et al. 2021).

Distinguishing core symptoms (glucose homeostasis) from diabetes-associated pathologies, such as those resulting from increased oxidative stress, such as liver steatosis, cardiovascular disease, retinopathy, nephropathy, or osteoporosis, is crucial in human investigations (Banerjee et al. 2021). Melatonin has been shown to have therapeutic efficacy in the majority of these related diseases.

CSF melatonin levels fall even in the preclinical phases of AD, when patients do not show any cognitive impairment, suggesting that CSF melatonin reduction could be an early trigger and marker for the disease (Colwell 2021; Liu et al. 1999). Although it is unclear if relative melatonin shortage is a result or cause of neurodegeneration, it is apparent that melatonin deficiency exacerbates AD and that early circadian disturbance can be a significant deficit to consider. Melatonin levels were found to differ significantly between mild cognitive impairment and AD patients, with a negative relationship between neuropsychological examination and melatonin levels (Şirin et al. 2015; Zhang et al. 2021a, b). Melatonin therapy is beneficial in improving sleep in dementia patients, according to meta-analyses and consensus reports (Xu et al. 2015; Trotti and Karroum 2016; Zhang et al. 2016; Fatemeh et al. 2021).

It is unclear whether melatonin can help people with fully developed AD. It should be highlighted that heterogeneity of the sample studied is one of the issues with AD patients with fully developed illness. Review of published evidence on the use of melatonin in the early stages of cognitive decline, on the other hand, consistently revealed that taking melatonin every night before retiring improves sleep quality and cognitive performance in this stage of the disease (see for ref. Cardinali 2019a, b, c; Liu et al. 2021; Sumsuzzman et al. 2021; Wade et al. 2014; Wang et al. 2017).

## 14.9 Concluding Remarks

NCDs linked to aging provide a significant public health challenge. Over 80% of NCD deaths are caused by cardiovascular illnesses, malignancies, respiratory diseases, diabetes, and neurological diseases, and NCDs are closely associated with disability, reliance, and long-term care demands. In this Chapter, we have covered two key etiopathogenic processes that contribute to NCDs: inflammaging and circadian disturbance, the latter of which is a result of living in a 24/7 society that affects sleep. As a result, dysregulation of the sleep/wake cycle causes a slew of pathophysiological alterations that hasten the aging process.

Melatonin emerges as a viable non-toxic chronobiotic/cytoprotective approach in this context. It is worth noting that melatonin has a very high level of safety. The lethal dose 50 for the intraperitoneal injection of melatonin was determined for rats (1168 mg/kg) and mice (1131 mg/kg), but the lethal dose for oral administration of melatonin (assessed up to 3200 mg/kg in rats) could not be determined and for melatonin subcutaneous injection (tested up to 1600 mg/kg in rats and mice) (Sugden 1983). Melatonin has an excellent safety profile in humans and is generally well tolerated (Schrire et al. 2021).

Melatonin, as discussed herein, combines two properties that are extremely important for the prevention and treatment of age-related NCDs: it is an effective chronobiotic that aids in the correction of circadian disruption, and it is a phylogenetically well preserved cytoprotective agent that addresses the treatment of inflammaging. Beyond melatonin's well-known antioxidant and anti-inflammatory properties, which have demonstrated its efficacy in the treatment of diseases/conditions in which excessive free radical-mediated oxidative damage and hyperinflammation are causative factors, the studies summarized herein support its use as a viable preventive agent in the low-degree inflammation found in age-related NCDs.

Numerous interrelated factors found in inflammaging, including the development of pro-inflammatory M1 macrophages, conversion to Warburg-type metabolism of immune cells, damage to mitochondria, release of cytokines, oxidative stress, etc. are counteracted by melatonin. A center piece of this series of cytoprotective processes may be the alterations in mitochondrial physiology and the shift of glucose oxidation to cytosol mediated via the melatonin effect on sirtuins, particularly SIRT3. This change in glucose handling markedly alters the metabolism of the mitochondria, which is critical to limiting cellular dysfunction, resisting disease and preventing organismal death. Indeed, there are numerous maladies that are specifically classified as mitochondria-related diseases (Chaiyarit and Thongboonkerd 2020; Cloonan et al. 2020; Kłos and Dabravolski 2021; Medala et al. 2021; Vaamonde-García and López-Armada 2019; Xin et al. 2021) with this category including viral infections such as SARS-CoV-2 (Swain et al. 2021).

When intracellular glucose metabolism is reprogrammed from the mitochondria into the cytosol, the mitochondria can no longer synthesize acetyl-coenzyme A (acetyl-CoA). This has high importance, since acetyl-CoA is a required co-substrate for intramitochondrial melatonin production, which normally occurs in these organelles of healthy cells but likely not in the mitochondria of inflamed cells (Reiter et al. 2021a, b, 2020c). Thus, in the absence of local melatonin synthesis, the loss of this locally produced potent anti-inflammatory and antioxidant agent, the mitochondria lose a major portion of their protection against ROS, inflammatory cytokines, etc., leading to their dysfunction. The ability of melatonin to reverse the Warburg effect in pathological cells in humans was recently documented, presumably allowing the mitochondria also to synthesize melatonin (Reiter et al. 2021a, b).

Melatonin is commonly used as a dietary supplement or dietary product to treat sleep disturbances in many countries. Melatonin reduces sleep onset delay, according to the European Food Safety Authority (EFSA). This allows for the introduction of melatonin as a meal to promote "sleep–wake cycle regulation," "relaxation," and "sleep patterns" (Agostoni et al. 2011). Melatonin, melatonin-rich foods, and bio-extracts of melatonin can now be developed as nutritional supplements, dietary products, and pharmaceuticals for the general population, as specified by the EFSA.

Melatonin is very effective to alleviate oxidative stress in plants (Anderson and Kim 2021; Tiwari et al. 2021), as it does in animal tissues. Since its discovery in plants two decades ago, researchers have made significant progress in understanding the effects of melatonin that contribute to the plant's ecological success (Back et al. 2021). Melatonin overexpression in plants promotes seed germination and increases

root development and maturation, protecting plants from biotic and abiotic stress (Anderson and Kim 2021; Tiwari et al. 2021). Melatonin's presence in plants has ramifications not only for plant development and crop productivity, but also for human and animal nutrition. Melatonin is easily absorbed and exerts its actions at the cellular level when plant products containing it are ingested. Melatonin is a beneficial chemical that neutralizes the physiopathological processes that undermine a healthy lifestyle in both animals and plants. Melatonin enrichment in foods is required to attain the levels that give efficient cytoprotection. As a result, the creation of functional meals containing high quantities of melatonin is a hot topic. The modest doses of melatonin usually utilized are not very advantageous if melatonin is supposed to be effective in enhancing health, especially in the elderly.

The question of whether melatonin has a therapeutic value in the prevention or treatment of NCDs deserves further analysis. Multicenter double-blind studies are needed to explore and further investigate the potential and utility of melatonin. The doses of melatonin used should be re-evaluated in view of the HED of melatonin derived from preclinical data. However, the failure of melatonin to attract attention as a potential treatment for healthy aging is somewhat disappointing considering the number of scientific/medical papers that have recommended its use. This may relate to several factors including the lack of promotion of its therapeutic use for this disease by any influential group. Since melatonin is non-patentable and is inexpensive, the incentive of the pharmaceutical industry to support its use is lost. Melatonin would be particularly helpful because it may be self-administered orally, is inexpensive, and has low toxicity. This is especially true in disadvantaged areas of the world, where people have fewer financial resources to spend on age-related NCD treatment.

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# Chapter 15
# Melatonin: A Saga of Health and Longevity


**Sameer Gupta, Amaresh Kumar Singh, Chandana Haldar, and Anirban Roy**

## 15.1 Introduction

Melatonin, *N*-acetyl-5-methoxytryptamine, is a hormone synthesized from tryptophan by the neuroendocrine pineal gland originating from the third ventricle of the brain. The secretory activity of the pineal gland is under the control of the biological clock residing in the hypothalamic suprachiasmatic nucleus (SCN). To maintain the diurnal rhythm of melatonin biosynthesis, SCN uses constant stimulatory signals via the paraventricular nucleus (PVN) pathway to pineal in the form of glutamate which is inhibited during the daytime suppressing the melatonin synthesis (Benarroch 2008). The nocturnally elevated levels of melatonin derived from the pineal gland act as an endocrine signal that conveys the circadian information and synchronizes the body's physiology to the changing environmental conditions (Reiter et al. 2014). Interventions like exposure to light at night, shift work, or certain drugs and medications, have been shown to disrupt the circadian system and the hormonal rhythms being governed by light–dark cycles resulting in altered sleep–wake patterns, psychological stress, and impaired physiologic and metabolic control leading to comorbidities like metabolic syndrome, cancer, and Alzheimer's disease (Reiter et al. 2020a, b).

Besides the vertebrate pineal gland, melatonin is ubiquitously expressed in bacteria to plants and other animal phyla and is synthesized as extra-pineal melatonin from various organs of the body (Acuna-Castroviejo et al. 2014). Unlike pineal melatonin, extra-pineal melatonin lacks rhythmicity and has been suggested to perform

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local cytoprotective functions via autocrine, intracrine, or paracrine signaling mechanisms (Acuna-Castroviejo et al. 2014). Melatonin is a versatile molecule and can exert multifarious effects via receptor depend or independent mechanisms that can be expanded to its anti-tumor, anti-mutagenic, anti-genotoxic, anti-cancer, anti-neurodegenerative, anti-apoptotic, and immunomodulatory and cardioprotective effects. One of the most significant effects of melatonin includes its antioxidant role and free radical scavenging capacity which directly or indirectly also regulates the anti-inflammatory and immunomodulatory potentials of melatonin (Hardeland 2018). The high levels of melatonin found in mitochondria, a site where most of the reactive oxygen species are formed during metabolism, support its cytoprotective merit in terms of preventing molecular damages that otherwise would accumulate and manifest into various pathologic conditions. The further sections of the chapter highlight the protective role of melatonin in the maintenance of cellular homeostasis and survival concerning various aspects of physiology.

## 15.2 Stress and Melatonin

The environmental conditions majorly influence the physiological activity of animals that are largely exposed to the environment. Moderate to extreme environmental conditions like extreme heat/cold, humidity, rain fall, and pathogenic invasion confront animals with an adversative situation that consequently activates the stress response. The stress response generally occurs to reduce the impact of stress (Charmandari et al. 2005), but under the lack of appropriate responses, this phenomenon costs the fitness and survival of an organism.

Stress is a constellation of actions that acts as a stimulus (stressor) to initiate the stress response in the physiological system (Dhabhar and McEwen 2001). Stress leads to suppression of immune functions and increases susceptibility to various infections (Glaser and Kicolt-Glaser 2005). The stress condition causes homeostatic imbalance by affecting the immune functions like reduction of immune cells activities, the decline in lymphocyte numbers, and proliferative capacity of NK-cells parallelly, with declined antioxidant response that leads to an immunocompromised state (Webster Marketon and Glaser 2008).

Stress condition activates hypothalamic-hypophyseal-adrenal (HPA) axis that modulates the activity of different target genes via glucocorticoids (GC) and GC receptor (GR) mediated actions (Sapolsky et al. 2000). Reports suggest that increased GC and its receptor expression activates immune cell apoptosis and declines antioxidant enzyme activity (Ashwell et al. 2000). The stress increases apoptosis by declining anti-apoptotic protein Bcl-2 and upregulating the level of Bax that ultimately reducing Bcl-2/Bax ratio (Singh and Haldar 2016). GR activation has also been reported to suppress antioxidant response (Kratschmar et al. 2012). The nuclear translocation of GR is precisely regulated by HSP90-based chaperone machinery where HSP90 plays an imperative role in regulating functional activation and inactivation of GR (Grad and Picard 2007).

Melatonin has been suggested to act as a potent anti-stress hormone. It downregulates GC and GR-mediated inhibition of immune responses (Gupta and Haldar 2013; Singh and Haldar 2016). Melatonin seasonal variation influences GR expression in human mononuclear leucocytes and in vitro melatonin treatment relieves the suppressive effect of GR and upregulates antioxidant response via Nrf-2-HO-1-mediated pathways in peripheral blood mononuclear cells (PBMCs) (Kratschmar et al. 2012; Singh and Haldar 2016). Nrf-2-HO-1 pathway upregulates the expression and activity of enzymes like superoxide dismutase (SOD), heme oxygenase-1 (HO-1) and catalase (CAT) to promote antioxidant repertoire (Singh and Haldar 2016). The downregulation of Nrf2 signaling has been suggested to increase apoptosis by influencing apoptotic proteins (Pan et al. 2013). Melatonin positively influences the Bcl-2/Bax ratio that protects the cells from apoptosis and increases the proliferative competency of PBMCs (Singh and Haldar 2016). The melatonin treatments also influence the secretory pattern of different pro- and anti-inflammatory cytokines to modulate the immune responses (Singh and Haldar 2020). Melatonin treatment ameliorates cold stress-induced immune suppression and prevents cellular death via upregulating HSF-1 and HSP-70 (Rastogi and Haldar 2020).

Oxidative and nitrosative stress is the major cause of disrupting various physiological activities like immune regulation. It has also been observed that declining melatonin levels with aging results in an increased level of oxidative and nitrosative stress conditions. The increased stress condition causes a decline in immune responses by inducing apoptosis in immunocompetent tissues and cells. Further, the administration of melatonin ameliorates oxidative and nitrosative stress in aging animals (Vishwas et al. 2013).

Consequences of stress could not be restricted to physiological disturbances rather psychological stress also plays a critical role in inducing the stress response. Our lifestyle has changed drastically in recent decades like shift-work (day-night) and target-oriented tasks that forces the individual to restrain on a chair for a longer period, which could be termed as restraint stress. Such conditions are very common in corporate culture, for the soldiers in barracks, nurses, doctors, etc. This restraint stress leads to psychological stress that adversely affects the health condition. It has been observed that night shift workers are more prone to mental and physiological stress conditions than day shift workers. The shift work disrupts the circadian rhythm resulting in a sleep deficit that compromises the work output and increased chances of accidents (Costa 2010). Melatonin being a potent anti-stress molecule could be used in clinical settings to regularize the endogenous circadian rhythms and its supplementation can be used to counterbalance the psychological and mental stress generated due to restrained conditions and shift work.

## 15.3 Oxidative Stress and Melatonin

Oxidative stress refers to the condition when body tissues are unable to adequately handle the endogenously generated reactive oxygen and nitrogen-based free radical

species. Oxidative stress is strongly linked to both local and systemic aging, as well as to a variety of health conditions like hyperglycemia, dyslipidemia, age-dependent neurodegeneration, inflammatory disorders, cardiovascular conditions, and so on (Liguori et al. 2018). Though free radicals are generally damaging, a bare minimum quantity of them is essential for the regulation of various cellular signaling mechanisms and maintenance of redox homeostasis. Melatonin is among such endogenous molecules that apart from exhibiting a prodigious functional diversity also makes oxygen metabolically more tolerable for the biological system (Manchester et al. 2015).

Melatonin probably evolved to neutralize the toxic oxygen derivatives in photosynthetic bacteria around 3.0–2.5 billion years ago (Tan et al. 2013). During evolution, the original antioxidant function of melatonin was topped up with a variety of other new roles some of which includes immunomodulation, geroprotection, oncostatic, and chronobiotic function (Reiter et al. 2016). Melatonin manifests its antioxidant actions either by direct detoxification of reactive oxygen and nitrogen species or indirectly by stimulating the antioxidant enzymes while suppressing the activity of pro-oxidant enzymes. Accordingly, melatonin could be metabolized in a variety of ways, including enzymatic, pseudo-enzymatic, and non-enzymatic free radical interactive processes (Reiter et al. 2016; Hardeland 2017). The uniqueness of melatonin lies in the fact that generations of metabolites, produced from melatonin also act as effective antioxidants thereby establishing a radical scavenging cascade reaction (Tan et al. 2000). Melatonin and its metabolite N1-Acetyl-5-methoxykynuramine (AMK), scavenges oxidizing free radicals and singlet oxygen, downregulates iNOS and nNOS, as well as cyclooxygenase-2(COX-2) (Mayo et al. 2005). Both AMK and melatonin are known to prevent the collapse of mitochondrial membrane potential and reduce electron leakage through the respiratory chain thereby avoiding the generation of superoxide anions (Hardeland 2017). The melatonin-mediated avoidance of radical formation seems to be a more significant chronobiological function in terms of maintaining low levels of oxidative damage during peak metabolic activity (Hardeland 2008).

Compared to classical antioxidants, melatonin was found to be four times more effective in scavenging ABTS [2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] cation radical and unlike other antioxidants, exhibited synergistic actions when used in combination with other antioxidant molecules like vitamin C, E, and glutathione (Tan et al. 2013). The free radical quenching property of melatonin is superior to that of glutathione against the hydroxyl radical (OH), whereas its activity against the peroxyl radical (ROO) involves single electron or hydrogen atom transfer for the creation of radical adducts (Galano et al. 2018). Apart from scavenging free radicals, melatonin can also interact with non-radical oxidants such as hydrogen peroxide ($H_2O_2$), singlet oxygen ($1O_2$), and peroxynitrite ($ONOO^-$) (Reiter et al. 2016). Melatonin was also found to stimulate antioxidative enzymes including CuZnSOD, MnSOD, catalase, glutathione peroxidase, and glutathione reductase while down regulating the pro-oxidant enzymes viz. nitric oxide synthases, lipoxygenases (LOX), and also regulating the activity of quinone reductase 2 (Boutin and Ferry 2019). Melatonin is also known to inhibit the activity and expression of

myeloperoxidase and eosinophil peroxidase. In addition to its role in alleviating oxidative stress directly or indirectly, melatonin is also involved in the chelation of transition metal ions involved in Fenton or Haber–Weiss reactions thereby reducing the incidence of oxidative stress by preventing the formation of toxic hydroxyl radical (Romero et al. 2014).

Subcellular concentrations of melatonin were found to be the order of magnitude higher than the concentration of melatonin present in blood suggesting its cytoprotective role (Acuña-Castroviejo et al. 2014). Unusually, higher concentrations of melatonin found in mitochondria suggest that these organelles apart from sequestering melatonin can also synthesize melatonin, implying that melatonin gains rapid access to the source where bulk free radicals are being produced (Suofu et al. 2017). In mitochondria, melatonin upregulates the activity of superoxide dismutase-2 (SOD2), by inducing sirtuin 3 (SIRT3), which deacetylates SOD2 rendering it active (Reiter et al. 2018). Forkhead box 03 (FOXO3a) is a direct target of SIRT3 which is also involved in melatonin's action against oxidative damages (Kumar et al. 2021). The antioxidant actions of melatonin on radical detoxification can also be mediated by Keap1-Nrf2-ARE (antioxidant response element) promoter located upstream of superoxide dismutase and glutathione peroxidase (Manchester et al. 2015; Yu et al. 2017) reported that melatonin activates AMPK-PGC-1α-SIRT3 signaling and increases SOD2, NRF1 and mitochondrial transcription factor A (TFAM) expression to protect the heart from the hypoxia and reoxygenation-induced (ischemia/reperfusion) oxidative damages. A recent report has demonstrated that melatonin improves cardiac capacity in the myocardial infarction rat model through the Sirt6-dependent antioxidant pathway (Wang et al. 2022). Melatonin also inhibits heamin-induced oxidative stress, ferroptosis, and platelet activation reducing the risk of thrombotic complications (NaveenKumar et al. 2019). Administration of melatonin in preterm neonates has been shown to inhibit free radical-mediated tissue destruction and prevent lung injury in neonates thereby protecting the high-risk newborns (Marseglia et al. 2021). Supplementation of pharmacological levels of melatonin (3 mg) has been reported to protect critically ill patients from oxidative injuries (Mistraletti et al. 2017).

Besides being potent antioxidant melatonin also acts as a conditional pro-oxidant. A higher concentration of melatonin (10 μM–1 mM) has been found to increase markers of oxidative stress and show moderate cytotoxicity (Büyükavci et al. 2006; Clapp-Lilly et al. 2001). However, the pro-oxidant effects of melatonin are mostly demonstrated in cancer cell lines and tumor cells which are either mediated by calmodulin-dependent PLA2 (phospholipase A2) activation and production of free radicals or via electron transport chain mediated free radical generation in mitochondria (Zhang and Zhang 2014).

## 15.4 Melatonin in Immunomodulation

Melatonin acts as a primary mediator of diurnal rhythmicity observed in the physiological functions including immunity. Almost every aspect of the innate or adaptive immune mechanism including the trafficking of immune cells, inflammatory processes, response to infection, chemokine and cytokine expression, and the activation of immune cell signaling exhibits diurnal variation (Man et al. 2016). This inherent rhythmicity in immune cell functions relies on neural and hormonal signals generated by the central clock, residing in the hypothalamic suprachiasmatic nucleus, in the form of glucocorticoid and melatonin (Córdoba-Moreno et al. 2020). Pineal ablation or other experimental approaches that inhibit melatonin synthesis (e.g., exposure to constant illumination, pineal denervation) depresses both cellular and humoral immunity that can be partly counteracted by exogenous melatonin administration (Luo et al. 2020). The night shift work in humans has also been shown to disrupt the relative phase of the rhythms of cytokine secretion and alter immune cell counts (Cuesta et al. 2016) thereby enhancing the risk of infections, exaggerated inflammation, and increased incidence of autoimmune disorders, cancer and cardiometabolic diseases (Morris et al. 2016). The disrupted sleep–wake pattern has been reported to suppress the magnitude of antibody response following vaccination while adequate sleep and time of vaccination can effectively improve antibody generation (Schmitz et al. 2022) suggesting the involvement of rhythmic melatonin levels in mechanisms related to an antibody response. Studies suggest that melatonin supplementation in a time-dependent manner or otherwise can promote antibody response either by enhancing antigen presentation to immunocompetent cells or by modulating the production of cytokines that regulate the cellular events critical for antibody generation (Cernysiov et al. 2010).

Melatonin exerts stimulatory effects on the cellular and humoral immune responses during immunocompromised states or under basal conditions. An early report from Maestroni and colleagues (1986) suggested that the night-time peak of plasma melatonin attenuates propranolol-induced cellular and humoral immunosuppression in mice. Several other reports from various groups also suggested the melatonin-mediated antagonism of steroid and age-dependent immunosuppressed conditions (Akbulut et al. 2001; Gupta and Haldar 2013). The functional spectrum of immunomodulation by melatonin is highly complex and involves various cytokines. Melatonin generally increases B-cell proliferation and the Th1 cytokines (IL-2 and IFN-$\gamma$) and decreased Th2 cytokines such as IL-10 production in aged mice. Pinealectomy-induced disruption in nocturnal melatonin rhythm was shown to polarize thymic Th1/Th2 cells toward Th2 type response which was reversed following melatonin treatment (Kelestimur et al. 2006). Melatonin modulates immune response by inhibiting the activation of inflammatory processes and regulating the proliferation and activity of immune-competent cells (Carrillo-Vico et al. 2013; Tarocco et al. 2019). In vitro treatment of melatonin increases splenic and thymic lymphocyte proliferation along with $CD4^+$ expression on the splenic cells (Kim et al. 2000; Gupta and Haldar 2013). Melatonin supplementation

increases peripheral levels of Th1, Th2, and Th17-related cytokines in pinealectomized mice and activates T- and B-cell signaling (Luo et al. 2020). Melatonin is also involved in T-cell development in the thymus. The T-cell-mediated immune responses protect mammals from cancer, infections, and various inflammatory and autoimmune diseases (Ren et al. 2017). Melatonin enhances Ki67 and Bcl-2 expression in antigen-specific T-cells suggesting its involvement in T-cell proliferation (Yoo et al. 2016). The most detailed studies have focused on the Th pathway where melatonin increases the number of Th ($CD4^+$) lymphocytes (Lissoni et al. 1995) and restores impaired Th-cell activity in immunosuppressed mice, and augments humoral response (Fraschini et al. 1998; Akbulut et al. 2001).

### *15.4.1 Melatonin and Immune Cells*

Melatonin influences the activity of different armaments of the immune system like neutrophils (NaveenKumar et al. 2020), macrophages (Xia et al. 2019), T-cells (Ren et al. 2017), dendritic cells, and natural killer cells NK-cells (Calvo et al. 2013) thus, playing an important role in modulating innate immune responses. A close association between night-time melatonin peak and proliferation of granulocyte and macrophage progenitor cells has been reported (Haldar et al. 1992; Guerrero and Reiter 2002). Melatonin also stimulates bone marrow and spleen-mediated production of monocytes (Currier et al. 2000). Monocytes serve two important functions, secretion of cytokines and production of reactive oxygen species (ROS) critical for monocyte functioning. Melatonin activates human monocytes to secrete IL-1, IL-6, and IL-12, thereby activating and inducing cytotoxicity in monocytes. Melatonin prevents ultraviolet irradiation-induced apoptosis by inhibiting the intrinsic pathway at the mitochondrial level in monocytic cell line U937 (Luchetti et al. 2009). Macrophages are a group of highly diversified and plastic cells derived mainly from circulating monocytes, except for the tissue-resident macrophages which are known by various names in different tissues. Macrophages express the major histocompatibility complex class I and II by the virtue of which macrophage acts as antigen-presenting cells (APCs) that display antigens to and activate T lymphocytes. Melatonin supplementation enhances the expression of major histocompatibility complex class II (MHC-II) in antigen-presenting cells and peritoneal macrophages (Luo et al. 2020) and augments the secretion of IL-1, IL-6, TNF-α, and M-CSF (Guerrero and Reiter 2002). One of the aspects of macrophage function is related to its phagocytic activity. The nocturnal circulatory levels of melatonin enhance the phagocytic activity of peritoneal macrophages and testicular macrophages (Pawlak et al. 2005; Sanchez et al. 2008). Melatonin influences anti-inflammatory (M2) polarization in macrophages by inhibiting nitric oxide (NO) production and inhibiting the expression of NF-kB and cyclooxygenase-2 (COX-2) and promotes NF-E2-related factor 2 (Nrf2) and haemoxygenase1 HO-1(Aparicio-Soto et al. 2014; Singh and Haldar 2016).

Dendritic cells are specialized APCs that link innate and adaptive immunity and are extensively found in the primary and secondary lymphoid organs except for bone marrow. A very recent study demonstrates that melatonin exerts a stimulatory effect on dendritic cell numbers and its secretory activity which may be correlated to increased immunity (Abd-Elhafeez et al. 2021). In vitro treatment of melatonin enhanced the intensity of oxidative burst in neutrophils but inhibited metalloprotease activity thereby inhibiting L-selectin cleavage (Recchioni et al. 1998). Exposure to constant light has been shown to decrease the phagocytic activity of the neutrophils which was regained following melatonin supplementation suggesting the involvement of melatonin in the maintenance of neutrophil-mediated phagocytosis (Hriscu 2005). Natural killer (NK) cells are the third-largest subset of the lymphocytes that possess the ability to kill or eliminate without undergoing clonal expansion and differentiation. Different studies suggest that melatonin in conjunction with IL-2 increases the number of NK-cells (Currier et al. 2000). Pinealectomized mice have been reported to show diminished NK-cell activity which was resumed following melatonin administration (Del Gobbo et al. 1989). The melatonin-mediated increase in NK-cell number and activity has been attributed to increased T-helper cell cytokines IL-2, IL-6, IL-12, and IFN-$\gamma$ (Lissoni et al. 1998; Currier et al. 2000).

### *15.4.2 Immunocompetent Cells and Melatonin Receptors*

Most of the immunoenhancing effects of melatonin on immune cells are either mediated by membrane-bound MT1 and MT2 melatonin receptors belonging to GPCR super family (Carrillo-Vico et al. 2013; Gupta and Haldar 2013) or through nuclear receptors belonging to RZR/ROR subfamily (Lardone et al. 2011; Gupta et al. 2015). Apart from canonical receptors many of the actions of melatonin are receptor-independent viz. scavenging of free radicals; interaction with cytosolic proteins and enzymes like calmodulin, calreticulin, metalloproteinase-9 (MMP-9), and quinone reductase 2 (Liu et al. 2019a, b). Lymphocytes, monocytes, and other immune cells widely express melatonin membrane receptors, and their expression depends on the maturation, physiological status, and age of the immune cells (Ahmad and Halder 2012; Carrillo-Vico et al. 2013). Studies by Drazen and colleagues (2001) indicated that melatonin receptor subtype MT2 is involved in melatonin-induced enhancement of cell-mediated and humoral function in mice. However, a report from our lab suggested the involvement of MT1 receptor in mediating the immunomodulatory roles of melatonin in a tropical seasonal breeder, *Funambulus pennanti* (Ahmad and Halder 2012; Gupta and Haldar 2013). Apart from expressing melatonin receptors, immunocompetent cells like monocytes, macrophages, neutrophils, mast cells, and lymphocytes including B and T-cells have been reported to express the biosynthetic machinery for the synthesis of melatonin (Maldonado et al. 2010; Carrillo-Vico et al. 2013; Calvo et al. 2013; Yoo et al. 2016). The melatonin derived from the immune cells through paracrine, autocrine, or intracrine mechanisms plays an important role

in the maintenance of cellular physiology and serves cytoprotective functions there by regulating the immune mechanisms.

### *15.4.3 Anti-inflammatory Potential of Melatonin*

Melatonin apart from promoting an effective immune response restrains the persistent inflammatory events which can cause tissue damage. However, melatonin does not act as a blunt anti-inflammatory agent, it rather modulates the immune response in a complex manner such that the body is protected from chronic and deleterious effects of inflammatory response. The antioxidant and anti-inflammatory actions of melatonin are of great importance for the maintenance of health and longevity. Melatonin has been reported to reduce symptoms of "inflammaging" (low-grade inflammatory processes during the progression of aging) in the senescence-accelerated aging mice model and counteracts the low-grade brain inflammation (Hardeland et al. 2015). The amyloid-beta (Aβ) peptide, a central player in the pathogenesis of Alzheimer's disease, acts synergistically with pro-inflammatory cytokines to promote astrocyte and microglia activation (LaRocca et al. 2021). The release of pro-inflammatory mediators is not restricted to microglia, even neurons respond to Aβ peptide by upregulating the expression of cytokines like tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and T-cell and monocyte chemo attractant factor (CX3CL1) (Hanzel et al. 2014). Melatonin has been reported to show anti-amyloidgenic effect and promote Aβ clearance and suppress pro-inflammatory mediators (Hardeland 2018). Melatonin administration in an experimental model of inflammation has also been shown to reduce pro-inflammatory cytokines like TNF-α and IL-1β while enhancing the levels of anti-inflammatory cytokines IL-4 (Carrasco et al. 2013). Melatonin supplementation inhibits transcriptional activation of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) and suppresses the expression of inflammatory mediators like leukotrienes, chemokines, and adhesion molecules (Deng et al. 2006; Liu et al. 2017). Melatonin-mediated reduction of inflammatory reaction involves degradation of IκBα thereby retarding the nuclear translocation and transcriptional activation of pro-inflammatory factor NF-κB (Li et al. 2005). To exhibit its anti-inflammatory actions, melatonin activates SIRT1 leading to upregulation of Nrf2 and downregulation of NF-κB (Negi et al. 2011; El-Bakry et al. 2018). The functional association between SIRT1 and melatonin seems to be overlapping as SIRT1 is known to enhance the circadian amplitude of SCN that may influence melatonin rhythm and SIRT1 and melatonin perform similar actions (Chang and Guarente 2013; Hardeland 2018). Likewise, inhibiting NF-κB melatonin is also reported to prevent gasdermin D (GSDMD) inducing pyroptosis in adipose tissue (Liu et al. 2017).

NLRP3 inflammasome activation and induction of inflammatory caspases can be induced by a variety of signals under different conditions. Melatonin has been reported to downregulate NLRP3 and inhibit inflammasome activation via a mitophagy-mediated reduction in levels of ROS (Cao et al. 2017; Liu et al. 2017). Melatonin has been reported to reduce lipopolysaccharide (LPS) induced

inflammation thereby preventing NLRP inflammasome formation in adipocytes by downregulating genes involved in inflammasome assembly, i.e., NLRP3, ASC, caspase-1, and IL-1β. Activation of TLR-4 (toll-like receptor4) is another pro-inflammatory pathway being targeted by melatonin. In lipopolysaccharide (LPS)-stimulated macrophages RAW264.7, melatonin downregulated interferon (IFN)-regulated factor-3 (IRF3), which was involved in TLR-4-mediated TRIF-dependent signaling thereby suppressing the expression of pro-inflammatory cytokines viz. TNF-α, IL-1β, IL-6, and IL-8 (Xia et al. 2012). Modulation of the mTOR (mechanistic target of rapamycin) pathway by melatonin has also been shown to manifest its anti-inflammatory effects. Melatonin inhibits mTOR expression thereby interrupting the mTOR signaling and activation of pro-inflammatory cytokines in the hippocampus in an experimental model of isoflurane-induced cognitive impairment (Yuan et al. 2019). Melatonin prevents ethanol-induced activation of mTOR, AMP-activated protein kinases (AMPK), mitogen-activated protein kinase (MAPK), and nuclear factor of activated T-cells (NFATc-1) pathway thereby alleviating the senescence-like phenotype and osteoclast activity in human periodontal ligament and cementoblasts cells via inhibition of PIN1 pathway (Bae et al. 2018). Anti-inflammatory properties of melatonin have also been extensively studied in sepsis. In experimental models of sepsis, melatonin has been shown to improve survival and prevent multiorgan failure through the restoration of redox homeostasis via regulation of ETC function, inhibition of iNOS expression and nitric oxide synthesis, and reducing cytokine production (Colunga Biancatelli et al. 2020). Furthermore, the overproduction of reactive oxygen species contributes significantly to the inflammatory process via the activation of pro-oxidant genes that eventually results in the activation of pro-inflammatory markers. Melatonin by its antioxidant properties counteracts inflammatory processes via direct or indirect purging of free radicals.

## 15.5 Melatonin and Metabolic Health

The earliest reference regarding the relationship between pineal neurohormone melatonin and energy metabolism was given by a Romanian group describing pineal peptide "pinealin" as being similar to insulin in its anabolic, hypoglycemic and anti-cholesterinemic effects (Milcu and Milcu 1958). Pinealin was reported to improve glucose tolerance, while pinealectomy was shown to inhibit insulin secretion and impair glucose tolerance (Diaz and Blázquez 1986). However, several contrasting reports were also published regarding the role of melatonin in the regulation of glucose metabolism (Bailey et al. 1974; Neacşu 1988). In recent decades, various experimental studies have recognized the involvement of melatonin in metabolic processes and regulation of energy balance in terms of food intake, energy storage, and energy expenditure (Cipolla-Neto et al. 2014). Melatonin dictates the daily rhythm of metabolic hormones like leptin, ghrelin, resistin, and adiponectin to modulate nutrient utilization and storage thereby synchronizing these metabolic rhythms to the environmental light–dark cycle to ensure metabolic homeostasis (Chakir et al.

2015; Challet 2015). Disruption of these functional metabolic rhythms, as in the case of shift workers, can lead to the development of obesity and metabolic syndrome. Melatonin supplementation has been reported to suppress body weight gain and reduce adiposity (She et al. 2009; Nduhirabandi et al. 2011). The reversal of body weight gain following melatonin supplementation was independent of food intake suggesting an increase in the energy expenditure mechanisms (Wolden-Hanson et al. 2000) while the rats with ablated pineal gland developed adiposity (Alonso-Vale et al. 2004). The development of adiposity was probably due to the induction of leptin resistance which was likely to affect the ability of leptin to influence body weight, food intake, and hypothalamic centers regulating satiety (Buonfiglio et al. 2018), suggesting the protective role of melatonin against leptin resistance during the obesity (Suriagandhi and Nachiappan 2022). Melatonin administration has also been shown to retard the body weight gain and restore insulin sensitivity in animal models of diet-induced obesity (DIO) (Sartori et al. 2009). Recent studies carried out in melatonin receptor MT1 knock-out (KO) mice suggest that melatonin through MT1R signaling exerts its protective effect on metabolic responses in the case of DIO. Thus, MT1R can be one of the important therapeutic targets for counteracting obesity (Owino et al. 2019).

Furthermore, melatonin supplementation has been shown to limit hypertrophic obesity and decrease the density of crown-like structures in adipose tissues thereby improving the inflammatory profile of the adipocytes in high-fat diet-induced model of obesity (de Farias et al. 2019a, b). Melatonin supplementation prevents morphological alterations in adipocytes, inhibits inflammatory cell infiltration, and attenuates the pro-inflammatory adipokines expression (Farias et al. 2019a, b), reducing the inflammatory response and improving the sensitivity of peripheral organs to insulin and leptin signals for better glycemic control (Favero et al. 2015; Oliveira et al. 2018). Melatonin promotes lipolysis in adipocytes and upregulates the expression of perilipin 1 (PLIN1) and enzymes like hormone-sensitive lipase (HSL), adipocyte triglyceride lipase (ATGL) via activation of MT2R signaling (Yang et al. 2017). Melatonin-mediated reduction of body weight gain may be associated with role in energy expenditure. In Zücker diabetic fatty rats, melatonin treatment induces browning of inguinal fat pads and increases brown adipose tissues (BAT) weight and expression of uncoupling protein 1 (UCP1), associated with energy expenditure through non-shivering thermogenesis (Fernández Vázquez et al. 2018). Melatonin reduces ectopic deposition of fat in muscles and promotes intramuscular thermogenesis by enhancing mitochondrial biogenesis and mitochondrial respiration (Liu et al. 2019a, b). Melatonin was shown to inhibit high-fat diet-induced oxidative damage to the liver and reverse the loss of mitochondrial membrane potential, prevented mitochondrial fission, and was shown to restore mitophagy to improve hepatocyte function in non-alcoholic fatty liver disease (NAFLD) (Zhou et al. 2018). In an experimental model of NAFLD and hyperlipidemia, melatonin decreases the activity of the hepatic lipogenic enzymes and enhances the expression of hepatic carnitine palmitoyltransferase-1 (Ou et al. 2019). Ablation of the pineal gland induces nocturnal hepatic glucose production and increases gluconeogenesis due

to activation of unfolded protein response (UPR) mediated by activating transcription factor 6 (ATF6) (Nogueira et al. 2011). Melatonin reduces the expression of fetuin-A (FETUA) and α2-HS-glycoprotein gene (AHSG), hepatokines involved in insulin resistance, and alleviates hepatic steatosis (Heo et al. 2018). Recent studies suggest that the impact of melatonin on the metabolic outcomes is also mediated by alterations in gut microbiota. Melatonin treatment has been shown to change the composition of gut microbiota in high-fat-fed mice (Xu et al. 2017). Melatonin supplementation decreased Firmicutes to Bacteroidetes ratio and increased Akkermansia while normalizing the diversity of gut microbes thereby inhibiting low-grade meta-inflammation and body weight gain (Yin et al. 2018).

### *15.5.1 Melatonin in the Protection of Cardiovascular Health*

The favorable effect of melatonin on serum cholesterol and lipid profile forms the very basis for its cardioprotective role in the metabolic disorders. Several experimental studies have shown that melatonin reduces the number and area of atheromatous plaques thus being effective in the treatment of atherosclerosis (Rodella et al. 2013). Melatonin has been shown to retard the progression of atherosclerosis and stabilize the rupture-prone plaques (Ding et al. 2019). Melatonin has been shown to improve the characteristic features of diabetic cardiomyopathy including reduced myocardial fibrosis, vascular endothelial cell death, oxidative, and endoplasmic reticulum stress and improves microcirculation and mitochondrial function (Huang et al. 2022). Melatonin by the virtue of its anti-inflammatory actions protects against obesity and ischemic stroke (Yawoot et al. 2021). Diminished levels of melatonin and its metabolite, 6-sulphatoxymelatonin, have been reported in various cardiovascular conditions like myocardial infarction, coronary heart disease, and nocturnal hypertension (Dominguez-Rodriguez et al. 2016; Baker and Kimpinski 2018). Exogenous melatonin supplementation has been found to exert a protective effect against ischemia–reperfusion injury in diabetic rats (Yu et al. 2017), increased heart rate (Simko et al. 2016), and postural tachycardia (Green et al. 2014). The melatonin-mediated cardioprotective mechanisms mainly includes its antioxidative and anti-inflammatory effects with activation of Nrf2, reperfusion injury salvage kinase (RISK), and survivor activating factor enhancement (SAFE) mediated pathways, and nitric oxide signaling (Song et al. 2020). Melatonin prevents arrhythmogenic remodeling of cardiac tissue and reduces fibrosis and apoptosis in rat hearts (Prado et al. 2018). Melatonin protects against oxidized low-density lipoprotein-(ox-LDL-)induced endothelial cell damage and mitochondrial dysfunction and prevents endothelial cell pyroptosis (Zhang et al. 2018; Li et al. 2021). Melatonin via activation of nuclear receptor retinoic acid-related orphan receptor-α prevents endothelial dysfunction in systemic lupus erythematosus (Huang et al. 2022). It has been suggested that melatonin, through breast milk during the early days in neonates influences body weight in the later part of life, limits the development of comorbid obesity

and promotes optimal conditions for the development of the cardiovascular system in infants (Gombert and Codoñer-Franch 2021).

### *15.5.2 Melatonin and Diabetic Nephropathy*

Various experimental models of chronic kidney disease suggest positive effects of melatonin in lowering blood pressure (BP) and normalization of diurnal rhythms in non-dipper to dipper type of BP variations highlighting its reno-protective role (Simko et al. 2016). Common features of diabetic nephropathy include enlarged nephrons, hypertrophied mesangial cells resulting in glomerulosclerosis, and hyperfiltration (Bherwani et al. 2016). Apart from ROS, several other factors are involved in the progression of chronic kidney disease related to diabetes like dyslipidemia, inflammatory cytokine production, pro-fibrotic signaling, and connective tissue growth (Pourhanifeh et al. 2020). Melatonin treatment during diabetic nephropathy showed beneficial effects on glycemic control, high-density lipoprotein-cholesterol (HDL-C), and total antioxidant capacity of the blood serum (Satari et al. 2021). Melatonin showed a synergistic effect when used with folic acid and significantly decreased the plasma levels of urea, uric acid, creatinine, TNF-α, IL-6, cholesterol, triglycerides, and low-density lipoprotein (LDL) along with renal malondialdehyde (MDA) and nitric oxide in the kidney of diabetic rats (Ebaid et al. 2020). Melatonin reverses the effect of oxidative stress-induced renal tubular damage and reduces the level of *N*-acetyl-β-D-glucosaminidase and albumin in the urine of diabetic rats (Oktem et al. 2006). Melatonin when used with rowatinex showed the most potent effects against the streptozotocin-induced diabetic nephropathy (Motawi et al. 2019). Melatonin activates SIRT1/Nrf2/HO-1 signaling pathway to protect from oxidative injury induced by acute kidney ischemia/reperfusion (Shi et al. 2019). Melatonin inhibits the accumulation of advanced glycation products (AGEs) and transforming growth factor-β (TGF-β) and attenuates the activation of the renin-angiotensin system to protect against kidney damage induced by diabetes (Guo et al. 2021). Most of the evidence suggests that melatonin can contribute beyond its well-known antioxidant and anti-inflammatory activity to reverse the kidney damage induced by diabetes, however, further studies are required to get better insights into the reno-protective mechanisms of melatonin.

## 15.6 Bone Health (Osteoporosis and Osteoarthritis) and Melatonin

Bone is a dynamic organ in which remodeling occurs throughout life. The remodeling process involves the initiation of bone resorption by osteoclasts, the transition from

resorption to new bone formation, and bone formation by osteoblasts (Florencio-Silva et al. 2015). There exists a fine balance between the osteoclast-mediated bone resorption and osteoblast-mediated bone formation throughout life. Bone remodeling is crucial for fracture healing, and repair of microscopic cracks as well for regulating skeletal calcium homeostasis. Osteoblasts under the influence of bone morphogenetic proteins (BMPs), wingless (WNTs), and runt-related transcription factor (RunX2) get differentiated from the mesenchymal stem cells. RunX2 upregulates the osteoblast-specific genes such as collagen type II (ColII), alkaline phosphatase (ALP), bone sialoprotein (BSP), bone Gla (gamma carboxy glutamic acid rich) protein (BGLP), and osteocalcin (OCN) (Florencio-Silva et al. 2015). Nowadays, a huge population beyond the age of 40 years is affected with bone diseases due to lifestyle changes. Osteoarthritis and osteoporosis are the two most common diseases that are seen in aged people and are the cause of major disabilities worldwide (Cui et al. 2020).

### *15.6.1 Osteoporosis and Melatonin*

According to the studies conducted among Indian women beyond the age of 50 years, 46 million women have osteoporosis (Pal et al. 2016). Osteoporosis is chronic, an asymptomatic skeletal disorder that increases the fragility and high risk of fracture specifically hip, spine, and wrist. It is a slow progressing, silent disease that does not display any symptoms till bones fracture. Osteoporosis is a condition that appears when there is a reduction in bone volume and bone mass. Studies suggest that osteoporosis patients have an imbalance between osteoblast differentiation and osteoclast production (Hart et al. 2020). Bone modeling is either formation of bone by osteoblasts or the resorption of bone by osteoclasts where these activities occur in sequentially coupled manner. The primary function of bone modeling is to increase bone mass and maintain or alter bone shape (Cui et al. 2020). Osteoclasts cells degrade bone by generating free radicals, such as superoxide and hydroxyl anions (Florencio-Silva et al. 2015) and melatonin inhibits the osteoclast activity by scavenging the free radicals (Munmun and Witt-Enderby 2021). Melatonin also inhibits bone resorption by inducing osteoprotegerin (OPG). OPG retards the interaction between receptor activator NF-κB (RANK) and receptor activator NF-κB ligand (RANKL) by binding to RANKL thereby inhibiting the bone loss (Wada et al. 2006). On the other hand, it is noted that melatonin stimulates osteoblasts to counterbalance bone loss (Sethi et al. 2010).

Melatonin via binding to its MT2 receptor on mesenchymal cells influences the osteogenesis by the formation of osteoblasts (Sethi et al. 2010). It induces osteoblast differentiation through ERK1/2-MAPK signaling pathway and expresses differentiation markers like alkaline phosphatase (ALP). Melatonin also induces osteoblast differentiation by influencing BMP-2 and Runx2, p38, and ERK1/2 signaling (Sethi et al. 2010). Therefore, melatonin prevents bone degradation and promotes bone formation via its receptor-dependent and independent mechanisms. As discussed previously, melatonin alleviates the glucocorticoid-mediated stress

condition. Reports also suggest that for the treatment of a variety of inflammatory condition and autoimmune disorders glucocorticoid-based medicines are being used that causes a significant decrease in bone mass and increased risk of fracture. Melatonin may impair osteoclast activity by its free radical scavenging and antioxidant property. Melatonin also has been suggested to induce osteoblast differentiation and proliferation (Li et al. 2019).

Melatonin could be a potential treatment for osteoporosis. Melatonin resists bone loss *by* eliminating the free radicals required for osteoclast activity. Reports also suggested that melatonin and combined fluid shear stress (FSS) enhances ERK/Akt/mTOR signaling in preosteoclasts, which activates the anabolic effect for the preservation of cell structure and function against osteoporosis (Kim et al. 2018). Increased bone resorption and low bone mass are accompanied by oxidative stress (Domazetovic et al. 2017). Osteoclast degrade bone by generating free radicals like hydrogen peroxide, superoxides, and hydroxyl ions (Florencio-Silva et al. 2015). Other experimental evidence suggest that melatonin increases short-term bone formation and improves the alveolar bone loss and fracture healing in a diabetic mouse model by reducing the oxidative load (Kose et al. 2016). Melatonin downregulates the iNOS expression to reverse the changes associated with osteoporosis in the ovariectomized rats (Oktem et al. 2006).

### *15.6.2 Melatonin and Osteoarthritis*

Osteoarthritis (OA) is a chronic disability characterized by progressive degeneration of articular cartilage (AC), which covers the ends of long bones (Xia et al. 2014). More than 60% of the population above the age of 65 years suffers from this disease. The increasing number of incidences causes a massive loss in workplace productivity. This disease is ranked as the 15th major cause of years lived with disability (Bitton 2009). The most striking and unfortunate part of osteoarthritis is that at present there is "no disease-modifying therapy" available to deal with it. Osteoarthritis is considered a multi-factorial disease of the whole synovial joint. The onset and progression of osteoarthritis are being studied for last three decades and observed that there are multiple factors involved in this disease like age-associated inflammation, cellular senescence, mitochondrial dysfunction, oxidative load, genetic factors, mechanical insult, trauma, obesity, and low-grade inflammation (Loeser et al. 2016). With accumulating evidence, it is suggested that at present there is only palliative care being provided to intervene in the disease, but unfortunately, these treatments do not stop the progression of the disease and ultimately the joint fails, that is being replaced with a prosthesis (joint replacement), however, there is a limitation as well, i.e., limited shelf-life of the prosthetic joints (Steinhaus et al. 2017). This disease causes a huge economic burden to the family as well as the country (Bitton 2009).

The only cells present in articular cartilage are chondrocytes that secret extra cellular matrix containing collagen type II and proteoglycans. The quality of articular cartilage is maintained by the fine balance between the anabolic and catabolic

activity of chondrocytes. The declined proliferative capacity of chondrocytes leads to a significant reduction in extracellular matrix production that ultimately compromises the quality of articular cartilage (Hou et al. 2018). It is well documented that melatonin concentration declines with aging and that disrupts the tuning of oxidant and antioxidant balance in the physiological system. This leads to increased inflammation that might be involved in the progression of the declined anabolic function of chondrocytes and downregulates matrix synthesis that ultimately leading to reduced quality of articular cartilage and onset of osteoarthritis (Karasek and Reiter 2002).

The cellular death of chondrocytes and loss of ECM leads to compromised quality of articular cartilage. The ECM contains collagen type II (ColII) which is a key feature of articular cartilage (Taniguchi et al. 2009). During osteoarthritis matrix, metalloproteinases (MMPs) are produced by hypertrophic chondrocytes. MMP-13 is responsible for the degradation of collagen type II, aggrecan and fibronectin. Another enzyme a disintegrin and metalloproteinase with thrombospondin motifs, ADAMTS4 and ADAMTS5, cleave aggrecan that also promotes articular cartilage degradation (Neuhold et al. 2001; Song et al. 2007). Experimentally, it was observed that melatonin restores the major component of articular cartilage, collagen type II, through the downregulation of MMP-13, pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, ADAMTSs, and catalytic transcription factors such as NF-κB in case of osteoarthritis (Zhang et al. 2019). Increased inflammatory cytokines generate NO by the chondrocytes and catabolic enzymes that cause progressive articular degeneration (Loeser et al. 2012). ROS are primary factor involved in the development of osteoarthritis. Mitochondrial dysfunction in osteoarthritic chondrocytes causes oxidative stress by increasing generation of ROS and RNS that inhibits ECM synthesis from chondrocytes (Lepetsos and Papavassiliou 2016). Further, the increased oxidative stress accelerates catabolism and initiates chondrocyte death, destroying articular cartilage and disturbs chondrocyte homeostasis (Lepetsos and Papavassiliou 2016).

In humans, it was observed that during osteoarthritis, the expression of endoplasmic reticulum (ER) stress associated downstream molecular players are positively correlated with cartilage degeneration (Rellmann et al. 2021). Articular cartilage being a hypocellular and avascular tissue is always at a risk of hypoxic and catabolic stress that may lead to activation of ER stress that contributes to cartilage degeneration via chondrocyte apoptosis. The expressions of phosphorylated protein kinase R like endoplasmic reticulum kinase (pPERK), ubiquitin (Ub), C/EBP Homologous Protein (CHOP), and phosphorylated c Jun N-terminus kinase (pJNK) are positively associated with the number of caspase-3 positive chondrocytes in in vivo and in vitro conditions (Takada et al. 2011; Price et al. 2010). ER stress induced by tunicamycin increased CHOP expression and reduced X-box binding protein 1 (XBP-1) mRNA splicing in high concentrations results in extensive apoptosis (Takada et al. 2011). Several studies show the induction of CHOP happens earlier than anti-apoptotic BiP, and this rapid upregulation of CHOP contributes to chondrocyte death (Price et al. 2010). Advanced glycation products (AGEs) induce ER stress in chondrocytes by specific receptors for AGE (RAGE), and activation of RAGE engages critical signaling pathways. In human chondrocytes, AGEs induce

ER stress and stimulate the expression of cyclooxygenase-2 (COX-2) and PGE2 through eIF2α, p38-MAPK, and NF-κB pathways (Oakes and Papa 2015). Melatonin treatment inhibits ER stress by attenuating ER stress mediators. Melatonin also mitigates glucose regulated protein 78 (GRP78) upregulation, phosphorylation of pulmonary eIF2a, cleaved activating transcription factor 6 (ATF6) elevation, and repressed inositol requiring enzyme 1a (IRE1a) phosphorylation and activation of XBP-1 and JNK, two downstream targets of the IRE1 pathway (Zhao et al. 2014).

Chondrocyte apoptosis and decline in autophagy results in reduced cellularity in the superficial zone of articular cartilage (Zhao et al. 2019). Melatonin performs its chondroprotective role via SIRT1 signaling and reverses the detrimental effect of sirtinol that blocks the activity of SIRT1 (Coryell et al. 2021). In chondrocytes, SIRT1 exerts an anti-apoptotic effect by regulating gene expression of the transcription factors RelA/p65 and p53 (Yeung et al. 2004). Melatonin via SIRT1 pathway protects chondrocytes against ROS-dependent p38 kinase activation and suppression of chondrocyte apoptosis (Lu et al. 2021). Melatonin induces autophagy to prevent extracellular matrix (ECM) degeneration via NF-κB pathway to ameliorate apoptosis and calcification by SIRT1-mediated autophagy. Melatonin increases SOX9 levels to promote chondrogenesis under inflammatory conditions induced by IL-1β. It also blocks the other mediators of inflammation including iNOS and COX-2, at transcriptional and translational level and also inhibit the secretion of TNF-α, IL-1β, and IL-8 from chondrocytes in in vitro condition (Hosseinzadeh et al. 2016).

Under osmotic stress, SIRT1 induces nuclear factor of activated T-cell (NFAT5) expression (Johnson et al. 2014), that acts on a specific set of targets, including TNF-α, IL-6, nitric oxide synthase 2, and MMP-13 in a spatio-temporal manner (Yoon et al. 2011). Melatonin decreases SIRT1-dependent NFAT5 expression in chondrocytes treated with IL-1β and its supplementation significantly reduces TNF-α, IL-1β, prostaglandin E2 (PGE2) in chondrocytes showing its suppressive effect on inflammation (Guo et al. 2017). Therefore, it can be suggested that melatonin exerts its effects in osteoporosis as well as different stages of osteoarthritis. Moreover, melatonin has therapeutic potential for bone regeneration and may also act as a potent therapeutic drug in osteoarthritis to prevent the exacerbation of articular cartilage damages.

## 15.7 Life Span Extending Benefits of Melatonin

Healthy aging and longevity have been one of the greatest pursuits of mankind. An unending search for an agent that could increase health expectancy and decrease the burden of age-related degenerative diseases has brought melatonin into focus. The declining nocturnal peak of melatonin in elderly associated melatonin to aging (Karasek and Reiter 2002; Tozawa et al. 2003) and based on this background melatonin supplementation was hypothesized to promote healthy aging and prolong life span (Anisimov et al. 2003). Unlike pineal melatonin, aging promotes the expression of enzymes related to melatonin biosynthesis in metabolically active tissues like

liver, intestine, and kidney. This locally produced, extra pineal melatonin activates antioxidant repertoire thereby defending these organs against age induced oxidative damages (Popović et al. 2018). Although scientists always remained doubtful regarding the clinical utility of melatonin, however, previous studies have reported the antioxidant, analgesic, anti-stress, and chronobiotic benefits of melatonin supplementation in counteracting age-related diseases and enhancing life span (Marseglia et al. 2015; Anghel et al. 2022). Initial studies demonstrated that pineal gland ablation induced senescence was reversed following melatonin supplementation in rats (Dilman et al. 1979; Armstrong and Redman 1991). Pineal of young animals when grafted into old animals delayed the development of senescence-like phenotype and prolonged the life span of old animals (Pierpaoli and Regelson 1994). Even lower dose of melatonin was shown to reduce the tumor incidence, especially the mammary carcinomas, thereby influencing the life span of the animal (Anisimov et al. 2003).

The prolongation of life span by melatonin has mostly been implied in terms of its immunomodulatory, antioxidant, and anti-stress properties. A recent study suggests that melatonin prolonged the life span of animals independent of the age at which the melatonin supplementation was started (Damiani et al. 2020). Melatonin supplementation effectively reduces age-dependent DNA damages exhibiting anti-genotoxic and anti-mutagenic potential thereby maintaining the genomic integrity (Damiani et al. 2020). Telomeres are considered as the guardian of genome stability and oxidative stress has been shown to negatively impact telomere length and promote its attrition, a hallmark of aging (Gavia-García et al. 2021). Reports suggest that melatonin facilitates telomere elongation probably through stimulation of telomerase activity, thus preventing age-related degenerative conditions in vascular endothelial and retinal pigment epithelial cells (Rastmanesh 2011; Xie et al. 2021). Melatonin interacts with numerous DNA repair and DNA damage response processes (Liu et al. 2013) and induces phosphorylation of p53 (Ser-15), a critical mediator of DNA protective effects of melatonin, responsible for regulation of cell survival, proliferation, and prevention of cancer (Santoro et al. 2012). Apart from enabling molecular defense mechanisms to prevent DNA damages melatonin also offers on-site protection to DNA through scavenging locally generated free radicals (Galano et al. 2018). Evidences indicate that there exists a direct connection between telomere attrition and mitochondrial dysfunction (Passos et al. 2007). Moreover, an aging axis has been proposed that links compromised genomic integrity to altered mitochondrial biogenesis and function via p53-mediated suppression of PGC1α and PGC1β (Sahin and DePinho 2012).

### *15.7.1 Melatonin and Mitochondrial Health*

Longevity is intimately related to mitochondrial function, while mitochondrial malfunction has been associated with a plethora of diseases collectively called as “mitochondrial diseases,” e.g., neurodegenerative disorders, cardiomyopathy, diabetes mellitus, and cancer. The connection between these diseased states and

mitochondria lies in the higher rate of accumulation of mutation in mitochondrial DNA (mtDNA), expansion of mutated mtDNA and age-related deterioration of the organelle-specific quality control mechanisms (Lionaki et al. 2022). In this context, the regulation of mitochondrial function by melatonin can be one of the mechanisms through which melatonin might promote health and longevity. Mitochondria, in fact, happens to be the most prominent target organelle for melatonin's pleotropic actions (Reiter et al. 2017). Mitochondria not only synthesize melatonin but also accumulate and metabolize melatonin (Reiter et al. 2021; He et al. 2016). Melatonin preserves mitochondrial function by retarding free radical generation at the level of electron transport chain, a process known as radical avoidance (Hardeland 2009). Melatonin stimulates ATP production without altering ATP synthase activity and ROS generation which is critical for prevention of various pathophysiological conditions related to mitochondrial diseases (Reiter et al. 2020a, b; Jauhari et al. 2020). Melatonin maintains mitochondrial membrane potential and prevents opening of the mitochondrial permeability transition pore (mPTP) (Petrosillo et al. 2009). Melatonin has also been demonstrated to prevent oxidation of cardiolipin, a phospholipid located at the inner mitochondrial membrane, thereby preventing cytochrome c release and subsequent activation of apoptotic pathway (Petrosillo et al. 2009). Melatonin by modulating mitochondrial dynamics (mitochondrial fission and fusion) has been shown to regulate redox homeostasis and bioenergetics (Paradies et al. 2010; Tan et al. 2016). Thus, melatonin supplementation can prove to be an effective therapeutic strategy against oxidative stress and age-induced mitochondrial dysfunction that could jeopardize cell survival and health.

### *15.7.2 Melatonin, Circadian Rhythm and Health*

Rhythmicity in the biological clock-controlled functions is also related to well-being of the organism and is among one of the aspects of melatonin physiology that may extend life span (Acosta-Rodríguez et al. 2021). Lack of rhythmicity results in loss of the adaptive ability and impairs the capacity of tissue regeneration (Acosta-Rodríguez et al. 2021; Paatela et al. 2019). Aging results in diminished amplitude of the circadian pacemaker as evident from the decreased melatonin secretion. The loss in circadian amplitude can lead to internal temporal disorder which may act as a prelude for diseased state that may manifest in the form of temporal crises related to sleep–wake cycle, cardiovascular activity, intestinal motility, asthma, and allergic attacks (Froy 2011). Exogenous melatonin supplementation feedback on the circadian pacemaker system to enhance the amplitude of circulatory melatonin thereby retarding the symptoms of aging and increase life span (Armstrong and Redman 1991). Evidence suggests that disruption of circadian system with advancing age is partly due to loss of sensitivity of the suprachiasmatic nucleus (SCN), to the entrainment signals (Chang and Guarente 2013). Inability to adapt to the entrainment signals affects the endogenous periodicity tau ($\tau$), by either shortening it or making it longer than 24 h (h). A positive association between tau close to 24 h and survival have been

suggested (Wyse et al. 2010). Indeed, it was shown that hamsters carrying 20 h period mutation tau, exhibit reduced longevity (Hurd and Ralph 1998). In another study, chronic disruption of circadian pacemaker by continuous reversal of light–dark cycle reduced the life span of cardiomyopathic hamsters (Penev et al. 1998). In fact, aged animals show higher mortality due to phase shifts induced by changing light–dark cycle while, fetal SCN implants in aged animals were shown to restore the higher amplitude rhythms and promote longevity (Davidson et al. 2006; Hurd and Ralph 1998). Thus, impaired circadian rhythmicity is associated with increased morbidity reduced life span, while melatonin supplementation may reset circadian rhythms and restore the pacemaker's amplitude thereby promoting survival.

## 15.8 Phytomelatonin: A Natural Nutraceutical for Health

D. van Tassel and O'Neill (1993) for the first time identified the endogenous melatonin in higher plants. The presence of melatonin in the Convolvulaceae ivy (morning glory: *Pharbitis nil*, syn. *Ipomoea nil*) and in tomato fruits (*Solanum lycopersicum*) was detected by radioimmunoassay (RIA) and gas chromatography-mass spectrometry (GC–MS), although the results were unpublished until 1995 (D. van Tassel et al. 1995). This melatonin identified in plants was named "phytomelatonin." In due course of time presence of melatonin was identified in coffee beans in 1970, it was isolated as a by-product during the processing of coffee beans (Tan et al. 2012). Since then, a variety of plant species were analyzed and it has been observed that different cereals and medicinal herbs contain a high concentration of melatonin (Hattori et al. 1995; Hardeland and Pandi-Perumal 2005). Surprisingly, the existence of melatonin was also noted in the edible plants and vegetables as well (Hattori et al. 1995; Reiter et al. 2007; Manchester et al. 2000). The presence of melatonin in plants modulates a range of physiological functions like flowering, fruit ripening, stress responses, morphogenesis, and photoprotection and antioxidant response (Arnao 2014).

The consumption of melatonin-rich plant products has been shown to influence the endogenous melatonin concentration (Reiter et al. 2005; Dragsted et al. 1993). Cheap and easily available economical cereals like corn (*Zea mays*) consumption have been shown to increase the endogenous melatonin concentration and improve the antioxidant enzymes status and proliferative potency of peripheral blood mononuclear cells (PBMCs) (Singh and Haldar 2017). The increase in endogenous melatonin concentration could be due to the high tryptophan content (32 mg/100 g of corn seeds) in corn seeds (www.ogtr.gov.au). The pineal gland has a high affinity for uptake of circulatory tryptophan for the synthesis of serotonin and melatonin (Paredes et al. 2009). Epidemiologic evidence suggests that the intake of vegetable has beneficial effects in protecting against cancer and cardiovascular diseases (Riboli and Norat 2003; Bazzano et al. 2003). Multiple studies have identified the beneficial effect of consuming vegetables which might be related to the presence of melatonin with other phytochemicals (Dragsted et al. 1993; Bazzano et al. 2003). The consumption of phytomelatonin-containing nuts like walnut (Reiter et al. 2005) and fermented

products like beer (Maldonado et al. 2009) has also been reported to increase the endogenous melatonin concentration and antioxidant capacity of the serum.

Although the content of melatonin in plant-based supplements is lower compared to exogenous sources containing chemically synthesized melatonin. However, phytomelatonin supplementation even at a low dose could improve circulatory melatonin levels up to 40 times within 5 min (Van Der Helm Vam Mil et al. 2003). Consumption of phytomelatonin-rich Japanese vegetables like sweet corn, bitter gourd, Japanese radish sprout, shimeji mushroom, and shiitake mushroom increases the endogenous melatonin concentration that has been suggested to protect from cancer and cardiovascular diseases (Oba et al. 2008).

Melatonin has a huge number of beneficial effects like antioxidant, anti-stress, oncostatic, and immunomodulatory impact but its supplementation is either subcutaneous or oral in the form of tables available over the counter. However, the general psychology of taking any medicine should be discouraged, and the general practice of preferring some dietary remedies be considered. Accounting for these concerns it is advocated to add phytomelatonin-rich plant products in our daily diet that may help to maintain the endogenous melatonin concentration in healthy and aged populations as well as in immunosuppressed individuals undergoing various treatment regimens.

The addition of phytomelatonin as a nutraceutical might be a promising non-invasive approach to improve health. Phytomelatonin supplementation might help protect against different bacterial and viral infections and can reduce low-grade inflammation thereby protecting against various age-associated cardiovascular diseases and skeletal complications. Therefore, it is suggested that naturally available resources rich in melatonin and other antioxidants should be included in our diet. This warrants further investigation that whether the consumption of phytomelatonin-rich food products could counterbalance the side effects of different drugs like glucocorticoid-based therapy, chemotherapy, etc., being used routinely in various clinical settings.

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# Part V
# Genetic Regulation of Sleep and Clock

# Chapter 16
# Circadian Rhythm Manipulations: Implications on Behavioral Restoration in Central Nervous System Insults

**Duttagupta Subhadeep, B. N. Srikumar, B. S. Shankaranarayana Rao, and Bindu M. Kutty**

## 16.1 Introduction

We live in a rapidly evolving 24 h society with hectic work schedules and mismanaged lifestyles. With the advent of electricity and blooming technology, humans nowadays are exposed to artificial light throughout the day. Night shifts and variation in work rotation schedules between day and night have become common across the globe. The environment plays a significant role in developing neural functions and adaptive flexible behavior in most living organisms. Ambient light is a prominent and crucial environmental cue (*zeitgeber*) that affects brain physiology and behavior. Apart from its role in visual image formation, light also has non-image-forming functions as well, such as entraining circadian rhythms, regulating physiological events, and influencing mood and cognition in mammals (Fu et al. 2005; Yan et al. 2019). The daily light-dark cycle is the major cue that entrains the mammalian circadian system (Daan and Aschoff 2001) and coordinates our bodily functions (Hastings et al. 2003). Intrinsically photosensitive retinal ganglion cells (ipRGCs) convey light information to the suprachiasmatic nucleus (SCN), the central clock in mammals (LeGates et al. 2014). Photoperiodism is the biological capability of an organism to measure and entrain to the environmental day-length, perceive the time of the year, and adapt physiologically and behaviorally to the seasonal change (Walton et al. 2012a, b). Prolonged exposure to either light or darkness desynchronizes the body's biological and behavioral clock, leading to negative consequences for health including mood and cognitive disruption.

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Depending on the activity patterns, mammals have been divided into two chronotypes: diurnal and nocturnal. Diurnal animals, including humans, orchestrate their physiological variables, metabolism, cognitive and behavioral functions during the daytime, whereas energy conservation, repair and consolidation occur in the inactive phase of rest at night. On the other hand, activities such as foraging, hunting, and mating by the nocturnal animals (like rodents and most mammals) peak during the night. Light synchronizes the diurnal and nocturnal circadian systems in the same manner. However, the circadian-independent direct influence of light on the brain (such as hypothalamus and brainstem regulatory structures- locus coeruleus and dorsal raphe nucleus) and behavior is different in both chronotypes (Challet 2007; Yan et al. 2018; Perrin et al. 2004). Interestingly, melatonin secretion peaks during the dark phase in both diurnal and nocturnal animals, but the nocturnal animals are active when the melatonin levels are high, whereas low levels of melatonin promote activity in diurnal animals (Challet 2007). Recent advancement in medical research has highlighted the effectiveness of bright light therapy and chronotherapy (e.g., time-restricted feeding and chrono-exercise) in combination with drugs for treating psychiatric and neurological disorders like depression, seasonal affective disorders, bipolar disorder, Alzheimer's, and Parkinson's diseases (AD and PD) (Avery et al. 1990; Terman and Terman 2005; Johnstone et al. 2016, Lee et al. 2021). In this chapter, we discuss the role of several circadian manipulation strategies in facilitating cognitive restoration in both clinical and pre-clinical conditions. We then consider some of the central hypotheses of the underlying changes and summarize the evidence for putative physiological and molecular mechanisms of action.

## 16.2 Circadian Rhythm Manipulation in Human Neurodegenerative Conditions

Researchers have extensively investigated the efficacy of bright light therapy (BLT) in neurodegenerative and neuropsychiatric conditions for the last two decades. A meta-analysis by Golden et al. (2005) reported a significant decrease in depressive symptom severity following BLT in both seasonal affective disorder (SAD) and non-seasonal depression. Longitudinal studies reported that almost two-thirds of the SAD patients respond positively following BLT (Loving et al. 2005). Numerous clinical studies have reported that BLT can consolidate rest and activity patterns in people suffering from AD (Fetveit et al. 2003; Dowling et al. 2005; Sloane et al. 2007). BLT during the morning (around > 1000 lux) has been shown to improve cognitive functions, increase night-time sleep, decrease daytime sleepiness, and reduce sundowning behavior in patients suffering from AD and related dementia (Lyketsos et al. 1999; Yamadera et al. 2000; Ancoli-Israel et al. 2003; Alessi et al. 2005; Sloane et al. 2007). In addition, PD patients showed noticeable improvement in sleep onset and continuity, mood and motor functions following 2–5 weeks of BLT (Willis and Turner 2007). In an RCT study, BLT (7500 lux for 30 mins in the morning for two weeks) significantly

improved the PD-related motor symptoms and moderately improved the mood (Paus et al. 2007) in PD patients. Furthermore, increased sunlight exposure reduces the rate and severity of symptoms in other psychiatric ailments, including bipolar disorder (Benedetti et al. 2001). Kent et al. (2009) reported that a low level of sunlight exposure is correlated with a higher chance of cognitive impairment. Light affects cerebral blood flow which further increases alertness and heuristic processing (Sinclair et al. 1994; Vandewalle et al. 2006) and shows a dose-response relationship with cognitive functions in depressed individuals (Kent et al. 2009).

Sleep deprivation therapy is another important circadian rhythm manipulation strategy reported to be used commonly for mood disorders for more than three decades. Studies have suggested that 45–70% of the depressed subjects had a 50% reduction in Hamilton Rating Scale for depressive symptoms following a combinatorial light and repeated total sleep deprivation therapy for a week (Giedke and Schwärzler 2002; Benedetti et al. 2005). A low level of adenosine is typically reported in depression [reviewed by Gomes et al. (2021)]. Sleep deprivation is known to regulate adenosine signaling by increasing vesicle-associated membrane protein (VAMP)-dependent ATP exocytosis from astrocytes (Hines et al. 2013; Dallaspezia and Benedetti 2015). In addition, sleep deprivation is associated with rapidly increasing serum brain-derived neurotrophic factor (BDNF) and normalization of decreased IL-6 levels in subjects with depression, which leads to rapid improvement in their depressive symptoms (Gorgulu and Caliyurt 2009; Voderholzer et al. 2012). However, the clinical value of sleep deprivation therapy is limited by the fragility of its response. Scant literature exists in understanding the antidepressant action of sleep deprivation in animals. Lopez-Rodriguez et al. (2004) showed that total sleep deprivation for 24 h decreased the immobility time in the forced swim test in adult rats which might be associated with increased extracellular serotonergic levels in the hippocampus (Lopez-Rodriguez et al. 2003, 2004). Taken altogether, circadian manipulation therapies have emerged as efficacious non-pharmacological biologically oriented treatment approaches in psychiatry today.

## 16.3 Evidence of Circadian Rhythm Manipulation to Restore Behavior and Cognition in Animal Models

To understand its fundamental mechanisms of action, circadian manipulation studies have been extensively investigated in both diurnal and nocturnal animal models. Photoperiod alteration triggers structural changes in the brain and influences the functional connectivity of regions regulating affective behavior (Salgado-Delgado et al. 2011). Exposure to bright light (3000 lux) for daily 1 h for 3 weeks resulted in anxiolytic and antidepressant effects in diurnal Sand rats (Ashkenazy et al. 2009a, b). These rats significantly spent more time in the open arms of the EPM and took more time to sink in the forced swim test. Short photoperiod (8:16 h light-dark cycle)

for 10 weeks increased associative fear memory in a fear conditioning task in white-footed mice (Walton et al. 2012a, b). These behavioral effects were associated with an increased dendritic spine density of the basolateral amygdala neurons. Reports suggest that rodents display increased social affiliation and reduced aggression during short days (Beery et al. 2008; Ashkenazy et al. 2009a, b). In wild conditions, rodents huddle up together in their nests to reduce thermoregulatory demand during winter or short-day conditions (Andrews and Belknap 1993). Increased oxytocin binding is related to increased affiliative behavior, whereas increased levels of vasopressin lead to aggression (Ferris 2005; Lee et al. 2009). The levels of oxytocin and vasopressin are modulated by pineal melatonin which gets affected due to photoperiod manipulation. Hence, the photoperiodic response of oxytocin receptors to short photoperiod needs to be studied to understand its effects on social behavior.

Exposure to a short photoperiod (5:19 h light-dark cycle) for one week resulted in decreased anxiety and depressive-like behavior in adult Wistar rats (Dulcis et al. 2013). Rats exposed to short photoperiod spent more time exploring the open arms of the elevated plus maze (EPM). They also spent a long time swimming before becoming immobile in the forced swim test. C57BL/6J mice with reduced dopamine transporter expression exhibit increased reward-responsiveness following two weeks of short photoperiod exposure (5:19 h light-dark cycle) in a probabilistic learning test (Young et al. 2018). These mice show decreased reward collection latency (milkshake vs. timeout) in a win-stay task suggestive of increased motivation and reward sensitivity following short photoperiod. Additionally, higher open arm percent entries in the EPM were also observed in these mice suggesting an increased risk-taking behavior (Young et al. 2018). In our laboratory, we have utilized this phenomenon of photoperiod-induced plasticity and studied its effects on the ventral subicular lesioned (VSL) rat model of neurodegeneration and cognitive impairment. Exposure to short photoperiod (6:18 h light-dark cycle) regime for three weeks reversed the VSL-induced anxiety-like behavior assessed using open field test, EPM and light-dark test (Subhadeep et al. 2017, 2020). The short photoperiod exposed VSL rats also showed an improvement in motivational and hedonic behavior (Subhadeep et al. 2020). The photoperiod manipulation with short photoperiod also restored the cognitive functions of the VSL rats as they performed significantly better than the normal photoperiod exposed counterparts in the Morris water maze (Subhadeep et al. 2021). Another study from our laboratory demonstrated that a combinatorial paradigm of wheel running and casein wheat diet in an enriched environment reverses spatial memory deficits and restores hippocampal neurogenesis in VSL rats (Kapgal et al. 2016). Wheel running is reported to have synchronizing effects on the entrainment of circadian systems (Tal-Krivisky et al. 2015). Recently, wheel running has shown to be beneficial in lowering anxiety-, and depressive-like behavior and improving recognition memory in diurnal Sand rats (Bilu et al. 2022).

Cuesta et al. (2014) studied the effect of photoperiod manipulation in a transgenic mouse model of Huntington's disease (HD). A combination of exposure to 10,000 lux for daily 1 h before lights-off and voluntary wheel running exercise resulted in delayed disintegration of the rest-activity rhythm and restored behavioral synchronization to the light-dark cycle in the R6/2 mice (Cuesta et al. 2014). In a similar

model, 14 weeks of chronic long photoperiod exposure (16:8 h light-dark cycle) significantly improved the survival and nocturnality of R6/2 Huntington's mice (Ouk et al. 2017). Similarly, six hours of daily exposure to blue light during the first half of the light phase for three months significantly improved the locomotor activity rhythm in transgenic HD mice models (Wang et al. 2017).

## 16.4 Possible Underlying Mechanisms for the Potential Role of Circadian Manipulation on Behavior and Cognition

The phenomenon of adult neuroplasticity is a boon as the adult brain can synthesize and reorganize its synaptic connections as a result of an experience in response to intrinsic and extrinsic factors. Photoperiod manipulation alters the balance between dopamine and somatostatin (SST) expression in the hypothalamic paraventricular nucleus (PVN). Exposure to short photoperiod for a week, increase the tyrosine hydroxylase (TH)-immunoreactive cells and decreased the SST-immunoreactive cells in the PVN of the hypothalamus, a phenomenon also referred to as 'neurotransmitter switching' (Dulcis et al. 2013). At the receptor level, there was an abundant increase in the D2R expression and a proportional decrease in the SST2/4R of the PVN. The SST and dopaminergic neurons of the PVN synapses on the corticotrophin-releasing factor (CRF) neurons which are located along the third ventricle (Kumar 2007). Interestingly, short photoperiod decreased the plasma corticosterone levels and CRF levels in the cerebrospinal fluid. In line with these findings, our study also reported that three weeks of short photoperiod housing optimized the levels of plasma corticosterone levels and hippocampal CA1 glucocorticoid receptors expression in the VSL rats (Subhadeep et al. 2020, 2021). Further, short photoperiod increased the expression of doublecortin (DCX, a marker for adult neurogenesis) and Arc (an immediate-early gene) protein in the dentate gyrus of the VSL rats, which might be possibly associated with the restored learning and memory functions observed in the VSL rats in Morris water maze (Subhadeep et al. 2021). Short day exposure is also reported to increase BrdU+ and NeuN+ cells in the olfactory bulb in the white-footed mice, which in turn facilitates the olfactory-mediated behavior (Walton et al. 2012a, b). Further, 10 weeks of short-day exposure enhanced cell survival in Syrian hamsters (Huang et al. 1998) and increased adult hippocampal neurogenesis in white-footed mice (Walton et al. 2014).

Sunlight exposure affects mood, learning and cognition (Kent et al. 2009; Beecher et al. 2016). However, the underlying molecular mechanisms are not well understood. An interesting study by Zhu et al. (2018) reported that moderate ultraviolet light B (280–315 nM; 50 mJ/cm$^2$) exposure for 120 mins enhances motor learning and object recognition memory in mice. This improvement in cognition is associated with elevated blood levels of urocanic acid which crosses the blood-brain barrier and facilitates glutamate signaling in the hippocampus, prefrontal cortex, and other

brain regions in a positive manner. Similarly, the precise mechanism of action of BLT remains unclear. However, it is speculated to act by increasing the availability of synaptic serotonin in the midbrain. BLT (2500–10,000 lux) treatment during the short days of winter lowers the binding potential of serotonin transporter, resulting in less uptake, and higher availability of serotonin in the synaptic cleft (Campbell et al. 2017). In addition, violet light exposure from 8 am to 8 pm for seven weeks significantly improved contextual fear memory, spatial memory, and social behavior in C57B6/J mice (Sasaki et al. 2021).

At the synaptic level, our study showed that short photoperiod (6:18 h light-dark cycle) exposure impacts the hippocampal Schaffer collateral-CA1 pathway in the VSL rats. Three weeks of short photoperiod improved the basal synaptic transmission but did not restore the long-term potentiation in the acute hippocampal slices obtained from the VSL rats (Subhadeep et al. 2021). Presumably, SPR might be modulating the postsynaptic mechanisms and thereby affecting the AMPAR-mediated synaptic component, which is responsible for input-output synaptic functions. Furthermore, photoperiod manipulation affects cellular properties. GABA is highly expressed in the SCN, and its receptors are known to control photic signals by presynaptically regulating glutamatergic signaling. Short days decrease the spontaneous postsynaptic GABA- evoked currents and affect its equilibrium potential in the SCN neurons, thus overall affecting GABA functioning (Evans et al. 2013). Overall, short days led to more inhibitory responses and lesser excitatory responses.

Melatonin is a neurohormone secreted by the pineal gland at night, whose circadian secretion is controlled by the hypothalamic SCN. It is a chronobiotic and an important *zeitgeber* that enables the transmission of photoperiodic information throughout the body. Manipulating the timing of melatonin secretion by exogenous administration or using melatonin receptor agonists are well-established potential strategies for synchronizing the circadian rhythm in neuropsychiatric conditions. For example, agomelatine has melatonergic agonist properties and is known to reverse behavioral deficits related to anxiety and depression in several animal models (Tuma et al. 2005; Fuchs et al. 2006). At the cellular level, chronic administration of agomelatine restored the adult hippocampal neurogenesis in mice (Rainer et al. 2012). Agomelatine has also demonstrated its efficacy in clinical trials in individuals with depression (Lemoine et al. 2007; Hale et al. 2010). It is now considered one of the strong candidates for circadian manipulation strategy to treat mood disorders. Another crucial neuropeptide that predominantly regulates the sleep-wake cycle, feeding behavior, arousal and metabolism is orexin (Tsujino and Sakurai 2009). The orexinergic neurons are heavily located in the lateral hypothalamus in vertebrates (Hurley and Johnson 2014) and are proposed to be involved in narcolepsy and mood disorders such as SAD (Scammell 2015; Bowrey et al. 2017). Exposure to short photoperiod (8:16 h light-dark cycle) increased the orexin gene expression in the lateral hypothalamus in sheep (Archer et al. 2002). Orexin influences mood and circadian rhythm by affecting the serotonin, norepinephrine, and melatonin systems (Liu et al. 2002; Bowrey et al. 2017; Sharma et al. 2018).

Age plays a crucial role in enabling the nervous system to adapt to photoperiodic changes. Photoperiod-induced neuroplasticity extinguishes with age and the 12 months old rats become less responsive to positive stimuli while still being susceptible to stressors. In these aged rats, the ability to increase the numbers of TH+ neurons in response to one week of short photoperiod (5:19 h light-dark cycle) exposure is lost (Pritchard et al. 2020). Due to the shift in the dopaminergic activity, these aged animals are less responsive to positive stimuli and more susceptible to stress. Aging also affects the calcium homeostasis in the SCN by reversing the rhythm of its intracellular levels (Farajnia et al. 2015). Future studies are needed to understand in detail how aging weakens the SCN network and how circadian manipulation can reverse these changes.

## 16.5 Conclusion

The field of chronobiology is rapidly evolving, and its advancements can result in substantial implications for human health and disease. Translational research in chronotherapy can offer insights into the diagnostic and therapeutic avenues of affective and neurodegenerative disorders. Although extensive literature exists on circadian manipulation strategies, we cannot make generalizations or overarching conclusions about the role of photoperiod across species. More studies are needed to investigate circadian manipulation effects in the longitudinal, species-specific, sex-specific, and chronotype-specific manner. It is also important to note that nocturnal physiology is not a phase-reversed version of diurnal physiology, and hence their responsiveness to light is different. Developing appropriate animal models is critical in understanding the effects of circadian manipulation. Therefore, a direct correlation of these results should not be directly extrapolated to develop therapeutic strategies in humans. Future studies are warranted to address how light affects mood, the neurotransmitter system, neuroplasticity, and the microbiota-gut-brain axis, which is gaining much attention recently (Fig. 16.1).

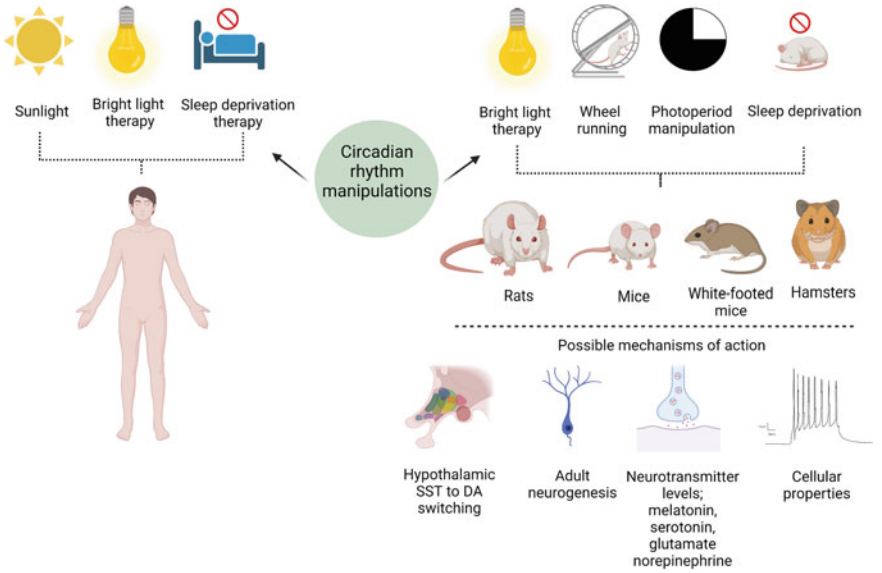


**Fig. 16.1** Different circadian rhythm manipulation strategies used in human and animal models. Exposure to sunlight, bright light or sleep deprivation therapy has proven to be beneficial in several mood disorders in humans. In addition, pre-clinical studies using different rodent models (rats, mice and hamsters) have used bright light therapy, wheel-running, photoperiod manipulation and sleep deprivation protocols to study the underlying mechanisms associated with circadian rhythm manipulation. These strategies are reported to cause hypothalamic neurotransmitter switching (between dopamine and somatostatin), affect neurotransmitter levels, olfactory and hippocampal neurogenesis and alter cellular properties in the suprachiasmatic nucleus. The figure is created with BioRender.com

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# Chapter 17
# Epigenetics of Altered Circadian and Sleep Cycle Induced Effects on Aging and Longevity

**Shashikant Patel, Vincy Vijay, Arvind Kumar, and Sumana Chakravarty**

## 17.1 Introduction

Aging is an inexorable process marked by pulmonary, renal, cardiovascular, endocrine, immune, gastrointestinal, and neurological alterations, eventually leading to the dissolution of consciousness and sentience of the living body. The electrification of the planet has attenuated the frontier between day and night, significantly impacting the circadian rhythms (Parameswaran and Ray 2022). Contributions from various environmental and biological aspects have pushed toward a society with desynchronized circadian rhythm leading to aging-related health and physiological disorders (Fig. 17.1). The age-associated disorders are often incurable, either medically or due to the frivolous physiology of the aged body. The physiological aging of an organism is synonymous to cellular or replicative senescence wherein cells subsequent to a limited maximal growth egress from the growth phase and exhibit decline in metabolic and functional activity (Ahmed et al. 2019). Genomic instability results in the accelerated aging process (Fig. 17.2). Higher order chromatin conformations are critical in maintaining the genomic stability by facilitating the packaging of nuclear DNA into a compact structure and stabilizing complex biomolecular interactions. Chromatin dynamics regulates the fundamental processes such as replication, transcription, repair, and recombination, thereby maintaining genomic stability. However, the compactness delineated through the chromatin condensation restrains the accessibility of regulatory enzymes to the genomic DNA, impeding the regulation of fundamental processes, yet more importantly chromatin dynamics is imperative in

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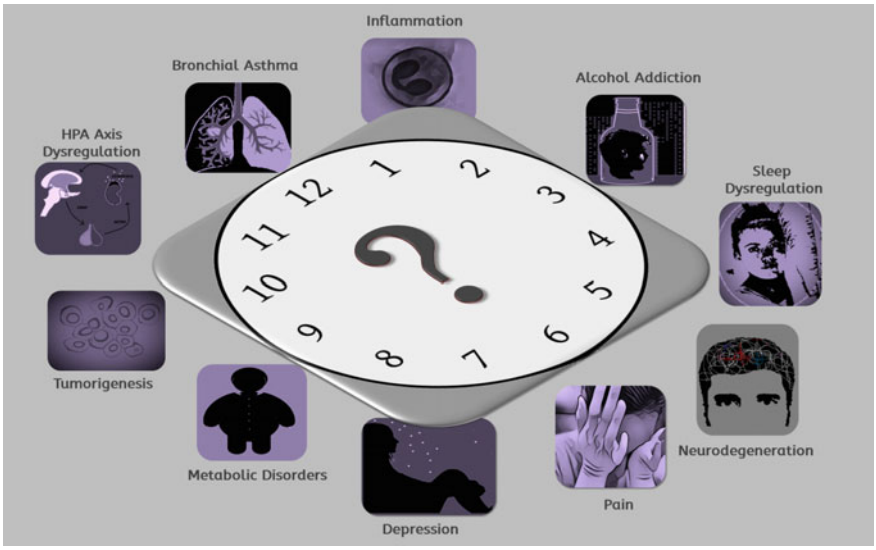


**Fig. 17.1** Diverse effects on physiology, cognition, and metabolism of the body, induced by altered circadian rhythms

preventing undesired gene activation. ATP-dependent chromatin remodeling mechanisms have been evolved across organisms to facilitate the accessibility of regulatory molecules to the chromosomal structure. These mechanisms were first propounded by Vincent Allfrey, who studied the acetylation and methylation patterns and associated them with the regulation of RNA synthesis (Struhl 1998).

Variations in the modulation pattern of chromatin organization have been associated with the aging genome. The epigenetic changes during aging are stochastic processes that are also influenced by environmental perturbations. Investigations underlying the role of epigenetic modifications in aging and longevity have rapidly evolved in the last few decades. Efficient transcription of a gene requires the accessibility of enhancer and transcription start sites that requires the transition from heterochromatin to euchromatin, mediated via chromatin remodeling and modifying enzymes (Klemm et al. 2019). The heritable and reversible effects mediated through an array of chromatin remodeling events comprise what is termed as epigenetics (Zhang et al. 2020). The term epigenetics was pioneered in 1942 by Conrad Waddington to define the biological changes during development that give rise to phenotypes based on the genetic programming. Further, the term was redefined by Arthur Riggs to formulate a more valid notion as the investigations pertaining to heritable mitotic and meiotic changes in gene expression without altering the DNA and protein sequences (Nicoglou and Merlin 2017).

Presently, the epigenetic regulation encompasses and is not limited to reduced levels of histone proteins, alterations in the pattern of DNA methylation (DNAm) and

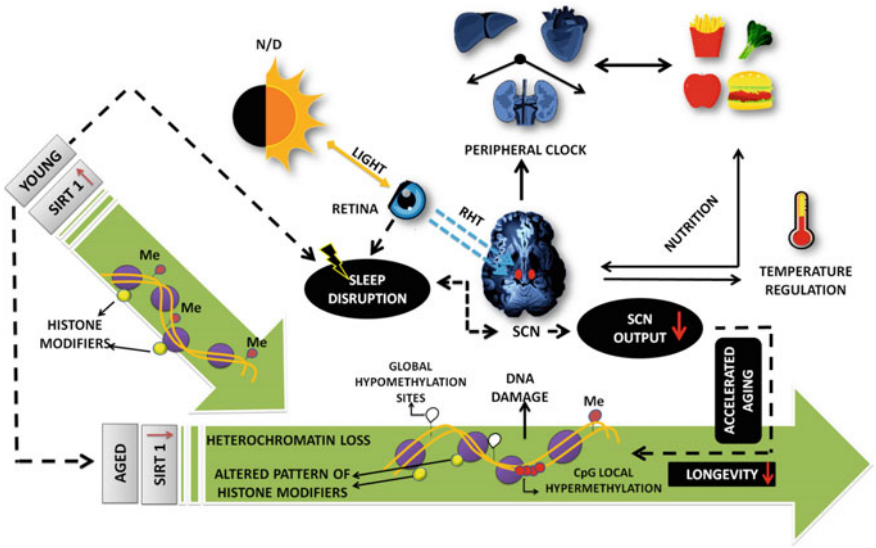


**Fig. 17.2** Altered circadian and sleep cycle negatively effects aging and longevity mediated via epigenetic dysregulation. Suprachiasmatic nucleus (SCN) is the master clock regulator in the body. It regulates multitude of physiological and biochemical processes of the body. The SCN output regulates the peripheral molecular clock activity present in the organs. Disruption of sleep and circadian cycle downregulates the SCN output and results in epigenetic alterations (global hypomethylation, CpG hypermethylation, altered histone modifications, and heterochromatin loss) accelerating the normal aging process

hydroxymethylation, variations in the expression of non-coding RNA, posttranslational modifications of histones, replacement of canonical histones with histone variants, and the chromosomal position effects (Zhang et al. 2020). Epigenetic dysregulation is one of the nine hallmarks of aging, and the discovery of DNA methylation opened windows to investigate this phenomenon in the process of aging. In one of the pioneering studies associating aging and epigenetics, it was discovered that 5-methylcytosine levels depreciate with age in spawning humpbacked salmon. Subsequent investigations implicated a global decline in the levels of cytosine methylation during aging in brain and heart. Lopez-Otin et al. had described the nine "hallmarks of aging," that includes telomere shortening, epigenomic alterations, genomic instability, loss of proteostasis, dysregulation of nutrient sensing, cellular senescence, mitochondrial dysfunction, alteration in the intercellular communication, and exhaustion of stem cells (López-Otín et al. 2013).

One of the major factors leading to circadian clock disruption and related epigenetic alterations is the prolonged sleep cycle disturbances. Sleep disruption is also an important criterion for major depressive disorder, post-traumatic stress disorder, bipolar disorder, and other mood disorders. Regulators of sleep–wake

cycle include ionic concentrations of potassium ($K^+$) and calcium ($Ca^{2+}$) channels (Yoshida et al. 2018), excitatory neurotransmitters such as serotonin, acetylcholine, norepinephrine, dopamine, histamine, orexin, neuropeptide S, glutamate, and humoral factors including cytokines and hormones. Recent studies have uncovered the substantial roles of microglia, astrocytes, and oligodendrocytes in sleep regulation (Steardo Jr et al. 2019). Sleep-modulating neurotransmitters including GABA and glutamate are produced by the glial cells, and these neurotransmitters play pivotal role in regulating the sleep–wake cycle. In the sleep regulation pathways, action potentials generated by astrocytes and oligodendrocytes are mediated by $K^+$ channels. In addition, humoral factors regulating sleep cycle including cytokines and energy regulating molecules like adenosine tri-phosphate (ATP) and adenosine are also produced by both neurons and glial cells. Sleep deprivation results in increased glycogen synthesis and dysregulated metabolic pathways. Increasing evidences indicate a close association between sleep deprivation, aging, and the related epigenetic mechanisms. Further, the rhythmicity in the expression of melatonin and glucocorticoids manifests the circadian rhythm (Falcón et al. 2007). Melatonin biosynthesis eventuates from serotonin via a two-step enzymatic process in the retina and pineal gland. The circadian clock machinery modulates the secretion of glucocorticoids from the adrenal glands. The glucocorticoid concentration is elevated during the active period, while the levels are low during sleep period. Exposure to dysregulated light intensities have tendency to negatively modulate the HPA axis, resulting in cortisol-associated mood disorders (Walker et al. 2020).

## 17.2 Circadian Rhythm: Regulation and Implications in Aging

Circadian rhythm (*circa* = about; *dies* = day) is an evolutionary conserved mechanism for autonomous regulation of periodic oscillation in behavioral and physiological activity of an organism. The rhythmic regulation of 24-h day/night cycle is observed in almost all organisms that enables them to harmonize biological functions with environmental stimuli. The endogenously regulated activities comprise sleep–wake cycle, glucose homeostasis maintenance, hormone release, oscillating body temperature, and others. Interestingly, circadian clock system parallels with the cell cycle as both depend on sequential events of transcriptional–translational regulation and posttranslational modifications followed by degradation. Further, both involve mechanistically autoregulatory loops. Cyclin D1, wee-1, c-myc, and few other cell cycle genes are also regulated rhythmically (Fagiani et al. 2022). Membrane bound cells more or less show periodicity in cell division roughly every 24 h.

Behavioral and physiological rhythms in a 24-h periodic cycle are regulated by autonomous system referred to as circadian clock. CLOCK–BMAL1-mediated transcription of the circadian genes climax during the day time while the feedback circadian repressor system pinnacles at night and is dependent on Period (PER)

and Cryptochrome (CRY). Furthermore, the circadian regulation is also susceptible to rhythmic epigenetic modifications. The core molecular circadian clock system consists of self-sustaining mechanisms comprising up of several gene networks which are operated by transcriptional and translational feedback loops (TTFLs). The positive regulation is mediated by two transcription factors (TFs) Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-Like 1 (BMAL1). The heterodimerization of CLOCK: BMAL1 complex increases its affinity to the E-box promoter on target genes having a consensus and canonical sequence of CANNTG and CACGTG, respectively (Wang et al. 2013). E-box drives the transcription of several oscillatory clock-controlled genes (CCGs). The late resting phase of CLOCK: BMAL1 transactivates the circadian repressors PERIODs (PER 1/2/3) and CRYPTOCHROMEs (CRY1/2). Further, the subsequent accumulation of PER and CRY leads to nuclear translocation where they interact with CLOCK: BMAL1 and repress CLOCK: BMAL1 mediated transcription (Kwon et al. 2006). E3 ubiquitin ligase acts upon PER and CRY and are then degraded by the proteasome, and a new transactivation cycle begins. Secondary feedback loop facilitates the activity of circadian gene network that comprises cis-regulatory transcriptional activator ROR (retinoid-related orphan receptor) response elements (ROREs) and the transcriptional repressor REV-ERBα which are responsible for modulating the rhythmic transcription of BMAL1 (Chatterjee and Ma 2016).

Epigenetic mechanisms are crucial in regulating the process of circadian transcription and maintenance of oscillating gene expression. The histone acetyl transferase (HAT) p300 acetylates the histone H3 at CRY and PER promoters. The intrinsic HAT activity of CLOCK acetylates BMAL1 which facilitates the recruitment of CRY1 to the CLOCK:BMAL1 complex and represses transcription (Feng and Lazar 2012). Further, the rhythmicity in the acetylation pattern of histone H3 in the core CLOCK gene promoters have been observed during the active phase. Histone deacetylase 3 has been associated with the repression of BMAL1, thus modulating circadian rhythms. HDAC inhibitors decrease the H3 lysine deacetylation and alter the expression of PER2 gene. Age-associated attenuation of the circadian system is known to contribute to the cognitive decline and aging-associated neurodegenerative disorders. In a recent study, mass spectroscopic-based approach was used to investigate circadian regulation at the proteomic level in hippocampal tissues collected from young and middle-aged mice. Middle-aged mice manifested reduced capability of learning and cognition (Shoji et al. 2016). There is a profound reduction in the functionality of the immune system with aging that significantly contributes to age-associated morbidities. With aging there is a decline in homeostatic polarization of macrophages and thus reduced phagocytic capacity. In a recent study (Blacher et al. 2022), it was shown that there is extensive decline in the circadian gene expression profiling of aged macrophages. Further, the loss of diurnal phagocytosis with aging was also demonstrated. Interestingly, they couldn't observe significant differences in the core clock genes in aged and young macrophages. They demonstrated that KLF4 was associated with distinctive binding to rhythmic genes and the oscillatory expression observed in young macrophages gradually declined with age.

Light is detected principally by photoreceptors (rods and cones in mammals). Rodent experiments have revealed that mice lacking rods and cones are still able to regulate the circadian rhythms; however, obliterating the eye significantly restrained circadian regulation (Foster 2020). Further, investigations discovered the presence of a population of intrinsically photosensitive retinal ganglion cells (ipRGCs) which possess a photopigment called "melanopsin" or OPN4. ipRGCs function through promulgation of neural stimuli through the retinohypothalmic tract to the mammalian suprachiasmatic nucleus (SCN) of hypothalamus (Fig. 17.2). SCN discharges functionality as the master regulator in mammals; however, subcellular regulators (Peripheral clock) are present in the liver, heart, adipose tissue, and certainly in every organ and tissue of the body. SCN is a bilateral structure located in the anterior region of hypothalamus. Despite comprising only about 20,000 neurons, the SCN is the principal pacemaker of circadian system regulation. The functionality of SCN has been reported to decline with age in a number of studies (Zhao et al. 2019). The SCN projects to several brain areas within the hypothalamus and regulates the hormonal release that play crucial role in maintaining molecular and physiological rhythmicity (Fig. 17.2). The pituitary hormones are also under tight circadian control.

## 17.3 Circadian Control of Sleep

Sleep is a universal behavioral phenomenon conserved among higher order living organisms. Humans dedicate one third of their lifespan to sleep. Albeit, our scientific knowledge of sleep is in the preliminary phase, several models have been developed to investigate physiological effects of sleep deprivation, and the results indicate that sleep deprivation crucially impacts the epigenome. However, it is often difficult to analogize and compare the results from these models with humans. Thus, human models of acute sleep deprivation have been largely investigated upon owing to the feasibility of these models. Sleep conditions have consequential impact on the brain epigenome. Some of the notable biological functions of sleep so far discovered include energy conservation, immune response modulation, memory consolidation, detoxification, repair, and regeneration. In animals, sleep states are determined by the complex amalgam of physiological and behavioral processes. Behavioral characteristics shown include reduced mobility, eye movements, sleep postures, reduced response to external stimuli, cognitive impairment, and reversible unconscious state. Even though sleep initiation and maintenance considerably changes as we grow, the sleep architecture at physiological level shows two alternating phases (REM and NREM) in a cyclic manner with independent functions and controls. In adults, the sleep cycle ranges from 4 to 6 alternating cycles, each series lasting about 90–110 min (Bah et al. 2019).

### 17.3.1 Sleep Physiology

Sleep induces a temporary shutdown of responsiveness toward external environment; however, it is necessary for the organism's ability to survive and propagate. Behaviorally, sleep disruption is the most characteristic consequence of altered in circadian rhythms eventually triggering neurodegeneration. Recently, plunging of rhythms in cortical excitability has been shown to be correlated with aging which potently contributes to age-associated cognitive decline. It is believed that these changes are necessary to compensate the energy spent while the individual was awake. As the sleep progresses, blood pressure and heart rate keep on changing. These fluctuations also depend upon the different stages of sleep cycle. These changes are governed by the autonomic nervous system. Similarly, ventilation and respiratory responses like cough reflex are maintained at minimum activity. A reduced urine flow is also maintained during the sleep cycle through reduced excretion of certain ions such as sodium, potassium, chloride, and calcium. Sleep also regulates the secretion of certain hormones typically melatonin, growth hormones, and thyroid hormones (Chokroverty 2010).

### 17.3.2 Sleep Architecture

Sleep is categorized into different stages based on the brain wave pattern as visualized through electroencephalogram (EEC) and muscle activity measured through electromyogram (EMG). Brainwaves are defined as the synchronized electrical impulses of particular frequencies produced by a cluster of neurons for the purpose of communication with other neurons. Sleep cycle is broadly classified into non-rapid eye movement (NREM) and rapid eye movement (REM) sleep. Of the total sleep duration, NREM accounts for the max duration (75–80%) which is further subdivided into four more stages: N1, N2, N3, and N4, respectively. The entire sleep cycle during night follows the cyclic order as: N1–N2–N3–N4–REM. This whole process lasts for approximately 90 min and alternates 4–5 times throughout the sleep duration. The characteristics of NREM sleep include slow wave activity associated with spindles and K-complexes. It is now well understood that NREM plays significant role in conservation of brain energy and memory consolidation. Further, research has identified that NREM has the ability to modulate synaptic weights in order to perform the above mentioned functions. A slow rolling eye movement is also recorded during this phase accompanied by other physiological changes like decreased muscle tone, reduced blood pressure, and decreased breathing and heart rate.

The hallmark of REM sleep is the rapid eye movement in all direction and highly diminished muscle activity. The EEG records measured during REM sleep revealed fast rhythms, slow alpha activity, and theta waves with saw-tooth appearances. In addition, phasic swings in the blood pressure and heart rate along with

irregular breathing pattern are also observed. Another notable characteristic associated with REM sleep is the process of dreaming. Scientifically, the complex interactions between the cholinergic, aminergic, and GABAergic neurons in the brain stem attributes to the process of rapid-eye-movement (REM). Since, REM sleep activates the limbic regions, it has been hypothesized that it may play significant role in the regulation of moods and emotions. Questions like why sleep cycle alternates between these two phases still remains to be deciphered. Irregular sleep cycle stages have been reported in several sleep disorders such as narcolepsy characterized by excessive sleepiness (Altevogt and Colten 2006).

### *17.3.3 Sleep Cycle Regulation*

Two internal biological mechanisms are associated with the regulation of sleep cycle in humans: the human body's homeostatic drive toward sleep and the internal circadian rhythm regulating the biological clock. Circadian rhythms regulate a wide variety of daily functions including time-dependent hormonal release, variations in the body temperature, and numerous metabolic pathways involved in maintaining an overall homeostasis during the 24-h cycle. This is achieved through the synchronization of many endogenous and exogenous factors known as zeitgebers such as temperature and light. However, all these activities can take place even in the absence of significant cues or signals. Usually, the drive for sleep gets accumulated throughout the day when the individual is awake. Accumulation of adenosine occurs in different brain regions throughout the day, which promotes sleepiness. Pineal gland plays an essential role in regulation of sleep cycle by releasing melatonin specifically at night. The hormone is known for its ability to stabilize and reinforce circadian rhythms by synchronizing with the day night cycle. Recent research has led to the discovery of sleep regulating brain regions and specific neuronal networks controlling the sleep–wake cycle in human. Similarly, neurons of the pons regulate the switching between NREM and REM sleep stages. These neurons facilitate the interaction between lower brainstem and spinal cord which further establishes physiological characteristics related to REM sleep. At the same time, they send outputs to the forebrain which activate the cholinergic signaling pathways to the thalamus. Cholinergic neurons present in the upper pons region, mediate the relaying of sensory information between thalamus, and cerebral cortex, while monoamine neurotransmitters in the upper brainstem enter the hypothalamus, traverses to the basal forebrain and perceives inputs from cells containing acetylcholine and GABA and eventually enters the cerebral cortex to activate the neuronal cells preparing them for interpretation as well as analysis of incoming sensory information (Zielinski et al. 2016).

## 17.4 Sleep Dysregulation: Aging and Epigenetics

Sleep dysregulation hinders the daily functioning and performance of individuals. This dysregulation significantly reduces the output of SCN and extends profound effect on the functionality of peripheral clocks and the normal metabolism of the body. Metabolic disturbance thus causes nutrient imbalance aiding a multitude of health disorders. If left untreated sleep loss leads to a wide range of detrimental health consequences. One of the principal contributors of sleep loss and related sleep disorders is the persistent stress. Sleep loss or sleep dysregulation is often seen followed by the disturbing life events, e.g., death, divorce, job loss, financial crisis, etc., and as one of the prominent symptoms of stress-related disorders such as depression or PTSD. Mutations in circadian clock dysregulate the rhythmic control of sleep and lead to sleep disorders. Familial advanced sleep phase disorder (FASPD) characterized by early onset of sleepiness around 7–9 pm and early awakening is caused due to a missense mutation (S662G) in the PER2 gene (Rijo-Ferreira and Takahashi 2019). Normal levels of PER2 is regulated by circadian pathway genes, casein kinases Iδ and Iε (CKIδ/ε), and the S662G mutation at CKIε binding site results in inadequate phosphorylation of PER2 leading to its nuclear accumulation (Toh et al. 2001). Missense mutation in the CKIδ gene (T44A) and CRY2 gene (A260T) is associated with FASPD. Another highly prevalent disorder is the delayed sleep phase disorder (DSPD) caused as a result of polymorphism in the CLOCK or PER3 genes.

The relationship between disruption of sleep and the underlying alteration of epigenome has recently emerged as a hot topic of interest among investigators. Shift workers tend to have lower levels of CLOCK gene methylation while elevated levels of CRY2 as observed from genome-wide studies (White et al. 2019). There is enough evidence to support that epigenetic modulations occur following sleep disruption. It has been observed that DNAm is significantly altered post the events of sleep disruption. It has been elucidated from experiments in mice that the expression levels of DNMT3A1 and DNMT3A2 (DNA methyltransferases) get upregulated after sleep deprivation and thus subsequent increase in DNAm (Gaine et al. 2018). Reduced sleep has been associated with accelerated epigenetic aging and increased disease risk in a number of studies. In a recent study with 6 month postpartum mothers, that reported reduced sleep (less than 7 h), exhibited older epigenetic age at 12 month post birth (Carroll et al. 2021). DNAm-based estimation revealed shortened leukocyte telomere length. The study imparted that sleep disruption of mothers in early postpartum period may have long lasting effect on the epigenome. However, sleep duration measured at 12 month postpartum did not had significant association with epigenetic aging. In another study through parallel sampling of human adipose tissue and skeletal muscle, investigations were done to delineate the tissue-specific mechanisms by which acute sleep loss affects metabolic tissues. Downregulation of the glycolytic pathway was observed in the skeletal muscle, while its upregulation was observed in subcutaneous adipose tissue. These changes were attributed to dysregulation of circadian system as a result of acute sleep loss that may have reprogrammed DNAm in adipose tissue resulting in increased adiposity (Cedernaes et al. 2018). In one of the

first systematic investigations deciphering the effect of total sleep disruption (TSD) on the genome-wide DNAm profile in blood and associated epigenetic marks, 269 gene probes and 184 CpG sites were found to exhibit altered methylation pattern post TSD (Nilsson et al. 2016). Studies have suggested of distinctive gene patterns exhibiting epigenetic modifications associated with sleep insufficiency in males below the age of 50. Impaired neuroplasticity and neurodegeneration were found to be triggered by insufficient sleep (Lahtinen et al. 2019).

In a study focusing on the effect of chronic sleep deprivation on the physiological state and accelerated aging processes of female mice of varying age groups, through long-term sleep deprivation modeling, it was observed that the food intake capacity of the adult mice were significantly increased during simulated stress. The result was attributed to the malfunctioned melatonin metabolism (Novozhilova et al. 2021) and elevated levels of cortisol, phenylalanine, and aspartic acid that impaired the homeostatic levels of neurotransmitters in the brain. On the contrary, the young mice despite the background of increased food intake showed reduced weight. This could be due to the variations in the levels of appetite-stimulating hormone. No alteration was observed in the expression of aging biomarker Perilipin 2 (PLIN2) and DNAm in young female mice suggesting resistance to accelerated aging. However in adult mice, increase in PLIN2 levels and decreased DNAm was observed (Novozhilova et al. 2021). Hypomethylation of DNA in adult mice indicated acceleration in aging processes and neurodegeneration (Mateus Brandão et al. 2022). Acute sleep disruption experiments with human contexts showed modest elevation in the levels of circulating FGF21 and altered DNAm levels in FGF21 promoter of adipose tissue (Mateus Brandão et al. 2022).

There are increasing evidences from studies that night shift work schedules result in impaired sleep–wake cycle followed by increased risk of serious disorders. In a recent study, CSNK1E (Casein Kinase 1 isoform epsilon) hypermethylation was observed in working shift workers while hypomethylation of NR1D1 (Nuclear receptor subfamily 1 group D member 1) in long-term night shift workers was observed. Further, hypermethylation of ARNTL (Aryl hydrocarbon receptor nuclear translocator like protein 1) was observed in the workers who worked $\geq 3$ consecutive night shifts within a week. The study implicated differential methylation of circadian genes depending on the exposure. Studies show that sleep deprivation leads to decrease in the acetylation of H3K9 and H4K12 in the hippocampus while HDAC2 is elevated. Specifically, sleep disruption impairs late-LTP and associative plasticity (Wong et al. 2020). The numbers of studies linking sleep, depression, and aging epigenetics have increased rapidly in recent years. A study to investigate the combined effects of disordered sleep and depression on DNAm pattern in blood leukocytes revealed of altered DNAm pattern in genes related to synaptic plasticity in adolescents (Ämmälä et al. 2019). Pediatric obstructive sleep apnea (OSA) patients experience episodic upper airway obstruction causing frequent sleep disruption, blood-gas alteration, and dysregulation in carbon dioxide levels. Adult patients with OSA show reduced levels of SIRT1 in peripheral blood cells (Gaspar et al. 2017). The subsequent alteration in histone chemistry causes damage to DNA and

contributes to accelerated aging. Indeed there are substantial evidences to proclaim that OSA is associated with shortened telomere length.

## 17.5 Epigentic Clock Theory and DNA Methylation

Aging inevitably impairs functional rhythmicity of body and leads to death. Aging researchers have since long focused on deducing biomolecular markers of aging, hoping that interventions on such biomarkers presumably may halt or slowdown the aging process. Present understanding culminates that among other plausible biomarkers, epigenetic clock leads the criteria of molecular estimator of biological age. Progressing age advances variance from normal epigenetic patterns, and the accumulation of these epimutations results in epigenetic drift convulsing homeostatic functionality of the body. Epigenetic drift essentially transcends as a result of the errors in DNA replication cycle that modulates epigenetic patterns. Early investigations in monozygotic twins deciphered that the divergences in epigenetic patterns are also mediated through varying environmental cues (Cunliffe 2015). Investigations to delineate genetic and epigenetic signatures of aging often used monozygotic twins as the subject of study. Young monozygotic twins were found to possess similar methylation signatures while twins of 50 years of age exhibited distinct methylation marks.

Genome wide methylation studies have indicated that chronological age is associated with modulation in DNAm. In DNAm, methyl group(s) is covalently added to the 5th position of a pyrimidine ring of cytosine usually in CpG dinucleotides. Typically, cluster of these CpG dinucleotides are profoundly located at the 5′ end of the DNA referred to as CpG islands. DNAm patterns are often described as epigenetic clocks. A general overview states that with age the number of CpG islands increase, that at younger age are unmethylated while an overall methylation level decreases (Maegawa et al. 2017). Studies by Maegawa et al. have shown that methylation patterns change with age in a number of species, including mice, human, and rhesus monkey. They showed that methylation drift is an evolutionary conserved phenomenon across different species, and this age-related epigenetic drift is delayed by caloric restriction (Maegawa et al. 2017). A summarized purview indicates an inverse relation between methylation drift and longevity. Expression pattern of DNA methyltransferases are also associated with aging.

Both prokaryotic and eukaryotic organisms display an evolutionary conserved mechanism of transcriptional repression through DNAm. DNA hypomethylation and hypermethylation of DNA are the key events driven in parallel with age. However, it is evident that global hypomethylation of mammalian DNA transpires with the aging genome. The paramount levels of methylation are observed in the DNA of embryonic tissues and newborn organisms. Gradually with chronological aging the levels tend to decline. The class of enzymes facilitating methylation is referred to as DNA methyltrasferases (DNMT). Embryonic DNAm is mediated by DNMT3A and DNMT3B (Okano et al. 1999). Rodent experiments have shown that inhibiting the function of

DNMT1, DNMT3A, or DNMT3B result in premature embryonic lethality (Johnson et al. 2012). Additionally, selective repression of DNMT3A results in neuromuscular deformities in mice with reduced lifespan. Studies involving administration of the demethylating agent 5-aza-2-deoxycytidine (5-aza-dC) showed decline in lifespan of human fibroblasts cells (Holliday 1986). Methyl-CpG binding domain protein (MBD) family mediates chromatin silencing through the recruitment of histone deacetylases. MBD proteins result in the suppression of transcription; however, mechanism of MeCP2 mediated silencing has been extensively investigated. MeCP2 was first identified by Lewis et al. in 1992 (Meehan et al. 1992). The MECP2 gene is localized at Xq28 and is exposed to X inactivation (Del Gaudio et al. 2006). MECP2 gene codes for multiple isoforms: MeCP2-e1 and MeCP2-e2 which differ at their N-terminal regions yet containing both MBD and transcriptional repression domain (TRD) (Rastegar et al. 2009). Expression levels of both isoforms are elevated in the brain. Frequently, documented mechanism of transcriptional silencing mediated by MeCP2 involves the recruitment of TRD of HDACs/Sin3A repressor complex resulting in the deacetylation. MeCP2 and CoREST (co-repressor for element-1-silencing transcription factor) complex function to repress the intended genes in the brain through the recruitment of SUV39H1 (suppressor of variegation 3–9 homolog 1) which is responsible for methylation of histones.

The age-associated modulations in DNAm to correlate them to epigenetic clocks are determined through either supervised machine learning or epigenome-wide association studies. Weidner and co-workers utilized only 3 CpG sites located in the genes Aspartoacylase (ASPA), Integrin alpha-IIb (ITGA2B), and Phosphodiesterase 4C (PDE4C) to develop an epigenetic clock, not as accurate yet reliable (Weidner et al. 2014). This clock was by far more accurate when compared with predictions based on telomere length. Hannum's epigenetic clock was developed by employing elastic net regression, evaluating 71 CpG methylation sites to decipher the chronological age with significant accuracy (Hannum et al. 2013). However, this clock was supervised specifically for whole blood samples. A multi-tissue chronological age predictor was developed by Steve Horvath and colleagues utilizing 353 age-related CpG probes (Horvath 2013). The correlation between chronological and DNAm age through this clock was impressively 96% despite considering multiple tissue types. However, some tissue types showed decreased correlation like tissues from breast, heart and uterine endometrium.

## 17.6 Heterochromatin Loss Model of Aging

Histones play an essential role in the packaging of genomic DNA in the nuclei; furthermore, they have a cardinal role in regulation of gene expression. N terminus of the histone tails readily undergo chemical changes leading to posttranslational modifications. These modifications alter the binding affinity of the histones to the DNA ultimately altering expression patterns and levels. A highly condensed form of

chromatin is referred to as heterochromatin and is transcriptionally inactive. Furthermore, the centromeric and telomeric region encompasses transposable elements and satellite sequences and is referred to as constitutive heterochromatin. This type of heterochromatin has more permanent heterochromatin domains. Contrary to constitutive heterochromatin, regions of DNA accounting to facultative heterochromatin can vary among different cell types and even within a species. A region packed as facultative heterochromatin may be packed as a euchromatin in a different subset of cells depending on the morphogenetic and differentiation signals.

Heterochromatin loss model is among the firsts to establish a link between epigenetics and aging (Lee et al. 2020). It proposes that the heterochromatin domains organized early in embryogenesis are disrupted during the aging process leading to altered gene expression patterns. Several model organisms have demonstrated of either decreased heterochromatin markers or reduction in molecular factors mediating maintenance and regulation of heterochromatin. H3K9me3 is known to promote stronger association between DNA and histones, thus mediating heterochromatin formation. With age the trimethylation at H3K9 is substantially diminished. Heterochromatin loss is also a major marker of cellular senescence. Facultative heterochromatins encompass a domain called senescence-associated heterochromatin foci, which gets increased in the senescent cells; however, an overall loss in heterochromatin is predominant, thus validating the heterochromatin loss model of aging (Aird and Zhang 2013).

## 17.7 Role of Non-coding RNA (NcRNA) in Aging

Among the three broad classes of epigenetic regulations, (Histone modifications, DNA methylation, and non-coding RNA) non-coding RNA-based epigenetic regulation is relatively recently identified and has been found to regulate aging in human beings. They represent a diverse class of structural and regulatory RNA species that doesn't encode proteins. Interestingly, the protein coding genes constitutes roughly 1.5–1.8% of the entire human genome. Non-coding RNAs play important roles in processes like chromatin regulation, splicing, translation of proteins, and gene expression regulation (Esteller 2011). Interestingly, only 1.5–1.8% of the mammalian genome is transcribed to proteins. Regulatory RNAs (e.g., miRNAs, lncRNAs) function at both transcriptional and post-transcriptional levels. Wide range of studies have reported significant roles of many ncRNAs notably microRNAs and lncRNAs in pathways regulating aging and longevity.

As the name suggests, miRNA's are short, ncRNA's which bind to the 3′UTR region of mRNAs, thus resulting in their degradation or translation inhibition (Hammond 2015). These miRNAs regulate different cellular pathways involved in cell differentiation and survivability, stress response, cellular death, and inflammatory pathways. As the age progresses, a progressive change in miRNAs regulating inflammatory responses, cell cycle regulation and cancer pathways are observed in several reports. Aging pathways regulated by miRNAs include insulin/insulin-like growth

factor (IGF-1) signaling, reactive oxygen species (ROS) signaling, sirtuin signaling, Target of Rapamycin (TOR) signaling and caloric restriction pathways (Smith-Vikos and Slack 2012). Also, it has to be noted that these differentially expressed miRNAs during aging appears to be generally tissue-specific. Various studies carried out in context of normal rodent liver aging have identified numerous miRNAs directly regulating aging pathways, including oxidative stress responses. Similarly, another study of miRNA expression profiling in aging brain identified about 70 miRNAs upregulated, most of which targeted components of the mitochondrial electron transport chain and $F_1F_o$-ATPase (Li et al. 2011). Region-specific miRNA profiling of mammalian brain have also identified various differentially expressed miRNAs which actively regulated aging pathways like neuronal atrophy. A gradual loss of muscle function has been observed during aging. Various researches have focused on the role of miRNA in muscle wasting during aging and discovered abundant miRNAs regulating cell cycle pathways, muscle cell proliferation, and myogenic precursor differentiation. Moreover, aging of mammalian reproductive system is at higher rate than other organ systems. Investigations have acknowledged miRNA-mediated regulatory mechanisms in mammalian reproductive systems and reproductive disorders (Gebremedhn et al. 2021).

RNA polymerase II transcribes lncRNAs from the intergenic and intronic regions of the mammalian genome. These ncRNAs undergo polyadenylation and 5′-capping similar to mRNAs. Most of the characterized lncRNAs are localized in the nucleus and perform regulatory functions in various cellular pathways related to aging including stress and immune response and quiescence and senescence pathway. Moreover, it also plays pivotal role in cell proliferation and differentiation signaling pathways. Senescence, a long-term irreversible growth-arrest of cells is one of the notable characteristics of aging. Present understanding of lncRNAs strongly implicates its association with senescence during aging. Functional analysis of aging-related lncRNAs revealed that these ncRNAs are also crucial in regulating immune system, protein synthesis machinery, and mRNA processing.

## 17.8 Histone Modifications in Aging Process

Genetic factors contribute around 25% to the disparity in lifespan. However, a greater proportion is accounted by non-genetic factors (70%). Epigenetic modifications lead to changes in expression levels without altering the DNA sequence. Histone modifications include acetylation, methylation, ubiquitylation, phosphorylation, sumoylation, deimination, and ADP ribosylation. Histone modifications essentially undergo modulations during the process of aging. However, it is still questionable whether these modifications are due to the aging process or the modifications intrinsically mediate aging to progress.

### *17.8.1 Histone Methylation in Aging*

The trimethylated lysine 4 on Histone H3 (H3k4me3) is known to be involved in activation of transcriptional process while trimethylation at lysine 27 of Histone 3 (H3K27me3) has been implicated in transcriptional repression of gene expression associated with aging. Although H3K36me3 is known to mediate activation as well as repression yet the activation function is more pronounced in normal expressing cells. Age-dependent decrease in activation marks (H3K4me3 and H3K36me3) and increase in repressive marks (H3K9me3) were indicated by experiments with fly heads. In mammals, increase in mark of constitutive heterochromatin, H4K20me3 was observed in cells that were derived from human patients of Hutchinson–Gilford Progeria, a premature aging syndrome; however, either reduction or complete loss of the heterochromatin mark H3K9me3 was observed in the same cells (Han and Brunet 2012). Thus, further investigations are required to decipher whether repressive and activating marks cause or are a consequence of aging.

Investigations in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* have implicated the association of H3K4me3 with expression of aging-related genes. In yeast, the downregulation of aging-linked genes as a result of H3K4me3-defective cells lead to reduction in the lifespan. Additionally, H3K4me has been observed to be involved in the initiation of DNA replication and maintaining the genomic stability (Chong et al. 2020). Mouse models of Alzheimer's Disease (AD) have evidenced that H3K4me3 and related catalyzing enzymes are upregulated in prefrontal cortex (PFC), and subsequent treatment with inhibitor of H3K4 methyltransferases (KMTs) resulted in significant recovery of PFC functions (Cao et al. 2020). RNAi mediated knockdown or mutations in ASH-2, SET-2, and WDR-5 resulted in H3K4me3 deficiency and eventually extended the lifespan in *C. elegans* (Yi and Kim 2020). However, RBR-2, a *C. elegans* lysine demethylase 5 (KDM5) restrained the lifespan extension resulted through ASH-2 knockdown. Studies involving the suppression of RBR-2 function have varying effect on lifespan and needs to be investigated further. In another study on *C. elegans*, it was shown that reduction in the function of LSD1/KDM1A which is a H3K4 KDM results in the increment of lifespan (Maures et al. 2011; McColl et al. 2008).

Suppression of LID, the homolog of RBR-2 in fruit flies, decreased the lifespan of male flies; however, similar effects were not observed in female flies (Li et al. 2010a). It is to be noted that modulation of H3K4me regulators do not always have an effect on lifespan as deciphered from experiments in *D. melanogaster* where TRR inactivation, a member of H3K4 KMT complex did not influence the lifespan of male flies (Siebold et al. 2010). Reduction in the levels of H3K27me3 through mutation of the Polycomb repressive complex 2 (PRC2) components E(z) and Ese lead to extension of lifespan (Karnani et al. 2007). Histone methylation studies in muscle and mesenchymal stem cells of rodents revealed that aging cognates with increased levels of H3K27me3 and transcriptional repression. It was interesting to observe that depletion or inactivation of the SET2-ortholog MET-1 which is responsible for functioning of the TOR pathway, shortened the lifespan in *C. elegans*, while

inactivation of the demethylase JMJD-2/ KDM4 enhanced longevity (Pu et al. 2015) (Ni et al. 2012). Further the deletion of the H3K36 dimethyltransferase SET-18 in *C. elegans* lead to the extension in lifespan. It was essentially mediated through alterations in the expression of DAF-16, which maintains the insulin/IGF conserved pathway. H3K79me3 level is known to be elevated with age in yeast and substitution of lysine 79 with glutamic acid (H3K79E) results in the reduction of replicative lifespan (Sen et al. 2015). In one of the experiments, the deletion of H3K79 methyltransferase DOT 1 also resulted in decreased lifespan (Ryu et al. 2014). Contrary to elevation of H3K79me3 in yeast, experiments with aged mouse brain showed opposingly decreased levels of H3K79me3 (Gong et al. 2015).

H3K4 trimethylation (H3K4me3) has been observed to be expressed in parallel with the core clock genes. Experiments investigating the clock gene expression in seedlings administered with H3K4me3 inhibitor reported longer expression in the circadian rhythms of *CCA1* and *TOC1*, thus indicating that H3K4me3 regulates the expression peaks of the clock genes. Previous works have established that histone methyltransferase MLL1 (Mixed lineage leukemia 1) which methylates H3K4 is recruited cyclically to circadian gene promoters. MLL1 and CLOCK interact in parallel with the cyclic peaks of transcription. MLL1 is known to facilitate the recruitment of CLOCK–BMAL1 to chromatin and thus engenders a chromatin state that is permissive for circadian transcription (Katada and Sassone-Corsi 2010). Downregulation of H3K4me3 augments the binding activity of circadian clock inhibitors. The histone methyltransferase SET domain group 2/Arabidopsis trithorax-related 3 (SDG2/ATXR3) have been observed to mediate the oscillatory gene expression and H3K4me3 accumulation, while dysregulated expression of SDG2/ATXR3 modulates the binding activity of clock repressors (Henriques and Mas 2013).

### *17.8.2 Histone Acetylation*

One of the major regulators of circadian rhythms "CLOCK" is a histone acetyl transferase (HAT) involved in chromatin remodeling functions. CLOCK readily acetylates histones and BMAL1. Increasing evidences indicate dysregulation in histone acetylation patterns of CLOCK genes significantly aids cancer progression. Upregulation of TIMELESS in colorectal cancer (CRC) has been recently reported. This upregulation was attributed to CBP mediated H3K27 acetylation of TIMELESS promoter. TIMELESS was observed to promote the proliferation and invasion of colorectal cancer cells (Cao et al. 2021). Furthermore, p300 a histone acetyltransferase is known to be highly expressed in hepatocellular carcinoma and significantly aids malignancy as observed from the studies of p300 inhibition, which lead to reduced invasion of malignant cells. Cancer chronotherapy thus is an emerging field in cancer research. Circadian rhythm defects have been implicated in several neurodegenerative disorders. The role of CBP histone acetyltransferase in Huntington's disease has been well studied. This acetyltransferase directly interacts with CLK/CYC transcription

factors which regulate circadian rhythms. CBP is sequestered into mutant huntingtin during the pathogenesis of Huntington's disease. Histone modifications such as H4K16 acetylation (H4K16ac) are known to be upregulated with age in yeast and human brains (Dang et al. 2009). Neighboring H4K12 acetylation (H4K12ac) was observed to be elevated in middle-aged drosophila and experimental reduction in H4K12ac increased the lifespan in flies (Peleg et al. 2016). H4K16ac and H4K12ac are altered in aged murine and human Peripheral Blood Mononuclear Cells (PBMCs) (Bux et al. 2020). Recent investigations revealed that H3K9ac was upregulated with age at regulatory regions of the cytochrome P450 2E1 gene while H3K27ac levels remained stable (Kronfol et al. 2020).

### *17.8.3 Histone Deacetylation: Role of Sirtuins*

Circadian regulations by sirtuins, which are class III Histone decetylases (HDACs), have been widely investigated in the last few decades (Fig. 17.2). The abbreviation "SIR" stands for silent information regulator. These have a role in both aging and regulation of circadian clock. Sirtuins have established roles in the regulation of circadian clocks, cell cycle, cellular homeostatsis, aging, stress resistance, and apoptosis. Commonly seven subtypes of SIRTs (SIRT1–7) are known to be present in mice and humans that differ in their cellular localization and function (Shah et al. 2021). SIRT1, SIRT2, SIRT3, SIRT5, SIRT6, and SIRT7 are known to mediate deacetylation and SIRT4 and SIRT6 regulate the ADP–ribosylation activity (Grootaert and Bennett 2022). SIRT1 is known to regulate the central circadian clock by activating the two principal regulators BMAL1 and CLOCK.

It involves a circadian loop comprising SIRT1, PGC–1α, and Nampt (Fig. 17.3). An enzyme known as Nampt is the major output target of CLOCK-BMAL1 association which is crucial for the biosynthesis of $NAD^+$ that acts as a cofactor for SIRT 1. Although sirtuins are well known histone modifiers, they have also been characterized to modify several transcriptional regulators such as NF–κB, p53, FOXO, PGC–1α, SOD–2, and α–tubulin (Khan et al. 2021) (Fig. 17.3). Sirtuins utilize $NAD^+$ to facilitate the removal of acetyl groups. Sirtuins are known to mediate circadian rhythmicity in brain as well as in peripheral oscillators (Fig. 17.2). Studies have shown that caloric restriction fostered lifespan increment is mediated by SIR 2 and its orthologs.

SIRT1 exacerbates the expression levels of CLOCK and BMAL in the SCN of the hypothalamus by deacetylating the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1-α) (Duszka and Wahli 2020) (Fig. 17.3). Through the deacetylation of H3K9 and acetylation of PER2, SIRT1 confers epigenetic control to circadian rhythm (Soni et al. 2021). It has been observed that the function of SIRT 1 is gradually tarnished with aging (Yuan et al. 2016). A possible explanation for this is the fact that levels of NAD+ systemically decline and in turn reduces the efficiency of sirtuins (Pardo and Boriek 2020). Furthermore, oxygen consumption rate of mitochondria is controlled by the activity of SIRT3. In one of the studies, it was found

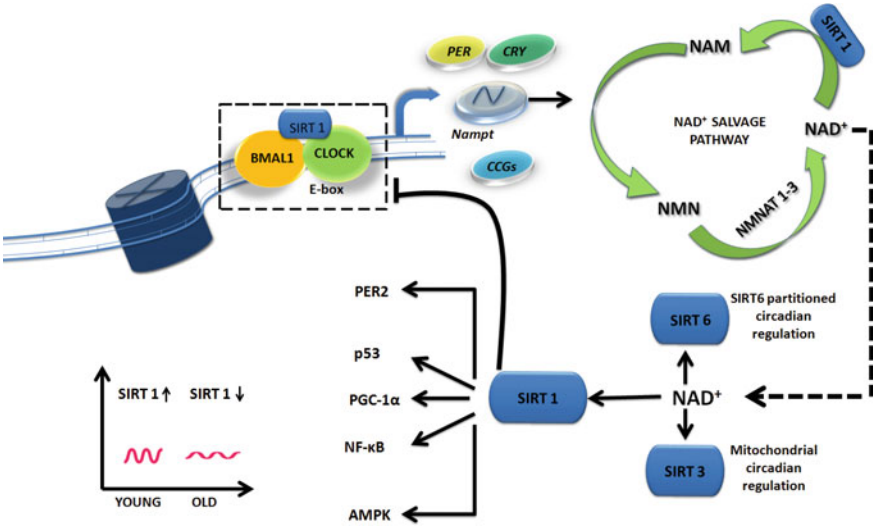


**Fig. 17.3** Role of Sirtuins in age-associated dysregulation of circadian rhythms and metabolism. Heterodimerization of CLOCK/BMAL1 leads to the activation of E-box motifs and advances the transcription-translation feedback loop which comprises PER, CRY, and other CCGs. CCGs and other transcripts exhibit circadian oscillations to maintain cellular functionality. Further, the *Nampt*-mediated salvage pathway generates $NAD^+$ which is a co-substrate for sirtuin family protein deacetylase. Sirtuins maintain circadian rhythms by modulating various biochemical pathways. Age-associated decrement in sirt1 results in dampening of circadian oscillations

that the hepatic circadian clock is regulated by the multifunctional enzyme SIRT 6. It is well known from rodent and fly studies that overexpression of SIRT6 increase the lifespan. SIRT6 essentially deacetylates H3K9 and is involved in the maintenance of telomere (Michishita et al. 2008). In one of the breakthrough studies, it was found that SIRT1 overexpression in lateral and dorsomedial hypothalamic nuclei delayed aging and amplified lifespan in mice (Satoh et al. 2010). Furthermore, investigations on Sirtuin knockout mice revealed that depletion of not all sirtuins lead to decrement in lifespan (Satoh et al. 2010). Essentially, SIRT3, SIRT7 and SIRT6 depletion reduces the lifespan unlike SIRT5. Mitochondrial SIRT 3 is a potent lysine deacetylase which is closely associated with the aging process as deciphered from knockout mice studies that resulted in progression of age-related disorders such as neurodegenerative disorders, cancer, and cardiac hypertrophy. SIRT3 deficient mice fail to regulate the ROS levels and sustain mild endothelial dysfunction. Mitochondrial MnSOD/SOD2 is a substrate of SIRT3 required for decomposition of ROS which malfunctions upon acetylation (Ansari et al. 2017). One of the most widely studied aspects of aging is caloric restriction mediated lifespan enhancement. The expression levels of sirtuins are found to be upregulated during caloric restriction.

## 17.9 Calorie Restriction: Rhythms and Implications in Aging

The concept of calorie restriction (CR) was introduced in the 1930s by McCay and co-workers, defining CR threshold as acquiring 20% lesser calories below ad libitum level (Lee et al. 2019). According to McCay, CR was significantly efficacious in extending health and longevity (McCay 1989). Our current understanding from years of studies ascertains that median and maximum lifespan of organisms is elevated through CR. Additionally, CR minimizes the risk of developing age-related disorders. One of the principal features of CR is the decreased temperature of the body which prevents dispensable energy expenditure and thus increases lifespan. It is widely accepted that circadian clocks are influenced by CR-modulated mechanisms. Rodent and fly studies reveal the role of CR in regulating the rhythms in expression of clock genes in the peripheral organs. Interestingly in mice deficient of BMAL1, CR fails to enhance the lifespan thus suggesting the link between circadian clocks, CR and aging (Kondratov et al. 2006). Notably, the conducive results through CR, especially increment in the lifespan is significantly altered depending on the sex (Astafev et al. 2017). In one of the studies, it was observed that 20% CR in female mice lead to increase in the lifespan significantly to a higher degree than in males, but 40% CR had unconvincing effects in females while males showed lifespan increment (Kane et al. 2018). Transcription factors PHA-4 and SKN-1 are known to regulate the extension of lifespan by CR in *C.elegans* as elucidated from RNAi-based screenings.

Evidences indicate a close association of modulation in chromatin functions during calorie restriction. In general words, CR exercises its age retarding effect through mechanisms that lead to increase in the genomic stability. CR is known to alleviate the repercussions of aging-induced aberrant DNAm patterns. CR leads to elevation in the levels of DNMT1 which functions to suffice the decrement of methylation level during aging (Li et al. 2010b). Reports suggest that HDAC activity is amplified during caloric restriction, implicating that global deacetylation might attenuate nutrition stress and may regulate the aging processes (Li et al. 2010b). Altered binding enrichment of HDAC1 on the promoter of p16INK4a and hTERT leads to modulations in the expression pattern of these genes resulting in enhanced lifespan (Li et al. 2010b). Regulatory role of the HDAC family in aging process during CR highlight the potential application of related epigenetic drugs or clinical strategies in aging and aging-related diseases. Calorie restriction has been observed to activate SIRT1 in various animal organs, while dysregulation of SIRT1 has been shown to reduce lifespan extension, thus indicating crucial role of SIRT1 in lifespan extension during CR.

## 17.10 Sex Differences in Aging Epigenetics

Previous experimental studies have established that females have greater susceptibility to sleep–wake cycle disorders. A number of species have demonstrated epigenomic instability with increasing age. While it is well accepted that epigenetic noise significantly contributes to aging, yet it still remains elusive that how epigenomic alterations are associated with the sex of the organisms. Generally among the sexes in humans, women have longer lifespan when compared to men as appraised by molecular biomarkers. However, it is a fact that women are associated with worsened health conditions at later half of their life, while males demonstrate healthier physical functions at equivalent age. It is widely believed that global DNAm levels decrease with age; however, this concept is often challenged by some scientists. There is a scarcity of research underpinning the sex-associated variability in the dynamics of methylation levels. Studies intending lifespan extension in drosophila through interventions in histone acetylation mediated through SIR2 overexpression showed an average extension of lifespan; however, the percentile extension in females (29%) was remarkably more than that of the males (18%) (Rogina and Helfand 2004). The sex-associated alterations in epigenetic age are perceptible in juveniles and adults (Horvath et al. 2016). Studies on brain DNAm patterns could not link the sex-age reciprocity in neurodegeneration in the samples of AD and controls (Pellegrini et al. 2021). In another study involving SIN3A knockdown, a structural integrant of histone deacetylase complex, knockdown through RNAi significantly reduced the lifespan of both male and female drosophila (Kadamb et al. 2013). In one of the fly studies, spermidine mediated histone acetylation repression led to significantly higher lifespan extension in females compared to that of the males (Eisenberg et al. 2009). However similar experiments in mice led to spermidine induced, increment equally in both the sex (Eisenberg et al. 2016). Studies on juvenile stress in the course of development, such as strident parenting, have been shown to be associated with changes in methylation (Non et al. 2016).

Sex differences in longevity, corporality, and aging-associated diseases are well documented in the literature. Alzheimer's disease (AD), one of the commonest dementia, affects more often females as compared to males (Laws et al. 2018). Sexual dimorphisms in AD pathologies have been investigated in mouse models, indicating females asseverate greater pathology (Jiao et al. 2016). Importantly, males are twice as susceptible to Parkinson's disease as are females (Jurado-Coronel et al. 2018). The epigenetic clock functions equally among both males and females, and it was customarily believed that age-associated epigenetic changes in DNAm are similar in the two sexes. However, recent findings in rodents suggest that less than 5% of the variations in DNAm with age overlap in males and females (Masser et al. 2017). A recent study discovered that gonadal hormones promote epigenetic aging. The castrated sheep showed DNAm at the androgen-regulated loci, thus promoting the epigenetic delay in aging (Sugrue et al. 2021).

## 17.11 Epigenetic Therapeutics

Aging is an inevitable biological process that encompasses nearly all organisms. People have since long endeavored of developing strategies to increase longevity. Scientists now believe that aging can be delayed with biological and lifestyle interventions (Zhang et al. 2020). Genomic instability and related alterations in gene expression accompany the aging process.

Epigenetic drugs refer to the molecules capable of modulating certain enzymes associated with inducing epigenetic changes. HDAC superfamily members are promising therapeutic targets reckoning their potential to repudiate epigenetic dysregulations. Several HDAC inhibitors are under clinical trials for targeting age-associated disorders (McIntyre et al. 2019). Sirtuin-based therapeutic interventions are also being explored owing to wide positive effects. One of the noxious age-related disorders is cancer, often lethal and incurable. Vorinostat, an HDAC inhibitor, is already in clinics to treat certain cancers. Other HDAC inhibitors are being investigated for their anticancer properties in many trials. In a study, class 1 HDAC inhibitor CI-994 was investigated for its efficacy to ameliorate the age-associated sensitivity to HAL-induced motor side effects. CI-994 was found to improve the expression and function of D2R mediated through histone acetylation at the DRD2 promoter (McClarty et al. 2021). HDAC inhibition leads to the acetylation of nuclear histones, thereby activating crucial tumor-related genes such as p53, GATA-1, and p21WAF1/CIP1, the expression of which impedes the proliferation of cancerous cells (Richon et al. 2000). Studies have shown that SIR2 activator resveratrol (3,4,5-trihydroxystilbene) enhances lifespan extension and thus modulates the aging processes (Lee et al. 2019). Fluorescence and mass spectrometry-based approaches revealed that SRT1460, SRT1720, and SRT2183 also exhibited SIRT1 activating properties (Milne et al. 2007). Experiments reveal that resveratrol retards cellular senescence in human diploid fibroblasts. Administering, mice on high calorie diet with resveratrol improved the mitochondrial number, increased sensitivity to insulin, decreased the levels of IGF-I, and increased PGC-1α activity, thus improving health and lifespan (Baur et al. 2006).

Depsipeptide, valproic acid, and phenylbutyrate are some of the well-explored HDAC inhibitors that have potently demonstrated significant antitumor properties along with low cytotoxicity. In recent years, natural bioactive dietary ingredients have been shown to possess natural HDAC inhibition properties and thus may be pivotal in cancer chemoprevention. Metformin increases insulin sensitivity and is a commonly suggested anti-diabetic drug that has also demonstrated to modulate molecular mechanisms of aging (Bridgeman et al. 2018). Evaluation of diabetic patients who received metformin exhibited enhanced lifespan as compared to non-diabetic individuals. The drug is known to obviate the onset of diabetes and ameliorates cardiovascular risk factors, thus preventing age-related disorders. Additionally, metformin reduces the risk of cancer and neurodegenerative disease (Sunjaya and Sunjaya 2021).

Metformin is known to interact with SIRT1, the HDAC associated with lifespan enhancement, and influences epigenetic aging. In one of the randomized trials,

metformin demonstrated an increase in the expression of SIRT1 in peripheral blood mononuclear cells (de Kreutzenberg et al. 2015). Banerjee and co-workers showed decrease in H3K9 and H3K27 methylation and increase in H3K4 methylation upon treatment with metformin, in breast cancer cells (Banerjee et al. 2016). The effects were both globally and specifically at the promoter of E-cadherin, a tumor suppressor gene. Inhibition of HMTs and reductions in the expression of SUV39H1 may be probable mechanism for obtaining these results; however, the molecular mechanism of inhibition of HMTs by metformin is not yet understood.

## 17.12 Concluding Remarks

Aging is an inevitable biological process associated with grievous physiological and mental health. Despite the evolution of a wide range of defense and repair mechanisms over the years, aging significantly alters the normal homeostasis of the biological system. Growth in the understanding of aging hallmarks has enabled us to delay the aging process through nutritional and biochemical interventions, though to a very limited extent. The prevention of age-related disorders necessitates further understanding of the mechanisms underlying the regulation and dysregulation of age-associated molecular alterations. Dysregulated circadian regulation has implications on the organism's systemic functionality including inefficient immune system functioning, cognitive impairment, sleep dysregulation, cardiovascular dysfunction, and reduced reproductive potential. At the genomic level, substantial epigenetic regulation is observed in aging and associated molecular pathways. The dysregulation of clock machinery has pronounced effect on diverse epigenetic landscape leading to accelerated aging and related disorders. The pattern of epigenetic modifications like CpG methylation, DNAm, histone acetylation and deacetylation of the clock, and associated genes gets altered. Despite the wide acceptance of sirtuins in longevity, it is unclear how exactly sirtuins facilitate the retardation of aging process. What is even more of primitive understanding is how sirtuins crosstalk the processes of aging and circadian rhythm. However, a plethora of studies on rodent and vertebrate models has indicated role of sirtuins in mediating the retardation of aging process, and the mechanistic purview still needs to be explored, specifically for development of therapeutics for aging-associated disorders.. Studies have since long vocalized the role of caloric restriction in the regulation of circadian clock genes and retardation of age-associated changes. Insight into the mechanistic purview of caloric restriction mediated positive health effects will foster the aim of developing delayed aging interventions. There is an utmost necessity of sex-specific studies of epigenetic basis of aging to develop unbiased restorative therapeutics. Several epigenetic therapeutics to delay the progression of age-associated diseases have been developed and are being tested; however, the efficacy of these is still debatable and compels for extensive research in this field.

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# Chapter 18
# Chronotype and Its Relation to Healthy Aging

**Meenakshi Sinha, Babita Pande, and Ramanjan Sinha**

## 18.1 Introduction

The revolution of earth on its own axis around the sun lead to diurnal and seasonal variation. Life on earth is synchronized to the periodic change in the environment which is apparent through cyclic occurrence of many biological rhythms with varied duration. The well-known biological rhythms observed in human are: the ultradian (with oscillation of <24 h period, e.g., sleep cycle with approx. 90 min of period of occurrence), circadian (≅24 h of periodicity, e.g., rhythm in adult sleep–wake times, melatonin and core body temperature) and infradian rhythms (periodicity >28 h, e.g., female menstrual cycle rhythm). Among these, the most prominent and ubiquitous biological rhythm in living organisms is *Circadian rhythm* which is derived from two Latin words "Circa" meaning "about/approximately" and "diem" meaning "a day/24 h". Circadian rhythm is the variation of 24 ± 4 h in any biological processes from gene to behavior levels.

The diurnal nature of the light–dark or the sunrise-sunset cycle broadly generates two types of activity pattern among the organisms with one having maximum activity during day/light like humans; while other being active mostly during night/dark (nocturnal) like the rodents. However, several other environmental factors like temperature and social behavior like feeding time, work schedule, etc. also have strong modulatory effect on the rhythmic behavior of the organisms. Thus, even humans who were mostly active in the day/light at ancient time, have altered their activity rhythm and thereby, all other functions of the body in the modern times due

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to availability of artificial light, leading to define this propensity in terms of various chronotypes.

## 18.2 Chronotype and its Distribution among Population

Chronotype or circadian typology (CT) is the behavioral trait in human about scheduling the preference for the sleep–wake time or activities (physical or mental) at specific time around 24 h.

According to morningness-eveningness preferences, three types of chronotypes (Fig. 18.1) are seen in human population (Montaruli et al. 2021). These are:

(i) Morning chronotype, also popularly known as "lark type" or "early chronotype" or "early birds" or "morning larks" are the people who prefer early sleep–wake time and avoid late night sleeping. These morning chronotype display better physical and mental performances during early hours of the day.

(ii) Evening chronotype, also known as "owl type" or "late chronotype" or "night owls" have the preference to sleep late in the night and wake up also late. Their ability to execute any physical or mental task in the early hours of the day is generally quite poor.

(iii) Intermediate chronotype (Neither type) are the individuals whose preferences lie in between morning and evening chronotypes. These individuals do not show any preference for scheduling their sleep–wake times early like morning chronotypes and/or also do not like to sleep late like evening chronotypes.

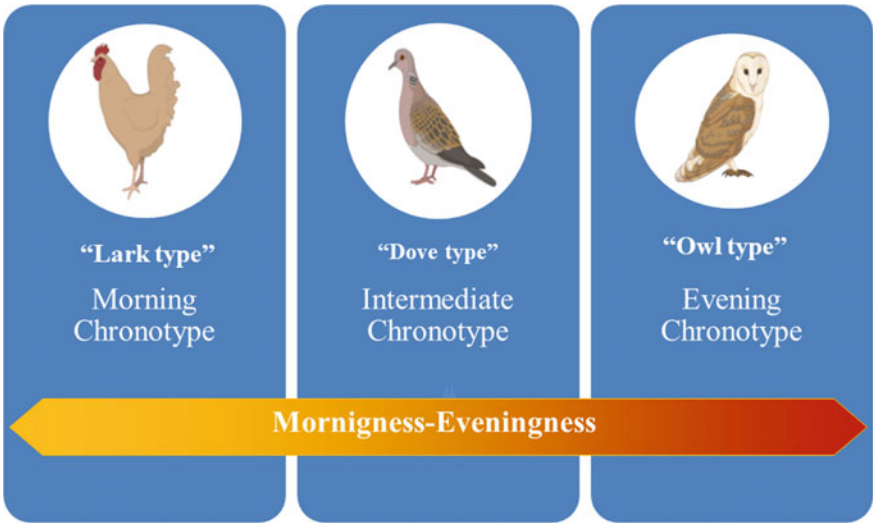


**Fig. 18.1** Morningness-eveningness is a continuum with distinct categories reflected as chronotype

**Table 18.1** Chronotypes in human population

| Preference | Chronotype | Nick name | Circadian rhythm (acrophase/peak) phase |
|---|---|---|---|
| Morningness | Morning | Morning lark | Early |
| Eveningness | Evening | Night owl | Late |
| Neither morning nor evening, rather prefer in between time | Neither or Intermediate type | Humming bird/Dove | Neither early nor late, in between |
| Morningness-eveningness as well as intermediate | Bimodal chronotype | Biomodal chronotype | Different from intermediate chronotype, also neither early nor late |

These individuals are also nicknamed as humming bird or dove type (Table 18.1).

A fourth type of chronotype has also been suggested known as "Bimodal chronotype". These individuals are broadly intermediate chronotype but also display preference of some selective behavior similar to morning or evening chrontypes. Their preference pattern has been linked to the influence of social and environmental factors (Tempaku et al. 2017). However, bimodality concept needs to be validated yet with circadian markers and other questionnaires that are used to quantify the morningness-eveningness behavior in human.

Chronotype is not a stationary/fixed behavioral trait but rather follow a near-Gaussian distribution during ontogeny/life cycle of an individual as observed in the population from Europe and USA (Roenneberg et al. 2007; Fischer et al. 2017). In the childhood, morning chronotype prevail, which shows shift to intermediate and then towards evening type in adolescent and then, morningness increases with advancing age.

However, individuals from countries with early sunrise, and/or culture of waking early in the morning (e.g., Indians) show skewed distribution with more morning preferences. Majority of rural and remote population from India show extreme morning orientation compared to urban. A mixed behavior is observed in school going Indian adolescent with preponderance of morningness (Pande et al. 2018) or intermediate chronotype (Jongte and Trivedi 2022). The difference observed could be attributed to the timing of school to which the students get entrained or could be because of use of different versions of morningness-eveningness questionnaires.

## 18.3 Assessing the Chronotype

Sleep–wake behavior is the outcome of the interaction between the homeostatic process and the circadian mechanism. The body homeostasis decides the amount of sleep requirement according to the accumulated sleep pressure or wakefulness while

the timings of sleep–wake is predominantly decided by the endogenous circadian system. Therefore, circadian preference for daily activities or the chronotype can be predicted or estimated from the sleep–wake timings.

While choice for daily physical or mental activities, timings of subjective fatigue, alertness, mood or drowsiness status also is able to predict an individual's chronotype. They can be assessed by using batteries (Table 18.2) of inventories/questionnaires (Montaruli et al. 2021) or with the help of automated devices worn by the individual.

*Use of devices for ascertaining chronotype:*

The objective assessment of chronotype has been carried out using wearable devices, such as Actiwatch, accelometer/band like Fitbit, Xiaomi, Microsoft, and Smartphone.

**Table 18.2** Popular and widely used morningness-eveningness questionnaires for chronotype assessment in human population

| Inventory | Main measures | Other variables |
|---|---|---|
| Morningness-eveningness questionnaire (MEQ) (Horne and Östberg 1976) | Three chronotypes (MT, IT and ET) | Higher value reflect higher morningnes, lower value indicate higher eveningness (from DMT, MMT, IT, MET to DET) |
| Diurnal type scale (DTS) (Torsvall and Åkerstedt 1980) | Three chronotypes morning active, intermediate type and evening active | Higher values reflect higher morningness, lower depict higher eveningness |
| Composite scale of morningness (CSM) (Smith et al. 1989) | Three chronotypes (MT, IT and ET) | Higher values reflect higher morningness and lower value indicate higher eveningness |
| Reduced morningess-eveningness questionnaire (rMEQ) (Adan and Almirall 1991) | Three chronotypes (MT, IT and ET) | Higher values reflect higher morningness and lower value indicate higher eveningness (from DMT, MMT, IT, MET to DET) |
| Munich Chronotype Questionnaire (MCTQ) (Roenneberg et al. 2003) | Chronotype or midpoint of sleep (as clock time) corrected for "oversleep" on free days (MSFsc) | Social jetlag, sleep–wake variables, sunlight exposure duration |
| Morningness-eveningness—stability-scale improved (MESSi) (Randler et al. 2016) | Three distinct dimensions viz. morning affect, eveningness, distinctness | Higher scores in each dimensions indicate higher morningness, higher eveningness and higher amplitude |

*MT* Morning type; *IT* Intermediate type; *ET* Evening type; *DMT* Definitely morning type; *MMT* Moderately morning type; *MET* Moderately evening type; *DET* Definitely evening type

These device can be worn on wrist, over chest, waist, etc. and work by recording and integrating the occurrence and degree of limb movement activity over time. Besides activity, these devices also measure sleep-wake timings, heart rate, blood pressure (Smith et al. 2018). Sleep latency, total sleep time, wake after sleep onset and sleep efficiency are some of parameters which can also be estimated by actigraphy and have implication in assessing the quality of sleep. Actigraphy is the more reliable approach to quantify morningness-eveningness behavior, measuring activity and sleep–wake variables that are well correlated with the circadian inclination of an individual used to classify individuals to different chronotypes. Among the circadian parameter of activity rhythm assessed by actigraphy of the extreme chronotypes, delayed acrophase in activity of around 2 h in evening type has been documented as compared to morning type individuals (Vitale et al. 2015). A positive and negative association of actigraphy-based acrophase with Morningness-Eveningness Questionnaire (MEQ) and the reduced Morningness-Eveningness Questionnaire (rMEQ) have also been observed (Montaruli et al. 2021).

Besides, body temperature or circadian markers like cortisol and melatonin rhythms are also reliable to measure the chronotype of an individual.

## 18.4 Chronotype and Variability in Biological Variables

Phase angle of entrainment of the circadian system, being influenced by environmental light- dark cycle, is expressed as chronotype of an individual. The circadian phase of different biological variables varies as function of chronotype. For example, the minima of body temperature rhythm appears earlier in morning chronotype (03:50 h) compared to evening type (06:01 h) (Baehr et al. 2000). Similarly, early DLMO (dim light melatonin onset, a marker of phase of circadian rhythm), early sleep patterns and earlier circadian phase is seen in rural inhabitants who have earlier light exposure (Ruiz et al. 2020). The evening-types individual have been seen to have sluggish parasympathetic reactivation response following exercise in the morning hours (Sugawara et al. 2001), while morning chronotype display better athlete performance with less perceived efforts in the morning hours (Vitale and Weydahl 2017). Also, better neuro-cognitive performance related to attention and alertness have been documented by evening chronotype in the evening hours and by morning type individuals in the morning hours (Venkat et al. 2020).

## 18.5 Determinants of Chronotype

### *18.5.1 Endogenous Nature*

Basically, chronotype is the indicator of circadian clock driven biological systems. It is a marker of individual's circadian inclination or the circadian phase/preference/behavior. A longitudinal study of one month invoking forced dysynchrony in 17 young men reported a significant correlation of individuals' chronotype with their endogenous circadian period and wake time. The morning chronotype are reported to have shorter circadian period compared to evening chronotype (Duffy et al. 2001). Several other similar studies have therefore, suggested that the behavioral trait of morningness-eveningness is correlated with fundamental property of the circadian pacemaker and thus, bear an endogenous basis for the origin of chronotype rather than any association with the ethnicity, gender and socioeconomic status.

### *18.5.2 Genetic Basis*

Chronotype also has heritable attributes, with 21–52% of transmission and thus its genetic basis has been studied in great detail (Kalmbach et al. 2017). The widely studied clock genes that exhibit circadian variation in their expression and regulate the circadian rhythm in living processes through transcriptional and translational autoregulatory feedback loops are period genes (such as *PER1, PER2* and *PER3*) and Cryptochrome (*CRY1* and *CRY2*) genes. In addition, Casein Kinase 1δ and 1ε (CK1) and transcription factors Circadian Locomotor Output Cycles Kaput Protein (*CLOCK*), Brain and Muscle ARNT-like protein (*BMAL1* and *BMAL2*), and Neuronal Pas Domain Protein (NPAS1 and NPAS2) are also involved in the generation of circadian rhythm.

Studies on *CLOCK* genes polymorphism and its association with morningness-eveningness behavior have given the evidences for the genetic basis of chronotype (Table 18.3). Subjects carrying 3111C polymorphic genes in the 3′-flanking region of a human *CLOCK* gene show evening preferences as assessed by using 19-items Horne-Östberg Morning-Evening Questionnaire (MEQ), irrespective of age, gender or ethnicity. A differential level of *CLOCK* gene expression or existence of polymorphic genes have been suggested to be linked with circadian phase differences in morning or evening type individuals.

Morningness behavior has been significantly linked with the PER2 (rs934945) genotype expression and the interaction among the three polymorphic genes namely PER2, ARNTL and GNB3 increase the propensity of PER2 polymorphism for the diurnal preference. Link between 5-hydroxy-tryptamine, serotonin receptor HTR2A rs6311 (−1438C/T) SNP and the morningness behavior has also been reported (Yeom et al. 2020).

**Table 18.3** Genetic basis of chronotype distribution

| Genes | Polymorphism | Chronotype attribution |
|---|---|---|
| *CLOCK* | *CLOCK* 3111C/T (SNP) rs1801260 | Associated with evening preferences (Mishima et al. 2005; Yeom et al. 2020) |
| *CLOCK* | *CLOCK and GNB (rs5443)* interaction | Associated with diurnal preference (Yeom et al. 2020) |
| *PER2* | G allele of C111G SNP of PER2 (rs2304672) | Extreme morning chronotype (Yeom et al. 2020) |
| *PER2* | PER2 G3853A polymorphism (rs934945) | Associated with diurnal preference in Korean sample (Yeom et al. 2020) |
| *PER2* | PER2 SNP (G2114A) | Associated with diurnal preference in Japanese (Matsuo et al. 2007) |
| *ARNTL, PER2 and GNB3* | G/A SNP in *PER2* (rs934945), C/T SNP in *ARNTL* (rs2278749) and *GNB3* (rs5443) genotype | PER2 is associated with diurnal preference in Korean; Interaction of *ARNTL* and *GNB3*linked with eveningness (Yeom et al. 2020) |
| *PER3, ARNTL2* | *PER3 G/T SNP (rs10462020), ARNTL2 T/C SNP (rs922270)* | Associated with diurnal preference in British Population (Parsons et al. 2014; Kalmbach et al. 2017) |
| *PER3* | *PER3* polymorphism (rs228697) | Associated with diurnal preference; Higher frequency of G allele linked to eveningness (Hida et al. 2014) |
| *Serotonin 2A Receptor* | HTR2A rs6311 (-1438C/T) polymorphism | C/C + C/T genotype associated to; morningness and T/T genotype associated with evening chronotype (Yeom et al. 2020) |
| *NR1D2 (Rev-erbβ)* | 22 SNPs in NR1D2 (Rev-erbβ); prominent SNP rs4131403 | Significantly associated with chronotype (Maukonen et al. 2020) |

Large-scale genome-wide association studies (GWAS) on large study group ($n = 8433$) in Finnish population of age 25–74 years male and female have also helped to trace the heritable nature of chronotype as trait inherited in members of family. These studies have identified clock gene NR1D2 (Nuclear Receptor Subfamily 1 Group D Member 2; (*Rev-erbβ*)) for the eveningness behavior (Maukonen et al. 2020).

Per3 polymorphism has been found to be significantly related to extreme diurnal preference as potential genetic marker, with association of longer allele to morningness and shorter allele to eveningness with higher frequency of minor allele rs228697 seen in evening chronotype. It has been suggested that differential PER3 phosphorylation might lead to phenotypic difference as morning-evening preferences. This

signifies the strength of the circadian oscillator in young individuals that decline in older people.

On the basis of above facts, it may be opined that the evolution of different genetic traits of chronotype through differential expression of clock genes and emergence of its variants (polymorphism) have increased the adaptive power and thus survivability and mortality of humans. As studied in ancient tribal community, the Hadza hunter-gatherers of Tanzania, the different chronotypes help to share the vigilance to increase the survival and reduce the risk of mortality during sleep at night in nature sharing habitat with wild animals (Samson et al. 2017). Further, different chronotypes differ in circadian regulation of sleep–wake timings, sleep pressure and thus neuro-cognitive performance like attention, decision making, etc. needed for successful survival and social integrity. The diurnal behavior is preferred over nocturnal behavior in case of human, because early rising has been considered as ambrosial hours for better cognitive functions through hormonal changes like melatonin and cortisol that create conductive milieu for a healthy body by increasing the sunlight exposure duration (Kumaran et al. 2012; Venkat et al. 2020). Therefore, humans are in great evolutionary benefit for adopting morning preference lifestyle, as it gives longer span to remain active and awakened to learn, doing creative work, socializing, defend and protect from predators (Nunn et al. 2016). Late timings of sleep–wake have been linked with an array of health problems such as increased risk of diabetes, cardiovascular diseases, psychiatric disorders, that elevate the chance of mortality (Didikoglu et al. 2019).

## 18.6 Moderators of Chronotype

Generally, the neither/intermediate chronotypes are found in majority among all types of chronotypes worldwide. However, age, gender, culture, geographical regions (latitude/rural or urban setup) are the prominent modulators of morningness-eveningness preferences leading to extreme chronotype (Table 18.3).

### *18.6.1 Age and Gender*

Though chronotype has genetic basis, it is a dynamic characteristic that changes from childhood- adulthood and between genders. Majority of studies from different parts of the world have shown similar trend of age and gender related variation in chronotype.

Allegedly, the first evidence of chronotype distribution on large a sample ~25,000, including German and Swiss population in majority, was conducted by Roenneberg et al. (2004). This group first documented the circadian typology changes from morning to evening type and then morning type, with the developmental stages passing from childhood to young age to elderly group (Fig. 18.2). The adolescent

showed maximum eveningness at about the age of 20 years (females 19.5 years and male 20.9 years). This eveningness inclination has been reported to start from the age of 13 years for Italian population. In a study spanning 12 years on morningness-eveningness behavior from the American Time Use Survey, the chronotype distribution in US population ($n = 53{,}689$) showed prevalence of intermediate chronotype in overall population. Majority of US adolescent also showed evening preference at around 19 years (Fischer et al. 2017). A Finnish study on a population of 10,503 adults aged 25–74 years documented that more younger population (25–34 years) are evening type, middle age groups (35–44 years) are intermediate chronotype, while morningness increases with advancing age (Fischer et al. 2017). Similar findings were reported for German, New Zealand population also (Paine et al. 2006; Fischer et al. 2017).

The obvious chronotype changes during adolescent have been linked to the changes in hormonal profile during puberty that could interact with the circadian system leading to delay in morningness-eveningness. Further, the study pressure or work pressure to excel in career forces the adolescent and young to work late at night exposing them to artificial light at night for longer duration, the changing

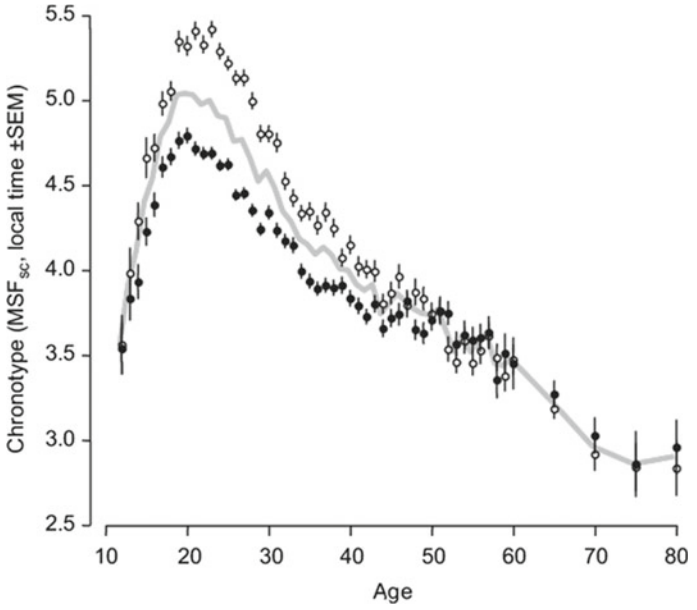


**Fig. 18.2** Chronotype depends on age. These changes are highly systematic and are different for males and females (filled circles, females; open circles, males; the gray line shows the averages for the entire population). The first data points represent the averages for subjects aged 12 or younger. Between ages 12 and 60 data were averaged for each year of age while those showing the mean chronotype for subjects above 60 years of age are averaged over groups of 5 years. Vertical lines represent SEM. MSFsc: Midpoint of sleep (as clock time) corrected for "oversleep" on free days. (*Reproduced with permission from Prof. Till Roenneberg* (Roenneberg et al. 2007))

habits like using mobile and laptops and other blue light emitting gadgets for reading, as well as entertainment or social communication, could also be the reasons for late sleep–wake pattern through delayed onset of melatonin, the signal for adjusting body clock to natural light–dark cycle. Older individuals have narrower preference of sleep time compared to other age groups, have less social responsibility or not engaged in job specifically night or shift work like young individuals which is responsible for less chronotype variability in older age group, thus limiting the variance in their chronotype.

It has also been purported that hormonal change during developmental stages could modulate the morning-evening trait. The concentration of testosterone in men has been positively associated with evening orientation in men. So, it has been stated that the decline in testosterone and estrogen levels in different ratio with aging in men and women might be the reason for changing morningness-eveningness behavior with age (Randler and Engelke 2019).

All these studies points toward a similar trend, i.e., after reaching the maximum eveningness, morningness increases with aging. This stage has been called as the "marker of end of adolescence" by Roenneberg et al. (2004).

These studies also come with the evidence that gender difference exist in morningness-eveningness preference (Fabbian et al. 2016). The gender difference discerned that females in reproductive stage show advanced sleep–wake timings compared to male which could be the influence of reproductive hormones, giving rise to gender difference in chronotype. It has also been reported that the gender difference in chronotype disappears after the age 50 years for German population, after 55 years in Italian population, 45 years in Brazilian and around 40 years in US population (Fischer et al. 2017). In this context, Duarte et al. (2014) stated that "*the ontogenetic development of the circadian timekeeping system is more plastic in men, as represented by the larger amplitude of chronotype changes throughout their aging process"*. Thus, gender difference is observed till the reproductive age of female and disappears after/during menopause with the change in the hormone repertoire which varies in between 40–55 years according to studies in different geographical location and ethnicity.

Changing concentration and release time of many hormones, e.g., maximum growth hormone release during growing age, adolescent to young age or the stress hormone cortisol or melatonin with aging, menopause related alteration in reproductive hormones in female can differentially interact with the circadian system (Roenneberg et al. 2004; Fischer et al. 2017).

The non-hormonal cause for gender difference is added family responsibility for female, compelling them to wake up early for doing household. Due to cultural believes or less economic privilege, females are not expected/allowed late rising in the morning in many parts of the world. It is also interesting to note that within a particular age group, all the three types of chronotype from very early type to late type are observed; however, the predominance of a particular chronotype varies according to the gender, while the variation in chronotype decreases with aging (Fischer et al. 2017).

However, as sunrise is the robust environmental time cues entraining the circadian pacemaker, the inconsistent findings on interaction between age and gender have been attributed to the studies being conducted at different geographical locations, with different sunrise and sunset time.

### *18.6.2 Entrainment to Environmental Light/Geographical Region*

Sunlight is the primary and robust time cue that reset the human circadian clock. Blind people with total retina degeneration free run in absence of any light cues living in societal condition. Predominance of exposure to social cues only and not exposing to daily sunlight progressively causes uncoupling of circadian clock and the solar time cues, leading to circadian misalignment and emergence of various types of health consequence. The difference in circadian alignment is easily visible between urban and rural people. Urban people mostly dwelling under artificial light and less in sunlight display evening preference compared to rural inhabitants who are more exposed to sunlight and entrained to it and thereby show morning preference in their daily activity pattern.

The effect of geographical location has impact on the morning-evening behavior, since the population near equator has been observed to be more morning inclined compared to population residing away from equator due to availability of sunlight. Moreover, in the same geographical location, people living farther east with early sunrise are more morning orientated compared to people inhabiting toward west and getting later sunrise (Randler et al. 2015).

### *18.6.3 Culture/Ethnicity/Work Schedule*

Work schedules are one of the important determinants of morning-evening preference. As discussed above, generally women due to cultural/ethnic believes and economic factors are found to be morning type. Night shift workers tend to show definite evening orientation compared to unemployed, who are mostly moderately morning type (Paine et al. 2006) (Table 18.4).

## 18.7 Chronotype as Determinant of

1. Physical activity and sleep health
2. Disease and health outcomes
3. Mental/cognitive performances

**Table 18.4** Chronotype distribution as function of age and gender across world

| Population | Chronotype distribution in population | Age related chronotype distribution | Gender related chronotype distribution | References |
|---|---|---|---|---|
| *Asian (Indian)* | • Predominance of MT<br>• More MT in remote areas followed by rural and then urban<br>• More intermediate types among urban teenagers | Morningness increases with aging | Inconsistency in findings; with no consistent gender difference | Pande et al. (2018); Jongte and Trivedi (2022) |
| *Asian (Chinese)* | • Normal distribution of MSFsc<br>• Inconsistency in finding related to prevalence of chronotype;<br>• IT or early types prevailed more compared to evening type | Eveningness in adolescent age that continued till 28 years; Children and aged above 55 years show morningness | Inconsistency in findings; females more IT preference than male | Liu et al. (2020) |
| *Asian (Korea)* | Prevalence of IT in young and middle age | Prevalence of IT, followed by MT/ET in young; predominance of MT in middle age and in sixties | No gender difference | Suh et al. (2018) |
| Asian (Japanese) | Morning preference compared to European population | Morningness increases with aging | Males more morning type compared to females; gender difference observed in sixties with males showing early chronotype compared to female | Komada et al. (2019) |

(continued)

**Table 18.4** (continued)

| Population | Chronotype distribution in population | Age related chronotype distribution | Gender related chronotype distribution | References |
|---|---|---|---|---|
| European (German, Slovakia, Italian; Finland) | Higher prevalence of IT | Evening type prevail in adolescent and young age, increasing morningness with advancing age, older adults morning type | Females more morning type than males; specifically younger women compared to older women | Randler and Engelke (2019) Roenneberg et al. (2004); Randler et al. (2015); Fischer et al (2017) |
| Americans (USA) | Prevalence of intermediate chronotype in overall population | Evening chronotype in adolescent age; Variability in chronotype declines with advancing age | Females morning chronotype than men before 40 years; decrease of variability higher in aging males | Fischer et al. (2017) |
| New Zealand | Prevalence of morning type in young and middle age group | Young age group (30–34 years) mostly evening type compared to middle age (45–49 years) being morning type | No gender difference reported | Paine et al. (2006) |
| Latin American (Brazilian) | Prevalence of morning type | Evening preference among adolescent; morningness increases with age | More morning type females compared to male till 30 years; females more evening type after 45 years | Duarte et al. (2014) |

*MT* Morning type; *IT* Intermediate type; *ET* Evening type; *MSFsc* Midpoint of sleep (as clock time) corrected for "oversleep" on free days

4. Social jetlag (Circadian Desynchrony)
5. Chronotype with reference to COVID-19 pandemic

### *18.7.1 Chronotype, Physical Activity and Sleep Health*

It has been observed that physically active elderly individuals including male and females have better overall sleep quality, less sleep disturbance, less dependence

on sleep medication, less daytime dysfunctions compared to physically inactive. Further the physically active elderly females with morning chronotype have lesser sleep related problems than physically inactive intermediate chronotype. Physically active morning chronotype males also report less sleep disturbances compared to intermediate/neither type physically inactive males. Further, active male with intermediate chronotype also display less complaints compared to inactive intermediate chronotype (Montaruli et al. 2021).

### *18.7.2 Chronotype, Disease and Health Outcomes*

Increased morbidity encompassing high risk of metabolic disorders and mortality due to cardiovascular diseases have been reported in individuals of evening preference and delayed sleep onset time. The evening chronotype people also report high rate of psychological disorders, diabetes, neurological disorders, gastrointestinal/abdominal disorders and respiratory diseases compared to morning chronotype. The tendency of eveningness behavior have been observed to be independently associated with a significant increased type 2 diabetes, arterial hypertension, faster heart rate and a significant decreased rate of systolic blood pressure (SBP) as well serum total cholesterol and low-density lipoprotein cholesterol compared to morning type people. Asthma patients of evening chronotype report increased breathing difficulties (shortness of breath, wheezy breathing with dyspnea) and more medication compared to morning or intermediate types with frequent awakening from sleep due to coughing compared to morning types (Montaruli et al. 2021).

The underlying cause for the increased risk of mortality in night owls has been assigned to the chronic social jetlag which is their inability to adjust their circadian timing system to the timing of the social obligation or work schedules (Montaruli et al. 2021). Chronic circadian misalignment in evening chronotype may be responsible for the age related poor health outcomes in middle, as well as in older adults. Night owls are associated with unhealthy habits like smoking or tobacco chewing, longer screen time and sedentary time with less physical activity, unhealthy diet, making them susceptible to high risk of cardiovascular disorders than morning larks. Evening types young and middle aged adults of both genders tend to have dyslipidemia, gain weight and are at risk of obesity compared with morning/neutral types.

Besides, due to late night sleep timings, the evening chronotype individuals are exposed to artificial light at night (LAN) in addition to blue light emitting from gadgets tike mobile, laptop causing suppression of melatonin secretion that desynchronized circadian rhythm (Tähkämö et al. 2019). This is associated with high insulin resistance, increased risk of metabolic disorders like diabetes and cancer of breast or prostate. Besides, irregular and delayed meal times which reduces the resting-energy expenditure, fasting carbohydrate oxidation, glucose tolerance, dampened daily free cortisol levels and thermal effect of food, also could be other reasons for the metabolic dysregulation and emergence of metabolic disorders in evening chronotype, since meal timing acts as a synchronizer for peripheral oscillators.

### 18.7.3 Chronotype and Mental/cognitive Performances

The evening chronotype have higher daytime sleepiness, poor psychomotor vigilance, executive function and isometric grip strength compared to morning chronotype (Facer-Childs et al. 2018). Depression and mood disorders have been frequently detected in evening oriented adults ≥50 years. The mental or psychological disorders such as depression, bipolar disorder are also linked with the morningness-eveningness (Montaruli et al. 2021). Circadian impairment and inclination toward eveningness is also noticed in bipolar disorder patients (Tähkämö et al. 2019).

### 18.7.4 Chronotype and Social Jetlag (Chronotypes and Circadian Desynchrony)

The night owls are more susceptible for social jetlag (Montaruli et al. 2021) which is the discrepancy in the mid sleep time on work days and free days that arises when on weekends the sleep–wake times is delayed compared to weekdays. The root cause for adverse health consequences faced by evening chronotype is the discrepancy between the circadian timing system and timing for work or social activities that is known as *circadian misalignment*. Further, the solar cycle or the natural light–dark cycle also cannot be ignored. Evening type people mostly miss the sunrise or morning light, the robust entertainer of the circadian clock that in long run delays the circadian phase. Evening chronotypes take longer time to recover from social jetlag compared to morning type. It has been well-known that social jetlag correlates with many health problems such as obesity and metabolic disorders, cardiometabolic risk and poor mental health such as emotional well-being and poor relationship.

### 18.7.5 Chronotype with Reference to COVID-19 Pandemic

COVID-19 imposed global shutdowns during its three major waves has opened the front gate of simulated lab revealing the impact of social factors, lack of sunlight exposure and irregular life style at population level. An array of observational and cross sectional studies during lockdowns reported altered sleep–wake behavior leading to many health consequences like sleep problems, psychosocial and emotional disturbances in different population across the globe (Sinha et al. 2020a; Blume et al. 2020). Majority of these studies documented that people under longer COVID-19 lockdown displayed evening proclivity for activities, e.g., delayed sleep–wake and meal timings. Such evening preference was more prominent in younger population but less common in elderly (Sinha et al. 2020a). Associated problems with the evening preferences of people during lockdown were increased screen time, reduced physical activity and decreased exposure to sunlight, which is the natural entertainer of

circadian system of an individual, causing social jetlag and circadian misalignment. But the important lesson emerging from the studies during COVID times was that individuals who maintained early sleep–wake timings, had more morning preference displayed least social jetlag (Sinha et al. 2020b).

## 18.8 Summary and Recommendations

The modern man has the compulsion of adjusting their endogenous circadian time preferences to societal timing and work schedule. The lifestyle of modern humans, especially in urban areas, varies a lot compared to ancient individuals who were closer to environment and followed sun and the moon for their activity and sleep behavior. This changed life style and increased exposure to environmental pollutants like light at night and noise emitting from modern gadgets cause "*evolutionary mismatch*", leading to health problems.

However, high association of eveningness with several pathologies leading to poor health and poor quality of life, signifies the impact of extreme morningness or eveningness behavior. In this context, it may be added that maintenance of morningness behavior in sleep–wake pattern could help to minimize circadian misalignment, promote sleep health and improve mental health also. It has been documented clearly that there exist positive significant correlation between healthy aging and sleep onset—waking times. Evening chronotypes show high vulnerability to delayed sleep phase disorder.

The imperative strategy to heal the circadian derailment is following natural solar cycle for waking and advancing the sleep schedule. Sunlight has always been known as the strong entertainer of the circadian system. This may be linked to the age old concept of early rising, i.e., at "Brahmamuhurtha", the last quarter of night, as described in ancient Indian tradition. Majority of individuals from primitive settlements were early risers, which is still evident in some civilizations and cultures like Indians in which awakening at "Brahmamuhurtha" (around 04:30 am) has been considered as ambrosial hours for better cognitive functions. Early morning riser (Brahmamuhurtha) students have been believed to have best concentration in the morning hours. The proposed scientific basis is that body temperature and level of melatonin is lowest in the early hours of morning coincides with increasing level of cortisol, which has direct bearing to process of attention and improved ability to recall (Kumaran et al. 2012). Therefore, man would be in great evolutionary benefit by adopting morning preference lifestyle, as it gives longer span to remain active and awakened to learn, doing creative work and socialize.

Exposure to bright sunlight and brisk exercise in the morning hours advances the circadian phase of the evening chronotype and helps to advance the later sleep onset times and thus the wake times (Fig. 18.3). As a part of chronotherapy, timed bright light therapy to advance the circadian phase or ingestion of melatonin at night can also help in adjusting the problems associated with chronic eveningness. Adopting good habits like reducing or stopping smoking, alcohol, late night eating, screen time,


**Fig. 18.3** Strategies for improving circadian entrainment and healthy aging

refraining from blue light emitters at night and adopting sleep hygiene could also minimize the social jetlag, adverse health consequences and increase the longevity of healthy physical and mental health.

The time-tested therapies for healthy aging professes the principles of harmonious living and being in tune with nature, universal consciousness, environment and individual constitution. Healthy aging would therefore require for the individual to incorporate healthy lifestyle practices and routines that synchronize an individual circadian phase with nature could promote good health and well-being, and encourage healthy transformation of the body and mind.

**Compliance with ethical standards:** Experiments described in the chapter involving humans or animals were conducted by respecting the corresponding ethical guidelines and that informed consent was obtained in case humans were involved.

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# Part VI
# Therapeutic Interventions in Sleep Disorders and Clock Misalignment

# Chapter 19
# Physical Exercise and Circadian Rhythm in Humans

**Yujiro Yamanaka**

## 19.1 Basic Characteristics of the Circadian System in Humans

Circadian rhythms are defined as those showing approximately 24-h fluctuations in physiology and behavior (sleep–wake cycle). In the real world, the plasma melatonin and the sleep–wake cycle circadian rhythms show stable rhythmicity within 24 h, equal to the same period of the environmental light–dark cycle. Subsequently, these 24-h rhythms persist and are free run within 25 h on average under constant conditions in a temporal isolation facility (Wever 1979). Then, an internal oscillator generates free-running rhythm, the central circadian pacemaker in the suprachiasmatic nucleus (SCN) of the brain's hypothalamus (Moore and Eichler 1972). However, since the free-running period is longer than 24 h, the phase-advance shift of the circadian pacemaker is needed to entrain to the 24-h environmental light cycle daily. Therefore, although the periodic exposure to 8-h bright light at ca.3000 lx is considered entrained during the free-running rhythms of the sleep–wake cycle, body temperature is maintained in subjects staying at an isolation facility without any time cues (Honma et al. 1987). Based on the timed exposure to bright light, this entrainment is called photic entrainment. The phase response curve (PRC) to a single pulse of bright light can explain the mechanism of photic entrainment. Furthermore, several PRCs to light have also been demonstrated in previous studies (Honma and Honma 1988; Minors et al. 1991) (Fig. 19.1).

The PRC to bright light has an advanced portion during the early subjective day, subjective late night (early morning), delayed portion during the subjective early night (midnight), and the no-shift portion (dead zone) during the middle of the subjective

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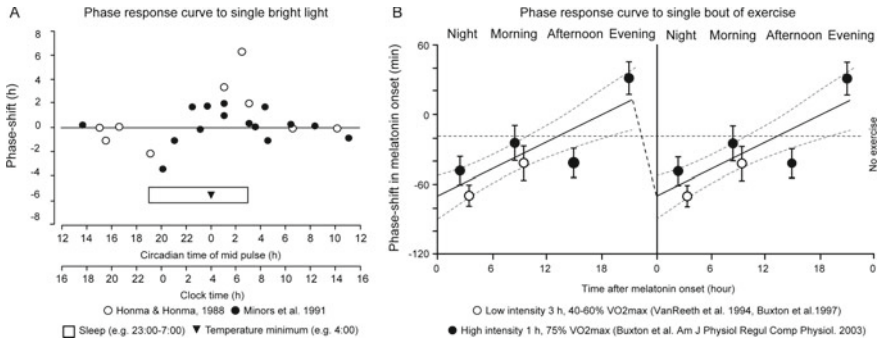


**Fig. 19.1** Phase response curve to single light pulses and single bouts of exercise. **a** Phase response curve to a 3-h single bright light pulse under free-running conditions (modified from Honma and Honma 1988; Minors et al. 1991). **b** Phase response curve to a single bout of 1-h high-intensity exercise or 3-h low-intensity exercise under a dim light condition (modified from Buxton et al. 2003)

day. Thus, our circadian pacemaker could be entrained by receiving natural sunlight in the morning every day in the real world. However, although the bright light is a primary zeitgeber for the circadian pacemaker in mammals, including humans, half of the blind persons who do not receive the light information from the eye show normal entrained rhythm in the real world (Sack et al. 1992). This normal entrained rhythm strongly proposes nonphotic time cues with a period of 24 h in the real world (e.g., regular sleep–wake schedule) act as a potent zeitgeber for the circadian pacemaker in blind persons.

The most unique feature of the human circadian system is the so-called "spontaneous internal desynchronization" between the sleep–wake cycle and circadian rhythms of the core body temperature (Aschoff 1965) and plasma melatonin (Honma et al. 1998). When subjects stay in an isolation facility under free-running conditions, even though the free-running period of the sleep–wake cycle and the circadian rhythm of the core body temperature have the same period for the first days (of approximately 25.0 h), it is occasionally observed that the period of the sleep–wake cycle desynchronizes from that of the circadian rhythm of the core body temperature—a phenomenon known as spontaneous internal desynchronization. Under this form of desynchronization, while the free-running period of the circadian rhythm of the core body temperature remains at approximately 25.0 h, the sleep–wake cycle becomes longer than 30 h or shorter than 20 h (Wever 1979). Therefore, spontaneous internal desynchronization supports the idea that the sleep–wake cycle and the circadian rhythms of core body temperature and plasma melatonin are regulated by two distinct, typically coupled oscillators (Honma et al. 1998). The oscillator site for circadian rhythms of core body temperature and plasma melatonin is located in the SCN of the brain hypothalamus. Although the oscillator site for the sleep–wake cycle is not fully elucidated, some animal studies support the idea that the dopaminergic neurons are candidates for the oscillator for the sleep–wake cycle (Fig. 19.2). Besides, based on the effect of exercise on the human circadian system, it is critical

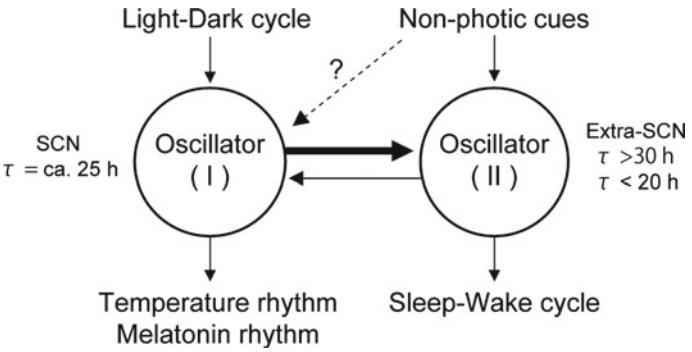


**Fig. 19.2** Two-oscillator human circadian rhythm models. In this model, oscillator I drives the circadian rhythms of body temperature and melatonin. It is located in the SCN, and the light–dark cycle entrains it. However, oscillator II drives the sleep–wake cycle, probably located in extra-SCN brain regions and entrained by nonphotic time cues (modified from Honma et al. 1998)

to evaluate whether exercise directly affects the circadian pacemaker in the SCN, the oscillator for the sleep–wake cycle, or both oscillators.

## 19.2 Effects of Single-Bout Exercise on Circadian Rhythm in Humans

Several previous studies have examined whether a single bout of physical exercise acts on nonphotic zeitgeber to elicit circadian rhythm phase shifts of core body temperature and plasma melatonin under dim light conditions (Van Reeth et al. 1994; Buxton et al. 1997, 2003). Therefore, the first study (Van Reeth et al. 1994) determined whether a single bout of exercise for 3 h could induce rapid phase shifts in circadian rhythms of plasma melatonin and TSH for three days under constant routine conditions. The exercise timing ranged from four hours to five hours around the minimum phase of the core body temperature. Furthermore, although the nocturnal exercise was associated with phase delay of both the melatonin and thyroid stimulating hormone (TSH) rhythms, the extent of delays in the shift was considered smaller when the exercise was presented in the latter part of the nighttime period and the early morning. These results propose the hypothesis that the direction of phase shift by a single bout of exercise can be similar to the shape of the PRC and a single pulse of bright light (Honma and Honma 1988; Minors et al. 1991). Therefore, the PRC to a single bout of exercise is valuable information to adjust (advance and delay shifts) ones' circadian rhythm. The reported PRC for a single bout of 1-h high-intensity exercise (75% $VO_{2\,max}$) was also established from the experiment in an isolation facility for three days under dim light conditions (Buxton et al. 2003) (Fig. 19.1). During the exercise, while the circadian phase of melatonin onset advanced in the evening exercise group by 30 ± 15 (SE) min and delayed in

the nocturnal exercise group by $-25 \pm 14$ min, the morning and afternoon exercise groups failed to produce a significant phase shift. Crossover points between advanced and delayed portions were also observed during melatonin onset. However, it is still a concern that the advanced shifts by evening exercise are attenuated by the next day. Also, the study did not examine whether the delayed shift by nocturnal exercise was still significant the next day. Nevertheless, phase shift by a single bout of exercise is proposed to be due to the so-called masking effect of exercise. Additionally, another group reported opposite results to the exercise PRC. For example, Miyazaki et al. (2001) examined whether a single bout of exercise in the morning, afternoon, and midnight under dim light conditions (<10 lx) elicited a significant phase shift of the circadian melatonin rhythm. However, the number of phase shifts in the exercise groups was not different from that in the no-exercise group. Furthermore, based on the effect of a single bout of exercise on circadian rhythm in older adults, one previous study examined this question by measuring the number of phase shifts in dim light melatonin onset based on nocturnal exercises between young and older adults (Baehr et al. 2003). They observed that the nocturnal exercise delayed the melatonin onset of both young and older adults on average. However, note that although all young subjects delayed the dim light melatonin onset, the direction and magnitude of the shift in older subjects showed a sizable inter-individual difference. Still, the effect of a single bout of exercise under dim light conditions on the human circadian rhythm is being debated on the basis of extant literature. In the real world, most people exercise outdoors or indoors at various times. One literature examined the effect of exercise for 30 min (70% $VO_{2\,max}$) at different times of the day and night throughout a 24-h period (Edwards et al. 2002). The effect on the phase of core temperature rhythm was also assessed by comparing the rhythms immediately before and immediately after the day of exercise with the participants living normally on these 2 days. As a result, while nocturnal exercise between 4 h before and 1 h after the minimum temperature phase was delayed by $1.03 \pm 0.78$ h, morning exercise between 3 and 8 h after the minimum temperature phase advanced by $1.07 \pm 1.23$ h. Nonetheless, the number of phase shifts did not differ from those in sedentary individuals exposed to domestic lighting. The results obtained from the controlled laboratory studies and under a normal living condition also showed that a single bout of physical exercise as the nonphotic zeitgeber is considered a weak synchronizer for the circadian pacemaker than bright light (photic zeitgeber). Moreover, unclear points in the effect of single-bout exercise on the circadian pacemaker in humans still exist (e.g., physiological, humoral, and neuronal signals associated with the exercise-induced phase shift of the circadian rhythm).

Based on the PRC to a single bout of exercise in rodents, exposure to a novel environment with a running wheel and forced treadmill running at various circadian phases under free-running conditions demonstrates the exercise PRC (Reebs and Mrosovsky 1989; Marchant and Mistlberger 1996). Results also showed that the shape of the reported exercise PRC was approximately 180° different from the PRC to light. Based on previous studies using a cultured SCN slice in vivo and in vitro, the circadian rhythm in the SCN showed the same nonphotic shift by applying a neuropeptide-Y (Biello and Mrosovsky 1996), GABA (Tominaga et al. 1994), and

Orexin (Belle et al. 2014). These agents and sites of brain nuclei are the candidates for an exercise-induced phase shift and therapeutic target to adjust circadian rhythms. Hence, although we should note species differences between the diurnal and nocturnal animals, further studies would be needed to demonstrate the steady-state PRC for a single-bout exercise in humans.

## 19.3 Effects of Repeated Exercise on the Circadian Rhythm in Humans

In nocturnal rodents, the free-running period of circadian behavioral rhythms under constant darkness influences the daily wheel-running activity (Yamada et al. 1988). Additionally, timed forced exercises with treadmill running or voluntary wheel-running activity entrain the free-running circadian behavior rhythm (Edgar and Dement 1991; Marchant and Mistlberger 1996; Yamanaka et al. 2013). Nevertheless, daily exercise's entrainability (zeitgeber strength) is considered a weak zeitgeber compared with the photic zeitgeber. However, based on the effect of daily exercises on the circadian rhythms of the SCN circadian pacemaker, daily wheel-running activity strengthens the amplitude of multiunit electrical activity in the SCN (van Oosterhout et al. 2012). Moreover, recent studies using the VIP or VPAC2 knockout mice revealed that regular voluntary wheel running recovered the disruption of circadian rhythms in behavior (Power et al. 2010) and reorganized clock gene expression rhythm in the SCN (Schroeder et al. 2012; Hughes et al. 2021). These findings in nocturnal rodents demonstrate that daily exercise acts as a potent zeitgeber for the circadian pacemaker in the SCN.

Compared to the effect of daily exercise on the circadian rhythms in nocturnal rodents, the effect of daily (repeated) exercise on circadian rhythms in humans is relatively complex due to the unique characteristics of the human circadian system (e.g., the two-oscillator model of human circadian rhythms). The role of daily exercise as the nonphotic zeitgeber for the circadian rhythms in humans can also be partly implicated from the evidence in blind persons (Sack et al. 1992). Interestingly, half of the blind people with no conscious light perception show normal 24-h rhythms in the real world. Alternatively, it has been expected that some nonphotic zeitgeber could entrain their SCN circadian pacemaker within 24 h (e.g., meal, exercise, sleep, etc.). Therefore, to argue the expectation of nonphotic entrainment in blind people, good evidence that circadian rhythm of core body temperature and plasma melatonin in them entrained to a strict 23.8-h sleep–wake schedule in an isolation facility (Klerman et al. 1998). It should also be noted that the protocol includes a single daily bout of 10-min bicycle exercise, six hours after wake up. Similarly, circadian rhythms in normally sighted subjects facilitate entrainment to a 23.6-h sleep–wake cycle schedule with 2-h daily exercises under dim light conditions less than 10 lx (Miyazaki et al. 2001). The sedentary group failed to entrain (phase delay) circadian rhythms to the 23.6-h schedule. Furthermore, these studies using a non-24-h sleep–wake

schedule address the possibility that daily repeated exercise acts as the nonphotic zeitgeber for the SCN circadian pacemaker and strengthens the feedback pathway through the oscillator for the sleep–wake cycle to the circadian pacemaker or both. Several lines of previous studies in isolation facilities have also been conducted to resolve this issue. For example, Yamanaka et al. (2010) examined the effect of a 4-day repeated exercise under dim light conditions less than 10 lx on the re-entrainment circadian rhythm of plasma melatonin and sleep–wake cycle to the 8-h advanced shift of the sleep schedule. In this study, the 8-h sleep period was advanced by eight hours from habitual sleep onset, after which the advanced sleep schedule was continued for four days. Afterward, the sleep schedule was terminated and free ran for six days. During the 4-day progressive sleep schedule, the exercise group subjects performed a 2-h bicycle exercise at 65–75% of HRmax twice a day. Then, the nonexercise group subjects sat on a chair during the exercise session. Under dim light conditions, timed exercise facilitated re-entraining (advancing) of the sleep–wake cycle to the shifted sleep schedule (Fig. 19.3).

In contrast, although circadian plasma melatonin rhythms were delayed in both the exercise and nonexercise groups, the number of phase-delay shifts was smaller in the exercise group. Moreover, note that while the no-exercise group subjects significantly delayed (free ran) circadian rhythm of plasma melatonin during the free-run session, the exercise group subjects did not further delay. Furthermore, Barger et al.

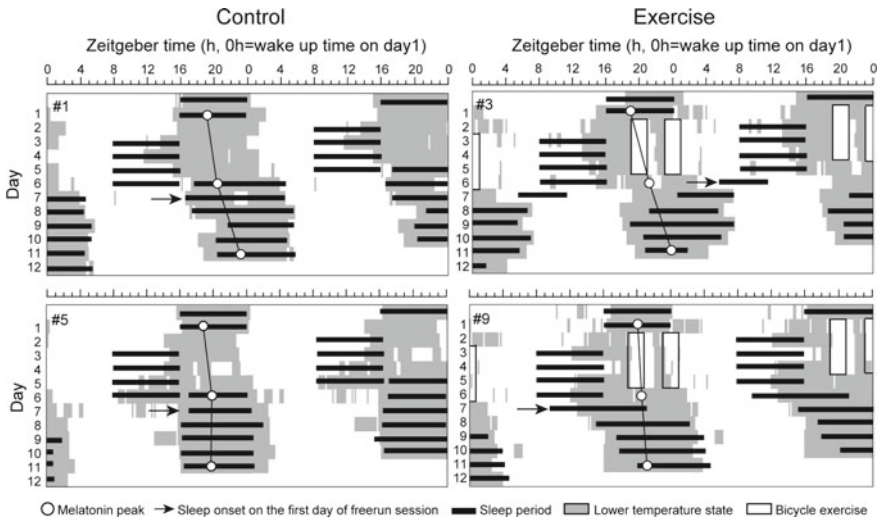


**Fig. 19.3** Physical exercise facilitates the entrainment of the sleep–wake cycle but not the melatonin rhythm under dim light conditions. Black horizontal bars indicate the sleep periods. Open circles indicate the peaks of the plasma melatonin rhythm measured at baseline, on the last day of shift schedule, and the last day of the free-running period, respectively. The rectal temperature rhythm is expressed as a raster plot. The shaded area indicates when the temperature was below the mean value for all values obtained during the experiment. However, open rectangles in the two right panels show the 2-h periods of intermittent exercises (modified from Yamanaka et al. 2010)

(2004) reported that daily physical exercises at night accelerated re-entrainment of the circadian rhythm of the plasma melatonin to a 9-h phase delay of the sleep–wake schedule (Barger et al. 2004). In these two studies, the exercise timing was midnight-early morning, when a single-bout exercise had previously been reported to elicit a phase delay of the circadian rhythm of plasma melatonin (Buxton et al. 1997, 2003). Thus, it was expected that the circadian rhythm of plasma melatonin would be more phase delayed in the exercise group than in the no-exercise groups. Still, the phase-shift results did not support this prediction. The significant difference in these two previous studies was the direction of the sleep–wake cycle shifts (8-h advance vs. 9-h delay). Nevertheless, the findings propose that the direction of the melatonin phase shift produced by exercise was different from that produced by the shift in sleep–wake schedule. Conversely, it was also reported that repeated exercise indirectly affects the circadian pacemaker (the circadian rhythm of plasma melatonin) through the oscillator for the sleep–wake cycle (feedback effect).

As noted above, although daily repeated exercise mainly acts as a nonphotic zeitgeber for the sleep–wake cycle rather than the circadian pacemaker, bright light acts as a primary zeitgeber for circadian rhythms. Therefore, the combined effects of bright light (natural sunlight and artificial light) and nonphotic cues (daily exercise) on circadian rhythms should be considered in the ordinary living world. Klein and Wegman (1974) reported that outdoor activity does not affect the time course of circadian rhythm resynchronization in body temperature and performance during translongitudinal air travel. However, Shiota et al. (1996) demonstrated that outdoor exercise helped to resynchronize to new environments of decreased jet lag on circadian rhythms of 17-hydroxy-corticosteroid (17-OHCS) in urine after an 8-h transmeridian flight (between Tokyo and Los Angeles) in airline crewmembers. Nevertheless, although the above studies focusing on the effect of outdoor exercise on circadian rhythm provided supporting evidence in practical and clinical fields, other time cues, such as artificial light and social contact, should also be considered to influence the circadian rhythm. Furthermore, this study investigated the combined effect of exercise and bright light on the circadian rhythm in strictly controlled conditions. Yamanaka et al. (2014) previously examined whether a 4-day bicycle exercise under bright light (>5000 lx) could accelerate the re-entrainment circadian rhythm of plasma melatonin and sleep–wake cycle to an 8-h advanced shift of the sleep schedule in an isolation facility. In this study, the sleep schedule was advanced by 8 h from habitual bedtime, and the advanced schedule was continued for four days. Afterward, the subjects were released into free-running conditions for six days. The exercise group performed 15 min of exercise/15 min of rest on a bicycle exercise for two hours twice a day. As a result, the exercise group showed an advanced circadian rhythm shift of the plasma melatonin and sleep–wake cycle. Still, although they also showed an advanced sleep–wake cycle shift, the no-exercise group did not show a significant phase shift from baseline values in their circadian rhythm of plasma melatonin (Fig. 19.4).

Internal desynchronization between the sleep–wake cycle and the circadian rhythm of plasma melatonin has been reported in the nonexercise group. Sleep polysomnography was also recorded during the baseline and advanced sleep periods

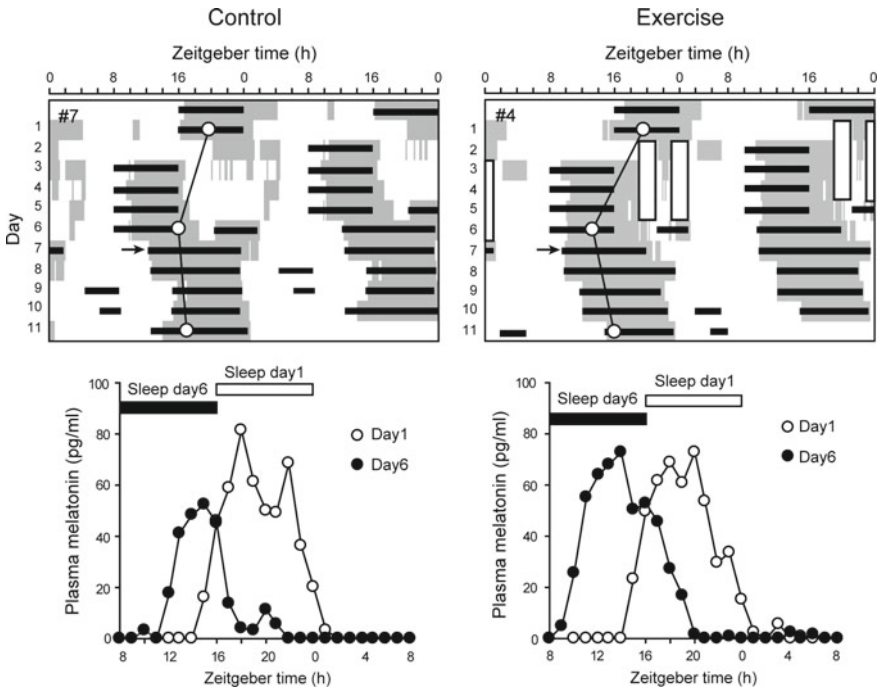


**Fig. 19.4** Physical exercise under bright light accelerates the re-entrainment of human circadian rhythms to an 8-h advance in sleep schedule. Representative recording of the sleep–wake cycle, plasma melatonin rhythm peak time, lower temperature state (upper), and the circadian profile of plasma melatonin measured on days 1 and 6 (lower). All data were taken from the same individual who participated, the nonexercise (left) and the exercise (right) groups (modified from Yamanaka et al. 2014)

in this study. We discovered that although the nonexercise group significantly decreased sleep efficiency with increased wakefulness after sleep onset, the exercise group maintained sleep quality during the advanced sleep. One possible explanation for the more significant phase advance is that exercise under bright light enhances the light perception of the circadian pacemaker, inducing a greater phase advance shift in bright light. Notably, exercise also increases sympathetic nervous activity, which increases pupil size (Ishigaki et al. 1991; Hayashi et al. 2010). Figure 19.5 summarizes the effects of repeated exercise under the two lighting conditions.

However, further studies are needed to assess whether timed repeated exercises under bright light are beneficial for promoting adjustments of the circadian rhythms in humans, especially elderly persons, in normal circumstances.

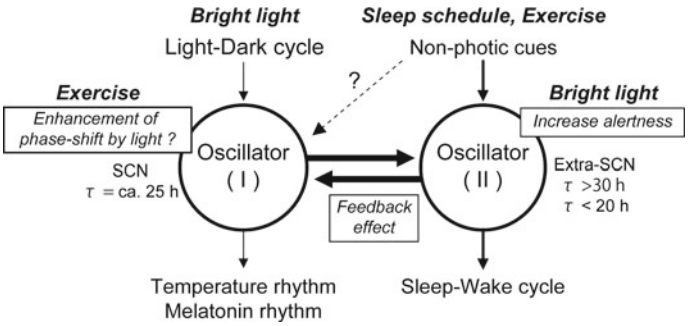


**Fig. 19.5** Two-oscillator models of the human circadian system and underlying mechanisms causing phase adjustment of the two oscillators by photic and daily exercise. Previous studies under dim light conditions (Yamanaka et al. 2010) show that strict sleep schedules and physical exercise serve as the nonphotic zeitgeber for the sleep–wake cycle. They also indirectly affect the circadian pacemaker through the sleep–wake cycle's oscillator. Besides, exercise under bright light conditions (Yamanaka et al. 2014) enhances the phase shift by bright light. Additionally, bright light increases alertness and facilitates the entrainment of the sleep–wake cycle to a social schedule

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# Chapter 20
# Circadian Rhythms and Time-Restricted Eating in Healthy Aging and Longevity


**Payal Bajaj and Gurcharan Kaur**

## 20.1 Introduction

Chrono-nutrition is an emerging area developing to elucidate the link between temporal eating timings/patterns and circadian rhythms and its impact on metabolic health (Queiroz et al. 2021). This concept is based on the idea that, in addition to the quality and quantity of food, meal timing is also important for the individual's well-being due to the complex interaction between nutrition, metabolism, and circadian clock (Asher and Sassone-Corsi 2015; Manoogian et al. 2019). Chrono-nutrition deciphers how the mismatch of food intake (meal size, timings, frequency, and composition) with biological rhythms of our body negatively impacts the body's internal clock system and impairs metabolic health. Alterations in energy metabolism are reported when food consumption is not in sync with the biological clock (Gooley 2016). Along with the consumption of energy-dense processed foods, the temporal patterns of eating over long hours in a day have also been identified as the root cause of deterioration of physical and mental health. Therefore, induction of robust catabolic circadian rhythms by temporal regulation of feeding and fasting may emerge as an innovative interventional strategy for healthy aging and longevity (Froy 2018).

The early onset and growing prevalence of lifestyle disorders are known to be influenced by modern societal pressures and lifestyle such as unhealthy dietary habits, lack of physical activity, excess of screen time, and sleep deprivation. The agricultural revolution, some 10,000 years ago, lead to the constant food availability round the year which is typical of modern societies. One major negative aspect of the modernization of the food industry over the last about 50 years is the inclusion of calorie-dense fast foodstuffs in daily meals. The dynamic changes in the molecular and cellular processes with biological aging cannot be considered as a disease (Rattan

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A. Jagota (ed.), *Sleep and Clocks in Aging and Longevity*, Healthy Ageing and Longevity 18, https://doi.org/10.1007/978-3-031-22468-3_20

2014) which necessitates to lay more focus on health-oriented and disease-preventive strategies in aging research. To promote healthy aging and to reduce the costs on the health care of the elderly necessitates that the increase in life span be accompanied by biological aging attenuation. Energy restriction by limiting caloric intake to 60–70% of the daily consumption and intermittent fasting by restricting meal timings to day hours may extend the health span by preventing or slowing down the onset of aging-associated lifestyle diseases like T2D, hypertension, and cancers.

## 20.2 Circadian Clock and Its Disruption with Aging

The "circadian" ("Circa Diem" or "about a day") cycle coordinates a series of behavioral, metabolic, and biological processes that take place throughout the day so as to anticipate and acclimatize to changes in daily rhythm (Queiroz et al. 2021). The circadian system in mammals consists of a master/central clock which is situated in the hypothalamus's suprachiasmatic nucleus (SCN), and various secondary clocks located in extra-SCN regions of the brain and other organs such as adipose tissue, skeletal muscle, liver, and pancreas (Queiroz et al. 2021; Stenvers et al. 2019). At the molecular level, circadian rhythm consists of a negative feedback loop driven by transcriptional factors brain and muscle ARNT-like 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK) (Chaix et al. 2019). BMAL1 and CLOCK heterodimerize and bind to circadian E-box elements site (CACGTG) present in the promoter region of negative repressors, CRYPTOCHROME (CRY1 and CRY2), and PERIOD (PER1 and PER2) to mediate their transcription. PER and CRY proteins in turn heterodimerize and inhibit BMAL1-CLOCK activity, thereby generating ~24 h rhythm in the transcription of PER and CRY (Chaix et al. 2019; Mattis and Sehgal 2016). SCN entrains and coordinates circadian oscillations in different peripheral organs such as the kidney, lungs, liver, and heart, known as peripheral clocks by sending outputs to different peripheral clocks and mediating the release of secondary messengers, glucocorticoids, nitric oxide, neuropeptides, and neurohormones. This results in an internal synchronization between different metabolic, physiological, and behavioral systems (Dibner and Schibler 2015).

Peripheral clocks are not just entrained by the master biological clock but also by non-photic cues such as different types of diets and dietary patterns, temperature, sound, and humidity (Jagota et al. 2019). Loss of neuronal and synaptic functions with aging alters SCN electrophysiology, reduces circadian amplitude, and lengthens the circadian period in mice (Barnard and Nolan 2008; Slotkin et al. 2005). Non-invasive disruption of circadian rhythmicity was found to reduce the lifespan of hamsters which was enhanced by implantation of neonatal SCN into older animals thus suggesting a link between aging and circadian rhythm (Hurd and Ralph 1998). Serotonin which is a precursor of melatonin is known to play an important role in the regulation of both photic and non-photic circadian rhythms. (Jagota and Kalyani 2010) reported that serotonin levels and rhythmicity were reduced with age but were restored after melatonin administration. Similarly, exogenous melatonin restored

the age-associated decline in antioxidant enzymes activity (Manikonda and Jagota 2012). Aging was also observed to cause desynchronization between clock gene expression and immunity regulating genes in the kidney which was restored by curcumin administration (Thummadi and Jagota 2019).

Molecular clock disruption, throughout the body or in specific metabolic tissue, results in lipid dysregulation and abnormal energy balance. Loss of Bmal1 function in mice has been shown to reduce fat-storing capacity in the adipose tissue thus increasing levels of triglycerides (TGs), cholesterol, and free fatty acids in circulation which resulted in ectopic fat in the skeletal muscle and liver of Bmal1 −/− mice (Shimba et al. 2011). Further, obesity, hyperlipidemia, hyperleptinemia, hyperglycemia, and hepatic steatosis were also observed in homozygous Clock and Bmal1 mutant mice (Marcheva et al. 2010; Turek et al. 2005). Mice lacking Per1 and Per2 function were observed to exhibit impaired glucose tolerance (Lamia et al. 2008). Furthermore, loss of Cry1 and Cry2 function has also been reported to cause glucose intolerance along with high corticosterone levels (Lamia et al. 2011). Together these studies provide evidence for the role of the circadian clock in regulating lipid metabolism and energy homeostasis.

Aging is associated with disrupted circadian rhythm due to multiple factors. Individual SCN neurons were observed to display rhythmic clock gene expression in older animals which were otherwise expressed constitutively in younger animals. Similarly, rhythmic expression of the Clock and Bmal1 gene in extra-SCN sites such as the hippocampus, hypothalamus, and amygdala was observed in young animals which were altered in older animals (Wyse and Coogan 2010). Although the total number of neurons remained unchanged in SCN of older animals, the percentage of neurons containing vasopressin, a coupling factor regulating circadian oscillations within SCN was reduced significantly by 31% (Mieda et al. 2015; Roozendaal et al. 1987). Another coupling factor within SCN, gamma-aminobutyric acid (GABA) was dysregulated suggesting age-related circadian disruption (Nygård and Palomba 2006). In response to GABA, a reduction in inhibitory postsynaptic potential (IPSP) was detected in aged SCN as compared to young SCN neurons. In addition to a decrease in electrical activity, aged neurons were found to have reduced peptidergic function, gastrin-releasing peptide, vasopressin, neurotensin, and vasoactive intestinal peptide (Farajnia et al. 2012). In humans, arginine vasopressin (AVP) neurons in SCN were found to exhibit diurnal oscillation showing peak value during early morning and lowest during the night in young subjects which was disrupted in older people (Hofman and Swaab 2006). Similarly, the number of vasoactive intestinal peptide (VIP) neurons were observed to be highest in the SCN of young healthy subjects (10–40 years), which were reduced by 60% in older subjects (Hofman et al. 1996). Altogether these studies suggest that the circadian clock is disrupted with aging therefore further misalignment of meal timings with circadian rhythms due to long eating hours of energy-dense food may be highly deleterious for maintaining good health in older adults.

## 20.3 Time-Restricted Eating as a Novel Dietary Intervention

Almost all cells of our body have inherent molecular clocks which help to anticipate and sense the predictable recurring changes occurring every day such as rhythmic nutrient availability and accordingly facilitate cellular functions adaptation. On the contrary, nutrient-sensing pathways judge acute imbalances in nutrient and modulate metabolism so that cells are able to optimally adapt to nutrients availability. Chrono-nutrition, which is developing at the interface of circadian rhythms and nutrient-sensing pathways, brings in a new and novel concept of meal timings alignment with biological clocks to maintain metabolic health (Chaix et al. 2019). The concept of time-restricted feeding (TRF) in animals or time-restricted eating (TRE) in humans came from studies investigating the impact of food intake timing on the circadian rhythm. It is a variant of intermittent fasting (IF) regimen which recommends to limit the eating period to 4–12 h while increasing the fasting window to 12–20 h. However, it differs from IF in two aspects: (1) It does not restrict the amount of calories intake during the eating window (2) it requires a consistent maintenance of eating window over time (Adafer et al. 2020). Current data in support of TRF are primarily based on animal experimentation although few recent pilot-scale human studies provide evidence for the possibility of translational benefit of TRE. (Jefcoate et al. 2021) reported their observations of a pilot study and identified some key factors such as cost, time availability, wake time, bedtime, and perceived health benefits (on workdays) which may motivate individuals to undertake time-restricted eating. Similarly, TRE was shown to improve glucose tolerance and also reduce body weight and blood pressure in obese adults and humans at risk of type 2 diabetes (Gabel et al. 2018; Hutchison et al. 2019). 5 weeks of early TRE (eTRE) were observed to improve insulin levels, blood pressure, β-cell responsiveness, oxidative stress, and insulin sensitivity in pre-diabetic men without affecting body weight (Sutton et al. 2018). Further, 11 overweight adults, in a randomized crossover trial, underwent eTRE (8:00–14:00) for 4 days resulting in reduced fasting glucose and insulin resistance in the morning and elevated fasting insulin levels in the evening with respect to control group who ate from 8:00 to 20:00 h. Before breakfast, levels of cholesterol, ketone bodies, and expression of stress gene were elevated in the eTRE group, while in the evening elevated levels of brain-derived growth factors (BDNF) and cell growth regulatory protein, mTOR was observed. Though the intervention lasted only 4 days, eTRE increased fasting levels of high-density lipoprotein (HDL), total cholesterol (TC), and β-hydroxybutyrate and reduced glucose levels in the morning as compared to the control group (Jamshed et al. 2019). Collectively, these studies suggest that limiting the eating window to the early hours of the day so as to align with circadian rhythms may prove beneficial for metabolic health.

A recent study investigated the effects of TRE on subjects suffering from metabolic syndrome. 10 h of self-selected TRE for 12 weeks reduced fat mass, body weight, TC, low-density lipoprotein (LDL), and blood pressure. However, no significant change in the levels of insulin, hemoglobin, glucose, HDL, TG, and sleep quality

was observed (Wilkinson et al. 2020). A similar pilot study found that 3 months of TRE resulted in a significant decrease in waist circumference, body weight and body mass index, waist-to-height ratio, and glycosylated hemoglobin levels in participants with abdominal obesity but no change in the lipid profile (Kesztyüs et al. 2019). Since both these studies lacked control groups, further studies are required to investigate the effects of TRE in people with cardiometabolic disorders. Another pilot study conducted in older people with mobility impairments although reported the loss of weight after 4 weeks of TRE, but there was no positive or negative effect of TRE on cognitive and physical functions, metabolic parameters, and quality of life (Anton et al. 2019). More such human studies are required to evaluate the impact of TRE on the metabolic health of elderly people because this dietary regime reduces the daily consumption of proteins and calories which can also aggravate age-related loss of muscle strength. Different studies investigating the effects of TRE on cardiometabolic health and related outcomes in humans are summarized in Table 20.1.

A recent study compared the efficacy of TRE and intermittent fasting regimen of alternate-day feeding (ADF) in rats and reported that TRE was equally effective in terms of redox homeostasis in rats (Bhoumik et al. 2020). Therefore, keeping in view the ease of compliance with TRE as compared to ADF regimen in our day-to-day life, TRE may be more viable strategy of dietary interventions for maintaining health in older adults. As mealtime is important to synchronize the biological clock to the central clock, so restricting the duration and timing of eating might reduce desynchronization between both and help maintain metabolic functions. Initially, some preclinical studies with TRF were performed by restricting food availability in rodents to a few hours (generally 4–8 h) during the day when rodents are asleep and resting and then analyzing their activity-rest cycle with respect to the new eating regimen (Escobar et al. 1998; Mistlberger 1994). Caloric intake is often reduced when the access to food is restricted to fewer hours. These studies suggested that rodents would get up several hours before the food arrived and begin ambulatory activities as if expecting food. Such meal anticipatory activities were also observed when the calories were limited and offered at night, and the intensity of ambulatory activity increased with calorie restriction (Mitchell et al. 2016). Based on these observations, it was suggested that CR did not reduce physical activity but rather improved food-seeking behavior which is essential for survival (Chaix and Panda 2016). Some landmark studies investigating the effects of TRF in rodents are summarized in Table 20.2.

### *20.3.1 Circadian Rhythms, TRE, and Energy Metabolism*

Recent studies on the integration of circadian rhythms with nutrient-sensing pathways have provided encouraging results and recommend that restricting the meal timings for 8–10 h during the day is beneficial not just for controlling body weight but also helps in glucose regulation, lipid homeostasis, maintaining healthy gut microbiome, cardiovascular functions, anti-inflammatory activity, sound sleep, and overall health

(Panda 2016). Hepatic lipid metabolism is associated with both nutrient-sensing pathways and components of the circadian clock (Asher and Sassone-Corsi 2015; Neufeld-Cohen et al. 2016). AMPK as the master regulator of lipid metabolism senses the decline in energy consumption during fasting and reduces the cellular ratio of adenosine triphosphate (ATP) to adenosine monophosphate (AMP) (Bhoumik et al.

**Table 20.1** Effects of TRE on cardiometabolic health and related outcomes in humans

| Study | Participants | Duration | Intervention | Findings |
|---|---|---|---|---|
| Carlson et al. (2007), Stote et al. (2007) | $n = 15$ (10 women, 5 Men) Age: 40–50 year BMI: 18–25 kg/m$^2$ | Two 8-weeks treatment with 11-weeks washout between two diet periods | TRE: One meal/day (16:00–20:00 h) Control: 3 meals/day | ↓ Body weight, blood pressure, fat mass, glucose tolerance, cortisol ↑ Fasting plasma glucose ↔ Leptin, insulin, and glucagon |
| LeCheminant et al. (2013) | $n = 29$ (men) Age: 18–26 years BMI: 24.4 kg/m$^2$ | 2 weeks | TRE: 13 h (06:00–19:00 h) Control: Ad libitum | ↓ Body weight |
| Gill and Panda (2015) | $n = 8$ (3 women, 5 men) Age: 34–37 years BMI: > 25 kg/m$^2$ | 16 weeks | TRE: 10 h (self-selected) Control: Ad libitum | ↓ Body weight |
| Moro et al. (2016) | $n = 34$ (Men) Age: 29.21 ± 3.8 Weight: 84.6 ± 6.2 kg | 8 weeks | TRE: 8 h (13:00–20:00) Control: 12 h (8:00–20:00) | ↓Fat mass, testosterone, Insulin-like growth factor-1, blood glucose, insulin, leptin, TG, TNF-α, IL-1β ↑ Adiponectin ↔ TC, HDL, LDL |
| Antoni et al. (2018) | $n = 13$ (12 women, 1 men) Age: 45–47 years BMI: 28–30 kg/m$^2$ | 10 weeks | TRE: Breakfast delayed and dinner advanced by 1.5 h Control: Ad libitum | ↓Low body fat, adiposity ↔ Body weight |
| Gabel et al. (2018) | $n = 23$ Age = 25–65 BMI: 30–45 kg/m$^2$ | 12 weeks | TRE: 8 h (10:00–18:00) Control: Ad libitum | ↓ Body weight ↔ Resting metabolic rate |

(continued)

**Table 20.1** (continued)

| Study | Participants | Duration | Intervention | Findings |
|---|---|---|---|---|
| Sutton et al. (2018) | $n = 8$ (Men) Age: 35–70 BMI: 25–50 kg/m$^2$ | 5 weeks | TRE: 6 h (Breakfast: 6:30–8:30 h, lunch and dinner: 10:00–13:00) Control: 12 h | ↓ Blood pressure, oxidative stress, appetite ↑ Insulin sensitivity, β-cell responsiveness ↔ HDL, LDL |
| Anton et al. (2019) | $n = 10$ (6 women, 4 men) Age ≥ 65 BMI: 25–40 kg/m$^2$ | 4 weeks | TRE: 16 h (Self-selected) Control: Ad libitum | ↓ Body weight ↑ Walking speed, cognitive functions |
| Jamshed et al. (2019), Ravussin et al. (2019) | $n = 11$ Age: 20–45 BMI: 25–35 kg/m$^2$ | 4 days | TRE: 6 h (8:00–14:00) Control: 12 h (8:00–20:00) | ↓ Body weight, Alters diurnal pattern of cholesterol, cortisol, ketones, circadian clock genes, BDNF, and SIRT1 |
| McAllister et al. (2020) | $n = 22$ (men) Age: 22 ± 2.5 years BMI: 28.5 ± 8.3 kg/m$^2$ | 28 days | Isocaloric TRE: 8 h (Self-selected) up to 300 kcal Ad libitum TRE: 8 h (No calorie restriction) | ↓ Body fat, blood pressure, ↑ Adiponectin, HDL ↔ plasma insulin, blood glucose |
| Wilkinson et al. (2020) | $n = 19$ (6 women, 13 men) Age ≥ 59 BMI: 33.06 kg/m$^2$ | 12 weeks | TRE: 10 h (Self-selected) Control: 14 h | ↓ Body weight, waist circumference, TC, HDL, LDL, blood pressure ↔ TG, fasting glucose |
| Hutchison et al. (2019) | $n = 15$ (Men) Age ~ 55 years BMI: 33.9 kg/m$^2$ | 1 week | eTRE: 8:00–17:00 dTRE: 12:00–21:00 | ↓ Body weight, fasting TG, glucose tolerance |
| Chow et al. (2020) | $n = 20$ (17 women, 3 men) BMI: 34.1 kg/m$^2$ | 12 weeks | TRE: 8 h (Self-selected) Control: Ad libitum | ↓ Body weight, visceral fat mass, and lean mass |
| Parr et al. (2020) | $n = 11$ (men Age: 30–45 years BMI: 27–35 kg/m$^2$ | 5 days | TRE: 8 h (10:00–18:00) Control: 15 h (7:00–22:00) | ↓ Glucose and insulin ↑TG, NEFA |

(continued)

**Table 20.1** (continued)

| Study | Participants | Duration | Intervention | Findings |
|---|---|---|---|---|
| Zeb et al. (2020) | $n = 80$ | 25 days | TRE: 8 h (19:30–3:30) Control: Ad libitum | ↓ TC, TAG, TNF-α, IL-1β ↑ HDL, BMAL1, CLOCK ↔ LDL |

*n*: number, BMI: Body mass index, TRE: time-restricted eating, TNF-α: tumor necrosis factor alpha, IL-1β: interleukin-1 beta, HDL: high-density lipoprotein, LDL: low-density lipoprotein, TG: triglycerides, TC: total cholesterol, BDNF: brain-derived neurotrophic factor, SIRT1: sirtuin1, eTRE: early TRE, NEFA: non-esterified fatty acids, TAG: triacylglycerides, BMAL1: brain and muscle ARNT-like 1, CLOCK: circadian locomotor output cycles protein kaput, ↓: reduced, ↑: increased, ↔ : no change

2020). AMPK activation tends to promote catabolic pathways by inhibiting triglycerides and cholesterol synthesis on one hand, and simultaneously increasing glucose uptake, energy expenditure, and rates of glycolysis and lipolysis (Waldman et al. 2020). One of the downstream targets of activated AMPK is acetyl-CoA carboxylase (ACC) which catalyzes the rate-limiting step of fatty acid synthesis by converting acetyl-CoA to malonyl-CoA. However, phosphorylated ACC is enzymatically inactive and cannot mediate lipid biosynthesis. TRF has been reported to increase AMP levels and activate AMPK which in turn, phosphorylates ACC, therefore, reducing de novo lipogenesis (Manoogian and Panda 2017). Further, TRF is also associated with the SIRT1 mode of energy regulation. During fasting, AMPK and SIRT3-mediated activation of ketogenesis and fatty acid oxidation in the liver may be a strategy to prepare the brain for reduced energy supply since ketones are the preferred source of energy for brain during fasting (Anton et al. 2018).

Further, TRF is found to increase the expression of circadian clock repressor, Rev-erb which represses genes implicated in lipogenesis (Cho et al. 2012). Increased Rev-erb expression is corroborated with reduced expression of lipid synthesis gene, fatty acid synthase (FAS) (Chen et al. 2019). Liver tissue produces approximately 20% of the total body cholesterol and regulates the breakdown of cholesterol for the synthesis of bile acids and many other sterols (Maxfield and Tabas 2005). Rev-erbα regulates the circadian cycle of hepatic bile acid and cholesterol biosynthesis by modulating rhythmic SREBP-1 and Cyp7a1 expression (Le Martelot et al. 2009). The expression of enzymes regulating committing steps of both classical and acidic pathways of bile acid synthesis, Cyp7a1, and Cyp7b1 was elevated in the liver of TRF mice as compared to ad libitum-fed mice (ALF). Further, expression of a transcription factor regulating cholesterol biosynthesis, SREBP-1, was also found to increase in TRF mice in comparison to ALF mice (Chaix et al. 2014). Change in the expression of Cyp7a1 and Cyp7b1 together with an increase in cholesterol breakdown suggests that TRE paradigm has the potential to counteract obesity and related co-morbid metabolic disorders.

**Table 20.2** Effects of TRF in rodents

| Study | Rodent strain | Duration | Intervention | Findings |
|---|---|---|---|---|
| Salgado-Delgado et al. (2010) | Male Wistar rats (5–6 weeks old) | 4 weeks | FD: food access from ZT0-ZT12<br>FN: food access from ZT12-ZT0<br>AL: ad libitum group | ↓ Body weight, abdominal fat<br>Restored TAG rhythm |
| Jang et al. (2012) | Male C57BL/6N mice (4 weeks old) | 4 weeks | RF: Food access (Normal chow diet + 60% HFD) from ZT0-ZT12<br>PF: Food access (Diet same as RF group) from ZT12-ZT0<br>AL: ad libitum group (Chow feed) | ↔ Body weight<br>Altered expression of BMAL1, PER2, CLOCK, SREBP1c, and FAS |
| Morris et al. (2012) | Male C57BL/6N mice | 6 weeks | FL: 10% fructose during day and water at night<br>FD: 10% fructose at night and water during the day<br>AL: ad libitum group (Normal water) | ↑ Plasma leptin and insulin in FL as compared to FD group<br>↔ cholesterol, TG, adiponectin, and glucose |
| Reznick et al. (2013) | Adult male Wistar rats | 3 weeks | HFD-ad libitum<br>HFD-day<br>Chow-ad libitum<br>Chow-day | Altered expression of genes BMAL1, DBP, TEF, PEPCK, and FAS |
| Gil-Lozano et al. (2014) | Male Wistar rats | 3 weeks | RF for 12 h | ↑ Plasma insulin, GLP-1, and glucose |
| Salgado-Delgado et al. (2013) | Male Wistar rats (4–5 weeks old) | 5 weeks | FRP: food access from ZT0-ZT12<br>FAP: food access from ZT12-ZT0<br>AL: ad libitum group | ↓ Body weight, glucose intolerance<br>Maintained expression of Per1, Per2, Bmal1, and Clock |

FD: food restricted during the day, FN: food restricted food during night, AL: ad libitum, TAG: triacylglycerides, TG: triglycerides, RF: restriction feeding in daytime, PF: pair-feeding in nighttime, BMAL1: brain and muscle ARNT-like 1, PER1: period circadian regulator 1, PER2: period circadian regulator 2, CLOCK: circadian locomotor output cycles protein kaput, SREBP-1: sterol regulatory element-binding protein 1, FAS: fatty acid synthase, FL: fructose light, FD: fructose dark, HFD: high-fat diet, DBP: (albumin D-box) binding protein, TEF: thyrotroph embryonic factor, PEPCK: phosphoenolpyruvate carboxykinase, FRP: food intake in the rest period, FAP: food intake in the active period, ↓: reduced, ↑: increased, ↔ : no change

### *20.3.2 Time-Restricted Eating to Align with Circadian Rhythms for Healthy Aging*

Dietary patterns and their therapeutic implications have taken pivotal place in the field of aging research over the recent years. Moreover, dietary restriction has been identified as a key strategy to maintain and improve mental as well as physical health of older adults (Currenti et al. 2021). Diet is a non-photic cue which is a vital aspect of maintaining good health. Therefore, both the nutrient composition and dietary pattern play important role in regulating metabolism by entraining as well as reinstating peripheral biological clocks. One of the most severe implications of chronic circadian disruption, like the one experienced by shift workers, is a higher risk for cardiovascular diseases (CVDs) (Lunn et al. 2017; Puttonen et al. 2010). In fact, it is the leading cause of mortality and disability among active firefighters at work (Donovan et al. 2009; Soteriades et al. 2011). At the same time, CVDs remain the major cause of death among the general population. Therefore, the circadian regulation of heart health is of utmost importance. Bmal1 knockout mice were found to be more susceptible to atherosclerosis, which was a result of metabolic perturbations (Chaix et al. 2019). Over-expression of dominant-negative $Clock^{\Delta 19}$ mutant protein in mouse cardiomyocytes disrupted cardiac gene expression and function as mutant mice exhibited increased lactate release, fatty acid oxidation, longer R-R interval, and bradycardia (Bray et al. 2008).

A substantial amount of experimental evidence generated from animals and few human studies clearly demonstrates link between circadian rhythms, nutrition, metabolism, and their impact on metabolic health. In a recent review article, Flanagan et al. (2021) compiled the available evidence from literature on chrono-nutrition and its underlying molecular and neuronal mechanisms. The modern lifestyle factors such as 24 h artificial light, shift-work professional demands, screen time, and excessive food availability put the individuals at risk of circadian and metabolic dysregulation. Based on the available literature reports from rodents and human pilot studies, it may be suggested that maintaining a regular temporal eating pattern may be helpful to restore circadian rhythm and reduce disease risk and promote healthy aging. TRE was conceptualized in view of its relevance to circadian rhythms, which influences daily 24 h rhythms of body's physiology, metabolism, and behavior (Xie et al. 2019). Several human pilot studies have highlighted the potential benefits of TRF regimen on metabolic health indicators (Jamshed et al. 2019; Sutton et al. 2018; Tinsley et al. 2017). In a recent study, Jamshed et al. (2019) reported the effects of early TRF, i.e., skipping dinner on 11 overweight adults and just 4 days of early TRF was observed to alter 6 circadian clock genes expression and also upregulated SIRT1 and LC3A expression which play key role in autophagy. These subjects showed improvement in 24 h glucose levels, lipid metabolism, and circadian clock as well as autophagy-related genes expression, and based on these observations, it was suggested that TRF regimen may have anti-aging effects.

Circadian rhythms help to optimize physiological functions to maintain health by temporal coordination between cellular and tissue function and behavior. The

endogenous circadian rhythms are dampened with age and thus compromise this temporal coordination. TRE strategy recommends to follow regular feeding-fasting pattern as an external cue that helps to maintain the robustness of daily biological rhythms (Manoogian and Panda 2017). Erratic eating timings derange the temporal coordination of physiology and metabolism with endogenous biological clock thus increasing risk and early onset of many chronic diseases and accelerate aging. In humans, epidemiological studies have reported that erratic eating behavior increases the risk of chronic lifestyle diseases, whereas consistent daily feeding-fasting cycles and overnight 12–14 h fasting provides protection. Possible interactions between circadian clock components with nutrient-sensing pathways and their health implications have recently been very appropriately elaborated by Chaix et al. (2019). It is suggested that these interactions may serve 3 major functions: (1) When food is ingested at an anticipated time of daily rhythm, then the integrated anticipatory response operated by the circadian clock, and the nutrient-sensing pathway's acute response, both act in a synergistic manner to maintain nutrient homeostasis; (2) On the other hand, when feeding is at an unanticipated time, the nutrient-sensing pathways tend to re-adjust the phase of the clocks in such a way that on the subsequent days; food availability is anticipated at the new feeding re-adjusted time slot; (3) Circadian regulation initially ensures that pathways involved in nutrients assimilation get activated in anticipation of food intake, so that the excess of nutrients can be handled by the organism. The activation of nutrient assimilation pathways lasts for few hours defined as limited time window for optimal metabolism of nutrients, and subsequently, there is misalignment of circadian rhythms and nutrient-sensing pathways. Based on this information about interactions between circadian clocks and nutrient-sensing pathways, it is suggested that TRF or TRE (when referring to humans) regimens to restrict food consumption within a consistent 8–12 h period during the day allow optimal utilization of nutrients and promote overall health (Panda 2016). This novel concept of TRF/TRE and the underlying molecular basis are being researched extensively using appropriate animal models, but currently, there are limited data available from TRE regimen-based human studies.

Several landmark studies using animal models have elucidated the adverse effects of feeding by defying the circadian rhythms. Nocturnal mice fed with a high-fat diet (HFD) during the light phase were found to gain weight more rapidly as compared to the mice fed only during the dark phase (Arble et al. 2009). However, time-restricted HFD fed mice showed improved circadian oscillations, elevated bile acids, low levels of serum cholesterol, and metabolites involved in fatty acid metabolism, implicating that consuming meals at an incorrect circadian time can have a detrimental effect on the metabolic health (Hatori et al. 2012). Consuming meals at odd hours on the postprandial lipemia response were found to increase plasma triacylglycerol (TAG) levels at night (20:00–4:00) as compared to day time (7:00–16:00). These findings suggested that the nocturnal impairment in lipid metabolism is a potential risk factor for cardiovascular diseases (Bonham et al. 2019). Another cross-sectional study found that high intake of energy in the form of fat at night (17:30–20:29) increased plasma levels of total cholesterol (TC) and low-density lipoproteins (LDL) which was reduced significantly by shifting 100 kcal of meal from night to morning, therefore,

indicating that lipid metabolism reduces at the end of biological active phase (Chen et al. 2019).

In the mouse heart, knockout or over-expression of Kruppel-like factor 15, a clock-dependent oscillator, resulted in abnormal repolarization, loss of rhythmic interval of QT, and increased susceptibility to arrhythmogenesis (Jeyaraj et al. 2012). These studies, therefore, suggest that the maintenance of circadian rhythm may reduce the risk of CVDs. The effect of TRF on the cardiometabolic functions and the underlying molecular mechanism by which TRF mitigates cardiac aging dysfunction in rodents have not been explored, but it has been well established in Drosophila (Melkani and Panda 2017). A study by Gill et al. (2015) found out that subjecting 2-week-old wild *Drosophila melanogaster* to TRF for 12 h every day was observed to show improved sleep–wake cycle, deceleration of cardiac aging as compared to Ad libitum-fed flies. Further, in the Drosophila heart, TRF resulted in the downregulation of a group of genes that encode for electron transport chain in mitochondria resulting in a decline in reactive oxygen species and in turn protecting the heart. Additionally, TRF also induced the expression of different subunits of ATP-dependent chaperonin complex, CCT. Upregulation of CCT expression was coupled with reduced expression of cytoskeletal monomers which in turn reduced the fraction of misfolded or unfolded cytoskeletal proteins, thereby, improving cardiac functions (Chaix et al. 2019; Melkani and Panda 2017). Overall. TRF studies in Drosophila indicated that the improvement in proteostasis and changes in mitochondrial functions may underpin the benefits of TRF.

Along with the physical health, mental health is also a major public health concern in the modern age due to its deleterious effects on quality of life. The circadian system is found to affect mood and reward-based circuitry, both of which are fundamental components of mental health (Siemann et al. 2021). Photoperiod exposure is found to promote plasticity and imprint circadian clock perinatally along with long-term impact on dopamine and serotonin systems which are known to regulate mood (Ciarleglio et al. 2011; Siemann et al. 2021). Circadian disruption has been associated with different psychiatric disorders such as depression, bipolar disorder, schizophrenia, anxiety, cognitive performance and increase negative emotions, attention deficit hyperactivity disorder, and autism (Finan et al. 2015; Hou et al. 2020). A 4 days-in-laboratory study conducted on young healthy adults showed that sleep deprivation that impaired circadian rhythmicity impacted cognitive flexibility and dynamic attention (Honn et al. 2019). Delayed sleep/wake and circadian timing was also correlated with poor academic performance in undergraduate students (Phillips et al. 2017). Functional MRI studies in humans have observed that light inhibits amygdala activity and strengthens functional connectivity with the prefrontal cortex (McGlashan et al. 2021), indicating the mood-elevating effect of light. Circadian disruption was also observed in genes such as BMAL1, REV-ERBa, PER-1, 2, 3, and DBP in different brain regions of patients with major depressive disorder (MDD) (Li et al. 2013).

Further, circadian rhythm has been found to play an important role in neurogenesis (Borgs et al. 2009; Tamai et al. 2008), and any disruption in circadian rhythm with age has been associated with neurodegenerative disorders such as Parkinson's

disease (PD) and Alzheimer's disease (AD) (Manoogian and Panda 2017). The amyloid hypothesis of AD suggests that the synthesis and deposition of amyloid β peptide initiates a cascade of events that destroys the neurites and synapses leading to the formation of neurofibrillary tangles comprising tau protein (Hardy and Selkoe 2002). Daily fluctuations in Aβ soluble interstitial fluid which were observed before aggregation were diminished after Aβ aggregation. Further, Aβ aggregation also affected molecules such as melatonin, orexin, and associated brain regions (Fronczek et al. 2012; Wu and Swaab 2007). Exposing AD patients to bright light has been observed to restore some behavioral modifications such as dementia and poor sleep (Ancoli-Israel et al. 2003; van Someren et al. 1996). Although many studies have reported the beneficial effects of TRE in non-neuronal tissues, but very few studies have been reported with mental health. Two recent studies have reported that TRF improved motor coordination, heart rate variability, activity/rest rhythm, and autonomic nervous system function in mouse models of Huntington's disease (Wang et al. 2018; Whittaker et al. 2018). A recent cross-sectional study on elderly Italian adults reported the association between time-restricted eating and their mental health and observed that individuals >70 years old following 8 h feeding time window were less prone to mental health distress (Currenti et al. 2021). These preliminary findings raise the hope that TRE could help delay the onset or reduce the severity of various neurodegenerative and psychiatric disorders in older adults.

## 20.4 Conclusion

Although dietary patterns and active lifestyle are very well known to influence metabolic status and disease trajectory of aging populations, but emerging data suggest that the beneficial effects of aligning meal timings with circadian clock may be much more effective to promote healthy aging. Figure 20.1 depicts the possible beneficial effects of TRE on metabolic indicators. Circadian rhythms constitute an integral part of our physiology and therefore essential for the maintenance of good health. Modern lifestyle comprising shift works, unhealthy, and untimely eating habits, aberrant sleeping patterns, jet lag, and inappropriate light exposure are associated with disrupted circadian rhythms which has made people more prone to different lifestyle diseases. Moreover, circadian rhythms naturally dampen with age, which further exacerbates the risk of age-related lifestyle diseases. Although more research is required to determine the optimal eating window and effectiveness of time-restricted eating in humans, but the available evidence from animal and human interventional studies indicates that chrono-nutrition may prove to be a novel approach to curb human epidemiology of obesity and co-morbidities and improve aging population's health.

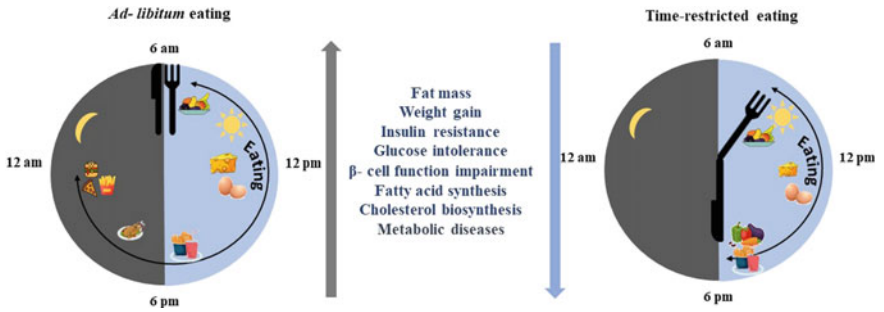


**Fig. 20.1** Impact of TRE on metabolic indicators

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# Chapter 21
# Achieving Healthy Aging in the Light-Polluted World


**Krystyna Skwarło-Sońta**

## 21.1 Introduction

Human civilization has developed under natural lighting conditions generated by Earth rotations. Beyond doubt, the consequences of this fact are described in the previous chapters dedicated to the endogenous circadian clock(s). Now, it is time to recall that our master clock must be synchronized day by day with the environment by the most potent *Zeitgeber,* i.e., by light. Circadian organization of human physiology and behavior is mainly related to the length of day (photoperiod), and only occasionally we are interested in the intensity of light, both during the day and at night. Most probably, only, few people are aware of the intensity of natural light, which, during a clear day can reach (outdoor) even 120,000 lx while at clear full moon, the maximal value of light does not exceed 0.3 lx, decreasing to near 0.001 lx on a moonless clear night (Grubisic et al. 2019). On the other hand, people living in cities are usually exposed in their offices/houses to the typical indoor lighting near 200 lx (seldom up to 500 lx), and only, occasionally, a citizen can see a bright blue sky with > 100,000 lx at midday (Wright et al. 2013). It means that most citizens in the developed countries (2/3 of EU population) live their everyday life under conditions where the days are not bright enough while the nights are much brighter than at full moon (Navara and Nelson 2007). Reduced exposure to sunlight during the

**Outline of content**

Sources and dangers of the contemporary ubiquity of artificial light at night are described. As light available in an unsuitable time and place acts as a potent circadian disruptor, it can have an impact in several civilization-related illness, often diagnosed in developed countries. Examples of light pollution related to the modern lifestyle are presented, as well as proposed measures that can mitigate its adverse effects, especially for elderly people.

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day, along with electrical city light, creates the environmental time cues not powerful enough to perfectly synchronize our circadian clock (Wright et al. 2013). Moreover, electrical lighting is present at times and places where the natural light does not normally occur and increases at 6% per year (Dominoni and Nelson 2018). This anthropogenic factor will be defined in subsequent sections as light pollution, and the consequences of this phenomenon will be discussed in the context of threats to human health and well-being with particular attention to aging individuals.

## 21.2 Light Pollution—What Does It Mean?

From a technical point of view, light pollution means an excessive and inappropriate use of light sources usually related to city life and resulting in any adverse effects. As global urbanization increases continuously, light pollution accompanying our everyday life in the developed countries is increasing too. Moreover, it has become a global problem. Light radiated from improperly installed city luminaires is directed toward the sky, and it is scattered in the atmosphere, creating a sky glow having a detrimental effect during dark nights, especially on night sky visibility. Other aspects of light pollution encompass glare, light trespass, light clutter, and a decreased visibility at night (Elsahragty and Kim 2015). The presence of an excessive number of luminaires, often being improperly managed, may extend the light to the areas where it is neither intended to be used nor needed, and for urban lighting professionals, it creates a relatively new problem, which should be resolved urgently at the interdisciplinary level (Perez Vega et al. 2022).

In other words, when artificial light is present at a wrong time, wrong place, and in excessive quantity, it creates **light pollution**. There are numerous definitions of this phenomenon, depending on the scientific interest of respective authors, pointing, on the other hand, to the complexity of this problem. Therefore, it seems worthwhile to present selected quotes from some recent papers, just to draw the readers' attention to the complex aspect of light sources and the effects of light pollution:

- **Astronomical light pollution**, i.e., degradation of human views of the night sky (where stars and other celestial bodies are washed out by light, either directed or reflected upwards) vs **ecological light pollution**, i.e., artificial light that alters the natural patterns of light and dark in ecosystems (Longcore and Rich 2004);
- **Interruptions in normal circadian light cycles and the resulting disruption of normal melatonin rhythms cause widespread disruptive effects involving multiple body systems** (Navara and Nelson 2007);
- **The circadian oscillator system and melatonin levels are both affected by perturbing light signals at night** (Hardeland 2014).
- **On an evolutionary scale** (…) **light pollution presents a novel stressor**, and it is unclear how organisms that evolved in stable cycles of light and darkness are affected by such changes (Grubisic et al. 2019);

- (Melatonin) can counter the **debilitating effects of modern lifestyle**: insufficient circadian time cues, especially natural bright light, and **exposure to artificial light at unsuitable times—the 24-h society** (Arendt 2019).

From above quotes seems to be particularly important to emphasize relative novelty of light pollution in the evolutionary dimension (Grubisic et al. 2019) what could be a new problem for the next generations as well. We should also be aware of the danger resulting from the omnipresence of electricity and ease of using it. However, it is worth noticing that excessive/disturbing lighting should be very easily switched out, needing neither any special effort nor additional costs, and virtually depends only on us and on our awareness of the problem.

Another aspect of the presence of light at unsuitable time is related with the modernity of our life, expressed by the activity 24/7/365. It means that people are active—professionally or just for fun—all round the 24 h, without stops for nights, weekends, holidays, etc. ("24 society," mentioned by Josephine Arendt, 2019). It is true in the case of shift work, work online, long distance travels and additionally—last but not least—unlimited usage of electronic devices: TV sets, computers, video games, smartphones, and e-books. In any circumstances, this means using artificial light at night (e.g., during night shift work) or the screens usually emitting the blue light, which is the strongest disruptor of the circadian system, mainly by the inhibition of melatonin synthesis (Hardeland 2014). Obviously, this light does not come from the external sources (considered to be classic light pollution), but for our circadian system, it makes no difference. What matters is the effect: light present at the unsuitable time always acts as environmental pollutant.

## 21.3 Effects of Light Pollution on Human Circadian Organization

As our circadian organization has evolved under the conditions set by the regular sequence of day (light) and night (darkness), it is obvious that the presence of additional light at the unsuitable time should certainly be profoundly detrimental to human physiology and behavior. When the light, a main *Zeitgeber* for human master clock, comes on additionally as artificial light at night (ALAN), it can exert two effects which are not mutually exclusive. The first is responsible for desynchronization of the circadian clock while the second one evokes a direct inhibitory action on melatonin synthesis. These effects will be briefly discussed in the following sections.

### *21.3.1 Desynchronization of the Circadian Rhythm by ALAN*

Desynchronization of the rhythm (circadian misalignment) describes a disorder of the clock and circadian system (Touitou and Point 2020). It results from the lack of

compatibility between the environmental light–dark cycle and the rhythms of such physiological processes as the sleep–wake cycle, i.e., external desynchrony (Skwarło-Sońta and Zużewicz 2021). If the situation continues, there is a disruption of the temporal organization of the body functions, i.e., internal desynchrony, resulting in negative health outcomes including sleep disorders, depression, metabolic syndrome and diabetes, obesity, and other civilization-related illnesses (Wyse et al. 2011).

In a healthy person, the diurnal rhythm of sleep propensity, body temperature, and melatonin synthesis are reciprocally synchronized in a way allowing to fall asleep easily. Namely, when the body temperature reaches its nadir being in antiphase with a peak of melatonin blood level (i.e., its maximum synthesis in the pineal gland), the metabolism level is low, and all these factors together are promoting falling asleep (Wichniak et al. 2017a). However, additional presence of light at night, i.e., at the dark part of the 24-h period, influences the diurnal rhythm by moving the phase of the rhythm differently, depending on the time of exposure (Lack and Wright 2007), what has been named phase-response curve (PRC). The presence of light pulses before the nadir of the body temperature (the first part of night period) delays the phase, while the light applied in the second part of night evokes an advance of the circadian cycle (Wichniak et al. 2017b). These light effects exerted on the circadian rhythms occur independently of the inhibition of melatonin synthesis. Moreover, the light effects on the magnitude of phase shift are dependent on the dose and time expressed by the number of nights with the light exposure and duration of each exposition (Lack and Wright 2007), while every light intensity can shift the phase and stop melatonin synthesis (Touitou and Point 2020). In everyday life, these desynchronizing conditions take place during the transmeridian travels or the night shiftwork and, unfortunately, also when the electronic devices are used with excessive frequency and/or in wrong time.

Unintentional penetration of the streetlights to the areas where they are not needed, quite recently taken into consideration by the architectural lighting designers (Perez Vega et al. 2022), has been already found to exert an adverse effect on human metabolism (McFadden et al. 2014). A cohort study conducted over several years and including more than 100,000 women in an age range from teenagers to centenarians revealed a very significant positive correlation between the obesity and the intensity of ALAN present in the bedroom. Even if some of the participants used the light at night because they wanted to, and some others were involuntarily exposed to the external lighting—it always indicates that the awareness of the harmful effects of light pollution is low among the population. However, the experiments conducted on laboratory rodents clearly demonstrated a stimulatory effect of ALAN on their weight gain along with the weakness of clock-controlled gene expression rhythmicity in the liver and the adipose tissue (Fonken et al. 2013) indicating an adverse effect of light pollution on metabolism regulation controlled by the circadian clock.

### *21.3.2 ALAN Affects Pineal Gland Function*

Considering that the previous few chapters are devoted to the pineal gland and melatonin, it is worth emphasizing here only an elevated level of nocturnal melatonin synthesis noted in all vertebrate species (Falcón et al. 2009), including humans (Pääkkönen et al. 2006), nocturnal rodents (Illnerova et al. 2000), and the diurnal species like birds (Piesiewicz et al. 2010). Thus, melatonin is a message of darkness and not of the rest or sleep period (Illnerova et al. 2000; Arendt 2019). Moreover, the magnitude and duration of the elevated melatonin synthesis depends upon the length of dark phase (Collin et al. 1989); therefore, melatonin is considered as "clock and calendar" for the entrainment of other biological activities (Reiter 1993). It will also be useful to remind that pineal melatonin synthesis in humans is strictly age dependent: in newborns, the diurnal rhythm of blood melatonin level establishes within the first three months, and thereafter, nocturnal peak increases until the end of childhood. After reaching maturity, it starts to decline and decreases continuously in the adult and advanced age (Stehle et al. 2011). Therefore, in the elderly, the diurnal rhythm of melatonin synthesis is not very well expressed leading to the desynchrony of the circadian organization of the body functions including the timing of sleep, meals, and other activities.

To become a transducer of the information about the external lighting conditions, the pineal gland must receive this message from eyes. This function is performed by the special non-vision-related photoreceptors located in the retina, i.e., the melanopsin containing intrinsically photosensitive retinal ganglion cells (ipRGCs), which convey information on light directly to the master clock (SCN) via the retinohypothalamic tract (RHT) (Berson et al. 2002). It is worth recalling here that ipRGCs are particularly sensitive to the short-wave (blue) light (440–480 nm) that exerts an inhibitory effect on the pineal melatonin synthesis. As this wavelength corresponds to the color of the clear morning sky, it seems essential to pinpoint the meaning of this sensitivity from the evolutionary point of view: nocturnal melatonin synthesis should be turned off because the day just starts, and a subject has to undertake a different type of activity. Thus, the nocturnal rest/sleep must be stopped, and metabolism should be directed at the pathway of energy production and expenditure (Stevens and Zhu 2015). However, when this blue light appears at an inappropriate time, i.e., other than early morning and particularly in the evening or night hours, it also inhibits melatonin synthesis being therefore an important component of circadian desynchrony.

In mammals, melatonin synthesis remains under the stimulatory control of the adrenergic innervation from postganglionic sympathetic fibers, active during the dark phase of the diurnal cycle. The neurotransmitter noradrenaline, released in darkness from these adrenergic endings, binds to the β- and $\alpha_1$-adrenergic receptors present on pinealocytes and activates molecular events leading to the increased melatonin biosynthesis (Zawilska et al. 2009). The knowledge of this regulation is particularly important for health of elderly people who frequently use medications acting as the β-adrenergic receptor blockers, additionally decreasing the nocturnal melatonin synthesis already weakened in the advanced age. It is essential to be aware that these

medications should not be taken in the evening so as not to counteract the adrenergic stimulation of nocturnal melatonin synthesis in the pineal gland.

### *21.3.3 Misalignment of the Circadian System by Light Pollution*

One of the best-known effects of circadian misalignment, very often due to the presence of ALAN, is the sleep–wake desynchrony. Again, it could result both from the streetlights penetrating to the houses, from the night shift work or even due to the use of electronic devices like e-books, TV sets, computers, and/or smartphones in the bedroom.

As has already been mentioned, for the circadian regulation, there is no matter where the light comes from—when the blue light is emitted by a computer screen while we are working at night, it also stops melatonin synthesis in our pineal gland. This finding has been confirmed in a study involving two groups of volunteers, using computers at night (from midnight to 1 am and from 1 to 2 a.m.) and submitted to the evaluation of melatonin levels in their saliva. When the volunteers sat in front of the blue light-emitting computer screen, melatonin concentration in their saliva was reduced during the 1st and the 2nd hour by approx. 40 and 70%, respectively. In the saliva of persons using the screens without the blue light emission, inhibition of melatonin synthesis was much weaker, indicating that for our circadian system pollution with the blue light is particularly dangerous (Wood et al. 2012). It seems unnecessary to remind the readers that very often we generate this light pollution ourselves, as people's awareness about this new pollutant is still very poor.

An important question arises how to avoid this new type of light pollution when the evening/nocturnal use of computers has become a daily habit of most of the population. Obviously, no one is willing to stop using computers, smartphones, and/or other electronic devices or even to minimize the exposure by spending less time using them, especially in the evening. Therefore, other user-friendly issues have to be proposed. The first, and probably the easiest issue, is to follow the recommendation of American Academy of Pediatrics that bedrooms must be "screen-free zones for children" https://www.healthychildren.org/English/family-life/Media/Pages/How-to-Make-a-Family-Media-Use-Plan.aspx), which should be extended to the adults as well. Another interesting option is using a screen filter reducing the effect of blue light or the blue light filtering glasses.

It is important to mention that blue light also influences the alertness of our brain, evoking a strong stimulation of brain structures which are responsible for the interactions between alertness and cognitive functions (Vandewalle et al. 2007, 2010). This is the reason why we do not feel drowsy when using electronic devices in the evening. When the effect of spending several hours reading a light-emitting (LE) e-book before going to sleep was compared with that exerted by reading a printed book, a profound and adverse effect of LE e-book on falling asleep was

noted, expressed especially by melatonin secretion (delayed DLMO[1] and decreased nocturnal peak of blood melatonin level), combined with a reduced next-morning alertness (Chang et al. 2015). These results strongly support the notion that reading paper books in evenings is much healthier, especially for people in advanced age.

Another aspect of light pollution results from the modern lifestyle related with the unlimited access to artificial light. It relies on the unrestricted timing of work (including night shift work), transmeridian travels including crossing time zones (very often several ones), various social activities, etc., all of them being related with irregular timing of sleep. Sleeping in an inadequate part of the 24-h period (e.g., during the day after the night shift) results in an impaired sleep quality or too short sleep length as well as poor level of alertness and performance at night (Arendt 2010). Additionally, very often it is related with the so-called "social jet lag," resulting from desynchrony between the individual clock and the social clock, and consisting in the difference between sleep on workdays and on days off, leading to circadian misalignment (Roenneberg et al. 2019). The workers most often affected by this type of circadian desynchrony include nurses (more generally—medical staff), flight crew, and workers of large industrial plants, personnel in intercity and international transport, security services and many others, all of them being the active participants of the modern 24/7 society. This lifestyle modifies not only the timing and quality of sleep, but also changes the hours of mealtimes, limits physical and social activity, and affects extra-occupational life, leading to measurable health consequences (Skwarło-Sońta and Zużewicz 2021). Epidemiological studies, mainly carried out on nurses, have revealed an association between sustained night work and a significantly higher incidence of breast cancer, and led the International Agency for Research on Cancer (IARC) to classification shift work into A2 group of "probable carcinogens to humans" since "they involve a circadian disorganization" (IARC 2010; Costa 2010; Touitou et al. 2017). Unfortunately, the surveys conducted in 15 European countries have demonstrated that the number of workers who do not work in shift system or at night, or on weekends, constitute only 24% of working population (IARC 2010). Therefore, it is not surprising that the incidence of the so-called "civilization-related illnesses" (including cancer, depression, obesity, type 2 diabetes, metabolic syndrome) increases progressively.

Extension of the working hours beyond the standard timing has currently become a common practice and is performed both by women and men in different age groups. On the other hand, the International Labor Organization (ILO 1990) recommends the upper age limit for shift workers not exceeding 50 and 55 years for women and men, respectively. Moreover, it is suggested that the number of years spent as a night shift worker beyond more than 10 years should not be extended as it makes a cognitive function of a person doing that job like a 6.5 year older (Rouch et al. 2005).

[1] DLMO – Dim Light Melatonin Onset – timing of the evening start of melatonin synthesis.

## 21.4 Light Pollution and Aging

### *21.4.1 Examples of Studies Involving Humans*

It is experimentally well proven that aging of an individual is related with a weakening of the circadian rhythmicity generated by the central pacemaker and peripheral oscillators. This seems to result from both the desynchronization of spontaneous activity of the clock neurons across the 24-h period and a progressive yellowing and thickening of the lens reducing sensitivity to light, what makes master clock less exposed to this most important *Zietgeber* (Hood and Amir 2017). All these changes lead to many age-related downstream functional modifications, such as a decreased amplitude of circadian rhythms of the core body temperature and hormone secretion (including those of melatonin, adrenal steroids, or growth hormone), altered phase relationships between wake-sleep cycle resulting frequent insomnia and daytime napping, metabolic alterations frequently resulting in negative health outcomes. The above brief reminder is a starting point for considering how the omnipresent light pollution further complicates the functioning of elderly people. The different sources of light pollution and the observed effects are summarized in Table 21.1 and briefly presented below. Among the available published data, the sample studies were selected to present various experimental approaches allowing to better understand the above-mentioned complex problems. Usually, the test of light pollution comprises a study of the cohort either submitted to the experimental exposure to ALAN of known intensity and duration (Duffy et al. 2007; Kripke et al. 2007; Obayashi et al. 2014), or the groups of people living in special environmental conditions (Park et al. 2019; Benedito-Silva et al. 2020), or finally—night shift workers (Suwazono et al. 2011). Various parameters were measured, and different effects were noted as well (see Table 21.1).

The curve of sensitivity to the light of increasing intensity presented in the paper of Duffy et al. (2007) was compared with that previously obtained in young subjects, and it indicated that the healthy older subjects were less responsive to low-to-moderate levels of light (50–1000 lx). Taking into consideration age-related changes in the pupil dynamics and lens opacity, effective retinal light exposure in older subject could be quite different from that noted in young adult ones, and the observed differences most probably do not result from an age-related reduction in the sensitivity to circadian light exposure (Duffy et al. 2007).

In other experimental approach (Kripke et al. 2007), additional light of 3000 lx imposed at various time over a 24-h period resulted in a maximal phase shift of about 3 h, not differing in amplitude among older and young women and men, while inflection from delays to advances were earlier 1.8 h among older participants as compared with the young ones. However, at baseline, in older adults, a significant phase advance in sleep, cortisol, and aMT6s onset was observed. An interesting observation is a dead zone of approx. 6 h duration surrounding the core temperature acrophase, when any light exposure at intensity employed would have minimal or any phase-shifting effect during much of the daytime (from approx. 1:40 pm until

**Table 21.1** Effects of ALAN: examples of circadian and metabolic alterations in humans at advanced age

| Age (y)/gender | Number of participants | ALAN intensity/duration | Parameter—effect | References |
|---|---|---|---|---|
| 68.3 ± 3.7 | 2 women<br>8 men | 1.35 to 8000 lx 6.5 h stimulus | – plasma MEL ↓<br>– MEL and CBT circadian phase delayed | Duffy et al. (2007) |
| 18–30 versus 59–75, both sexes | 50 young versus 56 old | 3,000 lx/3 h/3 days | – amplitude and sleep quality =<br>– aMT6s onset and cortisol acrophase advanced | Kripke et al. (2007) |
| av. 37 ± 10 at entry, male shift workers | 4,328 day versus 2,926 shift workers | 14 years | – excessive BMI value—alternating shift work as an independent risk factor for weight gain | Suwazono et al. (2008) |
| av. 72.5, both sexes | 145 ALAN versus 383 darker | ≥ 5 lx during in-bed period | – nighttime blood pressure ↑<br>– urinary aMT6s excretion = | Obayashi et al. (2014) |
| 55.4 ± 8.9 women | 43,722 | ALAN exposure while sleeping, over 6 years | – BMI ↑ by 10% or more, incident overweight and obesity | Park et al. (2019) |
| 50–70 both sexes | 52 non MetS<br>51 MetS | Approx. 3000 lx at day,<br>Approx., 300 lx at night | – motor activity and sleep characteristics =<br>– MetS significant in ALAN exposed | Benedito-Silva et al. (2020) |

Explanations: ↓ parameter decreased; ↑ parameter increased; = parameter unchanged; MEL—blood melatonin content; CBT—core body temperature; aMT6s—sulphatoxymelatonin; PRC—phase-response curve; MetS—metabolic syndrome

7:40 pm for a person getting up at 7 a.m.). This effect was neither related with age nor gender (Kripke et al. 2007).

Circadian misalignment between internal and environmental rhythms dysregulates blood pressure (BP) variability resulting in an increased nighttime BP, and this situation is frequently related with night shift work. Elderly persons exposed to ALAN ≥ 5 lx on two consecutive nights showed significantly higher nighttime systolic BP independently of overnight urinary melatonin excretion. As the increase in nighttime systolic BP is associated with an increase in total mortality, it corresponds to the excessive deaths in Japanese elderly population (Obayashi et al. 2014).

It seems important to emphasize that these measurements were performed on the subjects staying at home, and should constitute a serious warning to the public.

Literature review (17 studies) on shift work revealed a 40% increase in the risk of morbidity and mortality from cardiovascular disease observed in shift workers (Bøggild and Knutsson 1999). Most probably it is a multifactorial effect, but most studies have focused on the behavior and life style of shift workers and neglected other possible factors—this interpretation comes from 1999. Some years later, this unhealthy life style was also considered as a main cause of the weight gain in longitudinal (14 years) study in male Japanese shift workers (Suwazono et al. 2008).

Among a cohort of 43,722 women (mean age 55.4 ± 8.9 years), ALAN exposure while sleeping was positively associated with a higher prevalence of obesity. Compared with no ALAN, sleeping with a TV or a various intensity of light on in the room was related with an increase in BMI by 10% or more, incident overweight and obesity, not associated with a sleep duration and quality (Park et al. 2019). It should be worthwhile to elucidate this association and clarify whether lowering exposure to ALAN while sleeping can promote prevention of the obesity. These observations corroborate previously described association between the light at bedroom and obesity of the women in the larger age range, without, however, evaluation of the duration of the exposure (McFadden et al. 2014).

The last study presented in Table 21.1 seems to be the most interesting one because it concerns the natural (light polluted?) environmental conditions in which the surveyed people live and work permanently. Several parameters of the metabolic syndrome (MetS) were elevated in old persons exposed to the lower diurnal and higher light intensity; however, significant negative correlations were found only between the number of MetS components and diurnal light exposure. Moreover, increased daytime light exposure and a larger normalized difference between diurnal and nocturnal light exposure were significantly correlated with a reduced MetS risk. However, the direct association between light exposure and MetS does not appear to be attributed to disruption in circadian rhythm expressed by activity and sleep parameters (Benedito-Silva et al. 2020). These results agree with several previously published data, but the novelty is that the decreased diurnal light exposure correlated with the development of MetS. As a possible explanation of this effect, the authors take into consideration the effect of lighting conditions present in the participants' everyday life on vitamin D synthesis and melatonin level (supposedly, melatonin will be measured as a continuation of the study).

Indeed, another published study (Schmitt et al. 2018) describes a relationship between MetS and the serum vitamin D [25(OH)] level in the 463 postmenopausal women aged 45—75 years (therefore at comparable age), meeting at least 3 criteria of MetS. Serum Vit D was evaluated as sufficient in 32% of the examined women, insufficient in 32.6% and deficient in 35.4%. MetS was detected in 57.8% women with hypovitaminosis (insufficient and deficient) and in 39.8% of those with sufficient levels of Vit D. It has been concluded that Vit D deficiency in postmenopausal women creates a high risk of MetS and dysregulated lipid metabolism. This finding has not been observed in the participants with adequate levels of Vit D (Schmitt et al. 2018).

Anyway, MetS diagnosis was associated with exposure to less bright days and lighter nights without any difference in circadian parameters. It raises a question whether a small lifestyle modification, i.e., more light during the day and less at night should be effective in reducing MetS prevalence. Moreover—as these results come from the semi-rural environment in Brazil, it should be reasonable to suppose that such effects might be more pronounced in the big cities and might be more undesirable with increasing global urbanization (Benedito-Silva et al. 2020).

### *21.4.2 Examples of Model Studies on Animals*

As far as the experiments related with the effect of light pollution on the animal models are concerned, they are usually short lasting, while the human health issues are rather related with the long-lasting exposure to inappropriate presence of light. Therefore, only, two examples of this type of research will be presented below.

The short report of Davidson et al. 2006, presents the results of a study carried out on young (8–12-month-old) and old (27–31 month) male mice submitted to the standard LD conditions and to the changed lighting schedule (advanced or delayed by 6 h) once every 7 days. These rotating schedules were designed to mimic the changes to time zones while traveling or working in the shift cycles. It appeared that aged mice were significantly sensitive to the changes in light schedule, and after 8 weeks of light schedule rotations, only, 47% of animals survived the advanced cycle while 68% were delayed cycle survivors, and 83% of the sample were unshifted survivors. None of young mice subjected to rotating light schedule died. This phenomenon was not stress mediated as the fecal corticosterone level did not increase in aged mice. It is suggested that in such conditions either the immune system is perturbed, or, rather, that the internal desynchrony among functional oscillators may have an impact on the health consequences exacerbated in the advanced age (Davidson et al. 2006).

Another, rather fascinating recent experimental approach (Delorme et al. 2022), was performed for 1 year on mice kept in various lighting conditions. The light intensity of control lighting was 200 lx (*L*:*D* 12:12), while light pollution was caused by the low level of light, i.e., 20 lx during the dark phase of circadian cycle (dLAN). The idea was to imitate the circadian disruption period corresponding to the years of human shift work or an exposure to environmental ALAN lasting frequently for many years. Several circadian activities, motor functions, and the clinical chemistry were evaluated, also after some weeks of re-adaptation to the control LD conditions. Brain histochemistry was performed as well. The obtained results suggest that the long-lasting presence of dLAN conditions did not evoke any molecular or cellular changes within SCN, but it modified the anatomy of the dendrites in other brain regions. This creates a possibility that the observed changes in some aspects of the circadian function are resulting from the modified neuronal coordination outside of the clock. Moreover, long-lasting chronic, mild circadian disruption caused by exposure to dLAN can be corrected after a subsequent re-acclimatization to the standard LD conditions. However, some changes are still observed, especially those in the

dendritic spines. This previously unknown long-term impact of circadian disrupting dLAN exposure may influence the process of healthy aging (Delorme et al. 2022) and encourages more research to elucidate similar effects in humans.

## 21.5 Summary and Conclusion

To summarize the presented examples of the adverse effects of light pollution on human health and well-being, two levels of the problem should be pointed out.

1—limitation of the external light pollution resulting from the excessive urban illumination depends on the lighting professionals' awareness of possible measures to be applied in order to protect city nightscape simultaneously with that of naturally dark skies and ecosystems, and 2—limitation of the misalignment of circadian organization of human physiology and behavior by raising everyday users' awareness of the effect of electronic devices on the dangers resulting from using them late in the evening, just before going to sleep.

The first level needs reciprocal understanding and exchange of the knowledge and experience between the light designer professionals and ecologists (human physiologists should be included as well); this issue has been analyzed in depth in the recently published paper (Perez Vega et al. 2022). Moreover, we also should apply the appropriate protective measures, and to make it easier, Table 21.2 has been constructed.

**Table 21.2** Recommended measures to mitigate the adverse effects of light pollution on human health and well-being

| Number | Recommendation |
|---|---|
| 1 | Take care while maintaining high levels of the diurnal light (*D*) and low level of the nocturnal (*N*) light—the ratio of *D*/*N* is essential for the synchronization of the circadian clock |
| 2 | Control the level of vitamin D—its supplementation is recommended, especially for the elderly people living in the nursing houses or hospitalized, only occasionally exposed to the natural light during the day |
| 3 | Avoid unnecessary lighting of the garden, house walls and front door—in preference use the motion sensors to turn on the light when needed |
| 4 | Avoid the entrance of the external lighting to your bedroom—use dark curtains or window shutters |
| 5 | Do not sleep in the lit bedroom—for kids who fear the darkness use bedside lamps with weak orange or red light |

(continued)

**Table 21.2** (continued)

| Number | Recommendation |
|---|---|
| 6 | Do not use electronic devices in the bedroom (including TV sets, PC, smartphones, LE e-books) |
| 7 | Do not use electronic devices later than 1 h before going to sleep |
| 8 | If it is necessary to use a computer in the evening/at night, equip your PC with the screen filter eliminating the blue light or use the blue light blocking glasses |
| 9 | Finish your shift work after a few years of a such employment (if possible—do not work longer than 10 years) |

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# Chapter 22
# Disruptions of Circadian Rhythms and Sleep/Wake Cycles in Neurologic Disorders


**William H. Walker II, Jennifer A. Liu, and Randy J. Nelson**

## 22.1 Introduction

Circadian rhythms are internal cycles generated by biological clocks with a period of approximately 24 h; these clocks regulate physiological and behavioral processes of virtually all organisms. Biological clocks function to (1) coordinate individuals' interactions with the external environment (i.e., an internal temporal reference allows animals to avoid predation or find mates) and (2) synchronize individuals' internal physiological and biochemical processes (i.e., sleep and rhythmic hormone secretion in anticipation of food intake). Circadian rhythms are entrained to precisely 24 h by daily exposure to light during the solar day. Of the many circadian rhythms displayed by individuals, the sleep–wake cycle is perhaps the most salient of these temporal rhythms. Many studies have reported a relationship among disrupted circadian rhythms, especially sleep, and neurological disorders (e.g., Musiek 2015; Malhotra 2018; Fifel and Videnovic 2021). Importantly, the extent to which disrupted sleep is the primary outcome (or cause) of neurological disorders or whether disrupted circadian clock function is the primary outcome (or cause) of neurodegenerative disorders also remains unspecified (Colwell 2021). This chapter will review the relationship between disrupted circadian rhythms/sleep and neurological disorders.

Circadian clocks throughout the body (1) coordinate individuals' interactions with the external environment (e.g., internal temporal frame of reference allows animals to avoid predation or engage in mating) and (2) synchronize individuals' internal physiological and biochemical processes (e.g., sleep and hormone secretion in anticipation of food intake). The suprachiasmatic nuclei (SCN) of the hypothalamus is the master circadian clock in mammals (Zee et al. 2013) and functions at the top of a hierarchy of independent self-sustaining oscillators throughout the body. Light

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is the most potent entraining agent for circadian rhythms. In mammals, the SCN receives photic information from the retina via the retinohypothalamic tract, which projects from activated intrinsically photosensitive retinal ganglion cells (ipRGCs). Melanopsin is the photopigment in the ipRGCs; melanopsin is maximally sensitive to short wavelength (~460–480 nm; blue) light (reviewed in Blume et al. 2019).

In response to light activation of the SCN, the transcription factor cAMP response element binding protein (CREB) is activated which in turn binds and modulates transcription of the core clock genes *Per1* and *Per2* (Ashton et al. 2022). A signaling cascade induces the transcription of the core clock proteins including circadian locomotor outputs kaput (CLOCK), cryptochrome (CRY), period (PER), and brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) which are expressed in a rhythmic pattern over 24 h, driven by a transcriptional translational feedback loop (TTFL) (Takahashi 2017; Cox et al. 2019). Briefly, CLOCK and BMAL1 form heterodimers in the nucleus that promote expression of *period* (*Per1*, *Per2*, and *Per3*) and *cryptochrome* (*Cry1* and *Cry2*) via E-box enhancers (Takahashi 2017). PER and CRY proteins accumulate in the cytoplasm throughout the day. These proteins form a complex with a kinase, and after a certain threshold, these complexes translocate back into the nucleus to associate with CLOCK and BMAL1 and repress their own transcription (Takahashi 2017). One cycle of this process takes ~24 h to complete. In addition to the primary feedback loop, other regulatory loops influence the circadian clockwork (Cox et al. 2019). A number of extrinsic and intrinsic factors, associated with neurological disorders, can disrupt typical circadian rhythms.

## 22.2 Alzheimer's Disease

The World Health Organization estimates that worldwide over 55 million people live with dementia. The most common form of dementia is Alzheimer's Disease (AD) which accounts for ~60–70% of cases. AD is characterized by two hallmark pathologies: extracellular amyloid plaque deposition and intracellular neurofibrillary tangles of hyperphosphorylated tau (Weller and Budson 2018). Amyloid-β (Aβ) plaque depositions can be detected up to 15 years prior to symptom onset and initiate in the precuneus, medial orbitofrontal, and posterior cingulate cortices before spreading throughout the brain affecting regions such as the hippocampus, amygdala, and diencephalon (Bateman et al. 2012; Palmqvist et al. 2017). Notably, altered levels of Aβ in cerebrospinal fluid can be detected prior to abnormal amyloid-β within the brain in preclinical AD patients (Palmqvist et al. 2017). Abnormal Aβ plaque deposits induce phosphorylation of microtubule-associated tau protein, which leads to polymerization into insoluble neurofibrillary tangles (Tiwari et al. 2019). Consequently, this precipitates neuronal death, brain atrophy, and cognitive decline.

AD patients commonly display disruptions to circadian rhythms and experience altered sleep/wake states (Milán-Tomás and Shapiro 2018). Not surprisingly, studies demonstrate that the relationships among AD, circadian rhythm disruptions,

and altered sleep/wake states are bidirectional (Phan and Malkani 2019; Wang and Holtzman 2019). Approximately 30–60% of AD patients exhibit altered sleep/wake states (Bianchetti et al. 1995; Tractenberg et al. 2003; Guarnieri et al. 2012), which are manifested as increased sleep fragmentation, excessive daytime sleepiness, and presence of sundowning (increased confusion, anxiety, and agitation at dusk) (Phan and Malkani 2019; Wang and Holtzman 2019). Sleep architecture is also altered. Specifically, AD patients displayed decreased non-rapid eye movement (NREM) slow-wave sleep, alterations in K complexes and sleep spindles, decreased rapid eye movement (REM) sleep, and decreased total sleep time (Peter-Derex et al. 2015). Changes in sleep can proceed a diagnosis of AD by years (MacEdo et al. 2017). Studies demonstrate a clear association between altered sleep and AD pathology/onset. Indeed, increased sleep fragmentation is a risk factor for developing AD (Lim et al. 2013; Hahn et al. 2014). A recent meta-analysis concluded that sleep problems or disorders (defined by International Classification of Sleep Disorders version 2) were associated with an increased risk of preclinical AD (RR: 3.78, 95% CI: 2.27–6.30) and AD diagnoses (RR: 1.55, 95% CI: 1.25–1.93) (Bubu et al. 2017). Furthermore, a second meta-analysis concluded that subjects who reported sleep disturbances had a 1.49-fold higher risk of developing AD relative to subjects without sleep disturbances (Shi et al. 2018). In a cognitively normal population of older adults, Spira and colleagues (Spira et al. 2013) demonstrated that self-reported shorter sleep duration was associated with greater cortical and precuneus Aβ burden and reports of lower sleep quality were associated with greater precuneus Aβ burden measured via positron emission tomography (PET) imaging. In a subsequent study of cognitively normal older adults, Spira and colleagues (Spira et al. 2018) demonstrated that excessive daytime sleepiness was associated with more than 2.5 times the odds of Aβ deposition at follow-up 15.7 years later. A similar association between poor sleep and elevated total tau and phosphorylated tau within the CSF of cognitively normal subjects has been reported (Sprecher et al. 2017).

Notably, sleep deprivation significantly alters Aβ burden within the CNS. Aβ levels within the CNS display time-of-day oscillations in humans and rodents (Kang et al. 2009; Huang et al. 2012; Kress et al. 2018). Aβ peaks during the active phase in rodents and in human CSF, peaks 6 h after the zenith of wakefulness (Kang et al. 2009; Huang et al. 2012; Kress et al. 2018). The authors suggest that the delayed 6 h peak in humans is likely due to the lag from the time of labeling to the time of detection of labeled Aβ in the CSF (Huang et al. 2012). In a study of healthy middle-aged men, Ooms et al. (2014) replicated the time-of-day oscillations in Aβ concentrations within the CSF, demonstrating that Aβ concentrations are reduced in the morning following unrestricted sleep. However, sleep deprivation prevents this decrease (Ooms et al. 2014; Lucey et al. 2018). Similar increases in Aβ burden within the brain have been reported in human studies following sleep deprivation (Shokri-Kojori et al. 2018). Foundational science has further demonstrated the detrimental effects of sleep deprivation on Aβ accumulation (Kang et al. 2009; Qiu et al. 2016). Indeed, Kang et al. (2009) demonstrated that both acute sleep deprivation and chronic sleep deprivation significantly increase Aβ burden within the interstitial fluid and Aβ plaque deposition within the brain, respectively. Notably, administration

of αCRF9–41 (an antagonist of CRF receptors) did not reduce the increase of Aβ within the brain, demonstrating that the effect of sleep deprivation on Aβ is likely corticosterone independent (Kang et al. 2009). The effects of sleep deprivation are not specific to Aβ. Sleep deprivation increases unphosphorylated tau and alters site-specific phosphorylation in human CSF and alters tau phosphorylation with the brain in rodents (Di Meco et al. 2014; Qiu et al. 2016; Barthélemy et al. 2020).

The mechanisms underlying the relationship between sleep and Aβ are multifold (Lucey and Bateman 2014; Minakawa et al. 2019; Phan and Malkani 2019). First, the daily oscillations in Aβ concentrations within the brain are hypothesized to occur due to time-of-day alterations in neuronal activity and glymphatic clearance (Cirrito et al. 2005; Brody et al. 2008; Xie et al. 2013). Specifically, neural activity, which is highest during wakefulness, increases synaptic release of Aβ into the interstitial fluid (Cirrito et al. 2005; Brody et al. 2008). However, glymphatic clearance reduces Aβ burden within the brain during sleep (Xie et al. 2013). The net change of the two results in increased Aβ during wakefulness and reduced Aβ during sleep. Due to the decreased sleep time in AD patients, it is not surprising that Aβ accumulates at high rates. Additional mechanisms by which sleep distribution may promote and propagate AD include blood–brain barrier breakdown and neuroinflammation (Erickson and Banks 2013; Minakawa et al. 2019; Pak et al. 2020).

The disruptions to circadian rhythms in AD patients are not specific to sleep. Indeed, AD patients also display damped and altered melatonin rhythms, phase delays in body temperature rhythms, and altered activity rhythms (Harper et al. 2001; Hatfield et al. 2004; Wu and Swaab 2005; Weissová et al. 2016; Duncan 2020). In addition, studies have demonstrated differences in the phase of clock gene rhythms and phase relationships between genes and brain regions of AD patients relative to controls (Cermakian et al. 2011). Similar alterations in clock gene expression have been observed in rodent models of AD (Duncan et al. 2012; Song et al. 2015; Furtado et al. 2020). Notably, studies have demonstrated Aβ-induced degradation of BMAL1 and CBP, which may underlie the disrupted circadian rhythms in AD patients (Song et al. 2015). Circadian rhythm disruption may also enhance the risk of developing AD. Indeed, in a population of healthy community-dwelling older women phase delayed and decreased activity rhythms increased the odds of developing dementia (Tranah et al. 2011). Additionally, some studies have reported associations between single-nucleotide polymorphisms in BMAL and CLOCK and an increased risk of developing AD (Chen et al. 2013, 2015). Furthermore, rodent studies demonstrate that loss of central circadian rhythms accelerates amyloid plaque accumulation and disrupts daily interstitial fluid Aβ oscillations (Kress et al. 2018). Similar to the mechanisms underlying the relationship between sleep and AD, the mechanisms underlying alterations in circadian rhythms and AD are likely multiple. AD patients demonstrate neuronal loss within the suprachiasmatic nucleus (Swaab et al. 1985; Ferini-Strambi et al. 2020). In addition, Aβ deposits have been observed within the retina and melanopsin containing retinal ganglion cells, or ipRGCs, which is particularly important for circadian rhythms given the role of ipRGCs in regulating SCN entrainment (Koronyo-Hamaoui et al. 2011). Furthermore, post-mortem studies have

demonstrated reduced number of ipRGCs within the retina and depletion of axons within the optic tract (Hinton et al. 1986; La Morgia et al. 2016).

## 22.3 Parkinson's Disease

Parkinson's Disease (PD) is a neurodegenerative disorder first characterized by James Parkinson as "shaking palsy" over 200 years ago (Parkinson 2002). In the most recent global survey of neurological diseases, in 2016, it was estimated that 6.1 million people are living with PD (Feigin et al. 2019). However, this number today is likely much higher as updated numbers from the Parkinson's Disease Foundation estimated that more than 10 million people currently live with PD. Notably, PD incidence rates have risen dramatically over the past two decades for reasons not currently fully understood (Bloem et al. 2021). PD is characterized by hallmark pathology: intracellular α-synuclein aggregation into Lewy bodies or Lewy neurites, and loss of dopaminergic neurons of the substantia nigra (Mantovani et al. 2018). Several other dysfunctional processes have been described in PD, including mitochondrial dysfunction, altered protein clearance, and neuroinflammation (Kouli et al. 2018). Clinical PD patients present with bradykinesia, tremor, rigidity, and as the disease progresses postural instability (Kouli et al. 2018).

Altered sleep is one of the primary nonmotor deficits experienced by PD patients and was recognized in James Parkinson's seminal paper in 1817 (Parkinson 2002; Stefani and Högl 2019). Sleep disorders effect upwards of 60% of PD patients (Barone et al. 2009). Frequently, altered sleep is reported during the prodromal phase of PD prior to the onset of motor and cognitive dysfunction (Iranzo 2013; Tekriwal et al. 2017). PD patients commonly experience insomnia, excessive daytime sleepiness, sleep-related movement disorders (i.e., restless leg syndrome), and parasomnias (i.e., REM sleep behavior disorder (RBD)) (Stefani and Högl 2019). RBD is characterized as loss of muscle atonia during REM sleep and dream enactment (Fleetham and Fleming 2014). Notably, over 70% of patients presenting RBD will eventually develop α-synuclein neurodegenerative disease (i.e., PD, Multiple System Atrophy, or Dementia with Lewy Bodies) (Roguski et al. 2020). Less common parasomnias experienced by PD patients include NREM parasomnias (i.e., sleepwalking, confusional arousals, sleep terrors) and parasomnia overlap disorder (clinical features of both NREM parasomnias and RBD) (Fleetham and Fleming 2014; Stefani and Högl 2019). Insomnia, defined as difficulty falling asleep or maintaining sleep, early awakening or non-restorative sleep, is the most common sleep disturbance reported in PD patients and has been associated with disease duration, higher depression rating scale scores (Montgomery and Aasberg and Becks' Depression Inventory), female sex, fatigue, and age (Gjerstad et al. 2007; Chung et al. 2013). However, excessive daytime sleepiness in PD patients has been associated with male sex, disease duration, and anti-Parkinsonian medications (Mantovani et al. 2018).

Sleep architecture is also altered in PD patients (Zhang et al. 2020; Zahed et al. 2021). Consistency between studies, primarily studies examining NREM sleep, is

less than desired. Studies have demonstrated increased NREM sleep during stage N1, or the lightest stage of sleep, in PD patients ((Yong et al. 2011; Zhang et al. 2020); for a detailed review of NREM sleep see (Léger et al. 2018)). Increased N1 has been associated with the initiation of dopaminergic medications in PD patients (Brunner et al. 2002). N2 has been reported to be unaffected by PD (Brunner et al. 2002; Yong et al. 2011). However, a recent meta-analysis has concluded that the N2 stage of NREM sleep is moderately reduced (Zhang et al. 2020). Sleep spindles and K complexes, characteristics of the N2 stage of NREM sleep, are also altered in PD patients (Zahed et al. 2021). Studies have primarily reported reduced number of sleep spindles and K complexes (Emser et al. 1988; Comella et al. 1993; Latreille et al. 2015). Sleep spindles are further altered in patients with dementia. Indeed, Latreille et al. (2015) demonstrate that PD patients with dementia displayed reduced sleep spindles relative to PD patients without dementia and controls. The final stage of NREM sleep, N3, is reduced in PD patients (Zhang et al. 2020). The depletion in N3 is likely associated with disease progression as the reduction of N3 advances with disease duration (Diederich et al. 2005). Given the previously described RBD in PD patients, it is not surprising that REM sleep is reduced (Zhang et al. 2020). Indeed, studies demonstrate reduced total percentage of REM sleep, REM sleep time, and density of REM sleep (Diederich et al. 2005; Zhang et al. 2020). Similar to N3, reduced REM sleep is associated with disease duration (Diederich et al. 2005). In sum, PD patients demonstrated reduced total sleep time, reduced NREM sleep (note not all stages of NREM), and reduced REM sleep.

The effect of sleep dysfunction on PD is an area of ongoing research. Broadly, sleep dysfunction is thought to be detrimental to disease progression. Indeed, reduction in SWS is inversely correlated with motor dysfunction in PD (Zahed et al. 2021). Poor sleep efficiency and greater sleep fragmentation are associated with enhanced motor gait dysfunction (O'Dowd et al. 2017). In mouse models of PD, sleep deprivation increased α-synuclein deposition within the brain (Morawska et al. 2021). However, increasing SWS reduces α-synuclein burden (Morawska et al. 2021). Furthermore, there has been a reported "sleep benefit" defined as a period of lessened disability in PD patients (Currie et al. 1997). The underlying mechanism linking sleep dysfunction and PD progression is still being investigated. However, similar to AD, hypotheses include impaired glymphatic clearance and as a consequence increased α-synuclein burden within the brain due to reduced sleep in PD patients (Xie et al. 2013; Bishir et al. 2020; Zahed et al. 2021). Currently, mouse models support this idea (Morawska et al. 2021).

The effects of PD are not specific to sleep as PD patients also display alterations to other circadian processes. Notably, the clinical presentation of PD is influenced by circadian rhythms. Despite dopaminergic medications, symptoms of PD demonstrate time-of-day differences with patients displaying worsening of motor symptoms in the afternoon and evening (Bonuccelli et al. 2000; Van Wamelen et al. 2021). PD patients display altered melatonin secretion, disrupted activity rhythms, and altered

peripheral clock gene expression. Indeed, in a study of 20 PD patients and 15 aged-matched controls, PD patients exhibited blunted circulating melatonin rhythms relative to controls (Videnovic et al. 2014). Furthermore, PD patients reporting excessive daytime sleepiness had significantly lower amplitude melatonin rhythms relative to PD patients without excessive daytime sleepiness (Videnovic et al. 2014). Studies have also reported a phase advance in melatonin secretion (Fertl et al. 1991; Bordet et al. 2003). PD patients display reduced gray matter within the hypothalamus which is significantly correlated with melatonin concentrations. Additionally, melatonin levels were significantly associated with disease severity in PD patients (i.e., melatonin levels negatively correlated with disease severity) (Breen et al. 2016). Dopaminergic treatment of PD can also significantly affect melatonin levels. Bolitho et al. (2014) reported significantly enhanced secretion of melatonin in the medicated PD group relative to the unmedicated PD group. Furthermore, dopaminergic therapy delayed sleep onset relative to melatonin onset, suggesting possible uncoupling of circadian and sleep regulation (Bolitho et al. 2014). Patients demonstrate altered activity rhythms (Whitehead et al. 2008; Niwa et al. 2011). Specifically, PD patients have reduced activity during the day and higher activity at night (Whitehead et al. 2008; Niwa et al. 2011). Circadian rest-activity rhythms can predict cognitive dysfunction in PD patients independent of sleep (Wu et al. 2018). Altered circadian rest-activity rhythms are associated with worsening of the disease (Niwa et al. 2011). Clock genes alterations have been reported in PD patients, and single-nucleotide polymorphisms in clock genes have been associated with increased risk of developing PD (Gu et al. 2015; Lou et al. 2017). Cai et al. (2010) reported reduced BMAL1 expression in total leukocytes of PD patients. The expression of BMAL1 correlated positively with PD severity (Cai et al. 2010). Notably, treatment with melatonin increases BMAL1 expression in peripheral blood of PD patients (Delgado-Lara et al. 2020). BMAL2 is also significantly reduced in total leukocytes of PD patients (Ding et al. 2011). Similar alterations in clock gene expression have been reported in mouse models of PD (Shkodina et al. 2022). The mechanisms underlying alterations in circadian rhythms and PD are uncertain. However, a common hypothesis to explain these changes is altered SCN output (Willison et al. 2013). There is evidence to support this hypothesis as mice overexpressing α-synuclein display normal *Per2* oscillation in the SCN, but have damped electrical output from the SCN (Kudo et al. 2011a). Notably, the damped electrical output from the SCN is already present at the onset of motor symptoms (Willison et al. 2013).

## 22.4 Huntington's Disease

Huntington's Disease (HD) is a progressive autosomal-dominant neurodegenerative disease that affects approximately 10 people per 100,000 (Barnat et al. 2020; Crowell et al. 2021). HD is identified by CAG trinucleotide repeat expansion in huntingtin gene (HTT), resulting in a mutant huntingtin protein (Tabrizi et al. 2019). The disease

presents typically in the 3rd to 5th decade of life (Bates et al. 2015). HD is characterized by movement dysfunction, cognitive deficits, and psychiatric symptoms (Wilton and Stevens 2020). Clinically, patients display abnormal eye movement, involuntary muscle movement, and rigidity or dystonia (Jamwal and Kumar 2018). The hallmark pathology which underlies HD is the loss of GABAergic medium spiny neurons in striatum nuclei of basal ganglia (Jamwal and Kumar 2018).

Relative to AD and PD, the effects of HD on circadian rhythms and sleep/wake states are understudied. However, it is clear that HD patients suffer from altered sleep/wake states (Zhang et al. 2019). It is estimated that as much as 90% of HD patients display altered sleep/wake states (Fifel and Videnovic 2021). Patients can display sleep abnormalities during the premanifest stage of HD (Lazar et al. 2015). Additionally, there is evidence that sleep disorders progress as HD proceeds (Hansotia et al. 1985; Arnulf et al. 2008). Sleep abnormalities do not correlate with CAG repeat length (Arnulf et al. 2008). Similar to PD, the effects of HD on sleep are not homogenous in the literature (Herzog-Krzywoszanska and Krzywoszanski, 2019). Studies in HD patients report insomnia, frequent nocturnal awakenings, increased latency to sleep onset, reduced total sleep time, altered REM sleep, decreased sleep efficiency, and excessive daytime sleepiness (Wiegand et al. 1991; Arnulf et al. 2008; Videnovic et al. 2009; Moser et al. 2017). Similar to PD, RBD has been reported in HD patients (Videnovic et al. 2009). In a recent meta-analysis of polysomnography studies in HD patients, Zhang et al. (2019) reported significantly reduced sleep efficiency, increased wake time after sleep onset, and no significant change in total sleep time. Sleep macrostructure is also altered; HD patients displayed an increase in the N1 stage of NREM, no change in N2, and significantly reduced amount of slow-wave sleep (Zhang et al. 2019). Patients also demonstrated significantly reduced REM sleep and increased latency to REM sleep (Zhang et al. 2019). In addition, increased sleep spindle density has been observed in HD patients (Wiegand et al. 1991). Poor sleep is associated with depression, duration of illness, and severity of clinical symptoms in HD patients (Wiegand et al. 1991; Videnovic et al. 2009). Further, patients with sleep disturbances have significantly poorer neuropsychiatric outcomes and accelerated thalamic degeneration (Baker et al. 2016). The precise mechanisms underlying altered sleep in HD are unknown. However, it is likely due to the accumulation of the mutant form of huntingtin and neuronal loss in sleep regions. Indeed, cell death has been reported in the locus coeruleus and the hypothalamus (Zweig et al. 1992; Petersén and Gabery 2012).

Patients with HD display global circadian desynchrony. Indeed, HD patients have reduced melatonin rhythms, delayed onset of melatonin rise, increased cortisol rhythms, altered activity rhythms, and altered blood pressure rhythms (Aziz et al. 2009a, b; Goodman et al. 2011; Kalliolia et al. 2014; Bellosta Diago et al. 2017). Studies have demonstrated an association between altered melatonin and cortisol rhythms and motor and functional impairment (Aziz et al. 2009a, b). Studies examining alterations in clock genes in HD patients are not existent and represent an area of needed research. However, foundational science has demonstrated significant alterations to clock genes in mouse models of HD. Indeed, the R6/2 mouse model of Huntington's exhibits altered expression of BMAL1, PER1, and PER2 within the

SCN and CRY1, DBP, and PER2 in the liver (Morton et al. 2005; Pallier et al. 2007; Maywood et al. 2010). Activity rhythms worsen in this mouse model as the disease progresses leading eventually to arrhythmia. In the BACHD mouse model of HD, no change was seen in PER2 expression within the SCN. However, reduced electrical output from the SCN was reported (Kudo et al. 2011b). The mechanism underlying altered circadian rhythms is still inconclusive. However, changes in SCN structure likely play a role. Indeed, HD patients demonstrate significant neuronal loss within the hypothalamus (Petersén and Gabery 2012), and these effects can be detectable before clinical diagnosis (Soneson et al. 2010). In addition, significant D2 receptor loss and microglia activation within the hypothalamus have been reported (Politis et al. 2008). Two major regulatory neuropeptides of the SCN, vasoactive intestinal polypeptide (VIP) and arginine vasopressin (AVP), are significantly reduced, demonstrating an SCN-specific change in HD patients (Van Wamelen et al. 2013; Ono et al. 2021). These effects are further supported by foundational science. Indeed, decreased expression of VIP and the VIP receptor (VPAC2) was also observed in the R6/2 mouse model of HD (Fahrenkrug et al. 2007).

## 22.5 Stroke

Stroke is a disease that impacts blood flow to arteries in the brain resulting in reductions or interruptions of brain tissue receiving oxygen and essential nutrients leading to neuronal injury and death. Worldwide, this disease affects over 12 million individuals annually and situates itself as the second leading cause of death, and third leading cause of combined disability and death (Feigin et al. 2019). Stroke can be further characterized into two types: ischemic (accounts for 87% of all strokes) which develops due to alterations in blood circulation that result from lack of blood flow from the formation of an embolism in the vessel, or hemorrhagic, defined as the rupturing of vessels and bleeding into the surrounding brain (Donnan et al. 2008). Strokes are characterized by a sudden onset or display of neurological symptoms, including confusion, dysarthria, disrupted coordination or loss of balance, and numbness or weakness of the limbs and/or face. Hypoxia initiates the ischemic cascade, which consists of first reductions of ATP and lactic acid production that increases calcium and consequently glutamate, to over excite neurons and produce free radicals such as reactive oxidative species (ROS) and calcium-dependent enzymes. This excitotoxicity within neurons cause mitochondrial release of apoptotic factors and ultimately initiate the apoptotic cascade and/or necrosis pathway, initiating inflammatory response and damaging the blood–brain barrier to increase cerebral edema (Xing et al. 2014). Consequently, brain atrophy from this disease can result in both immediate and long-term progressive cognitive impairment and stroke-induced secondary neurodegeneration resulting in functional decline (Zhang et al. 2012; Brodtmann et al. 2021).

Sleep is critical during post-stroke recovery. Sleep and circadian rhythm disruption are often unrecognized modifiable risk factors and consequences of stroke (Bassetti

et al. 2006; Wallace et al. 2012; Gottlieb et al. 2019). Clinical studies investigating the relationship between stroke and sleep have predominantly focused on obstructive sleep apnea evaluating risk and outcome, but few studies have correlatively associated with circadian disruption and stroke. Greater than 50% of stroke patients display sleep disorders, consisting of sleep-disordered breathing (SDB) most commonly manifesting in the form of obstructive sleep apnea (OSA). OSA can be represented as both a risk factor for infarction and immediate consequence from brain damage (Bassetti 2005). Studies demonstrate that stroke patients with OSA have increased risk and greater predispositions for recurring stroke (Martínez-García et al. 2012) and increased mortality (Sahlin et al. 2008). Other sleep disorders reported in stroke patients include excessive daytime sleepiness, insomnia, restless legs syndrome, and REM sleep behavior disorder (RBD). However, the prevalence varies depending on the type of stroke that occurred (Hermann and Bassetti 2016). Insomnia is the most common sleep disorder in stroke patients. It exists in approximately half of stroke patients (Palomäki et al. 2003) and has been shown to extend up to 12 months after stroke. Notably, post-stroke insomnia is associated with higher incidence of stroke (Huang et al. 2018). Additionally, RBD has been correlated with poor functional long-term outcome and increased mortality (Wallace et al. 2012).

Sleep architecture is also impaired after ischemic stroke. In a meta-analysis of polysomnographic studies, post-stroke patients exhibited reduced sleep efficiency, total sleep time, and increased frequency of waking (Baglioni et al. 2016). Further, this study demonstrates that stroke patients have prolonged stage N1 sleep with reduction in stage N2 and slow-wave sleep compared to controls (Baglioni et al. 2016). Another polysomnographic study investigating sleep architecture in stroke patients with SDB found that in addition to the characteristic deficits listed above, they also observed reductions in REM sleep, suggesting that introducing sleep disturbances can further alter aspects of sleep architecture (Terzoudi et al. 2009). A pilot study analyzing sleep quality in patients after hyperacute ischemic stroke reported severe disturbances in sleep cycles; post-stroke patients had 30% sleep efficiency with frequent waking during the sleep/wake cycle within the first 48 h post-stroke. Six patients did not reach deep sleep and 10 patients did not reach REM sleep (Hofmeijer et al. 2019). Clinical evidence supports the positive relationship between sleep and rehabilitation, highlighting the importance of this biological function in this disease. Sleep can enhance memory consolidation and facilitates motor learning during recovery and rehabilitation post-stroke in humans (Siengsukon and Boyd 2009), and studies reported improvements in sleep slow-wave activity, important in synaptic plasticity and reorganization, in patients receiving targeted aphasia rehabilitation through language therapy and imitation-based speech (Sarasso et al. 2014). Notably, more clinical studies are needed to further characterize the long-term sleep consequences associated with stroke.

Chronic sleep and circadian rhythm disruption result in dysregulation of physiological function and mechanisms including inflammation (Zielinski and Gibbons 2022), hypothalamic–pituitary–adrenal axis activation (Buckley and Schatzberg 2005), and autonomic nervous system activation (Riganello et al. 2019), all of which can contribute to the pathogenesis and outcome of ischemic stroke. However, very

few studies have directly examined the relationship between circadian processes and stroke outcome. First evaluations into this relationship investigated the role of biological timing and periodicity on stroke onset in clinical settings, and first determined that the greatest incidence of stroke occurred during the morning time point, between 10:00 a.m. to noon, compared to any other time interval across the day (Marler et al. 1989). Several follow-up and meta-analyses have been conducted since then, compiling 31 publications to further support the presence of circadian influences on stroke onset. Notably, morning time points between 6:00 a.m. and noon have a 79% increased risk of occurrence compared to the normalized risk (Elliott 1998) along with greater risk of mortality during that time point (Turin et al. 2012). Clinical studies investigating melatonin in ischemic stroke patients demonstrate decreased urinary melatonin at night following acute (3 day) and chronic (2 weeks) time points in patients with extensive cerebral injury (Fiorina et al. 1999). However, this study only included one time point per light/dark phase. Therefore, this was followed up measuring urinary 6-SMT every 4 h, and the authors reported that in extensive cortical (and deep/lacunar) strokes, melatonin secretion is delayed during the acute injury phase, that normalizes after 10 days, while melatonin rhythms remain intact in less severe stroke patients (Beloosesky et al. 2002). Due to limited precision in timing for the method of analysis, further studies were conducted with animal models. Preclinical data identified that rats have immediate changes to pineal melatonin secretion post-transient middle cerebral artery occlusion (MCAO), with prolonged dysregulation to rhythms denoted through day-to-day alternating phase advances and delays in melatonin timing, which was determined through pineal microdialysis of melatonin timing profiles (Meng et al. 2008). Other preclinical studies investigating the mechanism underlying circadian rhythmicity for time-of-day and neuronal susceptibility to damage have been conducted in animal models of global ischemia, where rats were subjected to cardiac arrest at three different time points across a 24-h period (Zeitgeber time, ZT, 6, 14, and 20), and observed that hippocampal neurons exhibit variations in Caspase, a marker of cell death activation, with the greatest expression during the early night (ZT 14) compared to other time points. This coincides with human clinical data where the onset of the active period has the greatest extent of damage and injury. The authors further observed a coinciding 6-h shift in *Per1* following global ischemia, suggesting the potential role of the circadian clock (Tischkau et al. 2007). Notably, clinical and animal studies are limited in the context of research investigating the relationship between circadian rhythms and stroke. Further studies are necessary to investigate the contributing mechanism behind this neurological disease.

## 22.6 Conclusion

It is apparent that neurological diseases alter circadian rhythms and sleep–wake states. However, there is also a bidirectional relationship in which disruptions of circadian rhythms and sleep/wake cycles are detrimental and lead to the progression

of neurologic disorders. Importantly, as stated in the introduction, the extent to which disrupted sleep is the primary outcome (or cause) of neurological disorders or whether disrupted circadian clock function is the primary outcome (or cause) of neurological disorders remains unspecified. This is due to the inability to separate disruptions to circadian rhythms and sleep/wake cycles in diurnal species such as humans. However, these processes can be uncoupled in nocturnal species and provide further reasoning for studying circadian disruption and sleep/wake states in nocturnal rodents. Despite our understanding of the basic mechanisms governing sleep and the circadian clock, our knowledge of these systems during aging and neurological disease is still in its infancy. Future research should work to more fully understand these mechanisms in aging and neurological diseases to allow for the potential development of clock-specific neurotherapeutics.

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# Chapter 23
# Insomnia in the Elderly and Its Treatment


**Murat Özgören and Adile Öniz**

## 23.1 Definitions

The definition of insomnia is described in various dictionaries and encyclopedias as well as medical textbooks. The reasons can be attached to the fact that the word is at least 400 years old, not mentioning the concept being recorded in the ancient texts. Furthermore, it is interwoven into daily life, and hence, it has been acknowledged not only in health-related groups but also literature, art, and economics.

Commonly insomnia has been labeled as a noun relating to the condition with long-term inability to sleep. This prolonged incapacity results in difficulty in commencing or preserving a restorative sleep, which in return brings in fatigue. Proportional to the severity or persistence of the condition, it produces distress (at clinical levels) or loss in proper functioning. The condition arises from physical and psychological disturbances.

Various other terms have been also found to be addressing a similar broader area such as *agrypnia, ahypnia, ahypnosia, and anhypnia.* Lastly, the person suffering from the condition is referred as insomniac (n) (APA Dictionary of Psychology 2022).

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## 23.2 Setting the Scene

### *23.2.1 Historical*

The Pergamon Hospital Complex, Asclepios (Turkey), is a curious health-seeking center where body-mind and social interactions were interwoven. The common practices were brought to daylight from the eyes of a patient (acknowledged to be a hypochondriac himself) named Aristides (Steger et al. 2016). In light of some vague texts, the incubation facility was central to healing processes. To note, the herbal hypnotics were believed to be utilized by medical personnel one of whom is a well-known physician named Galen (Claudius Galenus). His role in careful experimentation of chemicals and herbal extracts later paves the way to Galenic medicine as a basis for modern medicine and historic Galenic Corpus). Galen is also believed to have prescribed valerian for insomnia (Blumenthal et al. 2000; Gärtner 2012).

When our scope of time frame reaches eleventh century, we observe that Avicenna (Ibn Sina, 980–1037) associates symptoms like pain, sleep environment conditions such as too much light, worries, and poor digestion (Feyzabadi et al. 2014). These parameters noted by Avicenna are not far from modern criteria for insomnia hinting at multidimensional therapeutical approaches.

For the nineteenth century, J. C. A. Heinroth is believed to be the person, addressing insomnia and sleep deprivation in 1818 (Steinberg and Hegerl 2014).

The rest follows the footsteps of foundations of sleep medicine, sleep laboratories, and sleep clinics. Surely, the availability of optimally controlled pharmaceuticals helped the way to the current date. Yet there is still the debate whether the biological models or cognitive behavioral approach or combinations would be the gift to next decades from current practices.

### *23.2.2 Demographics*

The aging population in the world has been recently marked by news from global markets indicating that there are more adult diapers sold than baby diapers (For Japan, the number is reported to be 2.5 times).[1]

From the source of European Statistics, Europe has witnessed a significant increase of aging population. It is reported that from 2021 to 2100, the working population is anticipated to decline, while elderly segment will display an increasing part of the overall population. 65 + will comprise 31.3% by 2100 with significant jump from current levels (20.8% in 2021). 80 + will increase by 2.5-fold in the same time frame (from 6.0% to 14.6%, respectively) (Fig. 23.1).

[1] https://9newsng.com/why-more-adult-diapers-are-sold-in-japan-than-baby-diapers/.

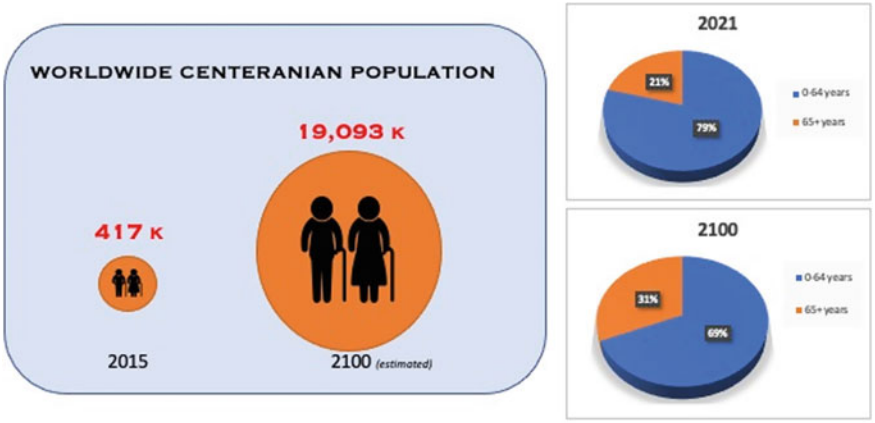


**Fig. 23.1** **a** Global centenarian (those who are 100 years and older) population by 2015 on the left and the projected numbers by 2100 on the right.[2] K indicates thousand. **b** Upper chart shows the elderly numbers (65 +) by 2021, and the lower one indicates the projection for 2100

Amid progressing countries, less developed countries discounting the least developed countries will host more than two-thirds of the global elderly (1.1 billion by 2050). Here, the least developed countries will face significant jump (225%) from 37 million in 2019 to 120 million in 2050 for 65 +. UN estimates the largest increase (312 M) to occur in Eastern and South-Eastern Asia (261–573 M). The same estimation addresses Northern Africa and Western Asia for fast growth of the elderly proportion. The current trends will provide further stress to the elderly group as they faced more isolation during the pandemic.[3]

Elderly population (65 +) face the serious issue of **insomnia,** and the ratio is multiplied with the centenarians. Interestingly, there will be approximately 45 times more 100 + toward 2100. Therefore, it is not false to focus on this problem as a major health disruptive factor.

[2] Modified from the projections (EUROPOP2019). Source: Eurostat (online data codes: demo_pjanind and proj_19ndbi).

[3] https://www.aa.com.tr/en/life/elderly-to-make-up-22-of-world-population-by-2050/2379462), https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Population_structure_and_ageing&oldid=549185#The_share_of_elderly_people_continues_to_increase).

#### 23.2.2.1 Centenarians

While in 2015 there were less than 0.5 M (app 417,000) centenarians globally, this number is projected to reach about 19 M by 2100. Unlike gradually increasing the elderly (65 +) population in general, the 100 + will increase by almost an exponential factor.

Globally, there were nearly half a million centenarians in 2015, more than four times as many as in 1990 (UN estimates). This change is expected to accelerate. Projections point to 3.7 million centenarians worldwide in 2050. The new consensus report from Canada points to a similar pattern to the US with an increase in very old population. Accordingly, the number of Canadian centenarians touched a new zenith in 2021. The number of these people, who are 100 years and older, has increased from just 1065 in 1971 to 9545 (Canadian 2021 census) while majority being females ($N = 7715$). Ratio-wise, the increase is also significant; in 1971, 4.9 people out of every 100,000 Canadians were 100 or older; in 2021, it was 25.8 per 100,000.

While centenarians contribute to a trivial portion of the world's older population, their percentage is rising. In 1990, there were 2.9 centenarians for every 10,000 adults ages 65 and older across the globe. That segment flourished to 7.4 by 2015 and is projected to escalate to 23.6 by 2050. Since 1990, the population of those ages 80 and older—the oldest segments of the 65-plus population—has increased more swiftly than that of the younger subdivisions, those ages 65–79. This faster growth is pushed by enhanced life expectancies among those 65 and older[4] (Fig. 23.1).

### *23.2.3 Socioeconomic Impact of Insomnia*

The social inclusion dynamics have influences on demographic developments. Hence, increased access to health care and basic services have contributed to mortality declines worldwide.

The aging population is a worldwide tendency with major social and economic effects that are portrayed by an ascending swing in age distribution. Worldwide, there were 728 million 65 + by 2020 or about 9% of the total population. This percentage is projected to stretch to 12% by 2030 and 16% by 2050; it could be nearly 23% by 2100, while women comprised 55% of 65 + globally (2020) and 62% of 80 +.[5]

In the scope of the elderly society, the sleep disorders including insomnia have many psychosocial etiologies like lifestyle, night shift, sporadic daily stressors, or environmental stress. On broad terms, insomnia is defined as a disorder manifested by difficulty in staying asleep, or going back to sleep after morning awakenings,

[4] https://www.pewresearch.org/fact-tank/2016/04/21/worlds-centenarian-population-projected-to-grow-eightfold-by-2050/, https://www.statista.com/statistics/996597/number-centenarians-worldwide/.

[5] https://documents-dds-ny.un.org/doc/UNDOC/GEN/N09/212/29/PDF/N0921229.pdf?OpenElement.

even when optimal environmental conditions are present. The overall occurrence of insomnia symptoms ranges from 30 to 48% in the elderly and occurrence of insomnia disorder has a smaller range of 12–20%, while up to 75% of older adults acknowledge symptoms of insomnia (Patel et al. 2018; Nguyen et al. 2019).

The sleep maintenance symptoms are most frequent among insomniacs (50–70%), trailed by difficulty in initiating sleep (35–60%) and nonrestorative sleep (20–25%). A study with the elderly population (65 +) detected an occurrence rate for insomnia symptoms of 5% per year, with a yearly incidence of 7.97% at 1-year follow-up. Nearly 50% of the patients with symptoms of insomnia will have a remission through the follow-up stage, with higher remission percentages among older males relative to females.

According to a study (Silva et al. 2017), elders who depended on others to perform regular and instrumental activities of daily life were frail and carried the risk of falls, and had insomnia, (strongest predictor) displayed inferior self-perceived health. All these parameters including the loss of old/young proper ratio as workforce will have an increasing socioeconomic impact on the societies. It is not hard to estimate that as the world population ages and environmental factors spiral, we should expect more elderly insomnia patients.

## 23.3 Clinical Parameters

The clinical parameters to diagnose insomnia are very similar across medical world. Luckily among the sleep societies, there is a tendency to use common classifications. Therefore, International Classification of Sleep Disorders (ICSD) has a central role in bringing the criteria toward other societies. Likewise, ICD-10 (International Statistical Classification of Diseases and Related Health Problems) addresses the issue as common denominator of sleep disorder. As the comorbidity plane of insomnia is commonly interwoven with other mental disorders, also comes the Diagnostic and Statistical Manual for Mental Disorders (DSM) classification. All these class criteria focus on the identification, differential diagnosis, comorbidity evaluation, and severity assessments. Table 23.1 is modified from review by Paul et al. (2022). DSM identifies episodic, persistent, and recurrent, and ICSD describes chronic, acute, and other categories.

### *23.3.1 Diagnostic Tools*

The review by Ali et al. 2020 systematically analyzed the instruments on Insomnia for diagnostic purposes (Ali et al. 2020). Accordingly, 38 tests were listed as the instruments for assessing consequences of poor sleep, screening for insomnia symptoms, assessing the cognitive aspect of insomnia, and measuring sleep hygiene.

**Table 23.1** International classification and diagnostic criteria for insomnia

| **ICD-10**<br>(International Classification of Diseases) | **ICSD-3**<br>(International Classification of Sleep Disorders) | **DSM-V**<br>(Diagnostic and Statistical Manual of Mental Disorders) |
|---|---|---|
| **A**. Complaints of increased sleep latency or poor sleep maintenance or generally poor sleep<br>**B**. Frequency of sleep-related complaints three nights a week for at least one month<br>**C**. Nocturnal ruminations about the day and night consequences of insomnia<br>**D**. Daytime disturbances due to disappointing quality and quantity of sleep<br>**E**. Waking up early in the morning with inability to go back to sleep<br>*Approximate Synonyms*<br>Insomnia<br>Insomnia disorder<br>Insomnia disorder related to known organic factor<br>Insomnia disorder, episodic<br>Insomnia disorder, recurrent<br>Organic insomnia<br>*Relevant Codes*<br>• G00-G99 Diseases of the nervous system<br>• G47 Sleep disorders<br>• G47.0 Insomnia<br>• Adjustment Insomnia F51.02<br>• Other insomnia not due to a substance or known physiological condition F51.09<br>• Primary insomnia F51.01<br>• Paradoxical insomnia F51.03<br>• Sleep Deprivation Z72.820<br>• Insomnia Due to Medical Condition G47.01 | **A**. A predominant sleep complaint by the patient or parent/caregiver accompanied by one or more of the following symptoms:<br>1. Delay in falling asleep<br>2. Dissatisfaction with the maintenance of sleep<br>3. Waking up before the desired wake-up time<br>4. Reluctance to go to bed to sleep on time<br>5. Trouble sleeping without a parent/caregiver<br>**B**. Sleep complaints as a result of difficulty sleeping at night with one (or more) of the following consequences:<br>1. Tiredness/uneasiness<br>2. Memory impairment/attention deficiency<br>3. Societal, family, occupational, or academic impairments<br>4. Irritable mood<br>5. Daytime somnolence<br>6. Behavioral troubles like hyperactivity, impulsivity, and hostility<br>7. Lack of energy for new beginning/reduced motivation<br>8. Error/accident-proneness<br>9. Sleep difficulty-related nighttime ruminations<br>**C**. Sleep–wake complaints despite adequate opportunity and environment for sleep<br>**D**. Sleep disturbance and significant disruptions at least three times a week<br>**E**. The aforementioned complaints persist for at least three months at a weekly frequency<br>**F**. The sleep–wake difficulty of insomnia cannot be explained better by the occurrence of other sleep disorders | **A**. Frequent sleep complaints related to the quality or quantity of sleep associated with one or more of the following insomnia symptoms:<br>1. Difficulty initiating sleep (In children, this may manifest as difficulty initiating sleep without caregiver intervention.)<br>2. Difficulty maintaining sleep characterized by frequent waking or problems returning to sleep after waking up (In children, this process may manifest as difficulty in maintaining sleep without the intervention of the caregiver.)<br>3. Early-morning awakenings accompanied by trouble in returning to sleep<br>**B**. Sleep complaints related to clinically significant daytime disturbances in social, behavioral, occupational, educational, academic, or other important areas of functioning<br>**C**. Difficulty sleeping occurs at least three nights a week<br>**D**. Sleep complaints should continue at the same weekly frequency for at least three months<br>**E**. Difficulty sleeping occurs despite adequate opportunity to sleep<br>**F**. Insomnia is not better explained by and does not occur exclusively during the course of another sleep–wake disorder (e.g., narcolepsy, a breathing-related sleep disorder, a circadian rhythm sleep–wake disorder, a parasomnia)<br>**G**. The occurrence of insomnia cannot be attributed to the physiological effects of a substance (e.g., a drug of abuse, a drug)<br>**H**. Co-existing mental disorders and medical conditions do not adequately explain the complaint of insomnia |

*Note* Acute and short-term insomnia (i.e., symptoms lasting less than 3 months but otherwise meeting all criteria with regard to frequency, intensity, distress, and/or impairment) should be coded as another specified insomnia disorder

The authors highlighted the increasing number of tests and pointed to the varying basis of insomnia pathophysiology as a possible culprit. Furthermore, the cultural, social, and different perspectives (i.e., non-Western perspective or socioeconomic evaluation of risk factors, etc.) would have been the reason behind the development of so many different tests. Lastly, from the comorbidity prospects to psychometric properties of insomnia tools across diverse populations is a possible research topic. Table 23.2 is the summary of the current diagnosis test and questionaries in relation to insomnia.

Besides the scales and questionnaires, there is number of tools as well (Schutte-Rodin et al. 2008). These include

**Table 23.2** The diagnostic tests and questionnaires

| |
|---|
| AIS, Arabic scale of insomnia |
| AIS, Athens insomnia scale |
| APSQ, Anxiety and preoccupation about sleep questionnaire |
| ASBQ, Athlete sleep behavior questionnaire |
| BIS, Bergen insomnia scale |
| CTIS, Catastrophic thoughts about insomnia scale |
| DSPS-4, Daytime sleepiness perception scale-4 |
| DBAS, Dysfunctional beliefs and attitudes about sleep scale |
| DCCASP, Daily cognitive-communication and sleep profile |
| ESS, Epworth sleepiness scale |
| FOSQ, Functional outcomes of sleep questionnaire |
| GSDS General sleep disturbance scale |
| H-Scale, Hyperarousal scale |
| IDWS, Insomnia daytime worry scale |
| InSS, Indian sleepiness scale |
| ICS, Insomnia catastrophizing scale |
| ISI, Insomnia severity index |
| JISS, The insomnia screening Scale |
| KSS, Karolinska sleepiness scale |
| LIS-18, Lebanese insomnia scale |
| MaSQuDI-17, Maugeri sleep quality and distress inventory |
| MISS, Minimal insomnia symptom scale |
| NRS, Non-restorative sleep scale |
| OISQ, Occupational impact of sleep questionnaire |
| PSQI, Pittsburgh sleep quality index |
| RSQ, Restorative sleep questionnaire |
| SCI, Sleep condition indicator |
| SDQ, Sleep disturbance questionnaire |
| SFIS, The sleep functional impact scale |
| SHAPS, Sleep hygiene awareness and practice scale |
| SHI, Sleep hygiene index |
| SHS, Sleep hygiene self-test |
| SQS, Single-item sleep quality scale |
| SPAQ, Sleep practices and attitudes Q |
| SPS, Sleep preoccupation scale |
| SQQ, Sleep quality questionnaire |
| SS, Jenkins sleep scale |
| WHIIRS, Women's health initiative insomnia rating scale |

- Physical and mental status examination (for comorbid conditions and differential diagnosis)
- Polysomnography (sleep apnea, movement disorders, etc.) and daytime multiple sleep latency testing (MSLT)
- Actigraphy (circadian rhythm patterns, sleep disturbances)
- Laboratory testing (e.g., blood, radiology) for comorbidity
- Sleep diary data
- Additional assessment for
  - Fatigue and sleepiness
  - Mood disturbances and cognitive difficulties
  - Quality of life.

### *23.3.2 Comorbidities*

Insomnia disorder commonly is also associated with psychiatric, neurological, or physical conditions. Historically pain has been a strong denominator for such comorbidity sign. Depression, apnea and other breathing problems, cardiovascular symptoms, hypertension, diabetes, hyperlipidemia, rheumatologic conditions, nausea, restless leg, and other sleep-disturbing conditions are among the many symptoms that accompany insomnia (Riemann et al. 2017; Ng and Ng 2021).

### *23.3.3 Neurobiophysical and Cognitive Background of Insomnia*

1980S insomnia diagnosis and treatment were not any inferior to today. Borkovec (1982) describes the physiological as well as psychological background for insomnia (Borkovec 1982). Hence, therapies from biofeedback and cognitive therapies to hypnotic medication were all listed in detail.

Kales and Kales (1987) reported that the insomniacs displayed a common pattern: Bedtime tense state, ruminations about unresolved issues, anxiety, worries, fixation, and elevated vigilance during the day were among the complaints (Kales and Kales 1987).

Edinger et al. (1988) investigated the personality types of insomniacs. The study also included the behavioral therapies in line with these subtypes (i.e., tendency to contain, minimize, and internalize stress and emotional conflicts, etc.) (Edinger et al. 1988).

The findings of research during this period revealed cognitive models for insomnia. Harvey, one of the leading researchers presenting the cognitive model, noted that loading in insomniacs with negative thoughts and concerns leads to both autonomic arousal and emotional dissection. The hyperarousal state with which

cognitive models are connected is a complex phenomenon. Inevitably, physiological processes also participate in the discussion. Therefore, when discussing insomnia etiopathogenesis, it is necessary to cover behavioral, cognitive, neurocognitive, and physiological models.

Physiological processes for arousal are evaluated such as basic psychophysiological measures, whole-body metabolic rate, heart rate variability, neuroendocrine measures, and functional neuroimaging. In fact, the first physiological measurements in insomnia began in earlier research (1960s). Heart rate variability, heart and respiration frequency, skin and core body temperature, muscle tone, skin conductance and resistance, and peripheral blood flow or vasoconstriction are among the parameters that have been utilized as biophysical factors. In these antecedent studies, it was stated that these biophysical and physiological markers were affected by arousal height. However, due to some methodological limitations, the results of these studies were not sufficient to define a single physiological model for insomnia.

One of the physiological measurements used in recent research is the whole-body metabolic rate utilizing oxygen uptake ($VO_2$) as a marker. Bonnet and Arand (1995) reported a high metabolic rate in insomniac patients (Bonnet and Arand 1995). Another finding that may be indicative of metabolic changes in insomnia may be the differentiation of body temperature. In insomnia, both 24-h core body temperature and body temperature in the application phases were reported to be higher (Lack et al. 2008). An easy method of evaluating arousal status is heart rate variability, which is controlled by the autonomic nervous system. Besides, Bonnet and Arand reported in their 1998 heart rate variability analysis in insomniac patients that the heart rate decreased especially in sleep and that the heart rate was better than that of those who slept well (Bonnet and Arand 1998). They reported that this was associated with reduced parasympathetic activity and increased sympathetic activity.

Likewise, the neuroendocrine responses to stress vary, especially in connection with hypothalamic–pituitary–adrenal (HPA) axis (Balbo et al. 2010). In addition, norepinephrine and melatonin are also important in insomnia as physiological parameters (Riemann et al. 2002; Takaesu et al. 2015; Dopheide 2020). Takaesu et al. conducted a study in 2015 on the relationships between diurnal melatonin secretion and sleep variables in patients at coronary care unit and pointed to the lower melatonin levels causing insomnia (Takaesu et al. 2015). The cortisol and norepinephrine levels in urine were found to be high in insomnia. Likewise, plasma levels have also shown that ACTH and cortisol levels in sleep and before sleep were elevated in insomnia patients (Johns et al. 1971; Vgontzas et al. 1998, 2001; Riemann et al. 2002). These findings demonstrate the relationship between HPA axis and sympathetic nervous system activation levels and sleep problems. One issue that is to clarify whether increased HPA activity leads to insomnia or whether insomnia leads to increased HPA activity. Stress-induced HPA axis activation can cause sleep disturbances, while chronic sleep disorders in turn display a persistent activation of the HPA axis.

Furthermore, various functional imaging methods were utilized in recent years (Spiegelhalder et al. 2013). In particular, PET and SPECT methods can show metabolic changes in the brain regionally. Much more commonly used fMRI can

address different tasks, to define the functioning of different brain regions, and changes in the brain can be examined in different phases of sleep.

The findings of these studies showed that bioenergetic processes in the brain of insomniacs at different periods of sleep were different from those of normal sleepers. Another remarkable finding is that morphometric features were in line with metabolic activity changes in limbic system connections such as hippocampus and anterior cingulate cortex, especially amygdala (O'Byrne et al. 2014; Bagherzadeh-Azbari et al. 2019; Schiel et al. 2020). Although the findings of neuroimaging studies include partial inconsistencies due to technical and methodological differences, they are promising in understanding the pathophysiology of insomnia, clinical evaluation, discrimination, and driving treatment. Additionally, a decrease in the hippocampal gray matter, the parietal and cingulate cortex, and the medial frontal lobes was noted (Riemann et al. 2015). Likewise, the review by Schiel et al. (2020) reported several neuroimaging studies focusing on amygdala, default mode network, salience network, etc. (Schiel et al. 2020). The outcomes indicated amygdala reactivity, and morphometry and adaptation to be altered. Furthermore, insomniacs displayed aberrant connectivity associated with individual sleep disturbances, hyperarousal, maladaptive emotion regulation, and disturbed integration of emotional states. The culprit was addressed to the limbic circuit besides the former.

In another domain, electrophysiology studies (i.e., electroencephalography-EEG) displayed a similar pattern to neuroimaging studies. EEG findings matched the worries and attentional emphasis of insomniacs (Harvey and Tang 2012). Additionally, other studies have described oscillation changes (delta, alpha, beta, gamma) during REM (rapid eye movement) sleep (Freedman and Sattler 1982; Merica and Gaillard 1992; Krystal et al. 2002). Whereas NREM (non-rapid eye movement) electrophysiologic frequency indices were thought to be physiologic correlates of sleep complaints.

Consequently, certain cognitive and behavioral factors need to be addressed such as attention, memory, problem-solving, and similar which are part of the processes involved in higher executive functions (Fortier-Brochu et al. 2012; Mukku et al. 2018; Wardle-Pinkston et al. 2019; Edinger et al. 2021).

Physiological, cognitive, behavioral, and cortical predominant hyperarousal mechanisms are moderated by age, gender, race, interpersonal relation, socioeconomic status, comorbidity, education status, self-rated health condition, physical incapacity, and sleep-disruptive medication. They are also related to environmental stimuli (temperature, light, noise), misbehaviors (alcohol use, nighttime caffein, substance abuse, smoking, reading, digital use, being active in bed, etc.), neurocognitive impairment (disrupted sleep-related rumination, life stress-related worries, etc.), and genetic factors (Perlis et al. 2005).

### 23.3.4 Nutrition and Insomnia

Diet quality and certain nutrients can change the quantity and quality of sleep by affecting hormonal pathways. In addition, sleep can change total energy intake by affecting the intake of certain foods and nutrients through biological and behavioral mechanisms (Frank et al. 2017). Older individuals have less quality and quantity of sleep compared to adults. Diet, one of the modifiable lifestyle factors, may affect sleep-related outcomes in the elderly (Gupta et al. 2021). It was stated that food diversity comprising eggs, meat, fish, milk products, fruits, and vegetables may affect sleep efficiency (Yamamoto et al. 2021). It has also been noted that quality sleep in the elderly individuals is associated with adherence to the Mediterranean Diet, which is based on dietary diversity (Mamalaki et al. 2018).

In fact, among other sleep disturbances, insomnia is a common sleep disorder in the elderly. With certain health consequences, insomnia symptoms are more common in women. When the causes of insomnia are investigated, it has been shown that anorexia is one of the important factors (Peng et al. 2021). In the study conducted by Kushkestani et al. older individuals with poor eating habits (malnourished) had lower sleep quality scores (Kushkestani et al. 2021). The prevalence of obesity is also increasing due to excessive food intake in the elderly individuals who have poor sleep quality, except for low food intake due to anorexia (Türkbeyler et al. 2021). For this reason, it is extremely important to determine the nutritional status of the elderly individuals and to properly regulate their appetite (Peng et al. 2021). As metabolic processes are also incorporated to sleep hormonal homeostasis, the connection between sleep quality and body composition exhibits a two-way street.

In the assessment of nutritional status in the elderly, Mini Nutritional Assessment (MNA) screening tool, anthropometric measurements (such as body weight, height, body mass index, waist, hip, calf, and upper middle arm circumferences), and food consumption should be evaluated together (Poda et al. 2019; Kushkestani et al. 2020, 2021; Türkbeyler et al. 2021). In addition, the Pittsburgh Sleep Quality Index (PSQI) should be used to evaluate sleep quality (Kushkestani et al. 2020, 2021; Türkbeyler et al. 2021). It should be aimed to establish the necessary policies to improve the quality of life by evaluating the nutrition and sleep quality of the elderly individuals.

### 23.3.5 Thermoregulation and Insomnia

There are several epidemiological studies reporting lower core body temperature of healthy men and women over 60–65 years of age than that of their younger adult counterparts. The current condition of the cardiovascular system, heat and cold exposure and responses, sweating, heat production, thermosensitivity, and behavioral thermoregulation are among the physiological parameters in the background. While there are few studies on the age-related changes in behavioral thermoregulation in

humans, the wide-ranging impression is that the elderly are less proficient in the thermoregulation respect than adults (Blatteis 2012).

Along with other factors for sleep disturbance, temperature has also been studied. In an experimental study, temperature manipulations were effective for promoting better sleep (Raymann et al. 2008). The authors postulated that skin warming would increase neuronal activity in brain areas that are critically involved in sleep regulation. Similarly, the temperature regulation of sleep environment (i.e., bed heating) was associated with improved sleep quality (Xia et al. 2020).

### *23.3.6 Behavioral and Physiological Conditions at Crosshairs*

The modern human life is getting more oriented with electronic devices, gadgets, and media devices.[6] In fact, The United Nations International Day of Older Persons 2021 theme "Digital Equity for All Ages" is a good example of this issue. The 2021 Pew Research Center survey found that 96% of the younger population (18 to 29 y) own a smartphone compared with 61% of the elderly (65 +). Regarding Internet use, younger ages extensively use, while the number is at 75 for the elderly (65 +).

A European study displayed the outcomes of this type of behavior in relationship to insomnia. Accordingly, they reported that computer usage for playing/surfing/reading was positively connected with insomnia and negatively associated with morningness. On the other hand, mobile phone usage was positively associated with insomnia and chronotype and negatively associated with morningness (Fossum et al. 2014).

In the background of this type of results, there are few facts that need to be discussed. Not only cognitive emotional factors but also physiological mechanisms might be responsible. To note, the modern electronic devices might expose no less than 500 cd/m$^2$ light even in the blue light spectrum. Accordingly, there have been some initiatives to counterbalance this factor by means of dimming the screen light, color shifting, etc. Yet the cognitive behavioral background is more complex and further interventions need to be planned.

### *23.3.7 COVID-19 Pandemic and Insomnia*

The pandemic has lasted approximately 3 years and approximately 15 million died worldwide. The initial stage brought extreme lockdown conditions, where mostly the elderly have been drastically affected. If not physically incapacitated during this period, the elderly population has complained about loneliness, worries, and

[6] https://pewrsr.ch/3HZd2ao.

depression. In an Italian study, for the elderly population during COVID lockdown, shorter sleep duration, lower habitual sleep efficiency, and increased sleep medication intake were reported (Amicucci et al. 2021). Similarly, a Turkish study emphasized the condition of the elderly with sleep problems as well as loneliness feeling during pandemic (Gezgin Yazici and Ökten 2022). It has been apparent that the pandemic conditions have presented immense stress on the populations and evidently displayed symptoms like insomnia in the elderly. Among the comorbidities are balance problems in the elderly, which have increased significantly during pandemic. Telemedicine was utilized to enable elderly engagement without bringing them out of their safe environments against the pandemic conditions (Bagkur et al. 2021; Yerlikaya et al. 2021).

## 23.4 Therapy: A Broadband of Different Disciplines

As indicated in the above sections, the etiology of the insomnia is depicting a rather complex landscape. The factors leading to insomnia are affected by life practices, sleep hygiene, biological and psychological comorbidities, and numerous other aspects that are inseparable. As such, any treatment approach would need to address these determinants. From 1960s, the insomnia treatment has been targeted at hypnotic medication as well as cognitive behavioral interventions. Currently, the debate of superiority of these methods is not concluded, accordingly in common practice combination of the therapy methods is applied. Naturally having insomnia brings further problems biologically, cognitively, and behaviorally, limiting the quality of life. Thus, the interventions target at correcting the person as-a-whole with the life parameters rather than only a particular symptom. Cognitive behavioral therapy for insomnia (CBT-I) is a common method before the administration of pharmacological agents.

The common therapy approaches are listed in Table 23.3. A number of prominent researchers as well as the clinical societies have published international guidelines to assist their health professionals treating insomnia (Riemann et al. 2017; Sateia et al. 2017; Hollsten et al. 2020; Scharner et al. 2022).

### *23.4.1 The Light Therapy for Insomnia*

Chronobiologically, intense light at evening time suppresses the production of melatonin. In the electromagnetic spectrum, short-wavelength section (blue light, 446–477 nm) has the highest biological response (Gooley et al. 2011; West et al. 2011). The optimal light spectra must be approximately 400–500 nm (blue-green) while this may compensate the need for higher lumens of light. Still, providing sufficient light intensity is critical particularly for patients with cataracts, which worsens light

**Table 23.3** The non-pharmacological Treatment and Intervention Protocols. Left column indicates the intervention, and on the right column, the description for the intervention is provided

| Intervention | Description |
|---|---|
| Biofeedback | Biofeedback is also known as neurofeedback. Commonly, a physiological parameter is monitored by the participant via an electrophysiological setup (such as EEG and EMG). The auditory cues or visual cues are provided to assist the patients with self-adjusting muscle tone, etc. Recently, a trial has found a solid effect of biofeedback falling short of CBT-I (Kwan et al. 2022) |
| Cognitive therapy and BTIs | BTIs include CBT-I and Brief Behavioral Therapy for Insomnia. emphasizing the behavioral components. CBT-I therapy pursues to readapt deceptive cognitive views and attitudes toward sleep. Recently, computerized cognitive training (CCT) is emerging in this field as well as digital self-assisting CBT and telemedicine |
| Exercise | Physical exercise as well as Tai-chi. Aerobic exercise improves self-reported sleep and quality of life in older adults with insomnia |
| Intensive sleep retraining | A rather intense and complicated intervention in a laboratory environment. Every half an hour if the patient falls asleep, the subject is awakened after three minutes while being instructed to stay awake for half an hour. The method is believed to regulate sleep drive and misperceptions |
| Light therapy | Adjustment and overall delivery of bright light to enhance sleep-related hormones and achieve better chronobiology cycles |
| Mindfulness | Mindfulness-Based Stress Reduction Program (MBSR) and the Mindfulness-Based Cognitive Therapy (MBCT) are among the protocols. The intervention focuses on (commonly in a group fashion) structuring emotions, awareness, and reactivity |
| Paradoxical intention | Rather than fixating at the sleep pressure to be produced, this intervention intends to approach from the opposite angle. The participant is coached to remain awake if conceivable after getting into bed. The patient is commanded to decisively involve in the feared task (staying awake) to reduce performance anxiety and intention to sleep (confounding associated goal-directed behavior). There is the presence of excessive focus to help decreasing anxiety |
| Relaxation therapy | Abdominal breathing, progressive muscle relaxation, autogenic training, guided imagery training, meditation, yoga, and hypnosis are among the techniques |
| Sleep hygiene | As environmental factors within the sleep quarters (bedroom) and prior to sleep are critical for a good quality sleep, this intervention aims at regulating them for a better standard. Thus, recommendations about lifestyle including dietary, activity, drug or alcohol use, and environmental factors such as optimal noise level, ambient temperature, and light are given |

(continued)

**Table 23.3** (continued)

| Intervention | Description |
|---|---|
| Sleep restriction therapy | Escalate the sleep drive and consolidate sleep by adjusting and limiting time in bed to meet sleep efficiency thresholds. Sleep compression is a similar but more gentle approach gradually reducing time in bed |
| Stimulus control | The intervention focuses on the elimination of (Pavlovian) conditions that are limiting the sleep, organizing and readjusting the internal and external cues associated with sleep, and consequently reducing the stress related to sleep |
| Thermoregulation | Intervention to achieve optimal temperature in bed and sleep environment directly or indirectly |

transduction. Generally, a prolonged light exposure would offer a greater circadian stimulus (Dewan et al. 2011; Cahan and Abbott 2020).

There is no single agreement on the optimum Bright Light Therapy (BLT) protocol. Most reports point to a light intensity of 2000–10,000 lx, for half an hour to two hours, for one to four weeks. Majority of the studies make use of an industrial lightbox, contrasting to natural light. There are potential side effects such as dry eye and skin, headache, nausea, anxiety, and agitation (Genhart et al. 1993; Gammack 2008). Besides the light source intervention, another study focused on blue light blockage (Shechter et al. 2018). They showed that wearing amber-tinted (blocking blue spectrum) lenses before bedtime advances better sleep quality in insomniacs.

## *23.4.2 Insomnia Pharmacology*

### 23.4.2.1 OTC Drugs and Supplements for Insomnia or Sleep Disorders

Over-the-counter (OTC) drugs are various drugs that can be taken without the need for a prescription. Although these drugs have a safer profile than prescription drugs, contrary to popular perception, they cannot be considered completely harmless. As with many diseases, these drugs should be used under the supervision of a health professional, which can also give effective results in sleep problems in the right person, at the right dose, and in short-term use. This section includes some examples of OTC drugs and food supplements used in sleep disturbances.

**Chemical Agents**:

**Doxylamine** is a first-generation Histamine ($H_1$) receptor blocker. It has anti-cholinergic and sedative effects. Anti-cholinergic effects can be listed as dry mouth, hazy vision, hardening of mucus secretions, or dry skin. The recommended dose and administration of use are in the form of a dose of 25 mg, to be taken 30 min

before bedtime, indicated for the treatment of short-term sleep disorders or insomnia (Culpepper and Wingertzahn 2015).

**Diphenhydramine**. Just like doxylamine, diphenhydramine is a first-generation Histamine ($H_1$) receptor blocker. It has anti-cholinergic and sedative effects. Anti-cholinergic side effects can occur in the form of dry mouth, blurred vision, hardening of mucus secretions, or coordination disorders, as in doxylamine. Diphenhydramine is used 30 min before sleep in doses of 25 mg or 50 mg (Culpepper and Wingertzahn 2015).

**Melatonin** is the neurotransmitter most familiar to sleep researchers, which is found naturally in our body and regulates the wakefulness-sleep cycle. Melatonin, which can be found in doses of 1, 3, 5, or 10 mg in the form of food supplements, creates an agonist effect on melatonin receptors found in many tissues of our body. Among the most common side effects are headache, excessive sleepiness during the day, and depression. Although the method and dose may vary, melatonin food supplements are used in the form of doses of 1, 3, 5, or 10 mg 3–4 h before bedtime (Culpepper and Wingertzahn 2015). It should also be noted that Melatonin is one of the most commonly used substances in cases where sleep–wake biorhythms are impaired due to shift work and jetlag.

**GABA** is the main suppressor neurotransmitter found in the human brain. When used as a food supplement, it has a sedative and stress-suppressing effect. There are also doubts that this effect may be placebo effect. There are differing opinions on whether GABA can cross the blood–brain barrier and have a central effect when taken externally. The use of GABA as a food supplement is very popular. The method and dose of use are in the form of doses between 100 and 300 mg per day in a period of at least 1–8 weeks. Long-term use is recommended for the treatment of short-term sleep disorders with its sedative effect (Hepsomali et al. 2020).

**Phytotherapy Agents**. This group of drugs is of natural and plant origin and has gained increasing popularity in recent years. There are many approaches where the sleeping environment is supported by using aromatic plants in beds with microcapsule. Here are some examples from this group.

**Valerian**. Although its pharmacology is not fully understood, it is known to influence central GABA, serotonin, and adenosine receptors. Many studies on Valerian's use in the treatment of insomnia have found that the mechanism of action of this plant is very similar to that of benzodiazepine group drugs. Instead of gamma sub-units on GABA-a receptors, the agonistic effect it shows in the beta subdivision is the main pharmacological difference between it and benzodiazepines. Dizziness, headaches, or stomach problems seen during the day are among the common side effects. The method and dose of use is an average of 600 mg of plant extract taken 1–2 h before sleep (Culpepper and Wingertzahn 2015).

**Passiflora**. Side effects of benzodiazepine group drugs or chemical alternatives, which are frequently prescribed in short-term sleep disorders, lead to the frequent preference of Passiflora Incarnata as a phytotherapy agent. The agonistic effect of

flavonoids, which are considered active substances in Passiflora plant extract, on various GABA receptor units leads to the emergence of sedative and anxiolytic results. The fact that it has much fewer side effects than its chemical alternatives paves the way for its frequent preference as OTC. It is used in various doses as different preparations such as capsules or syrup (Elsas et al. 2010; Guerrero and Medina 2017).

**Aromatherapy**

Essential oils obtained from plants can be used with three main methods in the phytotherapy framework: (a) inhalation, (b) massage, and (c) oral use.

In sleep disorders, anxiety, and depressive diseases, the role of aromatherapy as well as pharmacological agents is promising. The high side effect profile of pharmacological agents, especially used in intensive care units, increases the importance of aromatherapy methods and paves the way for its use in the form of a therapy method that promotes sleep. Lavender oil is undoubtedly one of the essential oils used when it comes to sleep.

**Lavender Oil**

Lavender oil improves sleep quality by having a sedative effect in addition to its relaxing, carminative effects. Linalool and Linalyl Acetate, contained in it, are responsible for the parasympathetic effect of lavender oil. The narcotic effect of Linalyl Acetate and the sedative effect of Linalool are well known. Lavender oil is the oil with the lowest toxic and allergenic effect profile compared to other essential oils. In addition to its sedative effect, it also has antiseptic properties and positively affects cardiological functions. It is recommended to use 4–5 drops of oil with different methods such as inhalation or massage (Karadag et al. 2017).

#### 23.4.2.2 Pharmacological Treatment Methods Used in the Treatment of Insomnia

The main pharmacotherapy agents used in the treatment of insomnia can be summarized by breaking down into 5 main groups. These are the ones that are categorized as Benzodiazepine Group Sedative Agents, Non-Benzodiazepine Sedative Agents, Antidepressants Orexin Receptor Antagonists, and Melatonin Receptor Agonists. Table 23.4 below summarizes the pharmacological properties of some of the drugs belonging to these groups and different OTC agents.

## 23.5 Final Note

Currently, our awareness of the scope of our biophysical being is broadened by the inclusion of microbiota, and environmental factors such as microplastics (unfortunately in our blood circulation), and digital era expanding into virtual

**Table 23.4** Pharmacological agents for insomnia treatment

| Drug name | Class | Studies and pharmacological features |
|---|---|---|
| Zolpidem | Non-benzodiazepine receptor agonists | Zolpidem is a pharmacological agent used to treat insomnia by connecting to GABA receptors with rapid onset of action and short-term hypnotic effect. This drug can be prescribed in normal or long/controlled release forms with doses of 5–10 mg. It is a chemical that should take into account side effects and addiction risks in long-term treatments (Patel et al. 2018) |
| Zopiclone | Non-benzodiazepine receptor Agonists | Zopiclone is a hypnotic agent that acts by connecting GABA-A receptors to sub-units α1 and α2. This agent, which does not fall into the benzodiazepine class, is indicated in the short-term treatment of sleep disorders in geriatric patients. Studies show that this pharmacological agent is well tolerated in short-term treatments at doses of 3.75–7.5 mg. In long-term treatments, the risk of side effects increases significantly (Pinto et al. 2016) |
| Doxepin | Tricyclic antidepressant | With FDA approval, doctrine is used to treat insomnia in doses of 3–6 mg. It is affected by selectively attaching to histamine 1 ($H_1$) receptors. Studies have reported that doctrine at doses of 1–3 mg bristled for 12 weeks provides significant improvements in sleep quality and sleep duration in sleep start measurements (Patel et al. 2018) |
| Mirtazapine | Serotonergic antidepressant | This antidepressant agent, which provides strong 5-HT2 receptor antagonism, is another example of antidepressant drugs used to treat insomnia. In a study involving participants aged 18–75 with an average age of 40.9 years, the mirtazapine group was only present after 2 weeks of treatment; significant improvements were detected in indicators of sleep duration, sleep quality, and frequency of awakening after the onset of sleep (Winokur et al. 2003) |

(continued)

**Table 23.4** (continued)

| Drug name | Class | Studies and pharmacological features |
|---|---|---|
| Lorazepam | Benzodiazepin | Lorazepam binds to benzodiazepine receptors in the postsynaptic GABA-A ligand-gated chloride channel neuron in various regions within the central nervous system (CNS). Drugs belonging to this group reduce sleep delay and reduce nighttime awakening, but also reduce rapid eye movement sleep (Pagel and Parnes 2001). They increase the risk of memory impairment, falls, fractures and motor vehicle accidents, and preventable emergency room visits and hospitalizations in older adults; therefore, their use should be avoided in older adults (Tannenbaum 2015). Prolonged use of benzodiazepines can promote psychological addiction, and over time, there is an increased risk of addiction and abuse. Tolerance may also develop, so higher doses are required to maintain effectiveness (Kamel and Gammack 2006) |
| Diphenhydramine | Antihistamine | Although diphenhydramine is traditionally known as an antagonist, it primarily acts as an inverted agonist of the histamine $H_1$ receptor (Khilnani and Khilnani 2011). Antihistamines reduce sleep delay; however, these over-the-counter sleeping drugs such as diphenhydramine cause rapid tolerance and are highly anti-cholinergic. Anti-cholinergic effects include blurred vision, dizziness, difficulty urinating, dry mouth, and constipation. Anti-cholinergic drugs can also increase the risk of cognitive impairment and decline; therefore, drugs with high anti-cholinergic profile, such as antihistamines, should be avoided in older adults (Fick et al. 2015) |

(continued)

**Table 23.4** (continued)

| Drug name | Class | Studies and pharmacological features |
|---|---|---|
| Ramelteon | Melatonin receptor agonist | Melatonin agony, a drug used to treat Ramelteon insomnia, is a drug. It is indicated for the treatment of insomnia, which is characterized by difficulties especially related to the onset/fall asleep of sleep. It is the only approved sleep-promoting drug (Neubauer 2008) that improves sleep with its effects on sleep-regulating mechanisms within the suprachiasmatic nucleus, with no direct soothing effect. In a study of older adults (ages 65 and older), ramelteon therapy significantly reduced patients' sleep duration during 5 weeks of treatment and showed no significant side effects or withdrawal symptoms (Roth et al. 2006) |
| Valerian | Herbal extract | Valerian lacks FDA approval and monitoring as a nutritional supplement. The mechanism of action is believed to occur through interaction with the neurotransmitter gamma amino-butyric acid and its receptors. There are a limited number of studies on valerian in older individuals, and data on its effectiveness in the treatment of insomnia are missing (Patel et al. 2018) |

The drug names, group classification, and the pharmacological features are provided in the respective columns

reality amid global immigration crisis (political and climate change among many factors) disrupting social structures. Therefore, "symptom hunting" will need to be replaced by a broader mind–body-environment approach within a socioeconomic and dynamic context. Interestingly, our not-so-favorable global facts (international political tensions, heat waves, floods, droughts, food security, etc.) are challenged by the prominent authors (Harari 2017) as humans would seek the "immortal life," thus pushing the centenarian concept to the limits. While these mind-boggling conditions, our task remains to find a suitable set of "insomnia diagnosis and therapy" tools with a challenge for fitting different socioeconomic status, race, gender, age, and living conditions.

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# Part VII
# Experimental Models to Study Sleep and Clocks in Aging and Longevity

# Chapter 24
# Invertebrate and Vertebrate Models in Sleep and Circadian Aging


**J. M. Hafycz and N. N. Naidoo**

## 24.1 Introduction

Sleep and circadian timekeeping are intrinsic to cellular and organismal function. While sleep has been shown to play a role in cellular health, consolidating memory, immune function, and restoring energy metabolism (Benington and Heller 1995; Buzsaki 1998; Diekelmann and Born 2010; Naidoo 2009; Toda et al. 2019), the explicit function of sleep remains largely unclear. Research has indicated that sleep is essential and that continuous sleep deprivation can even be fatal (Everson et al. 1989; Vaccaro et al. 2020). The current standing hypothesis for sleep regulation is the two process model (Borbely 1982). This model states that there is a circadian component to regulate the timing of sleep across the 24-h day, as well as a homeostatic process that regulates sleep based on the homeostatic regulatory processes of the cell (Borbely 1982). The circadian process is regulated by light across the 24-h day as well as the cycling of several genes, namely *Period*, *Clock*, and *Bmal* (Bae et al. 2001; Hastings 1998; Hendricks et al. 2003). This internal clock timing and regulation of circadian rhythms is intrinsic to the function of all organisms.

In humans, sleep and wake are regulated by groups of neurons in several key brain regions that can be thought of as switches, for example when wake-promoting pathways are active, sleep-promoting pathways are inhibited, and vice versa (Horner and Peever 2017; Scammell et al. 2017). Some of the key wake-promoting regions in humans are the locus coeruleus (LC) located in the brainstem, the tuberomammillary nucleus (TMN), and the lateral hypothalamus (LH) (Alexandre et al. 2013; Horner and Peever 2017), while the key NREM sleep-promoting region includes the GABA and galanin-containing cells of the ventrolateral preoptic area (VLPO) (Horner and Peever 2017; Scammell et al. 2017). Further, the neurons of the orexin neural circuit

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in the LH are activated during wake and inhibited during sleep (Sakurai 2007). Interestingly, the processes that regulate sleep as organisms age deteriorate, such that timing of sleep and quality of sleep are disrupted across aging in various species (Duffy et al. 2015; Koh et al. 2006; Kondratov 2007; Mander et al. 2017; Naidoo et al. 2008; Pandi-Perumal et al. 2002). The consequences of this disrupted sleep with age in humans are associated with cognitive decline (Helfrich et al. 2018; Nebes et al. 2009; Schmutte et al. 2007), and are even though to precede neurodegenerative disease progression, such as Alzheimer's disease (Malhotra 2018; Musiek et al. 2015).

Given the vital nature of sleep and the consequences of sleep loss, much ongoing research is dedicated to determining what mechanisms underlie these age-related changes in sleep and circadian rhythms and if these mechanisms can be modulated to restore sleep quality and circadian timing. Much of this research involves the use of diverse experimental models. In this chapter, we will discuss the different animal models used to study sleep, some of the experimental techniques for how that research is conducted, a few of the benefits and downsides to using each model system, and how this research has shed light on sleep and aging and potential therapies to improve age-related sleep and circadian disruptions.

## 24.2 Changes in Sleep Quality and Circadian Rhythms Across Aging

Sleep is a conserved biological process and has been found in all living creatures studied to date (Cirelli and Tononi 2008; Hafycz et al. 2021; Joiner 2016; Ly et al. 2018; Zimmerman et al. 2008a). The quality of sleep and proper timing of sleep–wake behaviors change across the healthy aging process in humans and animal model systems. There are several key alterations in sleep and circadian architecture, including increased sleep onset latency, shorter sleep duration, impaired sleep consolidation by increased awakening, increased daytime sleepiness, decreased melatonin levels, and reduced amount of deep slow wave sleep (Helfrich et al. 2018; Mander et al. 2017; Pandi-Perumal et al. 2002; Welsh et al. 1986; Wolkove et al. 2007). Importantly, these age-related changes in sleep characteristics are observable in many animal species studied (Brown et al. 2014; Koh et al. 2006; Mendelson and Bergmann 1999; Naidoo et al. 2008; Wimmer et al. 2013). Due to the prevalence and consistency of age-related changes in sleep and circadian behavior, research aims to understand the underlying mechanisms at play and how these can serve as targets for therapeutic intervention. Given the prevalence of sleep and circadian disruptions that occur with age and disease, using animal models to study sleep and circadian behaviors is vital to provide insight into how these disorders arise and potential therapies that could be used to treat them. While there are many animal models used to study sleep and chronobiology, in this chapter we will focus on well-established model organisms,

namely mice, fruit flies (*Drosophila melanogaster*), zebrafish (*Danio rerio*), and the roundworm (*Caenorhabditis elegans*).

## 24.3 Mice as a Model for Probing Sleep and Circadian Behaviors Across Aging

Measuring sleep in humans and mammalian animal models, like the mouse, is mainly conducted by using electroencephalogram (EEG) and electromyogram (EMG) recordings. In mice, this method requires surgical implantation of several electrodes into the skull and muscles, followed by a recovery period before the start of recordings. The EEG data files are scored in short 4-s to 10-s epochs, which requires intense effort to analyze and stringent quality control (Hafycz et al. 2021; McShane et al. 2012). Thus, while this method gives the most detailed information about brain activity during sleep and wake, it is impractical to use this method to conduct high-throughput assessments of sleep and circadian behaviors (Chuluun et al. 2020; Hafycz et al. 2021; McShane et al. 2012). In addition to EEG recordings, sleep behavior can be estimated in mice via beam break recordings. This method assesses mouse movements by tracking how often the mouse passes across infrared beams, and provides an approximation for sleep based on locomotor activity. Video recordings can also be used to capture behaving mice and provides another way to observe locomotion. Coupled with beam break recordings, video data can offer useful insight in distinguishing between inactive and sleep states, as video recordings allow researchers to examine and correlate behaviors and postures (Fisher et al. 2012; McShane et al. 2012). Both beam break and video recordings are appealing as they are not as invasive as EEG implantation, but neither provides as much information as EEG recordings. Another alternate system for examining sleep behaviors in mice is through the use of piezoelectric motion sensing (Yaghouby et al. 2016). This system uses piezoelectric mechanical sensors and transforms that mechanical information into electrical signals that are scored by relevant software programs as a measure of sleep and wake (Yaghouby et al. 2016). This system is appealing as it is non-invasive compared to EEG recordings, and has been validated to report accurate sleep and wake behavior compared with EEG data, as opposed to an estimate of the amount of sleep and wake obtained from beam break systems or video recordings alone.

Beyond measuring sleep, there are several techniques used in mouse models to manipulate sleep by altering neural activation or circuitry. These techniques include optogenetics and the use of chemogenetics, namely designer receptors exclusively activated by designer drugs, known as DREADDs (Hafycz et al. 2021; Roth 2016). Optogenetics allows sleep researchers to activate or inhibit neurons and neuron populations thought the use of light-sensitive ion channels that are artificially expressed in cells following a viral injection (Hausser 2014). Similarly, chemogenetics involves the use or virally-expressed artificial receptors that can manipulate neuronal activation when exposed to an exogenous ligand, namely clozapine-N-oxide (CNO) (Roth

2016). Another technique involves the injection of viruses containing genetic material that can cause cells to either silence or overexpress certain genes. These allow researchers to examine the role of specific genes in behavior, and can be precisely targeted to certain neuron populations (Dana et al. 2017; Kimura et al. 2019). Alternatively, mice themselves can be manipulated with transgenes that allow researchers to either knock out or overexpress different genes, leading to studies that probe what those genes are responsible for and how manipulating them can affect behavior (Haruyama et al. 2009). These techniques have been used to modulate and elucidate circuits involved in sleep–wake and circadian regulation.

The use of mouse models for studying sleep and circadian research is appealing in that they have similar analogous brain regions, circuits, structures, and neurotransmitters compared to humans. Indeed, mouse models have allowed researchers to uncover some of the mechanisms that coordinate sleep and circadian behaviors (Diniz Behn et al. 2010; Kroeger et al. 2017; Saper 2013). For example, some work has been dedicated to examining the homoeostatic response to sleep loss, which generally involves methods of sleep deprivation and subsequent examination of physiology (Franken et al. 2001; Mackiewicz et al. 2008; Nelson et al. 2013). Mouse studies have helped uncover some of the circuits underlying this homeostatic response, with some studies indicating that the preoptic area of the hypothalamus, specifically galanin neurons, are in part responsible for sleep homeostasis (Ma et al. 2019). In addition, mouse models are used to study an array of sleep and circadian disorders (Toth and Bhargava 2013). For instance, orexin knock-out mice displayed an inability to maintain state, suggesting that the orexin circuit is critical for behavioral state maintenance and could be involved in such disorders as narcolepsy (Chemelli et al. 1999; Mochizuki et al. 2004). It is clear that mice provide a vital model system to study sleep and circadian neurobiology.

In mice, much work has been dedicated to specifically examine age-related changes in sleep quality. Several studies show that aged mice have fragmented sleep (Hasan et al. 2012; Naidoo et al. 2008, 2011; Wimmer et al. 2013), one study showing that aged mice were less able to sustain longer bouts of wake or NREM sleep using a novel spike-and-slab analysis (Wimmer et al. 2013). More work has shown that aged mice have an impaired homeostatic response to sleep loss (Hasan et al. 2012), and an increased homeostatic sleep need (McKillop et al. 2018). Another study found that aged mice slept more than young mice during the dark phase (the normal active period for a mouse) and had more sleep fragmentation during the dark phase, akin to naps (Soltani et al. 2019). This work emphasizes that age-related changes in sleep are conserved across mammals and validates the mouse as a model to study age-related changes in sleep.

Beyond sleep and wake architecture, EEG recordings in mice allow researchers to examine spectral data. Spectral information from EEG recordings is divided into several ranges of frequency that are correlated with behavioral state. Deep slow wave sleep (SWS) is associated with delta brain waves, measuring roughly at 0–4 Hz, while wakefulness and REM sleep are associated with theta waves, ranging from 4 to 10 Hz (Carley and Farabi 2016; Jones 2020). Some work has shown that aging in mice reduces peak theta frequency, a marker for arousal intensity (Wimmer et al. 2013).

Interestingly, while mouse models allow researchers to obtain information about brain wave intensity, the genetic background of those mouse models can impact aged-related changes (Hasan et al. 2012). For example, one study showed that changes in delta power with age, a measure of sleep intensity, depends on the genotype of the mouse (Hasan et al. 2012). Further, while mice may have more homologous sleep–wake regulating brain circuits to humans than other models, there is still a limitation in using mice to study age-related changes in sleep and circadian behavior, for example, some research suggests mouse sleep increases with age, and research on consolidation and sleep intensity with age is inconsistent (McKillop and Vyazovskiy 2020). It is important to consider potential discrepancies between humans and model systems to help better address results and design experiments.

In addition to measuring behavioral changes, mouse models offer the potential to examine molecular mechanisms that underlie age-related changes in sleep quality. Interestingly, some work has linked protein homeostasis, or proteostasis, to sleep quality in aging (Naidoo et al. 2008, 2018, 2011). Specifically, it is now known that sleep loss or fragmentation that is seen in aged mice is coupled with an increase in cellular stress and the activation of the unfolded protein response (UPR) (Hafycz and Naidoo 2019; Naidoo et al. 2008, 2011). The UPR in part regulates protein translation under cellular stress conditions, and as protein synthesis is necessary for memory formation, could provide a link between age-related changes in sleep quality and age-related cognitive impairment (Brown and Naidoo 2012; Hafycz and Naidoo 2019; Havekes et al. 2012). Ongoing work is dedicated to further uncovering mechanisms underlying age-related changes in sleep as well as potential therapeutic interventions that could ameliorate these changes.

Mice are also used to probe age-related changes in circadian rhythms and longevity. Work examining genes involved in circadian regulation have shown that aged mice have reduced *Per2* expression in the superchiasmatic nucleus (SCN) (Weinert et al. 2001). Age-related changes in the expression of *Bmal1, Rev-erbα*, and other clock genes has been observed in the SCN of aged mice (Bonaconsa et al. 2014; Duffy et al. 2015). Another study using BMAL1 knock-out mice observed that these mice displayed premature aging (Kondratov et al. 2006). This premature aging has also been observed in mice deficient in PER1 and PER2 (Bae et al. 2001; Kondratov 2007; Zheng et al. 2001). Aged mice have been shown to have a delayed activity onset and slower entrainment following a phase advance of the light dark cycle compared to young adult mice (Valentinuzzi et al. 1997). Another study that recorded from the SCN of young and aged mice found that there was reduced neural activity in the aged mice, indicating that there are age-related changes in neural activity in key sleep–wake regulating brain regions (Nakamura et al. 2011). Another key aged-related change that is involved in sleep regulation is that melatonin secretion decreases across aging, and this is thought to contribute to age-related changes in sleep and circadian rhythm quality (Bubenik and Konturek 2011; Karasek 2004). However, the main mouse strain used for sleep and circadian studies, the C57/BL6 mouse, does not produce melatonin (Kennaway 2019; Pfeffer et al. 2022; Roseboom et al. 1998). It is important to consider this confound when choosing which mouse strain used to study sleep and aging (Pfeffer et al. 2022). Interestingly, a group of

researchers developed congenic mouse lines with the C57/Bl6 background that do produce melatonin, and could provide a more comprehensive model system for the study of sleep and aging (Zhang et al. 2021, 2018).

## 24.4 Non-mammalian Models of Sleep

While it could be argued that mice, as mammals, are more analogous to humans and therefore more meaningful to study, there are several important advantages to using small animal models to probe the mechanisms of sleep and circadian behaviors, particularly across aging. Namely, the life cycle for *drosophila*, zebrafish, and *C. elegans* is shorter than mice, and high-throughput analyses with these models are possible (Hendricks et al. 2000). Breeding in these models is also easier, as they have shorter reproductive cycles than mice. In addition, they have a simpler neural architecture than mice, allowing for more precise study of specific circuits that regulate sleep and circadian behaviors. For these model systems, non-EEG criteria for sleep are used to identify sleep behaviors. These are as follows: a period of quiescence associated with a specific posture, an increased arousal threshold, rapid reversibility to wakefulness, homeostasis, and interactions with the circadian clock (Hafycz et al. 2021; Ly et al. 2018). Using these criteria for sleep has allowed researchers to probe small model organism sleep–wake behavior and the mechanisms underlying sleep–wake regulation.

## 24.5 Studying Sleep and Circadian Rhythms Across Aging in Zebrafish

Zebrafish are another vertebrate model for sleep and they express genes that cycle, including analogs to *Bmal1*, *Period1*, and *Clock* (Rihel et al. 2010b). Further, zebrafish satisfy the non-EEG criteria for sleep (Yokogawa et al. 2007). Interestingly, zebrafish larvae are often studied due to larval transparency, short time to hatching, and ease of handling (Eisen 1996). Both adult and larval zebrafish exhibit the standard non-EEG criteria for sleep (Prober et al. 2006; Yokogawa et al. 2007; Zhdanova et al. 2001). Zebrafish sleep is measured mainly using video recordings and subsequent analyses of locomotor activity, similar to *drosophila* or *C. elegans* (Rihel et al. 2010b). While zebrafish lack completely analogous brain regions, such as a layered cortex, Zebrafish are also known to express the neurotransmitters that are important for sleep and wake, and respond similarly to mammals when exposed to sleep-promoting or wake-promoting agents (Panula et al. 2010; Rihel et al. 2010a, b; Sorribes et al. 2013). One study demonstrated that zebrafish sleep changes across development, as sleep does in mammals, suggesting that sleep and wake cycles in zebrafish develop in a way that can be meaningfully compared to mammals (Sorribes

et al. 2013). Zebrafish also exhibit a sleep–wake cycle across the 24 h day, observable through changes in locomotor activity (Prober et al. 2006; Rihel et al. 2010b). Zebrafish are diurnal and exhibit peak activity during the light phase, similar to humans, but unlike mice, which are nocturnal. Together, this evidence supports the relevance of a zebrafish model used to study sleep and circadian rhythms.

Importantly, zebrafish also display changes in sleep and circadian rhythms as they age. Some work has shown that zebrafish have increased fragmentation of circadian rhythms, a reduction in the duration of nighttime sleep, as well as a higher arousal threshold during the day (Zhdanova et al. 2008). Further, melatonin production, a key circadian hormone, declines across aging and aged zebrafish display alterations in the expression of circadian genes, Bmal1 and Per1 (Zhdanova et al. 2008). Thus, the zebrafish model provides another model system with which to study changes in sleep and circadian behaviors across aging.

## 24.6 Invertebrate Models for Sleep and Circadian Research Across Aging

Sleep in *drosophila* is typically measured with a combination of video recordings and beam break behavioral assays. The most common system for measuring for this is a single IR beam break system called the Drosophila Activity Monitoring System (DAMS), though it is important to note that using a single IR beam tends to overestimate sleep (Zimmerman et al. 2008b). The use of more infrared beams is preferred, and coupled with video recording, researchers are able to estimate sleep behavior in *drosophila* more accurately (Zimmerman et al. 2008b).

Invertebrate sleep circuits are analogous to humans, but are generally simplified, consisting of fewer regions and a smaller number of individual neurons. For example, in *drosophila*, there are about 150 neurons that express clock genes (Mezan et al. 2016). In *drosophila*, sleep is mainly regulated by a region known as the mushroom body, as well as a few neural groups including the dorsal fan-shaped body (dFSB) which is known to promote sleep (Artiushin and Sehgal 2017; Ly et al. 2018). Circadian regulatory neurons in *drosophila* consist of the ventral lateral neurons and the dorsal lateral neurons (Artiushin and Sehgal 2017; Ly et al. 2018). Some work has shown that sleep in *drosophila* is regulated in part by cyclin A (Rogulja and Young 2012). In this work, reducing cyclin A delayed the transition from wake-sleep, leading to increased arousals during sleep and a reduced homeostatic response to sleep deprivation (Rogulja and Young 2012). Further, the neurons in the *drosophila* brain that expressed cyclin A were intermingled with circadian clock neurons, indicating that there could be a functional relationship between sleep and circadian neurons in the *drosophila* brain (Rogulja and Young 2012). Interestingly, *drosophila* sleep is thought to be controlled by GABAergic signaling, suggesting that features of the *drosophila* sleep circuit are similar to mammalian sleep circuits (Agosto et al. 2008). A key regulator of sleep and circadian behavior in *drosophila* is the neuropeptide PDF (Ly

et al. 2018; Mezan et al. 2016). PDF is thought to be a wake-promoting aspect of *drosophila* circadian circuitry, as mutating the PDF gene or the PDF receptors results in flies that are hypersomnolent (Parisky et al. 2008). Together, this data suggests that not only are *drosophila* a useful model system for examining the basic circuitry of sleep and circadian regulation, but that these systems do, to some extent, mimic the circuitry and mechanisms of mammalian sleep.

With age, the strength of *Drosophila* circadian rhythm decreases (Koh et al. 2006), and *Drosophila* sleep is more fragmented (Brown et al. 2014; Koh et al. 2006). Further confirming that *drosophila* undergo similar age-related changes in sleep as humans, aged *drosophila* have reduced total sleep time, decreased arousal threshold, and less recovery sleep following sleep deprivation (Vienne et al. 2016). Interestingly, these age-related changes in sleep are linked to similar mechanisms seen in mice, specifically the UPR (Brown et al. 2014) and that intervening to reduce ER stress with a chemical chaperone serves to consolidate sleep in *drosophila* (Brown et al. 2014). This together indicates that *drosophila* are a validated and useful model to probe age-related changes in sleep and circadian behaviors.

The nematode, *C. elegans*, provides another small invertebrate model system that is useful for studying sleep behaviors. *C. elegans* have a connectome comprising 302 neurons that are fully mapped (Hobert 2003), allowing for precise examination of neurons and neural circuits. Measuring sleep in *C. elegans* consists mainly of video recordings and software to quantify locomotion and posture as a proxy for measuring sleep (Trojanowski and Raizen 2016). *C. elegans* has been reported to demonstrate periods of quiescence analogous to sleep between larval stages, stress, satiety, starvation and hypoxia (Hill et al. 2014; Lawler et al. 2021; McCloskey et al. 2017; Nichols et al. 2017; Raizen et al. 2008; You et al. 2008). One study probed the neural communication between sensory neurons and interneurons during sleep, and showed that sleep alters the link between sensory stimuli and motor neurons, further validating the *C. elegans* model as an effective one for the study of neural circuits involved in sleep behaviors (Lawler et al. 2021).

Interestingly, small invertebrate animal models serve an opportunity to study longevity, given their quick reproductive cycles and shorter lifespans. In *drosophila*, studies have focused on pathways that change with age and if intervening can affect duration of life (Vermeulen and Loeschcke 2007). One study showed that sleep and circadian changes are linked to lifespan, in that *drosophila* mutants for *Bmal1* had a reduced lifespan (Hendricks et al. 2003). More work in *drosophila* demonstrated that functional loss of the interaction between synaptic Homer proteins and the DmGluRA receptor, analogous to mammalian mGluR, resulted in reduced sleep and a shortened lifespan (Ly et al. 2020). Several studies have shown that a chemical chaperone and histone deacetylase (HDAC) inhibitor, 4-phenyl butyrate (PBA), reduced ER stress and extended lifespan in *drosophila* (Brown et al. 2014; Kang et al. 2002). More work supports this idea that proteostasis plays a role in survival and longevity, as another study demonstrated that overexpression XBP1s, part of the pro-survival UPR pathway, increased lifespan by 30% in *C. elegans* (Taylor and Dillin 2013). *C. elegans* provides another appealing model to study lifespan and several studies have linked *C. elegans* lifespan to food and metabolism (Tevy et al.

2013). A study demonstrated lifespan extension in *C. elegans* by reducing food intake (Kaeberlein et al. 2006). Another study showed that the *Sir2* gene, upstream of the insulin-like signaling pathway in *C. elegans*, also extends lifespan (Tissenbaum and Guarente 2001). Together, invertebrate models provide a unique opportunity to study how changes in sleep with age are linked to longevity.

## 24.7 Concluding Remarks

Animal models are a valuable tool with which to study sleep and circadian behaviors. Given the range of techniques and experimental models available, further research using these model systems is likely to elucidate a great deal of information about how sleep and circadian behaviors are regulated and how these change as organisms age. Use of these models in experimental research will provide insight into the age-related changes in sleep and circadian rhythms that occur in humans and inform potential targets for therapeutic intervention.

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# Chapter 25
# Melatonin, Circadian Rhythms, and Sleep: An Opportunity to Understand Mechanisms for Protecting Against Neurodegenerative Disease in *Drosophila*

**Hannah K. Dollish, Kathryn E. R. Kennedy, Michael A. Grandner, and Fabian-Xosé Fernandez**

## 25.1 Brief Introduction to Melatonin

Melatonin (5-methoxy-N-acetyltryptamine; MW: 232.2 Da) was first isolated from bovine pineal gland and structurally defined in 1958 (Lerner et al. 1959). The tryptophan-derived indolamine is synthesized through a common series of enzymatic steps in mammalian pinealocytes starting with the hydroxylation of its amino acid precursor to 5-hydroxytryptophan and subsequent decarboxylation of this product to serotonin. Melatonin is ultimately derived from serotonin by way of catalytic

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conversion with the enzymes arylalkylamine N-acetyltransferase (AANAT) and N-acetylserotonin O-methyltransferase (ASMT; formerly known as hydroxyindole-O-methyltransferase) using one of two possible intermediate pathways in which N-acetylserotonin (NAS) and 5-methoxytrypamine (5-MT) are substrates (Tan and Reiter 2020; Tan et al. 2015). The availability of tryptophan and the activity of these latter two enzymes serve as rate-limiting steps in melatonin production (Tan et al. 2014b; Klein and Moore 1979).

The identification of melatonin and characterization of its synthesis pathways soon led to corollary observations suggesting that melatonin is secreted from the pineal gland according to a circadian rhythm (Arendt and Broadway 1987; Lynch et al. 1975; Weitzman et al. 1978), most likely stemming from variation in AANAT expression, which changes 30- to 70-fold from day to night (Roseboom et al. 1996; Klein and Weller 1970). Findings that established the dramatic dark-phase rise in AANAT, pineal melatonin content, and the resulting circulation of plasma melatonin—along with their regulation by photohistory, acute light exposure, and the prevailing photoperiod—prompted an explosion of study from the 1960s onwards concerning melatonin's involvement in sleep, circadian timekeeping, and seasonality (Reiter 1985). This universal interest overshadowed a circumscribed niche of work that started small in the 1990s but has silently grown to become today's foremost area of melatonin research: melatonin's evolutionarily conserved function as a chemical antioxidant (Tan et al. 2010). In the current chapter, we review some of the canonical roles that have been assigned to melatonin and then describe the elaborate antioxidant ecosystem that it underpins. We end the chapter by suggesting that melatonin's antioxidant functions (1) may provide a treatment opening for several age-related neurodegenerative diseases and (2) might be best studied in *Drosophila*, a time and cost-efficient animal model for which there is currently very little known about melatonin biology.

## 25.2 Melatonin: Phase Marker and Chronobiotic

Circadian rhythms optimize life-sustaining processes such as metabolism and energy utilization. Cells throughout the body house molecular oscillators that time their intracellular activities to nearly 24 h, but their activities across tissues are organized according to a collective physiology that runs on an exact 24 h schedule imposed by the brain's master circadian pacemaker residing in the hypothalamic suprachiasmatic nucleus (SCN) (Golombek and Rosenstein 2010; Stephan and Zucker 1972). To achieve 24 h precision that is phase-aligned with the solar light–dark cycle, the SCN consults a number of zeitgebers (cues from the environment). However, it uses information about ambient light exposure routed directly from the retinohypothalamic tract as its primary means of calibrating humoral and hardwired output signals (Sadun et al. 1984; Moore and Lenn 1972). Among output signals, information about light exposure is relayed from the SCN to the pineal gland through a

well-defined (albeit sprawling) circuit that pivots through the hypothalamic paraventricular nucleus before rising among the superior cervical ganglia (Moore 1996; Teclemariam-Mesbah et al. 1999; Klein et al. 1983a). Using the neurotransmitter noradrenaline, the superior cervical ganglia stimulate melatonin production from the pineal gland while light remains undetected (Klein et al. 1970, 1983b; Sugden et al. 1985). Upon sensing light, this circuit shuts down melatonin synthesis at the level of individual pinealocytes by G protein-coupled noradrenaline-receptor signaling networks that either: (1) interfere with the transcription factors driving AANAT expression or (2) prevent AANAT phosphorylation, thus destabilizing the enzyme and leading to its rapid degradation (Klein and Weller 1972; Klein et al. 1978, 2002; Gastel et al. 1998). It is important to note that light can influence long-term rhythms of melatonin secretion when it is part of a stable photoperiod (where peak duration of circulating hormone will inversely scale with day length) (Kennaway et al. 1983; Rollag and Niswender 1976; Rollag et al. 1978; Wehr 1991, 1996), as well as short-term melatonin patterns when shown acutely during the night phase (Bojkowski et al. 1987; Brainard et al. 2001; Hoban et al. 1990; Kennaway and Rowe 1994; Lewy et al. 1980). In the case of acute nighttime light exposure, the recovery rate of melatonin post-pulse—that is, the time it takes to resume levels consistent with its circadian trajectory—will be species dependent, with rodents being suppressed for longer periods post-pulse than sheep or humans, for instance.

The pineal gland's secretion of melatonin represents a *bona fide* circadian process. Under free-running environmental or biological conditions that unmask the circadian pacemaker's rhythm (e.g., constant darkness in rodents or blindness in humans), the SCN and pineal gland will continue to direct alternating cycles of high and low melatonin secretion across the subjective day and night according to an endogenous schedule set by the animal or person's circadian period (Perlow et al. 1981; Ralph et al. 1971; Takahashi et al. 1980; Skene et al. 1999; Nakagawa et al. 1992a, b; Sack and Lewy 1993). Once unmasked, phase shifts in melatonin rhythms caused by a probe stimulus, such as light exposure, can be visualized in register with shifts that occur in other clock readouts or "phase-markers," including rhythms of cortisol secretion and core body temperature (Maeda and Lincoln 1990; Reppert et al. 1981; Broadway et al. 1987; Shanahan and Czeisler 1991; Laakso et al. 1993; Kennaway et al. 1987). The melatonin rhythm, in fact, is one of the most resilient indicators of the pacemaker's circadian phase position. Circulating levels of the hormone are not significantly influenced by food consumption, unlike other markers, which can be distorted by excessive carbohydrate intake (Pandi-Perumal et al. 2007; Krauchi et al. 2002). What's more, while nearly all circadian readouts are influenced by environmental illumination, proper dim light conditions (<10 lx) leave melatonin's daily secretion pattern intact. The melatonin rhythm's robustness is equally evident in plasma and saliva (where hormone concentrations are highly correlated (Leibenluft et al. 1996)), and in each of these biological fluids, only the *onset* of melatonin secretion preceding sleep needs to be quantified to assess small differences in circadian phase caused by internal or external stimuli; further precision will not be gained if one attempts to quantify the entire overnight melatonin profile. Because of these properties, measurement of the dim light melatonin onset (or DLMO) represents the

gold standard assessment many researchers use to infer the pacemaker's endogenous phase position (Lewy and Sack 1989; Lewy et al. 1999).

The melatonin system exhibits a unique property relative to other biological systems that are typically tracked during sleep and circadian experiments. The pineal gland's secretion of melatonin is under circadian control. However, once secreted, circulating melatonin can feedback onto the SCN to regulate its own rhythm as well as provide a larger entrainment cue for other physiological rhythms (Lewy and Sack 1997). Melatonin's properties as an internal zeitgeber are mediated by melatonin receptors expressed by SCN neurons (Dubocovich et al. 2005). Both major classes of melatonin receptor ($MT_1$ and $MT_2$) have been located to the mammalian SCN—including the SCN of humans (Lacoste et al. 2015; Song et al. 2000; Wu et al. 2006; Weaver et al. 1989; Rivkees et al. 1989; Reppert et al. 1988; Liu et al. 1997). Their activation is thought to be tied to a reset of electrophysiological output caused by changes in the molecular clock mechanism, in particular inhibition of the stabilizing proteasome for Bmal1 (i.e., a positive element in the circadian transcription-translation feedback loop) (McArthur et al. 1991). Evidence for melatonin being able to entrain biological rhythms, or at least entrain the sleep/wake rhythm, was first observed in experiments looking at supplemental dosing in rodents. In individually housed, free-running rats maintained under constant darkness, Redman and colleagues found that daily systemic injections of melatonin (1 mg/kg) entrained locomotor rhythms if the injections were timed to the approximate start of the circadian active phase (Redman et al. 1983). From these initial observations, additional studies in rats and mice confirmed that timed regimens of melatonin as low as 2–5 μg/kg could resynchronize circadian patterns of behavior and physiology (e.g., running wheel activity, water drinking, and body temperature) following placement of animals under isolated conditions with constant exposure to either darkness or light (Benloucif and Dubocovich 1996; Cassone et al. 1986a; Chesworth et al. 1987; Sharma et al. 1999; Thomas and Armstrong 1988). Moreover, exogenous melatonin was all that was necessary for entrainment to take hold; rodents with lesions of the pineal gland still entrained to daily melatonin injections and did so in a dose-dependent manner (Redman and Francis 1998; Warren et al. 1993; Schuhler et al. 2002). On the other hand, animals without intact SCN could not entrain (Cassone et al. 1986b; Redman and Francis 1998). Melatonin entrainment by way of the central pacemaker appears to be a fixture in metazoan evolution, as melatonin injections have been shown to organize the circadian rhythms of multiple amphibian and reptile species (Foa et al. 2002; Underwood and Harless 1985; Underwood 1986; Hyde and Underwood 1995).

The aforementioned corpus of work in laboratory animal models was soon complemented by a parallel line of work suggesting that melatonin also operated as a zeitgeber in humans. The first suggestions of this zeitgeber action were alternatively provided by Armstrong (Armstrong et al. 1986) and Arendt (Arendt and Broadway 1987; Deacon et al. 1994; Middleton et al. 1997), but subsequent investigations by Lewy and Sack defined a now universally acknowledged phase-response curve (PRC) that described the direction and magnitude of phase shifts caused by melatonin administration at different times of day (Lewy et al. 1992, 1995, 1996, 1998). The human melatonin PRC evinces a nonresponsive dead zone in the first half

of the night when endogenous melatonin secretion from the pineal gland is typically high. On either side of this dead zone are opposing regions where melatonin exposure will cause a phase advance or phase delay. Advances occur when administration is timed 2–7 h prior to DLMO in the afternoon, while delays occur after administration in the late-night or early-morning hours surrounding usual endogenous melatonin offset (Lewy and Sack 1997). Shifts in the pacemaker's circadian phase prompted by exogenous melatonin are reflected not only in the endogenous melatonin rhythm but also in the resetting of rhythms associated with cortisol secretion, core body temperature, and the sleep/wake cycle (Hashimoto et al. 1998; Dahlitz et al. 1991; McArthur et al. 1996; Lockley et al. 2000; Sack and Lewy 1997; Middleton et al. 1997; Hayakawa et al. 1998). The zeitgeber strength of melatonin in humans is sufficiently strong that it can capture and entrain the free-running circadian rhythms of totally blind individuals without access to light cues (Sack et al. 2000; Lewy et al. 2001). In such protocols, melatonin is given at a dose ranging between 0.5 and 10 mg one hour before habitual bedtime for several weeks. Melatonin can also be employed as an entrainment tool for individuals with non-24 h sleep–wake disorder (Non-24) (McArthur et al. 1996). While supplemental melatonin is recommended to treat Non-24 in both sighted and blind individuals (Standards of Practice Committee of the American Academy of Sleep Medicine), dual melatonin receptor agonists with high affinity for $MT_2$ have also been FDA approved for this indication. Animal studies suggest that $MT_2$ is the receptor subtype mediating melatonin's phase-shifting effects in the SCN (Hunt et al. 2001; Dubocovich et al. 1998). Most studies of Tasimelteon in patients with Non-24 indicate significant variability in the drug's efficacy, with success rates from 20 to 67% (Lockley et al. 2015). An important future question in melatonin research concerns identifying the individual-level health factors that might contribute to melatonin's ability or inability to phase-lock the central pacemaker's rhythm.

## 25.3 Melatonin's Role in Sleep

Many biological roles for melatonin have been highlighted since its discovery but none have been more widely discussed than its putative action as a hypnagogic hormone. Shortly after its characterization, melatonin was shown to be produced and secreted exclusively during the dark phase of the 24 h cycle. Examination of this secretion profile yielded a conspicuous relationship between the trajectory of circulating hormone and sleep: The nocturnal rise of melatonin preceded habitual bedtime, and levels peaked in the middle of the sleep period and then dropped precipitously around the time one usually woke up (Lavie 1997). Early studies in animals further suggested that melatonin triggered sleep after direct infusion into the preoptic regions of the hypothalamus, which function as important centers for sleep initiation in the mammalian brain (Marczynski et al. 1964). The narrative association between melatonin and sleep was ostensibly sealed in the early 1970s when multiple groups

reported that intravenous injections shortened sleep latency in neurotypical young adults without insomnia (Anton-Tay et al. 1971; Cramer et al. 1974).

The clinical work that has populated the literature since the 1970s has clarified a different role for melatonin in sleep—one that does not involve direct hypnotic actions (Mendelson 1997a; b). The lack of a direct effect is exemplified by several studies using polysomnography (PSG) or subjective self-report in cohorts with and without insomnia. Several of these studies used a randomized, double-blind design. By and large, oral nighttime administration of melatonin (upwards of 100 mg) before bedtime does not statistically change traditional indices of hypnotic efficacy, such as the latency to sleep onset (Pires et al. 2001; James et al. 1987, 1990; Ellis et al. 1996; Zhdanova et al. 2001; Almeida Montes et al. 2003), sleep duration (James et al. 1987, 1990; Ellis et al. 1996; Zhdanova et al. 2001; Almeida Montes et al. 2003), nor the amount of time spent awake after falling asleep (Stone et al. 2000; Ellis et al. 1996; Cajochen et al. 1997a; Zhdanova et al. 2001; Almeida Montes et al. 2003). PSG metrics related to slow-wave sleep (SWS), rapid-eye movement (REM) sleep, sleep architecture (time spent or transitions between non-REM and REM), and sleep continuity measures also show little change (Ferini-Strambi et al. 1993; Zhdanova et al. 1996; James et al. 1987, 1990; Cajochen et al. 1997a; Almeida Montes et al. 2003). Subjective assessments of sleep quality may or may not improve (Stone et al. 2000), but when they do can be attributed to placebo effects (half of a sample may report better sleep quality but might be unable to distinguish the treatment arm in which they received melatonin (Ellis et al. 1996)). It is important to note that a few of the investigations examining the effects of melatonin administration at bedtime are mixed with regard to the hormone's influence on nighttime sleep. Sleep efficiency or wake after sleep onset might be improved, for instance, but not measures of sleep latency or total sleep time (Garfinkel et al. 1995; Zhdanova et al. 2001; Waldhauser et al. 1990). Where available, statistically significant effects of melatonin are rarely clinically meaningful. Bedtime administration may change a measure of sleep efficiency or continuity but not do so to a degree that would likely affect perceptions of sleep quality (Attenburrow et al. 1996).

Unlike traditional hypnotics, there is no variable of sleep for which melatonin consistently demonstrates dose–response characteristics (e.g., the higher the dose, the longer the sleep duration or the better its consolidation) (Roth and Richardson 1997). This makes sense: By physiological standards, base circulating melatonin is already high at night. Even at several-gram doses that force blood levels to concentrations over 1000-fold above circulating levels, exogenous melatonin never produces an involuntary loss of consciousness. Some individuals may not even feel sleepy at these doses (Waldhauser et al. 1990). Consequently, the logic for why elevations in nighttime melatonin from exogenous sources might enhance sleep quality or quantity remains elusive beyond the circumstantial relationship between nighttime melatonin secretion and the timing of human sleep. From a broader ecological/biological perspective, the concept of melatonin as a sleep hormone ignores its association with wake and activity in nocturnal species (Sack et al. 1997).

An alternative perspective vis-à-vis melatonin and sleep begins to emerge when one considers human experiments that have timed melatonin administration specifically to the late afternoon or early evening several hours before habitual bedtime (Arendt 2000; Luboshizsky and Lavie 1998). When taken during this time block—one where circulating endogenous melatonin is low or absent—exogenous melatonin (1) triggers fairly consistent reductions in arousal (moderate sedation, feelings of tiredness) (Cajochen et al. 1996; Dollins et al. 1994; Mishima et al. 1997), (2) shortens sleep latency when a nap opportunity is provided (Reid et al. 1996; Dollins et al. 1994; Nave et al. 1995; Hughes and Badia 1997), and (3) produces hypnotic-like daytime cognitive impairments (Lieberman et al. 1984; Dollins et al. 1994). These effects have been associated with reductions in core body temperature as well as spectral power changes in the delta, theta, and spindle bands (Cajochen et al. 1996, 1997b, 2003; Reid et al. 1996; Dollins et al. 1994; Mishima et al. 1997; Hughes and Badia 1997). Oral melatonin at doses that place plasma levels at the range of endogenous melatonin or just several levels above (so-called low pharmacological doses; 0.3–5 mg) likely increases sleep by virtue of its daytime chronobiotic properties, which has the net effect of phase-advancing sleep propensity (Shochat et al. 1997). In that way, administration during the advance zone of the melatonin PRC enables the hormone to function as both chronobiotic and soporific (Wirz-Justice and Armstrong 1996).

The soporific and temperature effects of daytime melatonin administration open the door to a valuable perspective on what the hormone's role might be during the nighttime hours that contextualize human sleep. Normally, melatonin secretion steadily rises an hour or two before habitual sleep onset. Once synthesized, the hormone's high water and lipid solubility allow it to pass easily across cell membranes, gaining access to various biological fluids and tissues, including the cerebrospinal fluid, plasma, saliva, and tissue compartments across the periphery and brain (Shida et al. 1994; Costa et al. 1995; Reiter et al. 2013; Menendez-Pelaez and Reiter 1993; Venegas et al. 2012; Reiter 1991). Melatonin effectively permeates the body throughout the pre-sleep period and the first several hours of slumber. In doing so, the function of melatonin in sleep reveals itself: Elevations in circulating hormone signal a wholesale biological shift toward quiescence, integrating energy conservation strategies, and restorative processes that are best optimized when a diurnal organism is at rest (Saarela and Reiter 1994; Zisapel 2007).

Melatonin accumulation impacts a number of activities linked to digestion, including within the gut, where the hormone binds to $MT_1$ receptors to augment contractility (Ahmed et al. 2013). Across the periphery, it also instructs metabolism. Insulin sensitivity is ordinarily high during the daytime when melatonin levels are low, corresponding with wakefulness, vigorous energy demand, and food intake. Upon reaching peaks in physiological concentration, circulating melatonin increases insulin resistance at night while the body enters a period of fasting and slowing metabolism (Kampmann et al. 2021), thus optimizing energy balance at a time when endogenous stores are mobilized in lieu of calorie consumption. In that way, melatonin functions as a linchpin in maintaining the internal circadian synchronization/alternation that occurs between states of (1) activity/feeding and (2) rest/fasting

(Green et al. 2008). These states fall perfectly in line during entrainment with the solar light–dark cycle, which imposes a daily rhythm of energy harvesting and energy storage exemplified by cycles of photosynthesis in plants that create corresponding cycles of chemical energy transfer across food webs in animals. Decades of experimental evidence substantiate melatonin's role in metabolism. From the very first studies in animal models, it was clear that infusion of pineal extracts (and later melatonin itself) led to hypoglycemia, increased glucose tolerance, and hepatic and muscular glycogenesis after glucose loading (Vinogradova and Anisimov 2013; Wolden-Hanson et al. 2000; McMullan et al. 2013; Milcu et al. 1963; Csaba and Barath 1971; Diaz and Blazquez 1986). On the other hand, removal of circulating melatonin via pinealectomy flips these metabolic processes and causes a diabetogenic syndrome (Cipolla-Neto et al. 2014; la Fleur et al. 2001; Rodriguez et al. 1989; Mellado et al. 1989; Lima et al. 1998; Nogueira et al. 2011). Melatonin receptors are notably expressed within the vasculature (Viswanathan et al. 1990). Binding of melatonin to these sites at night causes vasodilation and lowers blood pressure (Zhao et al. 2017; Baker and Kimpinski 2018; Simko et al. 2013), thus providing a putative thermoregulatory valve that offsets heat production from increased breakdown of sugars and fats. Against this backdrop, quiescence is facilitated by melatonin's promotion of smooth muscle contraction in the airways at night (Sasaki et al. 2021).

Stepping back to look at the bigger picture, it comes as no surprise that many of the neural circuits involved in sleep, metabolism, and thermoregulation in the hypothalamus (Rothhaas and Chung 2021) are affected by melatonin and collaborate to shape the brain's sleep architecture (Cagnacci et al. 1997). During sleep, thermosensitive neurons within the hypothalamus signal to wake-promoting regions of the brain to coordinate cycles of non-REM sleep, REM sleep, and wake (Krilowicz et al. 1994). Non-REM tends to occur during the first half of the night when body temperature is lowest, synchronized with maximal melatonin secretion. As morning approaches, sleep becomes richer in REM, corresponding with spikes in sympathetic modulation and a gradual elevation in body temperature as melatonin declines (Boudreau et al. 2013). Far from the "darkness" or "tranquilizing" hormone it was originally envisioned to be, melatonin appears to be more of an organizing agent helping to tie together the elaborate physiologies that connect circadian changes in sleep/wake to those in metabolism and thermoregulation, thereby providing a clearing—a recurring biological context—set aside for recovery processes such as those described below.

## 25.4 A Primer on Reactive Oxygen Species

To appreciate melatonin's extraordinary reach as an antioxidant, one must first understand why and how oxygen byproducts are generated and neutralized in healthy cells. Aerobic organisms assimilate dioxygen ($O_2$) from the atmosphere to drive adenosine triphosphate (ATP) energy production from the mitochondrial respiratory chain (Tan et al. 2000b; Nathan and Singer 1999). While the vast majority of $O_2$ is consumed efficiently during this process (i.e., safely converted to water via

four sequential reductions), approximately 5% is only partially reduced, creating a series of reactive oxygen species (ROS) among which are free radicals containing an unpaired valence electron (Reiter 1998b; Kehrer 1993; Reiter et al. 2002b). $O_2$ is reduced one step at a time in a biological setting (Malmstrom 1982). Ergo, there are 3 possible ROS intermediates that can be generated during respiration: superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (•OH) (Reiter 1998b; Manchester et al. 2015). These intermediates are disruptive to the intracellular milieu to varying degrees but can trigger chain reactions that propagate molecular damage when they contact deoxyribonucleic acid (DNA), proteins, or lipids (Reiter et al. 2001c; Reiter 1998a). The interaction renders these biomolecules into secondary radicals that behave identically to their precursors.

Because ROS production became invariably connected to life-sustaining ATP production, organisms evolved enzymatic and non-enzymatic strategies to neutralize them by speeding their conversion toward water. Mitochondrial $O_2^{\bullet-}$ is the most significant source of intracellularly generated ROS (McCord and Omar 1993; Ames et al. 1993). The acceptance of a single electron by $O_2$ generates $O_2^{\bullet-}$ (Liochev and Fridovich 1994), which is metabolized to $H_2O_2$ by an enzymatic family of superoxide dismutases (SODs) (Fridovich 1983; McCord and Fridovich 1969; Fridovich 1975). Catalytic conversion of $O_2^{\bullet-}$ is especially critical under pathological conditions that cause acidosis like ischemia. $O_2^{\bullet-}$ is a base that readily accepts protons. In doing so, it forms the hydroperoxyl radical $HO_2^{\bullet}$, a much more lipid-soluble and powerful oxidizing agent that can damage the integrity of cellular membranes (Larosa and Remacle 2018). Other enzymes besides SOD also produce $H_2O_2$ as a byproduct of their activity, including monoamine oxidase (Simonson et al. 1993), L-amino acid oxidase (Izidoro et al. 2014), and glycolate oxidase (Li et al. 2021). The pool of $H_2O_2$ thus extends not just from mitochondrial respiration but also other processes linked to neurotransmitter recycling and detoxification. As $H_2O_2$ accumulates (half-life >4 s), it is not particularly reactive in vivo relative to other ROS, but readily crosses cell membranes, migrates to sites distant from where it was originally generated, and provides a reservoir for more damaging ROS (Reiter 1995; Reiter et al. 2002b). In the presence of cellular transition metals $Fe^{2+}$ and $Cu^{1+}$ (Floyd and Carney 1993), $H_2O_2$ is reduced to the dangerous hydroxyl radical (•OH) via the Haber–Weiss and Fenton reactions (Wang et al. 2021; Zeng et al. 2019; Reiter et al. 1995). These reactions are usually side-stepped by first converting $H_2O_2$ to water with two antioxidative enzymes, catalase (Dai et al. 2017) and glutathione peroxidase (GPx), which hold steady-state $H_2O_2$ levels in check at approximately $10^{-9}$–$10^{-7}$ M (Chance et al. 1979; Tan et al. 2000b). Glutathione is required for GPx to detoxify $H_2O_2$, resulting in the molecule's oxidation to glutathione disulfide (GSSG) (Brigelius-Flohe and Maiorino 2013). Another enzyme, glutathione reductase, recycles GSSG back to glutathione (Wu et al. 2004).

High cytotoxicity is observed in cases where •OH is converted from $H_2O_2$. The redox potential of •OH is more positive than any substance in a living cell (+2.31 V) (Allegra et al. 2003). Hence, •OH reacts rapidly with every macromolecule in the vicinity of its production, readily damaging nucleic acids in its wake when $H_2O_2 \rightarrow$ •OH conversion is primed by (1) transition metals bound to molecules situated close

to mitochondrial or nuclear DNA or (2) intracellular $Ca^{2+}$ spikes from pathological neural firing that activate nuclease enzymes (e.g., observed during seizures and stroke) (Pappolla et al. 1999; Halliwell et al. 2021). Besides its destructive actions on DNA, which involve electron transfers from guanosine nucleosides (Chatgilialoglu et al. 2021), •OH can also warp proteins and membrane lipids. When the radical attacks the amino acid residues of protein molecules, it induces extensive protein–protein cross-linking (Stadtman 1992). Within membrane lipids, it initiates lipid peroxidation, a self-propagating "branching" reaction started when •OH removes an $H^+$ from the side chain of the first membrane polyunsaturated fatty acid it contacts (i.e., the first peroxyl radical formed then snatches a hydrogen atom from the next fatty acid side chain, forming a second peroxyl radical and so on) (Niki et al. 1993; Cheeseman 1993; Pisoschi and Pop 2015). Once snowballing, this reaction chain may leave neurons especially vulnerable because their membrane phospholipids are enriched with easily oxidized polyunsaturated fatty acids such as linoleic and arachidonic acid (Rice-Evans and Burdon 1993). While there are no intracellular or extracellular enzymes of which we are aware that detoxify •OH (more than likely because the free radical would destroy them), mitigation strategies that directly scavenge •OH are available via dietary consumption of low molecular-weight chemical antioxidants such as vitamin C and vitamin E, a fat-soluble compound that concentrates within the hydrophobic interior of membranes. During cytotoxic events, vitamin E (in the form of α-tocopherol) is thought to be one of the major chain-breaking scavenger antioxidants maintaining regulated ion transport across cellular membranes and oxidative phosphorylation through the mitochondria (Miyazawa et al. 2019). Upon donating an electron, the vitamin E radical is inert and is eventually recycled to its non-radical form by ascorbate (McCay 1985).

Not all free radicals are oxygen-based or byproducts of mitochondrial respiration. For instance, nitric oxide ($NO^{\bullet}$) is a gaseous messenger used to convey retrograde and anterograde signals between cells. The molecule is synthesized endogenously from L-arginine with specific isoforms of nitric oxide synthase expressed in either neurons (neuronal NOS) or the vascular inner lining (endothelial NOS) (Gantner et al. 2020). Owing to its gaseous/hydrophobic nature, $NO^{\bullet}$ easily passes through cellular membranes and interacts with other radical species (Hardeland 2021). When it builds to unusually high concentrations, the gaseous molecule reacts quickly at diffusion-controlled limits with superoxide anion radical ($O_2^{\bullet -}$) to form peroxynitrite ($ONOO^-$), a toxic nitrogen intermediate functioning as both a nitrating agent and strong oxidant (Pryor and Squadrito 1995). The reaction of $NO^{\bullet}$ with $O_2^{\bullet -}$ is threefold faster than the dismutation rate of $O_2^{\bullet -}$ clearance by superoxide dismutase (Crow and Beckman 1995). Moreover, the toxicity of the ensuing product, $ONOO^-$, rivals that of •OH, presenting a danger to all major classes of biomolecule and overall cellular physiology through its irreversible inhibition of the mitochondrial electron transport chain (Pacher et al. 2007). Once propagating, the deleterious effects stemming from $ONOO^-$ can be long-lived. $ONOO^-$ is a relatively stable molecule and in the presence of iron- and copper-containing metalloproteins it eventually decomposes to •OH through homolytic cleavage of peroxynitrous acid (Radi 2018).

## 25.5 Melatonin: Innerworkings of a Powerful Antioxidant System

Oxidative phosphorylation is a highly efficient process worth protecting: Mitochondria can generate up to 30 molecules of ATP from each molecule of glucose oxidized into pyruvate. Owing to incomplete $O_2$ consumption, ROS are leaked during energy production, presenting a necessary cost balance to these efficiency benefits (Reiter et al. 1994). Working in the background, melatonin is one of the oldest countermeasures used in biological systems to neutralize ROS (Tan et al. 2010; Hardeland et al. 1995). It does so through a cascading series of actions as a direct free radical scavenger, preventative antioxidant, transition metal chelator, and lever arm for enzymatic antioxidative pathways (Galano and Reiter 2018).

The electron-rich parent structure of melatonin serves as a powerful broad-spectrum chemical scavenger, neutralizing (1) all three major oxygen side products ($O_2^{\bullet -}$, $H_2O_2$, and •OH) (Pahkla et al. 1998; Horstman et al. 2002; Stasica et al. 2000; Li et al. 2002; Matuszak et al. 1997; Tan et al. 2000a; Carampin et al. 2003; Zang et al. 1998; Sewerynek et al. 1995b), (2) radical nitrogen intermediates such as $NO^{\bullet}$ and $ONOO^-$ (Mahal et al. 1999; Noda et al. 1999; Escames et al. 1997; Guerrero et al. 1997; Gilad et al. 1997; Blanchard et al. 2000; Zhang et al. 1999a), and (3) the peroxyl radicals that form during lipid peroxidation (Antunes et al. 1999; Marshall et al. 1996; Pieri et al. 1994, 1995; Mayo et al. 2003a; Melchiorri et al. 1996; Daniels et al. 1995; Sewerynek et al. 1995a). For the more aggressive radicals, like •OH, each molecule of melatonin can detoxify two radicals (Seegar et al. 1997) with a rate constant ranging from $1.2 \times 10^{10}$ to $0.6 \times 10^{11}$ $M^{-1}$ $s^{-1}$ (i.e., with the same or, in some cases, greater rapidity than other scavengers, including N-acetylcysteine, β-carotene, and glutathione; (Reiter et al. 2001c)). Melatonin's scavenging activities are demonstrable in many subcellular compartments, protecting the nucleus from DNA oxidation and strand breaks (Tan et al. 1993, 1994; Davanipour et al. 2009; Fischer et al. 2008; Sliwinski et al. 2007; Romero et al. 1999; Cabrer et al. 2001; Yamamoto and Mohanan 2001a; Qi et al. 2000; Sewerynek et al. 1996; Perez-Gonzalez et al. 2019; Lai and Singh 1997; Morioka et al. 1999; Shaikh et al. 1997; Karbownik et al. 2000; Susa et al. 1997), the mitochondria from disturbances in the respiratory chain (Mohanan and Yamamoto 2002; Yamamoto and Mohanan 2002; Reiter et al. 2018a, b; Hardeland 2017), and the primary cell membrane from reductions in fluidity brought about by branching reactions (Pieri et al. 1994; Garcia et al. 1997, 2014).

Several products are formed when melatonin scavenges ROS (Reiter et al. 2002a; Tan et al. 2007). Cyclic 3-hydroxymelatonin (c3OHM) results from melatonin's neutralization of •OH (Tan et al. 1998a, 1999). $N^1$-acetyl-$N^2$-formyl-5-methoxykynuramine (AFMK) is yielded after further oxidation of c3OHM or after melatonin's neutralization of the early oxygen reactants, $O_2^{\bullet -}$ or $H_2O_2$ (The equilibrium between c3OHM and AFMK thus depends on the ratio of oxidants to melatonin.) (Tan et al. 2001; Rozov et al. 2003; Silva et al. 2005). Upon production, AFMK can be oxidized or enzymatically transformed by catalase to a third-generation metabolite,

$N^2$-acetyl-5-methoxykynuramine (AMK) (Hirata et al. 1974; Zang et al. 1998). In the case of radical nitrogen intermediate reactions, 6-hydroxymelatonin is one of several compounds yielded from melatonin's quenching of $ONOO^-$. Others include 2-hydroxymelatonin, c3OHM, 1-nitromelatonin, and 1-nitrosomelatonin (Reiter et al. 2002a).

Remarkably, metabolites of scavenging melatonin also function as antioxidants themselves, including c3OHM, AFMK, and AMK (Hardeland et al. 2009; Reiter et al. 2007; Galano et al. 2013). These primary, secondary, and tertiary metabolites are referred to as melatonin's "antioxidant cascade," enabling one 232.28 Da parent molecule of melatonin to neutralize up to 10 radical products. Such a ratio has little precedence (if any) in biological systems. By comparison, classic free radical scavengers typically neutralize a single ROS per circulating molecule invested. c3OHM has been shown to act through hydrogen atom and single electron transfer reaction mechanisms, which are important for preventing damage from oxidative stress as well as repairing damage to other antioxidants (Hardeland 2005). This versatility allows c3OHM to effectively scavenge •OH and hydroperoxyl radical at diffusion-limited rates, as well as to inhibit/reverse ROS modifications of DNA (Tan et al. 2014a). AFMK quenches $O_2^{\bullet -}$, •OH, and radical nitrogen species with a potency similar to melatonin, while also mitigating the effects of a variety of oxidative conditions on DNA and lipids (Tan et al. 2001; Burkhardt et al. 2001; Manda et al. 2007; Onuki et al. 2005). Data suggest that AMK, the last element of melatonin's antioxidant cascade, has an even greater capacity to neutralize and reduce the deleterious effects of ROS (Maharaj et al. 2002; Ressmeyer et al. 2003; Galano et al. 2013). It is 1.6-fold better than melatonin at scavenging singlet oxygen, for example, and a staggering 150-fold better than AFMK (Schaefer and Hardeland 2009). Adding to these multigeneration metabolites are melatonin's direct precursors, NAS and 5-MT, which are lesser known but equally important members of melatonin's antioxidant ecosystem (Garcia et al. 2001; Longoni et al. 1997; Ng et al. 2000; Wolfler et al. 1999).

Members of the melatonin antioxidant network bind heavy metals in addition to ROS, providing a complementary protective mechanism that prevents •OH formation via sequestration or removal of both metal and $H_2O_2$ precursors (Gulcin et al. 2003; Mayo et al. 2003b). The chelating action of melatonin is likely mediated through a coupled-deprotonation-chelation mechanism and is seen not only with prevalent transition metals (i.e., $Fe^{2+}$ and $Cu^{1+}$) but with many metals that may have natural origins in the body or act as environmentally derived toxins (so-called xenobiotics) (Galano et al. 2015; Esparza et al. 2003, 2005; Gomez et al. 2005; Said et al. 2021; Limson et al. 1998; Parmar et al. 2002; Lin and Ho 2000; Kim et al. 2000). For example, computational studies suggest that melatonin, c3OHM, and AMK form spontaneous complexes with lead (Pb), with the latter two metabolites possessing sufficient electronic and thermodynamic properties to act as clinically relevant lead-trapping agents (Diaz-Cervantes et al. 2019). Quantum mechanical modeling further suggests that the pool of melatonin and its metabolites might represent the body's most significant natural source of metal-chelating potential (Galano et al. 2015), a proposition strengthened by the fact that other metal-binding redox balance proteins, such as metallothioneins (Babula et al. 2012), are damaged by free radicals under

conditions of oxidative stress. The melatonin family's chelating abilities have been confirmed in basic molecular protection studies (e.g., reduction of iron and copper-induced lipid peroxidation and neurodegeneration), preclinical studies establishing that melatonin can buffer against the formation of toxic β-amyloid-metal aggregates (Zatta et al. 2003), and clinical studies in humans showing that melatonin supplementation can ameliorate the oxidative stress that occurs when anemic patients are treated with intravenous iron (Herrera et al. 2001).

Melatonin provides one last layer of defense against ROS by modifying the activity of enzymes that metabolize reactive oxygen and nitrogen species to inactive products (Rodriguez et al. 2004; Mayo et al. 2002). Under baseline physiological conditions, the nocturnal rise in circulating melatonin produced by the pineal gland is associated with a concurrent nighttime increase in the activities of SOD (dismutation of $O_2^{\bullet -}$ to $H_2O_2$), GPx ($H_2O_2$ conversion to water), and glutathione reductase (GRd; replenishment of glutathione) (Albarran et al. 2001; Pablos et al. 1998). The marked nighttime increase in GPx is prevented when animals are exposed to constant light, suggesting that increased antioxidant signaling does not occur without melatonin secretion (Pablos et al. 1998). In keeping with these trends, administration of exogenous melatonin enhances the mRNA expression of SOD and GPx, potentiates their activity, and promotes the de novo synthesis of glutathione by stimulating its rate-limiting enzyme, gamma-glutamylcysteine synthase (Pablos et al. 1995; Barlow-Walden et al. 1995; Ozturk et al. 2000; Fischer et al. 2013; Okatani et al. 2000; Kotler et al. 1998; Esparza et al. 2005; Ding et al. 2014; Antolin et al. 1996; Gomez et al. 2005; Baydas et al. 2002; Liu and Ng 2000; Martin et al. 2000; Urata et al. 1999). The co-factor for GRd, nicotinamide adenine dinucleotide phosphate (NADPH), might also be replenished via the reported stimulatory action of melatonin on glucose-6-phosphate dehydrogenase (G6PDH) (Hajam and Rai 2019). An extended literature documents melatonin's broad influence over antioxidant and prooxidant enzyme systems under constitutive and pathological conditions, including processes related to brain injury (Baydas et al. 2006; Tunez et al. 2003), cortical-hippocampal excitotoxicity (Floreani et al. 1997), heavy metal poisoning (Esparza et al. 2005), ultraviolet radiation damage (Fischer et al. 2013), and diabetes (Vural et al. 2001). Alongside the upregulation of antioxidative defense systems is down-regulation of pathways promoting free radical generation. Melatonin and its metabolites are significant inhibitors of NOS activity and $NO^{\bullet}$ production outside their roles as direct $NO^{\bullet}$ scavengers (Pozo et al. 1994, 1997, 1998; Bettahi et al. 1996; Leon et al. 1998), with such inhibition being shown to curtail the inflammatory challenges associated with carrageenan and lipopolysaccharide exposure (Cuzzocrea et al. 1997; Crespo et al. 1999). Suppression of NOS activity might be mediated through melatonin's binding and sequestration of calmodulin (Pozo et al. 1997; Tomas-Zapico and Coto-Montes 2005), a key factor regulating NOS function. The mechanisms behind melatonin's control over other antioxidative enzymatic pathways are not currently understood but may involve inhibition of Nrf2 degradation (Ding et al. 2014). Nrf2 (Nuclear factor-erythroid factor 2-related factor 2) is a transcription factor controlling the expression of an array of antioxidants, detoxifying proteins, and xenobiotic transporters in response to oxidative stress. It does so by binding antioxidant response elements (abbreviated ARE), which are

enhancer sequences embedded within the promoter regions of many cytoprotective proteins (Raghunath et al. 2018).

## 25.6 Melatonin: A Functionally Relevant Antioxidant

Melatonin is an amphiphilic molecule, allowing it to freely traverse the body, cross many macro-physiological barriers and cellular membranes, and to concentrate in subcellular compartments. Within minutes of its intraperitoneal or subcutaneous injection, melatonin is already detectable at high concentrations in the brain (Paterniti et al. 2016). These properties make the melatonin molecule—outside the contribution of a signal transduction agent like a receptor—a powerful tool for maintaining the homeostatic environment needed around the production and neutralization of free radicals. Melatonin likely contributes to ROS homeostasis in an opportunistic fashion. It is rhythmically secreted into the bloodstream at night. However, levels appear to be consistently higher in the cerebrospinal fluid relative to plasma (Hedlund et al. 1977), in lock-step with the increased metabolic demand (and ROS output) of the brain's neurons, glia, and vasculature, which use 20% of the body's collective $O_2$ supply (Halliwell 1992). Within individual cells, mitochondria are the primary sites of energy and ROS production (Cardinali et al. 2013). Not surprisingly, it is here that melatonin is most densely concentrated (Venegas et al. 2012), though appreciable levels of the indolamine are also detectable in many cellular nuclei (Menendez-Pelaez and Reiter 1993; Acuna-Castroviejo et al. 1994; Mennenga et al. 1991; Coto-Montes et al. 2003; Menendez-Pelaez et al. 1993), where melatonin's antioxidant properties might safeguard the genome's stability and cell cycle control (Finocchiaro and Glikin 1998).

Complementing this widespread biological distribution is the evolutionary conservation of melatonin between plants and animals. Melatonin survived evolution without any chemical structural modifications, is highly concentrated in edible plants (e.g., in the μg/g range of some fruits, vegetables, seeds, nuts, and medicinal herbs; (Hattori et al. 1995; Dubbels et al. 1995; Murch et al. 1997; Manchester et al. 2000)), and is readily available from plant foodstuffs (Tan and Reiter 2020). When plants containing melatonin are ingested, it materially affects plasma melatonin levels (Reiter et al. 2001b, 2005; Garrido et al. 2010; Sae-Teaw et al. 2013), whose circulation correlates with the total antioxidant capacity of the blood (Benot et al. 1998, 1999). Melatonin is biosynthesized within plant mitochondria and chloroplasts, enabling them to combat abiotic and biotic stressors (Arnao and Hernandez-Ruiz 2015; Zhao et al. 2019; Tan et al. 2013). In the larger scheme of the feeding/fasting cycle, the antioxidative actions of melatonin thus appear to be passed from one organism to the next in an environment with commonly shared challenges, including heat, drought, and soil contamination (e.g., with heavy metal). At the top of the food chain, humans unduly benefit from their brain's endogenous production of melatonin as well as the aggregate synthesis of melatonin across the ecosystems in which they inhabit.

Widespread neuroprotective effects are conferred by melatonin's potent and biologically accessible antioxidant properties. For example, systemic dosing of melatonin mitigates the fallout ensuing from cerebral models of focal ischemia/reperfusion injury. When administered near the onset of ischemia or before reperfusion in carotid artery and middle cerebral artery occlusion stroke models, exogenous melatonin reduces: (1) oxidative stress markers associated with depletion of glutathione, elevated malondialdehyde, and elevated myeloperoxidase (El-Abhar et al. 2002; Sinha et al. 2001; Cuzzocrea et al. 2000); (2) the resulting infarct volume measured by histochemical methods or early phase visualization techniques such as diffusion-weighted and magnetic resonance imaging (Pei et al. 2002a, b, 2003; Sinha et al. 2001; Kondoh et al. 2002); (3) immunohistochemical indices of cell survival in the ischemic penumbra as well as the core (Borlongan et al. 2000; Cho et al. 1997); and (4) neurological scores reflecting an animal's ability to walk, right themselves, and extend their limbs (Cuzzocrea et al. 2000; Wang et al. 2009; Sinha et al. 2001). Pinealectomized animals, by contrast, show aggravated stroke outcomes, developing more signs of oxidative stress, larger infarcts, and more memory problems than sham-pinealectomized animals (Joo et al. 1998; Kilic et al. 1999). Stroke outcomes can be improved or normalized if pinealectomized animals are first injected with melatonin (Kilic et al. 1999; Joo et al. 1998). Melatonin's neuroprotective effects are equally visible in other brain injury contexts like cortical impact injury and percussion trauma, where treated animals: (1) exhibit reductions in contusion volume that are optimized when exogenous melatonin is delivered along the nighttime peak of endogenous secretion (Sarrafzadeh et al. 2000) and (2) improved post-trauma grip strength and spatial memory (Ozdemir et al. 2005; Mesenge et al. 1998). What's more, melatonin-mediated neuroprotection is not developmentally regulated. Fetal or neonatal rats recovering from ischemia/reperfusion also evince fewer signs of oxidative damage and better cognitive function after exogenous melatonin administration (Berger et al. 2017; Wakatsuki et al. 1999, 2001; Carloni et al. 2008). The utility of melatonin in addressing post-stroke injury has been summarized in several reviews (Reiter et al. 2003; Cervantes et al. 2008; Paterniti et al. 2016; Maldonado et al. 2007; Cheung 2003). Meta-analyses covering the entire experimental stroke literature suggest that melatonin improves outcomes by approximately 43% (Macleod et al. 2005).

In vitro models have been consulted as well to study the functional implications of melatonin's neuroprotective-antioxidant properties. In primary neuron culture, where finer cellular changes pursuant to oxygen/glucose deprivation can be measured, melatonin incubation decreases cell death caused by $H_2O_2$ or NMDA, preserves the dendritic morphology of these cells, and inhibits the release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria, signals that otherwise trigger apoptosis (Wang et al. 2009). Results are observed in cerebrocortical cultures and in hippocampal slices maintained under hypoxic or excitotoxic conditions. In hippocampal preparations, melatonin prevents cell death and rescues failures in electrophysiologically measured synaptic transmission (Vlkolinsky et al. 1999; Vlkolinsky and Stolc 1999; Lezoualc'h et al. 1996; Skaper et al. 1998).

Some research suggests that melatonin might be naturally secreted in response to neural insults as a compensatory neuroprotective maneuver. Transient increases in plasma melatonin levels are seen after hemorrhagic shock in animal models (Wichmann et al. 1996) and in the cerebrospinal fluid (CSF) of human patients experiencing severe traumatic brain injury (Seifman et al. 2008). The rise in human CSF levels post-injury scales with molecular indices of lipid peroxidation. Other studies in patients suffering from acute ischemic stroke suggest, likewise, that melatonin's catabolism into active antioxidant metabolites might be accelerated during trauma (Ritzenthaler et al. 2009, 2013). An interesting parallel to this series of investigations is an alternative one that has examined changes in circulating plasma melatonin produced from demanding exercise. Plasma melatonin concentrations are significantly increased in long-distance runners after a daytime 10 km race (Ronkainen et al. 1986) or a 28.5 mile mountain race (Strassman et al. 1989), with levels doubling or in some cases growing fourfold between the start and end of the event. People participating in a several-week aerobic exercise training program (e.g., eliciting 85% of maximum heart rate) also saw increasingly larger plasma surges in melatonin during repeated intervals of treadmill running or high-intensity pedaling on a stationary bicycle (Skrinar et al. 1989; Carr et al. 1981). Elevations are observed during the day when light would otherwise be expected to suppress melatonin secretion. During the nighttime period, when endogenous melatonin levels are already elevated, high-intensity exercise will result in further acute spikes of melatonin secretion by 50% that are superimposed on the nighttime elevation (Buxton et al. 1997). Among several interpretations (Escames et al. 2012), it is thought that strenuous physical activity induces oxidative stress, cuing the secretion or release of melatonin from peripheral stores to combat the excess ROS.

## 25.7 Melatonin: A Functionally Relevant Antioxidant, Part II

The functional relevance of melatonin's antioxidant properties is in evidence in many brain pathologies extending beyond stroke. Vis-à-vis epilepsy, supplemental dosing has been repeatedly shown to improve neural survival and impede the development of epileptic discharges and behavioral clonic-tonic seizures caused by epileptogenic agents such as kainic acid (Yamamoto and Mohanan 2003; Giusti 1996, 1997; Tan et al. 1998b; Espinar et al. 2000; Mohanan and Yamamoto 2002; Uz et al. 1996; Chen and Chuang 1999), quinolinic acid (Maharaj et al. 2005; Southgate et al. 1998; Cabrera et al. 2000; Behan et al. 1999), pentylenetetrazole (Mohammadi et al. 2020; Peterson et al. 1981; Yahyavi-Firouz-Abadi et al. 2006; Champney and Champney 1992; Lapin et al. 1998), pilocarpine (Costa-Lotufo et al. 2002), cyanide (Maharaj et al. 2003; Yamamoto and Tang 1996; Yamamoto and Mohanan 2001b), and 3-mercaptopropionic acid (Golombek et al. 1992). Outcomes are similarly improved in rodent models of hyperthermic febrile seizures (Aydin et al. 2015)

and kindling models of epilepsy that simulate the limbic circuit changes that develop after repeated seizure episodes (Mevissen and Ebert 1998; Albertson et al. 1981). In a mirror image to these therapeutic benefits, pinealectomized gerbils, rabbits, and rats without circulating melatonin exhibit more spontaneous and drug-induced convulsions (Manev et al. 1996; Rudeen et al. 1980; Philo and Reiter 1978; Bindoni and Rizzo 1965; Reiter and Morgan 1972). Melatonin's anticonvulsant actions have been primarily studied in animal models but improvements in epilepsy symptoms have also been reported in human patients (Anton-Tay et al. 1971), including pediatric patients with lissencephaly, infantile spasm, Lennox–Gastaut syndrome, and myoclonic epilepsy (Peled et al. 2001; Jan et al. 1999). In some cases, use of melatonin as an adjunctive anticonvulsant therapy has been longitudinally documented over 2 years (Molina-Carballo et al. 1997).

Melatonin's general medical safety and efficacy are exemplified by its treatment effects in inflammatory conditions like sepsis (Acuna-Fernandez et al. 2020; Galley et al. 2014), where the body's exaggerated immune response to an infection—including explosions in free radical production—causes life-threatening changes to organ function. In one clinical study, septic newborns orally administrated melatonin (20 mg) showed decreased blood measures of lipid peroxidation and a better survival rate compared to untreated children. While all of the children in the melatonin treatment group survived, three out of ten newborns in the control wing of the study died within 72 h of diagnosis (Gitto et al. 2001). A systematic review of placebo-controlled clinical trials testing melatonin's anti-inflammatory properties suggests that exogenous dosing reduces several immune/neuroinflammatory markers. Across an aggregate sample of 1517 participants, reductions are observed in interleukin-1 and tumor necrosis factor, two quintessential cytokines that are elevated during immune challenge (Cho et al. 2021). Predictable anti-inflammatory effects in the brain and elsewhere are also seen in animal models after exogenous melatonin administration. Data have been collected under myriad experimental conditions associated with osteoarthritis (Hosseinzadeh et al. 2016), Aβ vaccination (Jesudason et al. 2007), liposaccharide exposure (Mayo et al. 2005), and biotic stressors (e.g., *Schistosoma mansoni* and *Leishmania* parasites, Venezuelan equine encephalomyelitis virus, Semliki Forest virus, West Nile virus, and Aleutian disease parvovirus) (Bonilla et al. 1997, 2001, 2004; Elmahallawy et al. 2014; Ellis 1996; El-Sokkary et al. 2002; Zhang et al. 1999b; Ben-Nathan et al. 1995). Melatonin's enhancement of biotic stress resistance appears to be a motif that is conserved between plants and animals (Hardeland 2016).

Germane to the present review is the possibility that melatonin can improve treatment outcomes for neurodegenerative disorders. There is no shortage of data to suggest this is the case. Melatonin studies related to Alzheimer disease (AD) have been done using neurotoxic drugs or transgenic mouse models in which animals have been genetically programmed to reproduce important hallmarks of AD neurohistopathology. In these contexts, administration of melatonin or its metabolites restricts the accumulation of amyloid-beta peptides into pathological aggregates (Matsubara et al. 2003), limits neural cell death (Shen et al. 2002b; Bachurin et al. 1999), increases behavioral indices connected to contextual or spatial navigation

memory (Bachurin et al. 1999; Shen et al. 2002a), and prolongs the lifespan of animals living with some facet of AD neurobiology (Matsubara et al. 2003). These effects are accompanied by reductions in proinflammatory cytokines and oxidative stress and might be mediated exclusively through melatonin's chemical structure without the added contribution of $MT_1$ or $MT_2$ receptors (Pappolla et al. 2002). Melatonin has been additionally explored as a drug candidate for treating neurodegeneration in amyotrophic lateral sclerosis (ALS). Animal models of this condition suggest that supplementing with melatonin can inhibit motor-neuron loss, reduce general spinal cord atrophy, delay neurological deterioration connected to muscle wasting (e.g., muscle strength, coordination deficits), and extend survival (Zhang et al. 2013). In a 2-year clinical safety study of patients with sporadic ALS, high daily dosing with melatonin (300 mg/day) was well tolerated and effective in normalizing serum protein carbonyls (Jacob et al. 2002; Weishaupt et al. 2006), which is a surrogate marker of oxidative stress (Reiter et al. 1999). A recent retrospective analysis of the Pooled Resource Open-Access Clinical Trials (PRO-ACT) database indicates that people with ALS who use melatonin regularly have a slower rate of decline in their ALS Functional Rating Scale scores and a decreased annualized hazard death rate compared with non-melatonin users (Bald et al. 2021). The upper and lower motor neurons that degenerate in ALS are resource-intensive cells and would logically benefit from largescale dosing regimens of melatonin.

## 25.8 Do Discoveries Await in *Drosophila*?

The sleep, circadian, and seasonal biology surrounding melatonin secretion have been studied in mammals for over half a century. Surprisingly, these phenomena have received scant attention in *Drosophila*, one of the most pervasively used animal models in biomedical research. The fly melatonin literature is largely restricted to two studies that have documented day-night variations in whole-body expression (Callebert et al. 1991; Hintermann et al. 1996). Unfortunately, these studies failed to address two important questions about the nature of the expression: (1) Did it occur under the direction of an endogenous circadian oscillator?; and (2) Could it be phase-shifted by zeitgebers such as light? The establishment of melatonin rhythms in *Drosophila* that are analogous to those found in vertebrates would be invaluable to the scientific community. *Drosophila* are ideal animal models for investigations that tie together research on sleep, circadian timekeeping, and neurodegeneration because the animals evince low genetic redundancy, short reproductive times, circumscribed lifespans (60–80 days), functional simplicity, and affordability (De Nobrega and Lyons 2020). Many sophisticated genetic tools are available in flies, where spatially and temporally restricted changes in specific genes can be examined in conjunction with circuit and system-level analysis. Biological manipulations can be studied in virtually any context related to oxidative stress or inflammation. Importantly, flies and humans share homologous genes for over 60% of their genome (Reiter et al. 2001a; Harrison et al. 2002; Pandey and Nichols 2011). Similarities are even tighter when

one considers genes implicated in human disease. Approximately 75% of human disease genes have homologs in the fly genome (Chien et al. 2002; Lessing and Bonini 2009).

Already, flies have provided important insights into how sleep deprivation worsens AD-related phenotypes in brain circuits (Tabuchi et al. 2015) and molecular insights into how specific signaling pathways influence the progression of ALS. The RNA-binding protein TDP-43 has been linked to ALS both at the level of pathology and as a causative factor. Ninety-seven percent of ALS patients exhibit cytosolic aggregates containing TDP-43, and 4–5% of them harbor specific mutations within TARDBP, the gene encoding TDP-43 (Bjork et al. 2022). Flies expressing human TDP-43, or an ALS-associated mutant variant, show alterations in multiple metabolic pathways (Bjork et al. 2022). However, particular upregulations are seen in intermediates from the tricarboxylic acid (TCA) cycle, a fundamental metabolic process that occurs in the mitochondrial matrix to produce NADH (i.e., the electron donor powering oxidative phosphorylation) (Loganathan et al. 2022). Data suggest that this upregulation operates as a compensatory mechanism in the mitochondria of motor neurons and accordingly that mitochondrial function might be an important treatment target in ALS.

Given that melatonin functions as a broad-spectrum chemical antioxidant and concentrates in mitochondria, it is a reasonable intervention to study in ALS fly models. Such investigations could tackle complex experimental questions that address the intersecting roles of sleep and melatonin in ALS neurodegeneration across the circadian day and night. Sleep fragmentation and mistimed sleep induce oxidative stress in *Drosophila*. In middle-aged flies, oxygen radicals remain elevated following recovery sleep (Williams et al. 2016). These observations suggest that recurrent sleep fragmentation can be a dangerous potentiator of pathological brain processes in ALS. Studies in *Drosophila* have the potential to quickly uncover important translatable findings regarding the therapeutic application of timed melatonin dosing and behavioral interventions to (1) restore sleep consolidation and (2) neutralize the ROS buildup. On the behavioral medicine side, cognitive behavioral therapy for insomnia (CBT-I) is the preferred non-medication approach for treating sleep fragmentation (Muench et al. 2022). CBT-I interventions that were first developed in humans have been successfully tested in *Drosophila*, where they have been shown to increase sleep efficiency in short-sleeping mutants (Belfer et al. 2021). Work in flies can provide multiple simulations of how melatonin can be integrated with CBT-I to improve outcomes in ALS patients with astounding mechanistic biological insights that might identify yet other treatment targets. The case study of melatonin in fly ALS research is just one example of the many opportunities that are available to researchers hoping to elucidate mechanisms for protecting against neurodegenerative disease. Undoubtedly, other case studies could be made for AD, Huntingtin disease, or Parkinson's disease.

## 25.9 Conclusion

The origin of melatonin's antioxidant properties likely dates back billions of years to the Great Oxygen Event (GOE). The GOE was a massive extinction event that occurred during the Paleoproterozoic era. Owing to uncontrolled aerobic respiration, toxic levels of oxygen accumulated in Earth's atmosphere (Manchester et al. 2015). Those organisms that survived, namely cyanobacteria, did so by evolving a means of sequestering and neutralizing ROS. Over time, more complex organisms evolved and absorbed cyanobacteria, retaining the prokaryote's $O_2$-adapted cellular processes as they did so. Assimilated alongside these properties was the ability to synthesize melatonin (just as it appears today) and use it as a primary antioxidant to maintain balance between energy production and ROS clearance. Melatonin has played a timeless role in living organisms but it has been only in the past 20 years or so that its antioxidant properties have received significant attention. Studies are now examining its therapeutic potential in a wide variety of medical conditions, including as an adjunct intervention with chemotherapy to treat cancer. What makes melatonin an attractive therapeutic option is the frequent comorbidity of sleep/circadian disruptions with oxidative stress. With a single molecule, both these issues can be addressed at once. While melatonin has been studied for decades in one context or another, very few investigations have been done in *Drosophila*, arguably the most prolific and efficient animal model available to biomedical researchers. Any watershed movement in the direction of fly melatonin research is sure to accelerate the development of effective neuroprotective strategies to improve neurodegenerative disease outcomes.

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# Correction to: Sleep Hormone Melatonin, Inflammation and Aging

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The correction chapter and the book has been updated with the changes.

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