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The circadian system throughout the seasons of life

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Chapter 2

A multi-level assessment of the bidirectional relationship between aging and the circadian clock

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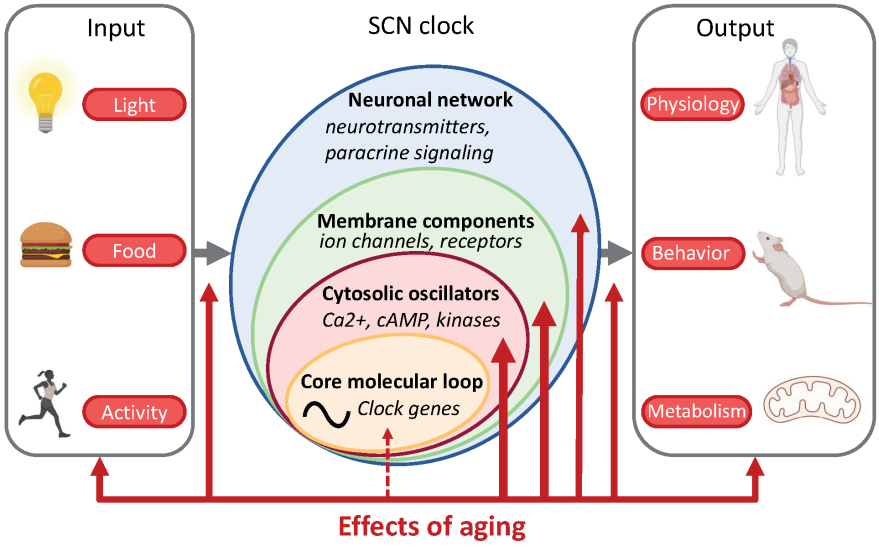
Abstract

The daily temporal order of physiological processes and behavior contribute to the wellbeing of many organisms including humans. The central circadian clock, which coordinates the timing within our body, is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Like in other parts of the brain, aging impairs the SCN function, which in turn promotes the development and progression of aging-related diseases. We here review the impact of aging on the different levels of the circadian clock machinery – from molecules to organs – with a focus on the role of the SCN. We find that the molecular clock is less effected by aging compared to other cellular components of the clock. Proper rhythmic regulation of intracellular signaling, ion channels and neuronal excitability of SCN neurons are greatly disturbed in aging. This suggests a disconnection between the molecular clock and the electrophysiology of these cells. The neuronal network of the SCN is able to compensate for some of these cellular deficits. However, it still results in a clear reduction in the amplitude of the SCN electrical rhythm, suggesting a weakening of the output timing signal., Consequently, other brain areas and organs not only show aging-related deficits in their own local clocks, but also receive a weaker systemic timing signal., The negative spiral completes with the weakening of positive feedback from the periphery to the SCN. Consequently, chronotherapeutic interventions should aim at strengthening overall synchrony in the circadian system using life-style and/or pharmacological approaches.

Graphical abstract



1. Introduction

One of the great achievements of modern medicine is the development of successful treatments of many diseases that meant a death sentence in earlier times. As a consequence, the average life span has increased steadily in the past century, creating new opportunities, but also many challenges. One of the biggest challenges is to abate age-related diseases and stay healthy, even productive, at an old age. Aging affects most physiological processes and parts of our body to some degree and we are currently unable to halt or reverse the aging process. Still, we can strive to achieve "healthy aging" by changes in lifestyle or smart medication. One of the potential interventions to achieve healthier aging is to support the regulation of daily rhythms of processes in our body. For instance, strengthening the circadian clock function will alleviate clock-related sleep disorders that often occur during aging and worsen in neurodegenerative diseases. The negative interaction between circadian clock and age-related disease often creates a downward spiral in health. For instance, for Alzheimer's and other neurodegenerative diseases, sleep disorders seem to be already present at the preclinical state (Musiek *et al.,* 2018) and worsen in the course of the disease (Leng *et al.,* 2019). Importantly, the sleep disruptions also aggravate the home situation for the patient and his family, often leading to early institutionalization and significant reduction in the patient's quality of life.

The suprachiasmatic nucleus (SCN), the central clock that organizes temporal order of many physiological processes including sleep/wake behavior is situated in the hypothalamus and – not surprisingly – is also affected by aging. Conversely, there is mounting evidence that the circadian clock in return is influencing the course of aging, the progression of age-related disease and general life-expectancy (Kondratova & Kondratov, 2012; Fonseca Costa & Ripperger, 2015; Musiek & Holtzman, 2016). A functional central clock in the SCN is beneficial to the performance of other brain areas, like the hippocampus, and organs like heart and liver. Understanding the impact of aging on the central clock and how to restore its function can therefore be advantageous for developing interventions for many age-related symptoms and disorders. After a brief overview of the circadian system, this review will focus on the age-related deficits in the SCN and underline the differential effect aging has at various levels of the SCN organization; from molecules to networks. We will then discuss the effects this compromised central clock has on so-called peripheral clocks, but also consider the consequences of age-related loss of local peripheral clock function. Lastly, we will briefly recapitulate the evidence for the interaction between the circadian clock and neurodegenerative diseases and close with some considerations of potential chronotherapeutic interventions.

2. The establishment of temporal order in our body

While there is no evolutionary advantage to an increase in life span, selection pressure was at first very high on environmental stressors with daily fluctuations, like ultraviolet radiation, extreme temperature difference and later the presence of predators. Consequently, molecular clock mechanisms were established in very early life forms, like cyanobacteria, and further developed into a specialized circadian system we now find in almost all organisms on this planet. In mammals, a dedicated central clock in the SCN seems to work as a conductor in an orchestra of clocks, controlling the temporal harmony in our body to ensure the optimal time of performance of all our physiological functions and behavior.

The endogenous circadian clocks run with a slightly different pace than the environmental cycle of 24 hours and therefore have to be synchronized or entrained by external zeitgebers like the daily cycles of light and darkness. The SCN receives information on the light-dark cycle from specialized retinal ganglion cells and distributes its "corrected" information on the time of day (and day-length) to other brain areas and organs. Importantly, the SCN is not just relaying light-information, but integrates this into its neuronal network, together with feed-back from the rest of the body, like sleep quality, physical activity, metabolic state and stress level. The resulting SCN timing signal therefore not only holds information on the time of day and season, but also of the state of many bodily functions. It is therefore inevitable that the effects of aging on brain and organ function will impact SCN function and vice versa, which often complicates the interpretation of the data on the aging circadian clock.

In addition to this clockshop of central and peripheral clocks, the circadian signal of the SCN is also shaped and influenced on a number of organizational levels, namely the molecular, cellular, membrane, and network level (Evans, 2016; Michel & Meijer, 2020).

As mentioned above, the basis for a circadian regulation of cellular function was established early on in evolution with a molecular mechanism creating an oscillation with a transcriptional-translational feedback loop (Buhr & Takahashi, 2013). The clock proteins BMAL1 and CLOCK enhance the transcription of *cry* and *per*, which will inhibit their own transcription with a sufficient delay to ensure the ~24-hour rhythms of the clock gene expression. This generates oscillations of proteins that either directly control other cellular functions, or control the transcription of other clock-controlled genes. An estimated 40-50% of all protein encoding genes in mice and humans are regulated by the circadian clock in an organ specific way (Ruben *et al.,* 2018; Zhang *et al.,* 2014). For instance, in the SCN, rhythmic regulation of intracellular signaling pathways like cAMP/Ca^{2+} seem to serve the stability and precision of clock function (O'Neill & Reddy, 2012), while in the liver circadian

timing of metabolic pathways is optimizing hepatocyte activity to food availability and energy demand.

3. The aging SCN

The SCN is a small brain area consisting of a relatively homogeneous network of neurons and glia, that is in appearance, its functioning and adaptability surprisingly diverse and remains the subject of a growing number of studies. Like all brain areas, the SCN is affected by aging (Farajnia *et al.,* 2014). However, because of its regulatory role in temporal cellular processes, a compromised SCN function influences the process of aging in other brain areas and organs. The importance of the SCN in aging is illustrated by the effect of transplantation of fetal SCN tissue in the third ventricle, alleviating aging deficits in circadian rhythms in rodents (Van Reeth *et al.,* 1994; Cai & Wise, 1996) and increasing life expectancy (Hurd & Ralph, 1998). In addition, manipulation of the central clock by for example phase shifts, SCN ablation and genetic manipulation results in health deterioration similar to that seen in aging, and often leads to a shortened lifespan (Hurd & Ralph, 1998; Davidson *et al.,* 2006). It is important to note that, when using animal models for aging, the lifespan and aging dynamics of each species and strain has to be known and evaluated. In mice (C57bl6), life stages have been defined as matured adult (3-6 months), middle-aged (10-14 months) and old (18-24 months) (Flurkey *et al.,* 2007). The specifics of age, sex and genotype for the relevant studies discussed by us can be found in table 1 (Table 1). Below we will discuss the effect of aging on different levels of the circadian system, from the molecular clock in individual SCN cells, via the SCN network, to the output signal of the SCN toward the periphery (Fig. 1).

3.1 Input to the SCN - Aging affects light transduction to the SCN

Environmental light information reaches the SCN through intrinsically photoreceptive retinal ganglion cells (ipRGCs) in the eye and the retinal hypothalamic tract (Hattar *et al.,* 2002). Aging is accompanied by an attenuated light response in the SCN, quantified by the induction of immediate early genes, like c-Fos (Sutin *et al.,* 1993). The decline in light sensitivity is likely a consequence of an overall loss of function of the light transduction pathway. First of all, aging is associated with a yellowing of the lens, which reduces the transmission of – mainly short wavelength - light to the retina (Kessel *et al.,* 2010). Secondly, in the course of aging, the number of ipRGCs and their dendritic arborisation are reduced (Semo *et al.,* 2003; Esquiva *et al.,* 2017; Lax *et al.,* 2019). Moreover, the loss of ipRGCs in aging is accompanied by a loss of projections to the SCN (Lupi *et al.,* 2012; Engelberth *et al.,* 2017). Despite the multi-level deterioration of light transduction to the SCN, there are several studies indicating that light is still capable of modulating circadian rhythms in humans (Najjar *et al.,* 2014; Benloucif *et al.,* 2006). Therefore, light therapy remains a

suitable candidate for chronotherapeutic purposes in the elderly, although possibly with adjusted light wavelengths or intensities (Rubiño *et al.,* 2020).

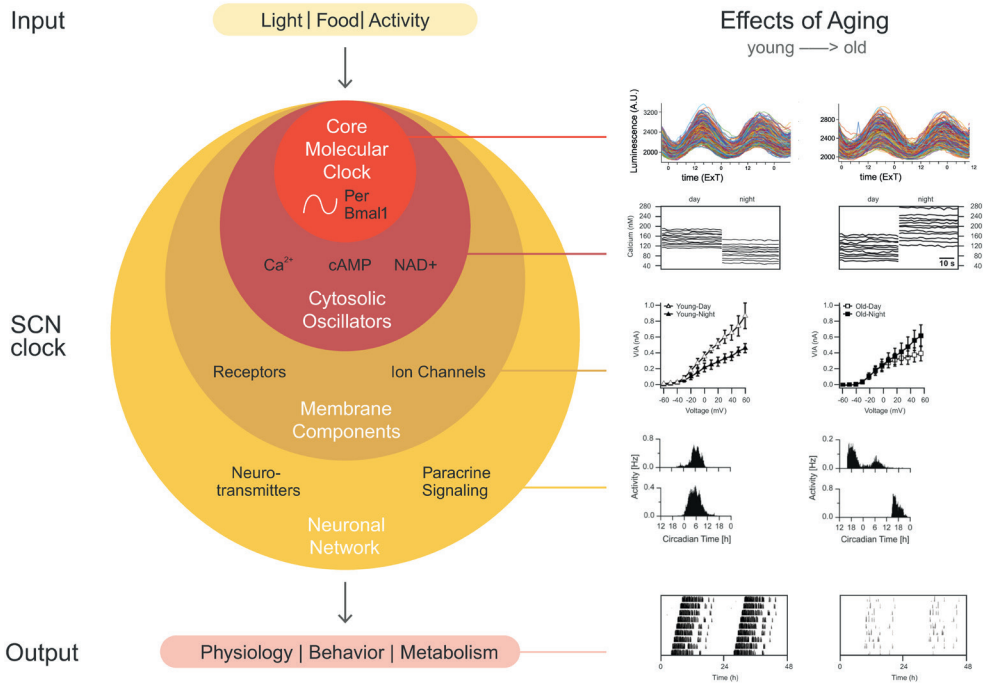


Figure 1. Aging affect multiple levels of the SCN differentially. The onion symbolizes different layers of the central circadian clock. The effect of aging on the different layers is symbolized on the right panels. Molecular rhythms seem to be less effected while diminished amplitude or reorganization can be seen in rhythms of intracellular Ca^{2+}, membrane ion channel activity, phase distribution of single SCN units of the neuronal network and behaviour (from top to bottom). Panels were modified after Farajnia et al 2014 (*Farajnia et al.* 2014).

3.2 The molecular clock of the SCN ages well

While the light-input from the eyes to the SCN is impaired in aging, it seems that the core molecular clock in SCN neurons remains largely functional., However, the results of studies on the effect of aging on the molecular clock components are ambiguous and only rather consistent for the core clock genes Period 1 (Per1) and Brain and Muscle ARNT-Like 1 (Bmal1). While almost all studies report no change in Per1 expression rhythm or level, aging related changes in expression of Bmal1 are consistently found (Per1: (Asai *et al.,* 2001; Weinert *et al.,* 2001; Kolker *et al.,* 2003; Davidson *et al.,* 2008; Bonaconsa *et al.,* 2014; Yamazaki *et al.,* 2002); Bmal1: (Kolker *et al.,* 2003; Bonaconsa *et al.,* 2014; Wyse & Coogan, 2010; Duncan *et al.,* 2013; Chang & Guarente, 2013a). There are only a few studies that have investigated the effect of aging on Cryptochrome 1/2 and Clock genes

and most do not report any age-related effects for the SCN (Asai *et al.,* 2001; Weinert *et al.,* 2001; Kolker *et al.,* 2003; Wyse & Coogan, 2010; Bonaconsa *et al.,* 2014). By contrast, Per2 has been studied extensively with respect to aging. Under an equinoxial light regime (Light-Dark; LD 12:12), almost half of the studies did not find an effect of aging on Per2 expression (Asai *et al.,* 2001; Kolker *et al.,* 2003; Leise *et al.,* 2013; Polidarova *et al.,* 2017; Buijink *et al.,* 2020), while others report a lower level (exclusively for mRNA; (Weinert *et al.,* 2001; Bonaconsa *et al.,* 2014; Chang & Guarente 2013a), or a lower amplitude of Per2 expression (Nakamura *et al.,* 2015; Nakamura *et al.,* 2012). One study actually reported an increase in Per2 amplitude (Sellix *et al.,* 2012). In a study from our laboratory we found that a moderate, and naturally occurring deviation from the LD 12:12 light regime, namely LD 16:8 and LD 8:16, resulted in a similar adaptation in Per2 expression rhythms in young and old mice, demonstrating that the aging molecular clock retains its plasticity (Buijink *et al.,* 2020). However, two other recent studies show that more extreme lighting conditions – constant light and constant darkness – did affect Per2 rhythms in aging (Nakamura *et al.,* 2015; Polidarova *et al.,* 2017). This suggests that there are limits to the flexibility of the aging molecular clock. Nonetheless, the currently available literature indicates that the effect of aging on the molecular clock is relatively subtle, and is unlikely to account for the severe deficits seen in other parts of the circadian system. Therefore, it is uncertain if strengthening the molecular clock with for example with pharmaceuticals, would benefit the elderly.

3.3 Other clock components in the cytosol of aging SCN neurons are affected by aging

Core clock gene expression in transcriptional-translational feedback loops were long considered to be essential for generating a circadian rhythm in circadian pacemaker cells of the SCN. Despite studies revealing cytosolic oscillators like Ca^{2+} (Noguchi *et al.,* 2017), cyclic adenosine monophosphate (cAMP; Doi *et al.,* 2011; O'Neill *et al.,* 2008) and nicotineamide adenine dinucleotide (NAD+/NADH; Huang *et al.,* 2016; Ramsey *et al.,* 2009) contributing to cellular clock function, the core clock genes are still the main players for initiating the circadian modulation of cellular metabolites and signaling pathways. For the SCN neuron and its network, this results in the circadian modulation of ion channels regulating membrane excitability (Harvey et al., 2020b), forming the basis for the rhythm in electrical activity, which is viewed as an important timing signal issued by the SCN (see 3.4.). However, the link between the molecular clock and channel regulation has been described for only a few of ion channels to date (Kudo et al., 2015; Meredith et al., 2006; Schmutz et al., 2014) and more work is needed to identify the direct and indirect pathways used by the clock genes to control the circadian modulation of ion channel properties.

One of the best studied intracellular signaling molecules known to influence ion channel activity is Ca^{2+}. Transmembrane Ca^{2+} influx serves to modulate spike frequency, but

can also activate (clock) gene expression, for instance to mediate light-induced phase shifts of the circadian rhythm (Golombek & Rosenstein, 2010). Interestingly, the level of intracellular Ca^{2+} concentration also shows a circadian rhythm with higher levels during the day compared to the night (Colwell, 2000; Jones *et al.,* 2018; Ikeda *et al.,* 2003; Aguilar-Roblero *et al.,* 2016). While the source and the function of this Ca^{2+} rhythm is still not fully understood (Enoki *et al.,* 2017; Ikeda *et al.,* 2003), there is even less known about the impact of aging; however, one study suggests a dysregulation of the Ca^{2+} rhythm in aging (Farajnia *et al.,* 2015). This lack of knowledge is surprising since there is a vast amount of literature on age-related Ca^{2+} homeostasis in neurons in other brain areas, especially the hippocampus (Uryash *et al.,* 2020; Calvo-Rodriguez *et al.,* 2020).

Importantly, intracellular Ca^{2+} signaling - often in concert with cAMP - comprises a cytosolic oscillator that reinforces SCN clock function (O'Neill *et al.,* 2008; Doi *et al.,* 2011). In addition, the paracrine feedback (e.g. of vasoactive intestinal protein; VIP) via g-coupled receptors, can amplify the cAMP/Ca^{2+} signaling and further stabilize clock function. There is evidence that the rhythm in cAMP (Gerhold *et al.,* 2005) as well as VIP is compromised in the aging SCN (Krajnak *et al.,* 1998) demonstrating again the complexity of the impact of aging on clock function.

Table 1: Effect of aging on SCN

molecular clock		results (aged compared to young)	model	light condition	target	age (m) young	age (m) old	# time points	Reference
Per1	–	No change in level and rhythm. Decreased induction of Per1 in response to light pulse in old.	rat	DD (2d)	mRNA	2-3	22-26	6	(Asai *et al.* 2001)
	–	No change in level.	mouse	DD (1d)	mRNA	4	15	2	(Weinert *et al.* 2001)
	–	No change in level. Shorter period in old.	rat	12:12	mRNA	2	24-26	≥24	(Yamazaki *et al.* 2002)
	–	No change in level and rhythm. Decreased induction of Per1 in response to light pulse.	hamster	DD (1-3d)	mRNA	2-3	17-20	4	(Kolker *et al.* 2003)
	x	Slightly reduced response to phase advance in old. No change in response to phase delay.	rat	12:12 & shift	protein	4-8	>24	≥24.	(Davidson *et al.* 2008)
	–	No change in level. Possibly slightly phase advanced.	mouse	12:12	mRNA	3	22	4	(Bonaconsa *et al.* 2014)
Per2	–	No change in level and rhythm.	rat	DD (2d)	mRNA	2-3	22-26	6	(Asai *et al.* 2001)
	↓	Lower level at ZT7, but not at ZT 21.	mouse	DD (1d)	mRNA	4	15	2	(Weinert *et al.* 2001)
	–	No change in level and rhythm. No change in response to light pulse	hamster	DD (1-3d)	mRNA	2-3	17-20	4	(Kolker *et al.* 2003)
	–	No chance in level. Reduced amplitude of 3rd cycle.	mouse	12:12	protein	3-6	13-16	≥24/4	(Nakamura *et al.* 2011)
	↑	Higher amplitude in old. Increased response to phase advance in old.	mouse	12:12 & shift	protein	3-6	22-28	≥24.	(Sellix *et al.* 2012)
	↓	Lower level at ZT4	mouse	DD (2d)	mRNA	6	21	1	(Chang & Guarente 2013)
	–	No change in amplitude at baseline. Changed response to phase shift in old.	mouse	12:12 & shift	protein	3.5	19	≥24.	(Leise *et al.* 2013)
	↓	Possibly slightly reduced level and phase advanced.	mouse	12:12	mRNA	3	22	4	(Bonaconsa *et al.* 2014)
	–	Faster decay of amplitude in old (12:12 & DD). Small phase delay and longer period in DD.	mouse	12:12 & DD	protein	3-5	13-15	≥24.	(Nakamura *et al.* 2015)
	–	No change in amplitude under LD. Higher incidence of arrhythmicity in LL.	mouse	12:12, LL & DD	protein	8-13	24-26	≥24.	(Polidarova *et al.* 2017)
	–	No change in rhythm under LD 12:12 and long and short photoperiod (16:8/8:16)	mouse	12:12, 16:8, 8:16	protein	4-8	22-28	≥24.	(Buijink *et al.* 2020)

Table 1 (cont): Effect of aging on SCN

neuropeptides		results	model	light condition	target	age (m) young	age (m) old	# time points	Reference
Cry1	↓	Lower amplitude.	rat	DD (2d)	mRNA	2-3	22-26	6	(Asai *et al.* 2001)
	–	No change in level. Rhythmic in old, not in young.	mouse	DD (1d)	mRNA	4	15	2	(Weinert *et al.* 2001)
	–	No change in level. Rhythmic in old, not in young	mouse	12:12	mRNA	3	22	4	(Bonaconsa *et al.* 2014)
Cry2	–	No change in level. Not rhythmic in young and old.	mouse	12:12	mRNA	3	22	4	(Bonaconsa *et al.* 2014)
Clock	–	No change in level. Not rhythmic in young and old.	mouse	DD (1d)	mRNA	4	15	2	(Weinert *et al.* 2001)
	↓	Lower level in old. Not rhythmic in young and old.	hamster	DD (1-3d)	mRNA	2-3	17-20	4	(Kolker *et al.* 2003)
	–	No change in level. Rhythmic in old, not in young.	mouse	12:12	protein	4	16	6	(Wyse & Coogan 2010)
	–	No change in level. Not rhythmic in young and old.	mouse	12:12	mRNA	3	22	4	(Bonaconsa *et al.* 2014)
Bmal1	↓	Lower level at CT2 and CT20. No changes in rhythmicity.	hamster	DD (1-3d)	mRNA	2-3	17-20	4	(Kolker *et al.* 2003)
	↓	Lower level. Not rhythmic in young and old.	mouse	12:12	protein	4	16	6	(Wyse & Coogan 2010)
	↓	Lower level at ZT4.	mouse	DD (2d)	protein	6	21	1	(Chang & Guarente 2013)
	↓	Lower level. Rhythmic in young and old.	hamster	14:10	mRNA	3-5	17-21	4	(Duncan *et al.* 2013)
	↓	Lower level in dark period. Rhythmic in young, not in old.	mouse	12:12	mRNA	3	22	4	(Bonaconsa *et al.* 2014)

Table 1 (cont): Effect of aging on SCN

neuropeptides		results	model	light condition	target	age (m) young	age (m) old	# time points	Reference
VIP	↓	reduced number of VIP neurons.	rat	-	peptide	7-8	32-33	-	(Chee *et al.* 1988)
	–	No change in number of VIP neurons. No effect of high/low light intensity.	rat	12:12	peptide	3	34-37	1	(Lucassen *et al.* 1995)
	↓	reduced number of VIP neurons in males, not females.	human	-	peptide	10-40y	66-99y	-	(Zhou *et al.* 1995)
	↓	Lower level and loss of rhythmicity of VIP.	rat	12:12	mRNA	3	24		(Kawakami *et al.* 1997)
	↓	Lower level and reduced number of VIP neurons. Loss of rhythmicity.	rat	14:10	mRNA	2-4	18-20	7	(Krajnak *et al.* 1998)
	↓	Lower level in neurons. Not rhythmic in young and old.	hamster	14:10	mRNA	3-5	19-22	5	(Duncan *et al.* 2001)
	↓	Lower level and loss of rhythmicity of VIP.	rat	12:12	mRNA	2-3	19-20	8	(Kalló *et al.* 2004)
	–	No change in level and number of VIP neurons. Shift (delay) in peak expression.	m. lemur	14:10	peptide	2y	6y	5	(Cayetanot *et al.* 2005)
	↓	Reduced number of VIP neurons.	rat	12:12	peptide	6	24	1	(Pereira *et al.* 2005)
AVP	↓	Reduced number of AVP neurons. SCN area and AVP neurons larger in aging.	rat	-	peptide	7-8	32-33	-	(Roozendaal *et al.* 1987)
	↓	Reduced number of AVP neurons under low light intensities, no change in high light intensities.	rat	12:12	peptide	3	34-37	1	(Lucassen *et al.* 1995)
	–	No change in level and rhythmicity.	rat	14:10	mRNA	2-4	18-20	7	(Krajnak *et al.* 1998)
	↓	Reduced number of AVP neurons.	vole	12:12	peptide	4-5	11-12	1	(Van der Zee *et al.* 1999)
	–	No change in level and rhythmicity.	hamster	14:10	mRNA	3-5	19-22	5	(Duncan *et al.* 2001)
	–	No change in level and rhythmicity. Changed expression of V1a and V1b receptor.	rat	12:12	mRNA	2-3	19-20	8	(Kalamatianos *et al.* 2004)
	↑	Higher level. No change in number of AVP neurons. Shift (delay) in peak expression.	m. lemur	14:10	peptide	2y	6y	5	(Cayetanot *et al.* 2005)
	↓	Reduced number of AVP neurons.	rat	12:12	peptide	6	24	1	(Pereira *et al.* 2005)

Table 1 (cont): Effect of aging on SCN

	results	model	light condition	target	age (m) young	age (m) old	# time points	Reference
neuropeptides								
Electrical activity							Rec. time	
↓	reduced averaged peak neuronal firing rates (single-unit activity)	rat	12:12	*ex vivo*	5-7	23-28	30h	(Satinoff *et al.* 1993)
↓	reduced averaged neuronal firing rates during day (single-unit activity)	hamster	12:12	*ex vivo*	2	24	7-12h	(Watanabe *et al.* 1995)
↓	reduced averaged peak neuronal firing rates (single-unit activity)	rat	12:12	*ex vivo*	3	28	-	(Ruby *et al.* 1998)
↓	slightly lower day-night difference of spontaneous firing rate (single-unit activity). Higher proportion of silent cells during the day.	mouse	12:12	*ex vivo*	2-3	14-21	<6h	(Nygard *et al.* 2005)
↓	reduced amplitude of neuronal firing (single-unit activity)	mouse	12:12	*ex vivo*	4-5	15-17	48h	(Biello 2009)
↓	lower amplitude of multi-unit neuronal activity (multi-unit activity)	mouse	12:12 & DD	*in vivo*	4	15	>7d	(Nakamura *et al.* 2011)
↓	lower day-night difference of spontaneous firing rate (single-unit activity), lower amplitude of multi-unit neuronal activity (multi-unit activity)	mouse	12:12	*ex vivo*	3	23	-	(Farajnia *et al.* 2012)
↓	lower amplitude of firing rates during the day (single-unit activity) *	mouse	12:12	*ex vivo*		18-20	8-12h	(Leise *et al.* 2013)

For the SCN clock the level of cell signaling and cytosolic oscillators seems to be gravely affected by aging, which is reflected in the consequences for the single-cell pacemaker described below. Given the bridging function between the molecular clock and the electrophysiological properties of SCN neurons, this level should be one of the important research-focuses for mechanisms and interventions for the aging clock.

3.4 Electrophysiological properties of SCN neurons and network are severely disrupted with aging

The SCN neurons express a circadian rhythm in electrical activity, with most neurons producing action potentials during the day and being relatively silent during the night (Gillette & Tischkau, 1999; Brown & Piggins, 2007). This rhythm is maintained in the absence of inputs from the eyes (light) or other parts of the brain, as demonstrated *in vivo* and in brain slices (Weaver, 1998). Together with the rhythmic release of neuropeptides like arginine vasopressin (AVP), the neuronal activity rhythm of the SCN is considered to be the major output to propagate the endogenous timing signal throughout other parts of the brain and control temporal order of the brain and other organs (Kalsbeek *et al.,* 2011; Colwell, 2011; Paul *et al.,* 2020b). Contrary to the rather mild effects of aging on the molecular clock, the amplitude of the electrical activity rhythm is severely attenuated in the aged SCN as shown by *in vivo* and *ex vivo* studies (Satinoff *et al.,* 1993; Watanabe *et al.,* 1995; Nygard *et al.,* 2005; Biello, 2009; Nakamura *et al.,* 2011; Farajnia *et al.,* 2012; Farajnia *et al.,* 2015; Leise *et al.,* 2013). It seems that, while the aging molecular clock keeps ticking, processes in the membrane clocks lose track of time. The majority of SCN neurons recorded from hypothalamic slices from old mice (>24 month) showed compromised rhythms in membrane potential and membrane conductance, suggesting loss of circadian control of passive and active ion currents (Farajnia *et al.,* 2012). In SCN neurons, the circadian modulation of ion channel activity is necessary to control membrane excitability and generate the rhythms in electrical activity (Brown & Piggins, 2007). The electrical activity is used for synchronization within the SCN network and functions as an important output timing signal to downstream targets (Harvey *et al.,* 2020a; Itri *et al.,* 2005; Brown & Piggins, 2007; Colwell, 2011). The activity of several voltage dependent K^+ channels express a circadian modulation and seem to be required for proper electrical rhythm, like the fast delayed rectifier current (Itri *et al.,* 2005), the A-type current (Granados-Fuentes *et al.,* 2012; Itri *et al.,* 2010) and Ca^{2+}-activated K^+ channels (Pitts *et al.,* 2006; Whitt *et al.,* 2016; Kent & Meredith 2008).

Aging was found to diminish the circadian modulation of a number of voltage-dependent K channels, but interestingly did not alter the channels' basic conductance (Farajnia *et al.,* 2012; Farajnia *et al.,* 2015). The loss in circadian rhythm in channel activity of the fast delayed rectifier channel and the transient K^+ channel can contribute to a change in firing frequency modulation (Farajnia *et al.,* 2012), while the impact on the rhythm in

large-conductance Ca^{2+}-activated K^+ current led to change in the waveform of the action potential (Farajnia *et al.,* 2015). Is seems that the age-resistant molecular clock becomes uncoupled from the downstream processes, which are needed to control membrane rhythmicity.

As we have discussed above, there are several mechanisms that are responsible for the transmission of information from the electrical activity at the membrane to the molecular clock and vice versa. It is likely that aging associated deterioration of one or more of these coupling mechanisms is responsible for the discrepancy seen between the effect of aging on the molecular and electrical clock. Environmental light information may reach the molecular clock, whereby it retains the capability of entraining to the light-dark cycle, including adapting phase distributions to long and short photoperiods (Buijink *et al.,* 2020). However, this information fails to reach the electrical components of the central clock, both directly via projections from the eye, as well as indirectly, through communication with the molecular clock, resulting in an uncoupling of the electrical and molecular clock components. This is partially corroborated by the finding that the aging molecular clock is unable to remain stable under constant lighting conditions (Nakamura *et al.,* 2015; Polidarova *et al.,* 2017). Irrespective of the underlying cause, since electrical activity is one of the two main outputs from the SCN to the rest of the brain and body, it is likely that the reduced amplitude has consequences for the signaling of the SCN to other brain areas and the periphery.

3.5 SCN network synchronization – neurotransmitter and peptide signaling

The SCN employs not only electrical signaling, but also multiple neurotransmitters and neuropeptides for communication between neurons of the SCN, as well as towards the periphery. There are several ways these means of communication are known to be affected by aging. For instance, synaptic terminals and signaling for the neurotransmitter gamma-aminobutyric acid decline in aging, which would affect the control of synchronization between SCN neurons (Nygard *et al.,* 2005; Palomba *et al.,* 2008; Biello, 2009; Farajnia *et al.,* 2012). Aging is also accompanied by a reduced expression of the NR2B subunit of the N-Methyl-d-aspartic acid receptor, affecting glutamatergic signaling, which is the main input signal for the SCN (Biello, 2009; Biello *et al.,* 2018). Another important "synchronizer" in the SCN, the neuropeptide VIP, displays a reduction in its mRNA rhythm and fewer cells in the old SCN express VIP (Kawakami *et al.,* 1997; Krajnak *et al.,* 1998; Duncan *et al.,* 2001; Zhou *et al.,* 1995; Chee *et al.,* 1988; Kalló *et al.,* 2004; Pereira *et al.,* 2005) Similarly, a significant reduction in cells expressing AVP – another essential neuropeptide in the SCN - was described in the aging rodent SCN (Roozendaal *et al.,* 1987; Van der Zee *et al.,* 1999), although several other studies did not find a difference in AVP mRNA levels and its rhythmicity (Krajnak *et al.,* 1998; Duncan *et al.,* 2001; Kalamatianos *et al.,* 2004). In the mouse lemur, a small primate, VIP and AVP protein levels are not affected, but their

rhythms are phase delayed (Cayetanot *et al.,* 2005). Importantly, exposure to increased light intensities could prevent the loss of AVP, but not of VIP neurons in rats (Lucassen *et al.,* 1995). It is remarkable that, to our knowledge, no recent study has been conducted on neuropeptides in aging SCN. The emergence of new techniques, enabling the cell-specific tracking of activity by targeted labelling and imaging of these neuropeptides (Jones *et al.,* 2018; Shan *et al.,* 2020), should facilitate new studies on the impact of aging on peptidergic signaling in the SCN and will generate more in-depth knowledge of this system.

3.6 From cells to network – A role for astrocytes?

Although neurons are still supposed to play the main role in timekeeping, recent studies have highlighted the importance of glia cells, specifically astrocytes, in the functioning of the SCN (Brancaccio *et al.,* 2017; Tso *et al.,* 2017; Brancaccio *et al.,* 2019). Furthermore, the importance of astrocytes in aging, and specifically the requirement of a functional clock in astrocytes was illustrated by the finding that the selective knock-out of Bmal1 in only astrocytes shortens the lifespan in mice (Barca-Mayo *et al.,* 2020). The circadian clock in astrocytes is found to be capable of driving circadian rhythm in the absence of a functional molecular clock in SCN neurons (Brancaccio *et al.,* 2019), as well as modulate circadian period in the SCN and behavior in a normal functioning SCN (Tso *et al.,* 2017). Therefore, it is likely that under normal circumstances astrocytes play a role in fine-tuning circadian clock function in the SCN. In the aging brain, astrocytes show signs of cellular senescence and undergo transcriptional changes that indicate neuroinflammation, a process detrimental for the correct functioning of these cells (Salminen *et al.,* 2011; Clarke *et al.,* 2018). Unfortunately, there is a shortage of studies on astrocytes (and other glia cells) in the aging SCN, so it is unclear to what extend they are involved in its malfunctioning. One study at least indicates that the number of astrocytes is increased in the aged SCN, which warrants additional research into the functioning of astrocytes in the old SCN (Deng *et al.,* 2010).

4. Aging of clocks outside the SCN

The SCN orchestrates circadian rhythms in de rest of the brain and body through different pathways. The output pathways of the SCN are affected by aging in different ways, in turn resulting in various disturbances of circadian rhythms throughout the brain and body. In this part of the review we will discuss the effect of aging on target areas of the SCN, namely the hypothalamus and the pineal gland, as well as how dysregulated circadian rhythms in aging affects the rest of the brain and body.

4.1 Communication with the periphery – Outputs of the SCN within the hypothalamus

Most of the direct output areas of the SCN are located in other parts of the hypothalamus, a crucial brain region for regulating a wide range of physiological processes (Kalsbeek & Buijs, 2002). This makes the location of the SCN within the hypothalamus ideal to relay non-visual light information from the eye to the rest of the brain and body for circadian regulation of their physiological processes. The importance of circadian rhythms for functions of the hypothalamus is illustrated by the relatively strong autonomous rhythms in several hypothalamic areas outside the SCN (Guilding *et al.,* 2009). The processes in the hypothalamus that require a circadian regulation are supported by input from the SCN. In turn, rhythmic processes in the periphery, like feeding and physical activity feed back to the SCN mainly through other areas in the hypothalamus (Buijs *et al.,* 2017; Buijs *et al.,* 2019). These reciprocal processes are essential for energy preservation and for hormone homeostasis. Many of the regulatory functions of the SCN that are affected by aging, like energy homeostasis, sleep-wake rhythms and hormonal balance, are mediated through areas within the hypothalamus (Kim & Choe 2019). Indeed, the hypothalamus is even suggested to be a key regulator in systemic aging (Zhang *et al.,* 2013).

One of the hallmarks of aging is a deregulation of nutrient sensing, which is essential to control the organism's energy balance (López-Otín *et al.,* 2013). A complex network of neurons in the dorsomedial, ventromedial and lateral hypothalamus, as well as the paraventricular nucleus and arcuate nucleus of the hypothalamus has developed to sense and regulate vertebrate energy expenditure and food intake. The essential circadian regulation of this network is achieved by the SCN, which has direct projections to the paraventricular nucleus and the dorsomedial hypothalamus (Kalsbeek *et al.,* 1993). Two key proteins in nutrient sensing – the mammalian target of rapamycin (mTOR) and sirtuin 1 (SIRT1) – are critical in the aging process and are directly linked to the molecular clock.
The mTOR pathway is able to detect changes in energy status, by monitoring levels of ATP and amino acids, and modify a wide variety of essential cellular processes (Wullschleger *et al.,* 2006). In the hypothalamus of old mice mTOR signaling in pro-opiomelanocortin neurons is increased, which contributes to an aging phenotype (Yang *et al.,* 2012). On the other hand, pharmacological inhibition of mTOR activity, for example with rapamycin, promotes longevity and health span (Vellai *et al.,* 2003; Harrison *et al.,* 2009). Importantly, mTOR activity is also inhibited by a calorie restricted diet (Kaeberlein *et al.,* 2005), which to date is the most efficient life extending intervention in multiple organisms, from yeast to primates (Colman *et al.,* 2009; Lin *et al.,* 2002). The mTOR pathway is linked to the timekeeping system in multiple ways. In the SCN, the mTOR pathway can be triggered by light (Cao *et al.,* 2008), while the core clock gene Bmal1 negatively regulates mTOR signaling (Khapre *et al.,* 2014). Inhibition of mTOR signaling reduces the amplitude and lengthens the period of Per2 expression rhythm in the SCN and liver (Ramanathan *et al.,*

2018). Another essential regulator of cellular energy status is sirtuin 1 (SIRT1). Sirtuins are a class of proteins that detect their surrounding energy status by sensing oxidized NAD+, and respond with the regulation of a multitude of metabolic processes (Houtkooper *et al.,* 2012). Like mTOR, SIRT1 is a candidate target for life extending agents, since overexpression of its monologue in yeast, Sir2 was found to increase lifespan (Kaeberlein *et al.,* 1999). SIRT1 is similarly thought to be involved in the effect of calorie restriction on life span, since calorie restriction enhances SIRT1 expression (Cohen *et al.,* 2004). Interestingly, SIRT1 influences the transcription of the core clock genes Bmal1, Clock, Per2 and Cry, and SIRT knockout or overexpression modulates circadian behavior (Chang & Guarente 2013a). Moreover, SIRT1 levels decreases in the aged SCN, which results in a longer free-running period in behavior, while overexpression of SIRT1 in the hypothalamus slows down the aging process (Chang & Guarente 2013b; Satoh *et al.,* 2013). The function of mTOR and SIRT1 in nutrient sensing and their potential to directly modify core clock genes in both the SCN, as well as in peripheral tissue points at an important role in conveying information on energy status to the circadian system.

Disturbances of circadian rhythmicity at the level of the SCN, the SCN target brain areas, or the periphery can cause diverse health issues, mostly based on changes in energy metabolism. The close entanglement of aging with the system regulating energy homeostasis and with the circadian system, highlights the role of energy consumption in modifying circadian rhythms and influence the aging process. Healthy and moderate food intake at the correct time of day will likely support peripheral oscillator functions as well as provide appropriate timing feedback to the hypothalamus, promoting energy homeostasis, overall health, and longevity.

4.2 Circadian rhythms and memory impairment – Aging of the hippocampus

One of the most debilitating consequences of the senescent brain is the diminished memory forming and retrieval., Even healthy aging is often accompanied by memory deficits, but aging is also the prominent risk factor for developing dementia. One of the brain centres playing an important role for declarative memory is the hippocampus and aging related memory deficits have been linked to changes in this brain area (Fan *et al.,* 2017). The hippocampus, like many other brain regions (Paul *et al.,* 2020a), exhibits circadian rhythms in clock gene expression and physiological functions. One example hinting how clock genes in the hippocampus may contribute to neurodegeneration is demonstrated by the deletion of clock genes (e.g. bmal1, clock and Npas2) leading to neuronal oxidative damage, seemingly through a disturbance in the redox homeostasis in the brain (Musiek *et al.,* 2013).

Circadian rhythms in learning and memory have been described (Chaudhury & Colwell 2002; Snider *et al.,* 2018) which seem to persist in VIP-KO mice with compromised SCN

function (Chaudhury *et al.,* 2008). Interestingly, the circadian modulation of long-term potentiation, which is thought to be a physiological correlate for learning, persists in hippocampal brain slices thus demonstrating that hippocampal rhythms may be independent of inputs from the central clock in the SCN (Wang *et al.,* 2009). Together the data suggest that local oscillators can control daily rhythms of neuronal performance in the hippocampus, however, detailed studies on these independent circadian oscillators and their function are still sparse.

Similar to the SCN, aging seems to affect the clock genes in the hippocampus less severe (Duncan *et al.,* 2013) compared to the more drastic effects on downstream rhythms in metabolites or membrane and synaptic properties as seen in a recent proteomics study (Adler *et al.,* 2020). However, while the impact of aging on hippocampal function is well described, the contribution of the circadian clock to aging related dysfunctions still needs further studies. It is important to realize that this effect of the circadian system on the aging hippocampus is not necessary a direct inpact from the central clock. One of the systemic timing cues, corticosterone, showed a lower amplitude of daily variation in plasma and hippocampus in aged rats (24 months), but its concentration was in both still higher during the night compared to the day. (Barrientos *et al.,* 2015). The plasma rhythms in old rats showed a reduction of their peak values during the night, while the corticosterone rhythms in the hippocampus were blunted by an increase of the through levels during the day. It is important to note that this study used automated blood-samplers, which avoided stress-induced corticosterone release in aged animals. Interestingly, the elevation of corticosterone in the hippocampus during the inactive phase of the old animals led to a stronger activation of glucocorticoid receptors, activation of microglia, increasing neuroinflammation and potentially contributing to cognitive decline (Barrientos *et al.,* 2015). A recent study on adult hippocampal neural stem cell pools, which represent a reserve for maintaining plasticity through neurogenesis also shows a negative effect of the presence of glucocorticoid receptors (Schouten *et al.,* 2020). However, using a mouse model for accelerated aging, this study also finds benefits of circadian modulation in corticosterone, which, together with ultradian oscillations, helps to sustain this reserve pool of neural stem/precursor cells. And finally, another example for the interconnectedness of the different players involved in aging is the influence of aging on microglia function in the hippocampus. These first responders of the brain immune system also have surveying function in their "resting" state and contribute to maintenance of synaptic function in brain networks like the hippocampus (Schafer *et al.,* 2012). In aging, microglia will change morphology and physiology and their immune response is compromised (Nissen, 2017). In addition, microglia in the aging hippocampus has been shown to have altered rhythms in period gene expression and lose their circadian rhythm in immune challenge induced cytokine response (Fonken *et al.,* 2016). Again, it seems that dampened amplitude of the corticosterone rhythms contributes to the disruption of circadian rhythms in microglia

and an exaggerated immune response in the aged hippocampus. It can be expected that the resulting increase in chronic inflammation will affect neuronal circuits in the hippocampus and the rest of the brain, as discussed below.

4.3 The interaction between circadian rhythmicity and chronic (neuro)inflammation

The link between the circadian clock and the peripheral immune system has been well established (Labrecque & Cermakian, 2015; Scheiermann *et al.,* 2018) and the clock also plays a role in CNS immune responses (Fonken *et al.,* 2015; McKee *et al.,* 2020), which can be of particular interest for aging and neurodegenerative diseases (Lananna & Musiek, 2020). The peak of immune responsiveness occurs at the end of the inactive period suggesting a preparation for potential attacks on the immune system during the active period of the organism (Curtis *et al.,* 2014). Chronic inflammation is a key hallmark of aging in periphery and the CNS, caused by malfunctioning autophagy, damaged cells and cellular debris lingering within tissue, sending out pro-inflammatory signals (Di Benedetto *et al.,* 2017). In the brain, interactions between neurons, astrocytes and microglia regulate the immune response (Castellani & Schwartz, 2020) and this crosstalk is significantly affected by aging (Santoro *et al.,* 2018; Rodriguez-Arellano *et al.,* 2016). In addition, astrocytes and microglia both have circadian clocks modulating their function (Fonken *et al.,* 2015; Prolo *et al.,* 2005) which are altered in aging (Fonken *et al.,* 2016). The astrocyte clock function has been best described in the SCN where it acts in concert with neurons to ensure the stable generation of circadian rhythm in neuronal activity (Prolo *et al.,* 2005; Brancaccio *et al.,* 2017) even in the absence of a clock in SCN neurons (Brancaccio *et al.,* 2019). There is some evidence that the astrocyte clock also plays a role in regulating neuroinflammation, by regulating Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) proinflammatory signaling (Sugimoto *et al.,* 2014) and brain-derived neurotrophic factor (BDNF) dependent protection of neurons from oxidative stress (Ishii *et al.,* 2019). The circadian clock in the brain's early responders to an immune challenge, the microglia (Bilbo & Stevens 2017), modulates gene expression of cytokines and response to an immune lipopolysaccharide challenge as found in isolated hippocampal microglia (Fonken *et al.,* 2015). Interestingly, these rhythms of cytokine release and immune response of microglia are blunted in 24 months old rats (Fonken *et al.,* 2016). Given that clock deficient monocytes show higher lethality after immune challenge (Nguyen *et al.,* 2013), the reduction of clock function in aging immune cells may be contributing to the loss of function in neuronal network in the SCN and the rest of the brain.

Aging-associated inflammation is not only a problem for the central nervous system, but also a systemic problem, and there are several ways the circadian system is known to contribute to (chronic) inflammation and a subsequent diseased state in peripheral tissue. For instance, forced circadian disruption in mice causes an exaggerated inflammatory

response and even death in response to an immune challenge (Castanon-Cervantes *et al.,* 2010). In a recent study, a causal link was found between disruptions in circadian rhythms, and the development of atherosclerosis through an aberrant inflammatory response (Schilperoort *et al.,* 2020). Inflammation and aging are often linked to melatonin and glucocorticoid, both of which display a strong circadian rhythm. Not surprisingly, the link between aging, aging-related inflammation and the circadian system is often made. However, the question remains to what extend the connection is (bi)directional, and thus, if purely strengthening circadian rhythmicity could lower inflammatorily burden in aging. Nevertheless, there is strong evidence that several interventions that strengthen the circadian clock, either directly or indirectly, also decreases inflammation markers.

4.4 Communication with the periphery – The aging pineal gland and its product, melatonin

An important relay station between the SCN and the periphery is the pineal gland. The pineal gland is innervated indirectly by the SCN, through a pathway leading from the SCN to the PVN in the hypothalamus and via ganglia in the spinal cord and to the pineal gland (Teclemariam-Mesbah *et al.,* 1999). The main function of the pineal gland is the production and release of melatonin, acting as a systemic circadian timing signal (Pang & Ralph 1975; Pelham 1975; Lewy *et al.,* 1980). Melatonin production by the pineal gland normally peaks during the dark period, and induces sleepiness in humans.

The role of the pineal gland in the aging process became clear from studying the effect of pinealectomy and pineal transplantations. Pinealectomy at a young age induced an aging phenotype, and transplanting young pineal tissue to aged animals caused rejuvenation (Pierpaoli & Regelson 1994; Pierpaoli & Bulian 2005; De Butte & Pappas 2007). However, it is still unknown to what extend the pineal gland itself is actually affected by aging. The pineal gland undergoes clear aging-associated calcification, but it is not certain if this affects its function (Tan *et al.,* 2018). One indication for a functional deficit is a significant advance in the phase of Per1 rhythms in the aging pineal gland (Yamazaki *et al.,* 2002). Moreover, there are many studies that found a dampened circadian rhythm in melatonin, because of lower melatonin levels during night-time (Iguchi *et al.,* 1982), which could be due to dysfunction of the pineal gland, or to reduced innervations from the SCN (Jengeleski *et al.,* 1989). The age-related decline in melatonin was questioned by a study using a rigorous method with a constant routine, and taking into account several non-prescription drugs, like ibuprofen and aspirin, which suppress melatonin and are often used by the elderly (Zeitzer *et al.,* 1999; Duffy *et al.,* 2002). However, the same researchers did find a reduced melatonin level when subjects were allowed to sleep, meaning that under real life conditions, melatonin rhythms do deteriorate in aging (Zeitzer *et al.,* 2007). In addition, melatonin receptor expression in peripheral tissue is found to decrease in aging (Sánchez-Hidalgo *et al.,* 2009).

The decline in melatonin amplitude as well as the decreased melatonin receptor expression could well be contributing to a diminished synchronization of the central and peripheral clocks (Pfeffer *et al.,* 2018). Importantly, aging had also been associated with a decline in melatonin sensitivity of the SCN (Wu *et al.,* 2007a; von Gall & Weaver 2008), which may partially account for the reduced rhythm strength of the SCN observed in aging (Pfeffer *et al.,* 2017). Melatonin is linked to several aging associated disorders, like cardiovascular diseases (Dominguez-Rodriguez *et al.,* 2010), type 2 diabetes (Karamitri & Jockers 2019), neurodegenerative diseases (Wu & Swaab 2005) and (neuro)inflammation (Hardeland 2018). However, it is currently unclear if this is a direct consequence of the attenuated melatonin signal, or indirectly acts through a weakening of the circadian system.

Melatonin, both from the pineal gland, as well as from other sources, has an important role in the immune system (Markus *et al.,* 2018), and administration of melatonin is found to counteract age-associated inflammation (Rodríguez *et al.,* 2007). Although most cells of the body can produce melatonin, it is the pineal gland derived melatonin that plays a role in the circadian rhythmicity of the immune response (Lopes *et al.,* 1997). The idea of melatonin as a contributor and possible treatment for neurodegenerative disease is currently receiving much attention (Cardinali, 2019; Socaciu *et al.,* 2020) and see below). This is on the one hand based on the finding that neurodegenerative diseases are associated with, and possibly preceded by a decline in melatonin levels (Wu & Schaap, 2005). On the other hand, melatonin has a proven neuroprotective role through its anti-oxidant and anti-inflammatory function (Alghamdi, 2018). And finally, melatonin supports the function of the SCN and may strengthen circadian rhythmicity in aging. However, the contribution of compromised systemic level or circadian rhythm amplitude of melatonin to the development of neurodegenerative diseases is still unclear (Zhang *et al.,* 2016; Cardinali 2019).

Thus, aging goes along with a decline in circadian rhythmicity of melatonin produced by the pineal gland. The decline in melatonin levels and rhythm amplitude is associated with several processes that are at the core of health deterioration during aging, like chronic inflammation and reduced synchronization of peripheral clocks (Rodríguez *et al.,* 2007; Pfeffer *et al.,* 2018). Concordantly, melatonin has many properties that can delay the aging processes, like its strong anti-oxidant function, promotion of neurogenesis and mitochondrial health (Tan *et al.,* 2016; Ramírez-Rodríguez *et al.,* 2009). More research is needed to confirm if treatment with melatonin leads to improve overall health in aging, and it will be very interesting to see to what extend a synchronization of central and/or peripheral clocks is involved.

4.5 Aging clocks in peripheral tissue

The discovery of the molecular clock has had a major impact on the subsequent discovery of self-sustaining clocks in a wide variety of tissues, from the liver (Yamazaki *et al.,* 2000) to the cochlea (Park *et al.,* 2017) and beyond (Brown *et al.,* 2019). It has led to the detection of differentially phased rhythms in a variety of tissues, painting the picture of a complex hierarchical system of central and peripheral pacemakers and oscillators (Brown *et al.,* 2019). Studies using bioluminescent reporters for clock genes have shown that the molecular clock in the SCN is relatively unaffected by aging, while the rhythmicity and phase shifting capacity of the molecular clock in the periphery is considerably more affected (Sellix *et al.,* 2012; Davidson *et al.,* 2008). For instance, Davidson and colleagues show that the Per2 expression rhythm in the aging liver is uncapable of following a 6-hour shift in the LD cycle within the six days measured in the experiment, while the aging SCN showed an almost similar re-entrainment as the young SCN (Davidson *et al.,* 2008). Even under normal lighting conditions, circadian rhythms drift out of phase in aging, affecting the whole-body synarchy of temporal processes (Yamazaki *et al.,* 2002; Wyse & Coogan, 2010; Bonaconsa *et al.,* 2014).

From deteriorating of whole-body homeostasis to impaired molecular processes within single cells, the effects of senescence are noticeable on all levels. The affected processes often concern metabolism, from aging-associated obesity, to mitochondrial deficiency. Most, if not all, metabolic processes are highly rhythmic, for example lipid and glucose homeostasis, and these rhythmic processes are often found to be disturbed in aging. A recent study shows that aging severely affects the levels and rhythmicity of lipids in brown and white adipose tissue, indicating a disturbed lipid metabolism (Held *et al.,* 2020). Aging is also associated with changes in rhythmic gene expression in the liver, affecting genes associated with metabolic processes (Sato *et al.,* 2017). Interestingly, both this study, as well as several others find that the rhythmic expression of the core clock genes in peripheral tissue is relatively unaffected by aging, compared to other classes of transcripts. This indicates that the molecular clock in the periphery is more resilient to aging than their target genes, which shows parallels to the aging SCN, discussed in paragraph 3.2 and 3.3.

In line with the fact that many of the processes affected by aging concern metabolism, dietary interventions that consist of caloric restriction boost longevity, and improve overall health (see 4.1). Importantly, time restricted feeding – allowing food intake only during certain hours of the day - can have a similar beneficial health effect, which is likely a consequence of a stronger temporal regulation of metabolism. For example, a study by Hatori and colleagues showed that the detrimental effects of a high-fat diet on health, like metabolic disease, type-2 diabetes and liver damage – diseases also prevalent in aging humans – could be counteracted by only adjusting the time-window of the availability

of the high-fat diet. It could even prevent weight gain, while the daily caloric intake was the same for the ad libitum and time-restricted feeding group (Hatori *et al.,* 2012). Furthermore, time-restricted feeding is capable of restoring gene expression rhythms in the liver and WAT when circadian rhythms are disrupted (Yamamuro *et al.,* 2020). Similarly, overall lowering of caloric intake also leads to a considerable upregulation of circadian rhythmicity in the aging liver, through the important metabolic pathway NAD^+/SIRT (Sato *et al.,* 2017). Beneficial effects are also found with diets that mimic continuous calorie restriction with a periodic reduction of caloric intake (Brandhorst *et al.,* 2015). Both these so-called fasting-mimicking diets as well as time-restricted feeding do not have clear negative side-effects, and are proposed a feasible intervention with health span increasing potential (Longo & Panda 2016). It is possible that the health benefits from these diets are partially attributable to the strengthened circadian rhythmicity, since there is a direct interaction between the main proteins involved in the effect of calorie restriction and the molecular clock (see 4.1). It will be interesting to investigate what paradigms lead to the best balance between health benefits and are also feasible to implement in daily life.

5. Circadian clocks and neurodegenerative disease

In addition to the general hallmarks of aging like mitochondrial dysfunction, dysregulated energy metabolism, oxidative damage, impaired proteostasis, inflammation, deficiencies in DNA repair and stem cell exhaustion (López-Otín *et al.,* 2013), brain aging is marked by impaired cellular stress response, aberrant neuronal network activity, and dysregulated neuronal calcium homeostasis (Mattson & Arumugam 2018). Non-pathological brain aging leads to compromised brain function, with a reduced cognitive function as one of the most common consequences. However, aging is accompanied by an increased risk for developing neurodegenerative diseases, with growing prevalence because of the increase in life-expectancy.

The relationship between the function of the circadian system and neurodegeneration is bidirectional; neurodegeneration can impair the SCN function (Bellanti *et al.,* 2017; Leng *et al.,* 2019) and the functional state of the circadian clock can affect the progression of the disease (Duncan, 2020). While this constitutes a downward spiral, the timely strengthening of clock function may activate an upward spiral, slowing the process of neurodegeneration (Fig. 2). Indeed, using bright light with melatonin (Riemersma-van der Lek *et al.,* 2008) or just melatonin (Wade *et al.,* 2014) as chronotherapeutic interventions in the elderly diagnosed with dementia resulted in improved cognitive function. In addition, the interventions also improved sleep disorders, which are often associated with neurodegeneration. Similarly, bright light also strengthened sleep-wake rhythms in Parkinson's patients (Videnovic *et al.,* 2017). However, given the small number of studies

and inconsistencies, more studies need to be conducted to determine the value for the treatment of neurodegenerative disease.

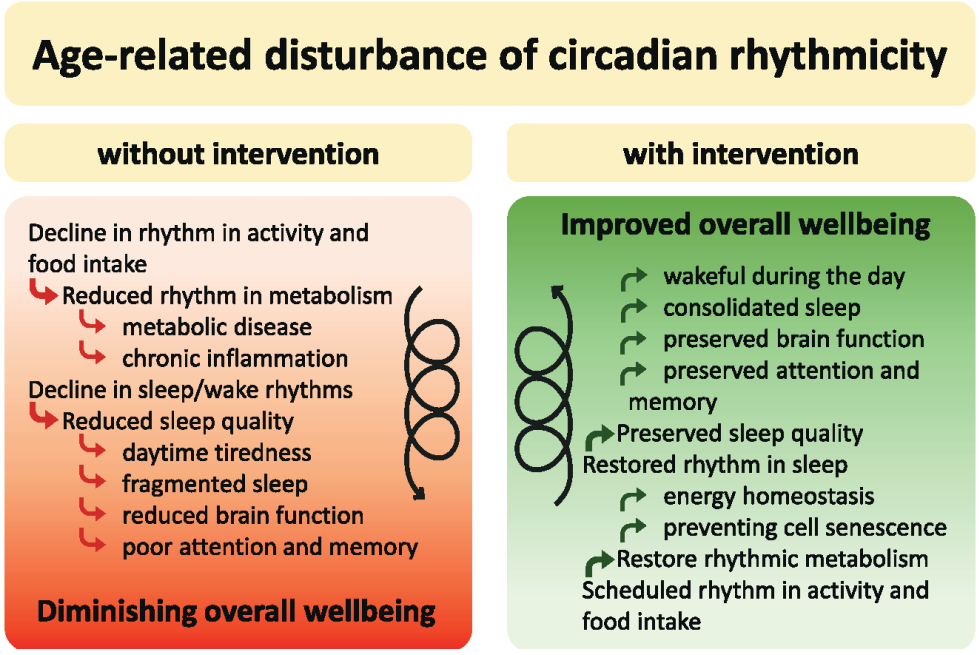


Figure 2. Effect of aging on the organism can produce a downward spiral while chronotherapeutic interventions can counteract this trend.

The impact of aging on circadian clock function is not necessary the result of neurodegeneration. Senescent-related alteration in clock function are often milder compared to deficits seen in neurodegenerative diseases like Alzheimer's disease (AD) (Chauhan *et al.,* 2017; Leng *et al.,* 2019). At the level of the SCN, there are ambiguous reports on neuronal loss in healthy aging (Madeira *et al.,* 1995; Tsukahara *et al.,* 2005; Roberts *et al.,* 2012) and the clock dysfunction is more likely caused by changes in cell physiology and network properties (see above). In contrast, volume and cell count of the central clock are reduced in AD (Swaab *et al.,* 1985) and specifically neuropeptidergic neurons are diminished in the SCN of AD patients (Zhou *et al.,* 1995; Ishunina & Swaab 2002). In addition to cell loss, a reduction of melatonin receptor expression (MT-1) has also been observed in AD patients, which reduces the positive feedback of melatonin on SCN function (Wu *et al.,* 2007b). Furthermore, the number of astrocytes in relation to neurons is increased in these patients, which indicates that another culprit of AD - neuroinflammation – is also contributing to the pathogenesis of AD in the SCN (Castellani & Schwartz, 2020). One general problem here is the distinction of senescent-related effects of neuroinflammation

and the additional effect of AD, since markers of neuroinflammation are also reported in the SCN of healthy aging rats (Deng *et al.,* 2010). Studies with AD models or AD patients has yet to reveal direct evidence for the link between neuroinflammation and AD in the SCN, although the prerequisites for this – increase in astrocytes and neurofibrillary tangles have been found in the SCN (Roy *et al.,* 2019). However, the SCN shows a somewhat untypical AD pathology with no mature plaques present, while the surrounding hypothalamus does show typical plaque development (Stopa *et al.,* 1999).

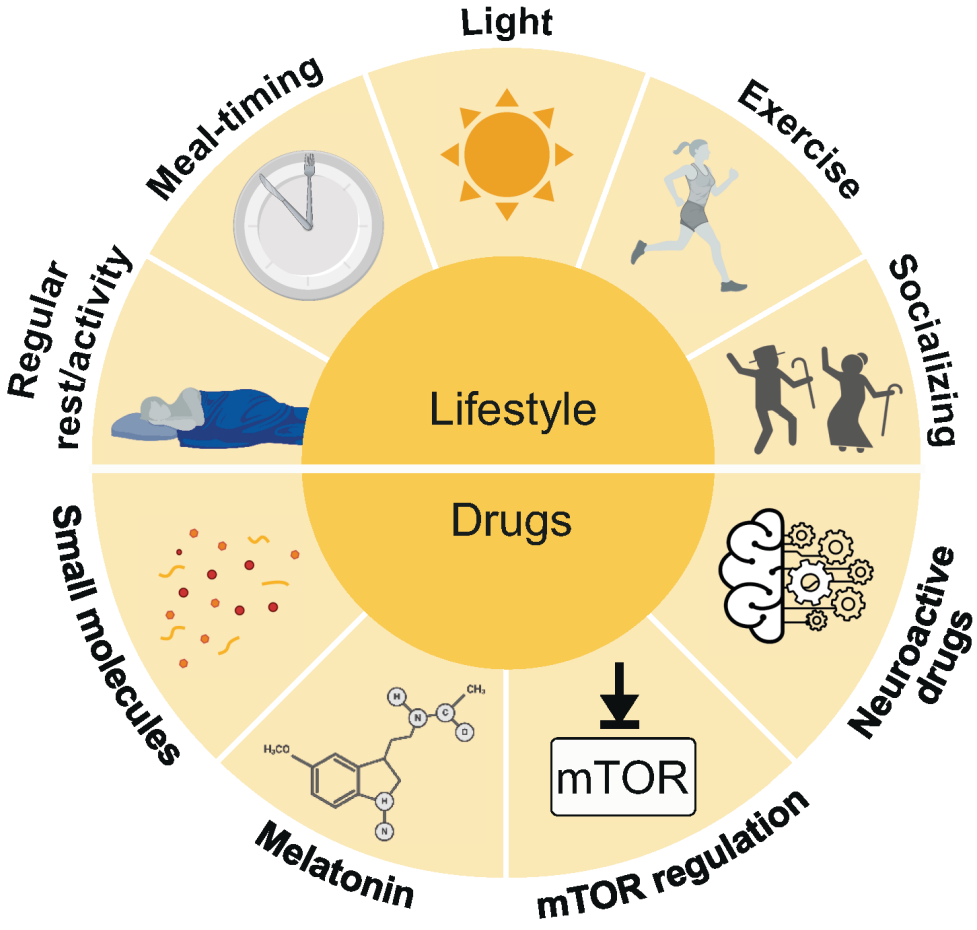


Figure 3. Potential intervention strategies to strengthen the circadian clock in aging. Among the lifestyle interventions regular bedtimes and mealtimes, exercise, social interaction and (day)-light exposure can synchronize the body clocks, when applied at the 'right' time. Pharmacological interventions with small molecules and melatonin may boost the amplitude of the cellular circadian rhythms, while neuroactive compounds may target network synchronisation. Inhibitors of mTOR like rapamycin acting on the cellular metabolism, may reduce the effect of aging on clock function.

An interesting question from both the mechanistic, as well as therapeutic view is if a dysfunctional clock promotes AD or if clock deficiencies are only caused by AD at a later stage. The current data of patients with preclinical stages of AD indicated that circadian disruptions set in very early if not preceding the AD pathogenesis (Musiek *et al.,* 2018). In addition, several studies confirm the correlation of sleep- or rhythms-disorders and AD pathology markers like amyloid-beta levels or neuronal atrophy (Sprecher *et al.,* 2017; Ju *et al.,* 2017; Van Someren *et al.,* 2019) and even cognitive decline (Waller *et al.,* 2016). The further investigation of this link may offer not only new mechanistic insights, but could also be useful for diagnostic purpose as well as chronotherapeutic approaches. The interventions discussed in the next paragraph could therefore not only promote healthy aging of the brain and our clock, but may also delay the onset and/or progression of neurodegenerative diseases.

6. Chronotherapies

Aging per se is not a disease and therefore interventions to counteract aging-related limitations in performance of the elderly can be questionable. However, aging can also present a health risk for many individuals, increasing their chances for cardiovascular and neurodegenerative diseases, among others. The degree of interventions is therefore dependent on the level of impairment caused by aging and/or aging-related diseases. The circadian clock with its unique position to regulate the temporal efficiency of many physiological functions in our body is in any case a good mediator for interventions with systemic impact.

One of the most efficient and non-invasive ways to influence the clock is light and, indeed, as mentioned above, light has been successfully used to strengthen the clock in the elderly and partially restore dysregulated sleep patterns. A second important gate to strengthen the clock is lifestyle. Regular and right timing of behaviors like eating, exercise and sleeping can help in turn to synchronize central and peripheral oscillators with positive effects on physiological function especially in aging and disease (Fig. 3).

Physical activity - even at a moderate level - seems to strengthen clock function in general (Hughes & Piggins, 2012; Leise *et al.,* 2013) and in aging in particular (Dupont Rocher *et al.,* 2016; Gu *et al.,* 2015). Similar to light, the timing of these activities is important. For instance, eating at the wrong time of day would have adverse effects on the clock. This has been shown in a number of animal experiments using scheduled feeding (Mukherji *et al.,* 2015) and is also evident from studies in humans (Wehrens *et al.,* 2017). Lifestyle-adjustments like regular bedtimes are also a tool to increase temporal order in the circadian system as misalignment can promote serious diseases like metabolic syndrome

(Leproult *et al.,* 2014). A third venue of interventions are substances that impact circadian clock function. One of the earliest agents used to influence clock function is melatonin or a synthetic agonist of it (Zisapel 2018). Studies in both shift-workers and the elderly have shown improvement of sleep-wake rhythms after administration of melatonin (Skene & Arendt 2006; Riemersma-van der Lek *et al.,* 2008). More recently, high throughput drug screenings have identified so called clock enhancing molecules: synthetic small molecules with little to no side effects which can influence phase, period or amplitude of the rhythm in central or peripheral clocks (Chen *et al.,* 2018). Currently some of these agents have been shown to successfully target the interface between circadian clock and serious diseases (Miller & Hirota 2020) like diabetes (He *et al.,* 2016), immune disorders (Solt *et al.,* 2011) and also age-related metabolic challenges (Nohara *et al.,* 2019) in animal models.

While controlled (day)light exposure as well as life style approaches are appropriate to assist the organism in healthy aging, the role of these and other chronotherapies in neurodegenerative disease has to be thoroughly investigated. As mentioned earlier, we need to know more about the interaction between circadian system and neurodegenerative diseases. Is it a mere correlation or are they causally connected? More studies using multiple markers of the circadian clock like body temperature and melatonin profile in addition to behavior measurements will give better insights into the mechanisms of circadian disruption in these diseases and their potential (chrono)treatment.

7. Conclusion and future developments

The central clock has many ways to confer temporal information to the rest of the body. It is the collective effort of molecular, cytosolic, membrane and network oscillators that make up the precision, strength and flexibility of the SCN rhythm, but there is some level of redundancy (Fig. 1). In addition, the function of the SCN is even better adapted and tuned to the challenges in environment and physiology by feed-back loops from its target organs and behavior. As a result, the SCN takes the role of a conductor of a well-rehearsed orchestra of peripheral clocks that efficiently regulates the temporal order in our body. The impact of aging on the clock function is therefore as complex as the intertwined symphony of rhythms. Therefore, strengthening of the rhythm of the SCN alone might not be sufficient to restore circadian rhythms in the rest of the body, and a multi-level intervention should be warranted. Supporting proper rhythmicity in both the SCN, the rest of the brain, as well as the periphery by means of correctly timed (and dosed) light exposure, food intake, exercise, and possibly treatment with drugs acting on clock function could contribute to healthy aging and slow down the progression of neurodegenerative disease (Fig. 3). While the lifestyle interventions are readily applicable to humans and mostly work on a systemic level, it is still unclear which cellular or subcellular targets would be most effective for drug

interventions. Aging has minor impact on the molecular clock therefore this level may be a less rewarding target for interventions. Overall, improving synchronisation within the cell, the SCN network and between central and peripheral clocks may re-instate precision and stability in the aged circadian system.

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