THE CLOCK IS TICKING. AGEING OF THE CIRCADIAN SYSTEM: FROM PHYSIOLOGY TO CELL CYCLE

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Abstract

The circadian system is the responsible to organise the internal temporal order of all physiological processes, stablishing a phase relationship between them producing an optimisation of the energy and resources. From a basis level, all these processes are controlled by genetic positive and negative auto regulated transcriptional and translational feedback loops of clock genes forming the cellular temporal machinery. It has been described about 10% of the genome are controlled by clock genes, with a special interaction with the cell cycle. Ageing is a deleterious process which affects all the organisms' structures including circadian system, producing a disorganisation between circadian rhythms which result in a harmful situation to an organism. Also environmental conditions which alter the circadian system could produce that disorganisation, also called chronodisruption, resulting in accelerated ageing. This review try to gather recent advances in the chronobiology field related with the ageing process, from physiology to cell cycle, including the neurogenesis process.

1. Introduction

From the life's origin, Earth has been rounding on its axis and around the sun, generating day/night and annual cycles. In these conditions, the organisms had evolved and the presence of a biological clock allows them to improve their survival rate by predicting the periodical environmental changes. Thus, possible damage on biomolecules produced by the most energetic electromagnetic waves could be diminished by predicting the daytime, metabolic processes could be scheduled improving the efficiency and cyclic events like breeding, hibernation or predator/prey habits can be prepared in advance.

All the living beings have a biological clock, which predicts, adapts and prevent the organism to the external cyclical environment changes. The circadian timekeeping system (from Latin *circa* and *diem*, means approximately one day) is responsible for predicting environmental cyclical cues, also called *zeitgebers* ("time giver" in German), and organising the internal temporal order. The circadian system is constituted by a series of structures hierarchically organized like a clock with inputs to wind-up the clock, the main pacemaker as the central machinery, and the circadian rhythms as the clock hands.

2. Structure and organisation

The circadian system differs in some characteristics among species. However, its general structure is very similar consisting of: 1) inputs, which receive the information from the main *zeitgebers* as light, environmental temperature or food availability ; 2) central pacemaker, which is in charge of keeping the temporal structure of the organism by inputs reception, its integration in useful information and the transmission of this information to the organism; 3) outputs, which are in charge of temporal signal transmission from the main clock to every cell of the organism (Figure 1A). In addition to the central pacemaker, there are other oscillators in brain and other organs such as kidney, liver, intestine or adipose tissue, whose function is to aid the main clock. Some of the outputs present also a synchronizer effect such as scheduled sleep, activity and feeding time [1-3]. These overt rhythms with both components (synchroniser and overt rhythm) are known as *zeitnehmer* ("time taker" in German), which is defined as an input pathway that is itself rhythmically regulated by feedback from an oscillator [4].

<u>2.1. Inputs</u>

Living beings appeared and evolved in a cyclic environment with the light-dark cycle as the most important environmental cue and, thus, the organisms used light information as the main *zeitgeber*. However, there are non-photic synchronisers that send information to the main clock in order to maintain the organism synchronisation [5].

In mammals, circadian photoreception occurs mainly by a subgroup of intrinsically photosensitive ganglion cells in the retina (ipRGCs) due to the presence of melanopsin [6,7], which shows a maximum sensitivity *in vivo* from 440 to 480 nanometres [8]. In addition, these ipRGCs receive also information from rods and cones [9,10], completing the information sent to the central pacemaker [11-13]. This information is transmitted to the master clock in the hypothalamus by the retinohypothalamic tract [14-16].

Thermocycle is also an important *zeitgeber* to the circadian system, which is capable to entrain cellular cultures *in vitro* [17], core body temperature of mice *in vivo* [18] and ectotherm organisms [19]. In addition, some overt rhythms present also a synchroniser effect such as feeding time, scheduled sleep and activity [4]. Food availability has an important role in the circadian physiology, since digestion, motility, nutrient absorption and mobilisation are organised around the feeding schedule to improve the efficiency of the processes. Regular exercise exerts a synchronising effect on the human circadian system improving physical health and affective disorders [1], while sleep habits are a weak synchroniser probably due to its ability to determine light exposure and to drift melatonin secretion and core body temperature by a fixed schedule [20]. Although social contacts were considered long time as a synchroniser [21], recent researches did not find any synchronising effect [3,22].

2.2. Central pacemaker

The master clock is embedded in specialised neural structures with specific anatomical organisation in different organisms: for example, the optic and cerebral lobes in insects; the eyes in certain invertebrates and vertebrates; the pineal gland in low vertebrates (several fish, amphibians, and reptiles). In mammals, the master clock is comprised of two small groups of around 20.000 heterogeneous and small neurons together with different populations of glial cells, the suprachiasmatic nucleus of the hypothalamus (SCN). Each single suprachiasmatic nucleus consist of two regions in terms of anatomical and physiological differences, the dorsomedial “shell” and the ventrolateral “core” [23-25]. The dorsomedial area has low neuronal density and expresses argininge-vasopresin (AVP) as main neuro neurotransmitter. The ventrolateral area is highly packed and expresses vasoactive intestinal polypeptide (VIP) as

main neurotransmitter [24]. The light information from the retina via retinohypothalamic tract to SCN is mainly received in the ventrolateral region, where immediate genes are induced by light, while dorsomedial region is more involved in gene expression; therefore, it is the place of reception of ventral projections and the origin of projections to other brain nuclei [26]. Thus, ventrolateral area is the responsible for light synchronisation, while output regulation is mediated by dorsomedial area [13,24].

At cellular level, each neuron is an independent oscillator with its own period, although all together are coupled to show a unique periodicity [27,28]. A molecular clock resides in each neuron, which consists in a series of genetic positive and negative auto regulated transcriptional and translational feedback loops (TTFL), evolutionarily conserved in metazoans [29], showing 24 hour rhythms in mRNA and protein levels of key clock components even in the absence of external rhythmic inputs [30]. The transcription factors BMAL1 and CLOCK (alternatively NPAS2 in the SCN) constitute a heterodimer, which activates the expression of clock genes *Period*, *Cryptochromes* (*Per* and *Cry*, respectively) *Rev-Erbα* and *Ror*, as well as other clock controlled genes (CCGs, representing approximately a 10% of the complete genome), by binding to E-box enhancement elements [29]. PER and CRY dimerize and inhibit their own expression by translocation into the nucleus, interfering with the CLOCK:BMAL1 heterodimer with a delay of several hours [31]. Also exist a second regulatory loop induced by CLOCK/BMAL1 heterodimers which activate the transcription of retinoic acid-related orphan nuclear receptors, *Rev-erbα* and *Rorα*. Afterwards, REV-ERBα and RORα compete to bind retinoic acid-related orphan receptor response elements (ROREs) present in the *Bmal1* promoter. REV-ERB proteins repress *Bmal1* transcription [32], while ROR proteins activate it [33] (Figure 1B). However, transcription oscillation of the gene *Clock* is very weak if not arrhythmic in the mammalian circadian system [34]. Members of the Ror family (α, β and δ) also present strikingly different expression patterns across tissues with varying circadian peak times [33]. Data from several studies ultimately suggest that ROR proteins may contribute differently to promote rhythmic *Bmal1* expression in a tissue-dependent manner [35], implying a Bmal1 essential role in a variety of functions depending on the tissue in which it is activated [36-38]. Finally, several isoforms of Casein Kinase I are involved in the degradation of PER by the proteasome, regulating the negative TTFL [39].

Once it is demonstrated that the molecular clock is ubiquitous in every cell-type studied [40], a question emerges about which is the difference of the master clock and all the other peripheral clocks. In this situation, it was suggested that the SCN generates oscillations

autonomously and synchronise the peripheral clocks thanks to its higher coupling level [41]. Most peripheral oscillators follow the circadian pacemaker with a 6 to 8 hours delay and the coupling between peripheral oscillators and central pacemaker is affected and modulated by the cellular energetic metabolism, in a manner that can overcome the SCN pacemaker action. At present, the cellular processes that allow such a fine-tuned control of the central pacemaker on the peripheral oscillators of the different organs and tissues are far from being totally understood and they represent a field of current intense investigation.

2.3. Outputs

The SCN drives the organism by means of neural or humoral mediators which synchronise peripheral clocks inside and outside the brain. The SCN have connections to the subparaventricular zone (SPZ), dorsomedial hypothalamus (DMH) and paraventricular nucleus (PVN) [42]. The SPZ regulates core body temperature by projections to the medial preoptic area [43]. In addition, SPZ sends connections to DMH, which controls sleep-wake pattern through projections to the ventrolateral preoptic area [44]. Feeding behaviour and activity are also controlled by DMH by connecting with the lateral hypothalamus (LH) and the orexygenic system [45-47]. Moreover, SCN has preautonomic neurons which regulate the sympathetic-parasympathetic balance innervating LH and PVN [48]. At last, SCN drives the release of a variety of releasing hormones as corticoid, gonadotropin and thyrotropin releasing hormones [49].

For the study of the human circadian clock it is not possible a direct measure of SCN, thus, a reliable overt rhythm is necessary for assessing the SCN functionality (known as circadian marker rhythm). From the wide range of circadian rhythms only a few accomplish the required characteristics (high amplitude, reliable, easy-measure, specific phase relationship with the circadian clock). The most commonly used marker rhythms are core body temperature, cortisol secretion and melatonin secretion patterns [50-54]. From these, the melatonin pattern is considered the gold standard because its synthesis and release is directly dependent of the SCN [55]. Melatonin (N-acetyl-5-metoxy tryptamine) is a pineal hormone which transmits the timekeeping signal [56]. The key enzyme in the regulation arylalkilamine N-acetil transferase (AA-NAT) is modulated by nervous input from SCN to the pineal gland through PVN [57,58]. During night, PVN neuronal activity promotes melatonin secretion while light increases SCN electrical activity, which inhibits PVN neurons and, thus, melatonin secretion [49,59]. Melatonin is produced during the subjective night whenever light is absent. Thus, melatonin pattern shows higher values at night and low values during daytime. These characteristics

converts melatonin into the endocrine daily clock since high melatonin levels in blood indicate night phase while low values of melatonin mean daytime. Cortisol secretion pattern is also considered a marker rhythm because it has a stable phase relationship with melatonin and shows a circadian pattern with the peak in the morning related to the usual awakening. Another marker rhythm is core body temperature, which shows higher values during daytime improving neurons' transmission velocity and muscular strength [60], suffering a decrease before the sleep necessary to initiate sleep [61]. Finally, core body temperature nadir coincides with the peak of melatonin pattern [51].

In addition, distal skin temperature is increasingly considered as a marker rhythm because is more comfortable for the experimental subject than other marker rhythms, it has a stable phase relationship with other marker rhythms as core body temperature, which is slightly delayed and it is in antiphase [61-63], or melatonin secretion since distal skin temperature evening increase coincides with the onset of melatonin release [64]. In addition, distal skin temperature depends directly on master clock by the sympathetic balance [48] and favours the heat loss producing the core body temperature fall to initiate sleep onset [54,61,65]. Finally, distal skin temperature is maintained during constant routine protocols [66], persists after mathematical demasking procedures [67] and it has been demonstrated its utility to assay the circadian phase [64,68,69].

3. When the age sets the rhythm. Circadian system ageing

Ageing is a universal progressive deleterious process that affects all the living beings. This process is endogenous and its last step is the organism's death. The circadian system is also affected by the ageing process at all the structures, from the inputs to the outputs through the circadian clock, and affects all levels: morphological, physiological and biochemical [70-72].

3.1. Inputs ageing

As it was mentioned above, the retina through the retinohypothalamic tract is the main input pathway. Ageing affects the light reception by pupillary myosis or crystalline lens yellowing impairing specifically blue light transmission, which is the most important to the circadian entrainment [72]. Interestingly, a 55 years old adult receives less than half the circadian photoreception of a 25 years old adult. This fact produces a general weakening of the circadian system light input [73]. Thus, elderly people should exposure longer to bright light (higher than 1000 lux) in order to counteract the light transmission impairment. However, duration of bright light exposure in young adults receive less than 120 minutes [74], whereas in the elderly

exposure time is only around 30-60% of this value [75]. Furthermore, light exposure levels are related to subjects' well-being; in this way, individuals with low light exposure experience sleep disorders, depression and rhythm alterations [76].

3.2. Central pacemaker ageing

The main disturbances of the SCN during ageing process are the reduction in the number of neurons [77] the alteration and/or reduction of synapses among them [78], functionality impairment measured by electrical activity [79] and attenuation of the firing rate pattern reducing day-night contrast [80]. In addition, there are alterations in neurotransmitters secretion such as the neuropeptide AVP [81]. Furthermore, using advanced imaging systems for PER2::LUCIFERASE reveals that each SNC individual neuron shows a normal rhythm, but there is strong rhythm dissociation among them [82]. Thus, each SCN individual neuron produce an attenuated rhythm together to the lack of synchronisation among them impairs the temporal signal. In addition to the alteration of the molecular clock, biochemistry and morphology of the suprachiasmatic nuclei occurs, the main output, melatonin, is dampened by pinealocyte receptors changes and pineal size reduction and calcification [71,83,84]. In mammalians, the molecular clock experiences a general dampening during ageing. *Clock*, *Bmal1* and *Per2* experience an amplitude reduction while *Per1*, *Cry1* and *Cry2* remains without change [85-87].

3.3. Outputs ageing

Since this is the last link in the chain of the circadian system is noteworthy that output changes could be due to the direct ageing effect and/or indirect by the deleterious ageing effect on inputs and master clock. As it was mentioned above, the main output of the SCN is the SPZ, which undergoes a similar ageing process to the SCN [80]. Thus, the main output of the master clock is affected by the ageing process.

In the overt rhythms, the main changes observed are a phase advance, rhythm fragmentation, amplitude dampening and period shortening [71]. However, regarding circadian period there is no total agreement in literature, so it has been reported that period length is similar to young people in forced desynchrony protocol for melatonin and core body temperature [88]. Melatonin secretion pattern experiences a consistent decrease as ageing progresses and phase advanced [89], while activity pattern shows a dampening due to the increase of night time values and the diminishing of daytime values [90,91]. Sleep-wake cycle and the core body temperature pattern ageing process are characterized by a dampening, fragmentation and

phase advance [71,92]. The ageing of distal skin temperature rhythm shows a clear phase advance [93]. In addition, animal models experienced the loss of anticipation capacity (lights on/off or feeding time for example), which is an evidence of the circadian system affectation [94,95]. Recently, a gradual and sequential process of circadian system disconnection of the environment during the last days of the organism's life has been described. This process ends with total disconnection and disorganisation of the circadian system leading to death of the individual [96]. Finally, and in contrast to the general behaviour, there is a group of oxidative stress inducible genes named as late life cyclers (LLC) which gain rhythmicity or even show a circadian rhythm for the first time in aged individuals [97,98].

4. Circadian rhythms and cell cycle

The two main cyclic regulatory mechanisms affecting biochemical reactions in cells are the cell cycle and the circadian clock (Figure 1B and 2). Although these two regulatory systems are characterized by distinct mechanisms, many evidences indicate that these cycles are indeed linked. For example, most mammalian diploid cells show a 24h cell cycle period, and the involvement of the circadian clock in the regulation of the cell cycle phases has been widely demonstrated in literature [99]. Based on that, the emerging field of chronotherapy aims to identify the optimal daytime of chemotherapeutics delivery to minimize side effect s and optimize the efficacy of the treatments [100].

Cell cycle is characterized by well-defined biochemical phases finely tuned by a pool of regulatory factors, that are synthetized activated and degraded during precise phases of the cell cycle (Figure 2), and a tight regulation of the cell cycle is crucial to the survival of a cell, including the detection and repair of genetic damage, as well as the prevention of uncontrolled cell division. As a demonstration of the essential role of this process, eukaryotic cell cycle is controlled by a regulatory network whose features are evolutionary conserved from yeast to humans [101-103]. A fundamental role in this regulation is played by the two protein families of Cyclins and Cyclin-dependent Kinases (CDKs), that are responsible for the progression of the cycle by acting in the form of Cyclin:CDK complexes, where they represent the regulatory subunits and the catalytic subunits of an activated heterodimer, respectively [104-111]. On the other hand, cell cycle progression is prevented by the repressive action of several phase-specific inhibitors: two major groups of inhibitory factors are known: the *cip/kip* and the INK4 families (Figure 2). Cip/kip family includes the genes p21, P27 and p57 which stop cell cycle in G_1 phase, by binding to, and inactivating, cyclin-CDK complexes [112,113]. Indeed the regulatory action of the Cip/Kip family members results to be essential for the normal

developmental processes in mammals [114]. The second group of CKIs is represented by the INK4 family, whose members specifically bind CDK4 and CDK6 and inhibit cyclin D association: among them we find $p15^{INK4B}$, $p^{16INK4A}$, both having an important role in checkpoint control rather than differentiation [115,116], and $p18^{INK4C}$, $p19^{INK4D}$, that emerged as a subset of CKIs essential in preventing terminal differentiation, at least in a cell line model system [117].

Cell cycle is characterized by three main "checkpoints", where the cell cycle can be stopped if specific molecular/cellular requirements are not fulfilled [118]: the G_1/S checkpoint (or "restriction point"), where the amount of raw materials necessary to fully accomplish DNA replication is assessed; the G_2/M checkpoint, where the presence of the right amount and composition of cytoplasmic material to sustain the formation of two daughter cells (and define symmetrical/asymmetrical division, for example) is assessed; finally, the metaphase (mitotic) checkpoint, where the correct alignment of chromosomes along the spindle is assessed before anaphase. The above mentioned checkpoints are characterised by a network of regulatory proteins that check and regulate the progression of the cell through the different cell cycle phases. Regulation of cell cycle checkpoints plays an important role in many physiological processes, such as development, or embryonic and adult neurogenesis, and their dysregulation is frequently involved in a number of pathologies, such as several types of cancer [119-123] and developmental defects in many experimental organisms [124,125]. Therefore, they also represent ideal candidate entry points through which the cell cycle can reciprocally interact with other key-pathways of the cell metabolism, such as the circadian cellular clock.

4.1. Role of clock genes in the cell cycle

More generally, we can state that interaction of clock/cell cycle genes takes place at the level of specific cell cycle checkpoints: more in detail, the mammalian period paralogues *Per1* and *Per2* seem to be part of the molecular network involved in the repression of G1-S transition, while the circadian transcription factors *Bmal* and *Clock* take part to the molecular network which regulates tG2-M transition [126-129]: indeed, Per1 and Clock1 involvement in the cell cycle control has been recently confirmed in diurnal low vertebrates as well (Figure 3) [172]. ~~I~~Moreover, it has been reported~~, for example~~, that per1 associates with both Chk2 and ATM proteins and directly participates to the signaling of ATM3-Chk2 DNA damage pathway [130].

Cell proliferation is synchronized under physiological conditions and often shows asynchrony between normal and malignant tissues, so highlighting the importance of the circadian clock in cancer context and providing a strong theoretical support to the field of the emerging cancer

chronotherapy approaches [131]. In particular, the tumor suppressive action of *Per2*, mediated through its cell cycle control action – as indicated by the induction of apoptosis, inhibition of cell growth, reduced colony formation and growth in soft agar – has been clearly shown in several studies [132-134]. In cancer (which obviously includes a prominent a dysregulation of the cell cycle) it has been reported that many CCGs, such as *c-Myc*, *p53*, and cyclins, are involved in the regulation of cell cycle and apoptosis [132]. Absence of *p53*, for example, is associated with impaired cell cycle regulation, apoptosis inhibition and genomic instability in many tumoral conditions and the main way by which *p53* mediates tumor suppression is through elimination of abnormally proliferating cells [129,133,135]. It has been shown that *Per2* expression in MCF-7 cells significantly raised *p53* levels and in *Per2* expressing breast cancer cells there is an elevation in p53 expression, which contributes to promote G1 arrest and apoptosis [133]. Indeed, it has been described that the overexpression of *Per1* leads to cell death in numerous cancerous cell lines, presumably through the activation of the ATM3-Chk2 signaling pathway, by halting proliferation of cell and stimulating apoptosis [130].

Finally, although *Per2* can function as a tumor suppressor independently, its activity is significantly enhanced in the presence of *Cry2*, its clock partner [133].

4.2. Interaction with other molecules of interest: *Wee1*, *p21*, *c-Myc* and human *Timeless* (*h-tim*) protein

Clock pathway controls the expression of a huge number (up to 10%) of all mammalian genes, most of which are tissue- and/or organ-specifically expressed. However, some genes of the cell cycle/DNA repair pathways are expresses in more than one organ [136] and are functionally and molecularly linked with specific clock genes: these interactions are essential for the maintenance of the genome integrity and stability [137].

The cell cycle and circadian clock are two main regulatory systems of the cellular and organismic physiology and therefore, the existence of reciprocal interactions between different components of the two pathways is not surprising (Figure ~~3~~4). Indeed, the two systems interface at some critical points. For example, it has been demonstrated that in proliferating cells, main clock components affect the cell cycle by controlling *Wee1* expression, a kinase that regulates G2-M phases transition by acting on Cdc2 activities, and vice-versa *Wee1* is positively regulated by BMAL1-CLOCK heterodimers [126,138,139]

Another molecule playing a key role in the interconnection between the two pathways is p21: this protein belongs to the Cip/Kip family of cyclin-dependent kinase inhibitors and represses

cell cycle progression by inhibiting cyclin E-cdk2 complexes activity during G1, as well as inhibiting DNA replication via binding to proliferating cell nuclear antigen (PCNA). Moreover, p21 is activated by p53 after DNA damage, and its mRNA levels result dramatically increased in *Bmal1*-null mice. In particular the last observation suggests that p21 is directly repressed by *Bmal1*, playing to all effects the role of CCG [140-143].

Also the protypical onco-gene *c-Myc*, a major positive regulator of cellular proliferation, behaves in many contexts as a clock-controlled gene: it is repressed by *Bmal1-Clock* and, consequently, its expression is significantly increased in *Per2* mutant mice, where the *Bmal1* expression is down-regulated, due to loss of its transcriptional activator per2 [132].

The connection between cell cycle and circadian clock pathways occurs not only at transcriptional level, but also at the protein–protein interaction and signal transduction level. For example, TIMELESS protein is known to be essential for a normal circadian rhythm in mice and human [144,145]. TIMELESS directly interacts with the checkpoint proteins ATR and Chk1 of the cell cycle, so that downregulation of the circadian TIMELESS protein interferes with the action of Chk1-ATR3 complex on DNA damage checkpoint response. TIMELESS acts in all respects as a cell cycle checkpoint protein [145]. In contrast with previous studies disclaiming the role of TIMELESS as clock protein [146-148], it has been recently shown that TIMELESS represents a circadian variable essential for the molecular clock pathway in the SCN [144]. The existence of two splicing forms of mammalian TIMELESS (the full-length *Tim* showing circadian oscillations, and the most abundant truncated form which does not oscillate and does not have any clock function) can explain the strong discrepancy between experimental findings reported in the literature -TIMELESS act definitely as a key-element in the connection between the two pathways, showing a bivalent role, on one side as important cell cycle checkpoint protein [149-151], and as active circadian clock protein on the other side [152,153]. Although the cellular mechanisms driving the coupling of cell and circadian cycle are far from being completely elucidated, it seems that TIMELESS protein has all the essential characteristics to play the role of a fundamental connection-factor between the two cellular processes, actively participating to the molecular machinery of both pathways and connecting them by the contemporary interaction with molecules specific of the two processes [145].

5. Circadian rhythms, neurogenesis and ageing

During embryonic development, all neuron derive from a dynamic process during which neuroepithelial cells of the neural crest and primary neural stem cells (NSCs) give rise to

several non-neuronal and neuronal cell types, through intense proliferative events, migration, establishment of synaptic contacts and subsequent massive neurons elimination by programmed death (apoptosis).

Although the general knowledge is that terminal-differentiated cells, such as neurons, finally exit from the cell cycle, entering a quiescent phase (G0) and lose the ability to divide [154], the incorporation of [3H]-thymidine into DNA of dividing neural stem and progenitor cells provided clear evidences for the generation of new neurons in postnatal mouse brain regions, such hippocampus and olfactory bulb (OB) [155]. Postnatal neural proliferation is modulated by physiological and pathological stimuli such as running and seizures and induction of this process is envisaged as a promising strategy for regenerative medicine as opposed to stem cell transplantations [156]. Conversely, NSCs also are deeply embedded in the etiology of gliomas, one of the most lethal forms of cancer [157]. According to what stated above, we can easily understand the importance of a strict and finely-tuned cell cycle regulation, to ensure the realisation of the correct central nervous system (CNS) formation process during mammalian development. Indeed, the deep comprehensions of the cellular mechanisms underlying neuronal proliferation during adulthood is an essential factor to develop further therapeutic strategies to prevent brain damages under many different context, such as age-related neurodegenerative pathologies, stroke-induced brain injuries, etc…

In the past, many studies focused the attention on the role and importance of the cell cycle components in controlling the proliferation and differentiation of NSCs during the embryonic development of the nervous system, by providing a deep level of knowledge of the cellular and molecular mechanisms underlying their involvement in the control of these developmental processes. However, more recently, the interest of research in this field was attracted by the regulatory processes carried by cell cycle control of adult NSCs. For details, all the most recent and fundamental findings in the topic of cell cycle machinery involvement in the regulation mechanisms on embryonic and adult neurogenesis are exhaustively reviewed in Cheffer et al. (2013) [158]. However, despite a steady progress in understanding cell cycle control in the adult brain, several questions still lack answers. In this context, an aspect which certainly deserves attention is the analysis of the interactions between cell cycle machinery and the circadian clock in the organ that is best suited to integrate any form of external inputs: the brain.

5.1. Interaction between neurogenesis and clock genes

In the last two decades, the introduction of nucleotide analogs, such as bromodeoxyuridine (BrdU), as lineage tracer [159] led to the detection of a life-long continuous neurogenesis process in almost all mammals examined, including humans [160]. Active adult neurogenesis is normally restricted to two "neurogenic" brain regions, the subgranular zone (SGZ) placed in the dentate gyrus of the hippocampus, and producing new granular neurons, and the subventricular zone (SVZ) of the lateral ventricles which produces new GABAergic and Dopamenergic interneurons migrating to the OB through the olfactory migratory stream (OMS) [161]. Several aspects related to the adult neurogenesis molecular processes have been studied and exhaustively reviewed in literature [162].

Adult neurogenesis can be regulated by intrinsic and extrinsic mechanisms at different levels, and many molecular factors and signaling pathways have been recognized to play a prominent role in neurogenesis regulation, including niche-specific factors/receptors, cytoplasmic molecules, transcriptional factors and epigenetic regulators (reviewed by Ma et al., 2010; Mu et al., 2010; Ninkovic and Gotz, 2007; Sun et al., 2011 [163-166]).

In the last years, it has been extensively demonstrated that the circadian molecular clock plays an essential role in the regulation of adult neurogenesis in vertebrates, both in physiological and altered conditions, such as ageing, spontaneous diseases, associated or experimentally-induced neurodegenerative processes [167-175].

It has been described that the quiescent NSCs of the hippocampus SGZ, which are able to reenter in the cell cycle to produce newborn neuron during adulthood, express molecular-clock components, such as PER2 and BMAL1, and show a rhythmic proliferative behaviour, with higher proliferation during the subjective night of the animals [168]. In particular, absence of PER2 has been shown to prevent the gating of cell cycle entrance of NSCs, whereas genetic ablation of *Bmal1* caused constitutively high levels of proliferation, together with a delayed cell cycle exit. These data establish a clear connection between the circadian clock and the cell cycle control during adult hippocampal neurogenesis. On the one hand, PER2 action limits the overall number and timing entrance of quiescent neuronal progenitors (QNPs) into the cell cycle. On the other hand, BMAL1 is essential not only for maintaining rhythmicity in cell-cycle entry of QNPs, but also for limiting the number of stem cells that leave the quiescent state. The role of clock genes in the regulation of hippocampus adult NSCs has also been demonstrated *in vitro*, using *Bmal1*$^{-/-}$ and *Cry*$^{-/-}$ Dentate Gyrus (DG) neurosphere cultures [167]: the absence of these clock genes slowed down the neurospheres growth and suppressed

neuronal fate commitment (this last effect residing indeed in the non-clock *Bmal1* function), whereas increasing apoptosis.

The molecular mechanisms by which the clock might regulate neurogenesis are manifold, and could explain, at least in part, the presence of heterogeneous results in the literature. Circadian clock could directly affect differentiation by acting on E-box elements in the promoter of neurogenic transcription factors, such as NeruoD1, Pax6, etc. [175], or else regulate fate commitment by modulating miRNAs. For example, the CLOCK/BMAL1 heterodimer regulates miRNA 219, involved in oligodendrocyte differentiation [176,177]. Finally, differences could be due to the use of animals with different genetic background, age, and/or housing conditions.

It is well documented that enhanced hippocampal neurogenesis correlates with better cognitive performances in animals under different experimental conditions [178-180] and the contribution of hippocampal adult neurogenesis to vertebrates behavioural plasticity is extensively reviewed in Gonçalves et al. (2016) [181]: indeed, circadian rhythms enable animals to prepare for cyclic events important for their survival. Impairing the normal expression of core circadian clock proteins, typically expressed in the hippocampus, causes deficits in habituation, exploratory behaviour and learning [182].On the other hand, reduction of adult neurogenesis in the hippocampus correlates with learning deficits and impaired memory functions in experimental settings, neurodegeneration and ageing [183-185]. For example, Bmal1$^{-/-}$ mice showed reduced learning performances and displayed accelerated ageing phenotypes [186]. Further, *Per1*$^{-/-}$ and *Per2*$^{-/-}$ mice showed impaired trace-fear memory, suppressed long-term potentiation (LTP), and diminished CREB phosphorylation [187-189].

In conclusion, a deep understanding of the NSPC proliferative activity rhythms can be fundamental to determine when the cells are least sensitive to negative effects of specific pharmacological treatment, as in the case of cancer chemotherapies that could be cyclically administered at a specific NSCs cell cycle phase, in order to minimize toxic effects on NSCs and adult neurogenesis.

5.2. Neurogenesis and ageing

A large body of studies in mammals, based on incorporation of thymidine analogs, retroviral tracing, genetic labeling as well as ^{14}C quantification and post-mortem immunohistochemical investigations in humans, demonstrated that neurogenesis persists in the adult brain with a clear age-dependent decrease [156,190-192]. In humans, mice and dogs the number of

dividing cells in the hippocampus decreases exponentially throughout postnatal life therefore resulting in an early decay of adult neurogenesis [191,193-195]. Finally, age-dependent reduction of adult neurogenesis has been demonstrated also in teleosts despite a much larger population of adult neuronal stem cells [196]. Increased radial glia quiescence, decreased reactivation upon injury and unaltered neuroblast behaviour underlie decreased neurogenesis in the ageing zebrafish telencephalon [197].

Finally, adult neurogenesis is highly plastic and can be enhanced by physiological stimulation such as sensory stimulation, physical exercise and learning (reviewed in Kempermann, 2011 [156].

Although in the last two decades the research on neurogenesis and correlated mechanisms has intensified significantly, the neurobiological changes that contribute to the age-dependent neurogenesis decrease are not yet totally understood. Quantitative analyses of cell division in both SGZ and SVZ of young adult and older animals, (by BrdU labelling of S-phase cells) have demonstrated a huge decline in BrdU-labeled cells number present in the neurogenic regions, shortly after BrdU injection, indicating that reduced mitotic activity contributes to age-dependent decline [159,198-206]. Nevertheless, several studies support the idea that a large proportion of quiescent stem cells exist in the aged brain and this population can be reactivated to restore neurogenesis at a youthful level, as demonstrated by the fact that aged brains respond to many stimuli, by inducing levels of neurogenic activity comparable to young brains [199,200,203,204,207-211]. Other studies, always based on the BrdU labeling method, support the conclusion that ageing does not diminish the survival of newborn cells, but rather that the decline in neurogenesis is mostly attributable to a decreased proliferation [159,200,207]. However, although the survival of newborn cells in neurogenic regions appears to be unaffected by age, the percentage of terminally differentiated neurons is much lower in middle-aged and old animals than in young adults [198,200,207], and the critical steps seem to be not the neural commitment phase (in fact a comparable percentage of newborn cells expresses the neuroblast marker doublecortin (Dcx) 24 hours after BrdU labeling, between young and old subjects), but rather the migration and maturation phases [212].

Given the circadian control of mitotic activity in neuronal precursors, it is highly likely that impairments of circadian rhythms in the ageing brain affect neurogenesis. It remains to be elucidated whether also commitment, survival and integration of newborn neurons is under control of circadian core genes.

6. Chronodisruption and circadian system ageing

Circadian disruption or chronodisruption is the result of the misalignment between the internal clock and *zeitgebers* or the unstable and/or wrong phase relationship among circadian rhythms (a combination of them is also possible). This syndrome appeared favoured by artificial light [213] and it is frequent in people exposed to bright light at night, darkness during daytime, chronic and/or social jet-lag and shift-work. However, other input anomalies as meal shift or frequent snacking may also result in chronodisruption [214]. In fact, citizens of modern societies live most of their life indoors, a very chronodisruptive environment, characterised by dim light, warm temperatures, irregular sleep time, low physical activity and frequent meals or constant snacking [215]. The ageing process or lesions of the SCN can also produce internal misalignment, supressing overt rhythms, from activity to core body temperature [216,217]. Chronodisruption is associated to a predisposition to metabolic syndrome, cardiovascular diseases, cognitive and affective impairments, sleep disorders, premature ageing, prostatic, mammary and colorectal cancer and, in general, higher mortality [218-225].

Ageing is a source of chronodisruption since affects the circadian system at all levels as we mentioned above. In summary, the ageing process produces: 1) light entrainment impairment by reducing light reception and blue light transmission [72]; 2) master clock degeneration due to the reduction of the number and functionality of neurons and synapses together to the attenuation of SCN firing rate by the uncoupling of individual neurons [77-82]. In addition, pineal size reduction and calcification impairs the main SCN output [71,83,84]; 3) overt rhythms such as sleep-wake cycle or activity fragmentation, phase advancing and amplitude reduction provoke a lack of day-night contrast in aged people [90,91], which will cause chronodisruption in the short-term.

7. Chronoenhancement: a new strategy

Chronodisruption is becoming a new health concern in the XXI century. Thus, modern societies need countermeasures to reduce its impact on human health. There are three main intertwined strategies to prevent chronodisruption consisting in:

1) Circadian resonance: consist in the perfect entrainment of the internal clock to the environmental cues [226]. In this sense, ageing with the circadian pacemaker not entrained with the environment is clearly deleterious to health [227]; ageing in absence of environmental cues seems to be healthier [228]; ageing with the master clock perfectly entrained to environmental cycles is the healthiest approach [82].

2) Physiological and behavioural chronoenhancement: based on the enhancement of the circadian inputs to obtain an increase in day-night contrast [215]. Because of that, the number of possible strategies is as large as the number of circadian system inputs. As main *zeitgeber* the solar light exposure shows a strong effect increasing day-night contrast [74] and bright light therapy reduces cognitive and affective disorders [229,230]. As antagonist of light, darkness is also necessary since constant light provokes chronodisruption in short-term [231] and it is related to some types of cancer in long-term [232]. Regular exercise helps to synchronise the human circadian system in humans [1] and regular meal schedule synchronises circadian rhythms in animal models [2]. Social interaction is able to entrain animal models, however these results are not confirmed in humans [3]. Finally, sleep habits have a weak synchronising power, but in humans it partially determines light exposure and drives melatonin secretion and core body temperature [20].

2) Pharmacological and genetic chronoenhancement: circadian clocks empowering by means of chronobiotics such as melatonin is a worldwide known strategy to delay or reduce ageing symptoms [95,233]. In addition, dexamethasone is able to synchronise the cell clock by activating *Per* expression in tumour cells, which would be useful for cancer chronotherapy [234]. However, there are new chronoenhancement strategies like genetic engineering. In this sense, overexpression of *Cry* delay the apparition of age-related symptoms in the fruit fly [235]. This topic is in the cutting edge of the chronobiological research and opens a new promising objective in cancer therapeutics.

Declaration of Interest

The authors report no conflicts of interest.

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