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REVIEW

A Crosstalk between the Biorhythms and Gatekeepers of Longevity: Dual Role of Glycogen Synthase Kinase-3

Gregory A. Shilovsky[1,2,3,a]*, Tatyana S. Putyatina[2], Galina V. Morgunova[2], Alexander V. Seliverstov[3], Vasily V. Ashapkin[1], Elena V. Sorokina[2], Alexander V. Markov[2], and Vladimir P. Skulachev[1]

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119991 Moscow, Russia*
[2]*Faculty of Biology, Lomonosov Moscow State University, 119234 Moscow, Russia*
[3]*Institute for Information Transmission Problems, Russian Academy of Sciences, 127051 Moscow, Russia*
[a]*e-mail: gregory_sh@list.ru; grgerontol@gmail.com*

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Abstract—This review discusses genetic and molecular pathways that link circadian timing with metabolism, resulting in the emergence of positive and negative regulatory feedback loops. The Nrf2 pathway is believed to be a component of the anti-aging program responsible for the healthspan and longevity. Nrf2 enables stress adaptation by activating cell antioxidant defense and other metabolic processes via control of expression of over 200 target genes in response to various types of stress. The GSK3 system represents a "regulating valve" that controls fine oscillations in the Nrf2 level, unlike Keap1, which prevents significant changes in the Nrf2 content in the absence of oxidative stress and which is inactivated by the oxidative stress. Furthermore, GSK3 modifies core circadian clock proteins (Bmal1, Clock, Per, Cry, and Rev-erbα). Phosphorylation by GSK3 leads to the inactivation and degradation of circadian rhythm-activating proteins (Bmal1 and Clock) and *vice versa* to the activation and nuclear translocation of proteins suppressing circadian rhythms (Per and Rev-erbα) with the exception of Cry protein, which is likely to be implicated in the fine tuning of biological clock. Functionally, GSK3 appears to be one of the hubs in the cross-regulation of circadian rhythms and antioxidant defense. Here, we present the data on the crosstalk between the most powerful cell antioxidant mechanism, the Nrf2 system, and the biorhythm-regulating system in mammals, including the impact of GSK3 overexpression and knockout on the Nrf2 signaling. Understanding the interactions between the regulatory cascades linking homeostasis maintenance and cell response to oxidative stress will help in elucidating molecular mechanisms that underlie aging and longevity.

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Keywords: GSK3, Nrf2, oxidative stress, aging, biological rhythms, aging programs, anti-aging programs, antioxidants

INTRODUCTION

Attaining longevity *per se* is not a top-priority evolutionary goal for living organisms. From the evolutionary viewpoint, the priorities are adaptation to the environment, survival, food acquisition, and reproduction. Regulation of homeostasis and repair processes, the efficiency of which decreases with aging, involves a complex network of interrelated processes [1-9]. Under specific circumstances, nonetheless, aging can hold adaptive value [4, 5].

The major goal of biogerontology is elucidation of the pathways that link aging/anti-aging programs with

Abbreviations: AD, Alzheimer's disease; ARE, antioxidant response element; Bmal1, brain and muscle ARNT-like 1 protein; CK, casein kinase; Clock, circadian locomotor output cycles kaput protein; CRY, cryptochrome protein; D3T, H3-1,2-dithiole-3-thione; Dbp, albumin site D-binding protein; GSK3, glycogen synthase kinase 3; Keap1, Kelch-like ECH-associated protein-1; LPS, lipopolysaccharide; MEF, mouse embryonic fibroblast; Npas2, neuronal PAS domain-containing protein; Nrf1, nuclear factor erythroid 2-related factor 1, NFE2-related factor 1; Nrf2, nuclear factor erythroid 2-related factor 2, NFE2-related factor 2; NQO1, NAD(P)H:quinone oxide reductase; Per, period protein; Rev-erbα, reverse erythroblastosis virus α protein; RORE, RAR-related orphan receptor response element; ROS, reactive oxygen species; SCN, suprachiasmatic nucleus of the hypothalamus; β-TrCP, β-transducin repeat-containing protein; WT, wild type.

* To whom correspondence should be addressed.

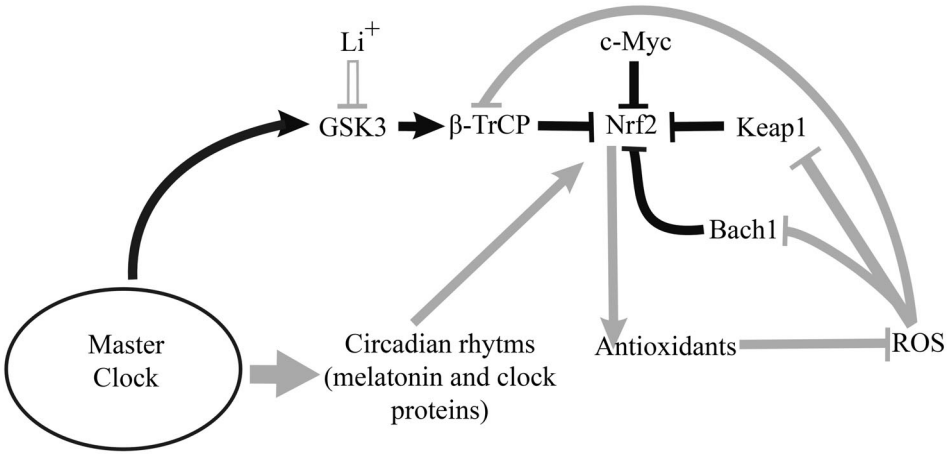

Fig. 1. Master Clock regulates cellular antioxidant status by acting on the transcription factor Nrf2, which controls the expression of more than 200 cytoprotective enzymes responsible for detoxification and antioxidant defense. The Master Clock promotes body adaptation by regulating circadian rhythms via melatonin and circadian clock proteins. Nrf2 is activated by reactive oxygen species (ROS). Simultaneously, glycogen synthase kinase 3 (GSK3), activated through a variety of signaling pathways, functions as a suppressor by inhibiting Nrf2 activity. Preparations containing lithium salts produce a positive effect by inhibiting GSK3. Gray lines, interactions resulting in the stimulation of Nrf2 activity (and subsequent expression of antioxidant enzymes), including suppression of Nrf2 inhibitors; black lines, interactions resulting in Nrf2 inhibitions; arrows, direct stimulating effect (including catalysis); blunt lines, inhibition. Bach1, BTB domain and CNC homolog 1 protein 1; Keap1, Kelch-like ECH-associated protein-1; β-TrCP, β-transducin repeat-containing protein.

adaptation and homeostasis maintenance mechanisms, including those providing biorhythm regulation. Interaction between diametrically opposed aging and anti-aging programs determines the shape of survival curves and their alterations in the course of biological and, in the case of humankind, cultural evolution. Over the last 150 years, the human mortality dynamics has drastically changed due to the scientific and technological revolution. These changes have resulted in a substantial lifespan prolongation. The survival curve has become almost rectangular, which clearly distinguishes it from the survival curves of chimpanzees, hunter-gatherers, and even the population of developed European countries in the 18th and 19th centuries (see [1] for details). However, there are also internal factors that influence the lifespan and the pattern of survival curves. They include aging and anti-aging programs, which, in terms of current concepts, represent a set of signaling cascades regulating gene expression [1-6]. If longevity is supported by natural selection (e.g., in the case when "longevity assurance genes" are associated with an adaptive trait and, therefore, become fixed in a population), living organisms can develop special defense and repair systems that slow down chronic phenoptosis. Since lifespan is a stable species-specific trait, similar to the body size and fertility, its duration (i.e., the time of death) and the mechanisms involved should be at least partly programmed in the genome [4, 7, 8]. Long-lived species typically possess more powerful damage repair systems, including antioxidant defense mechanisms. By enabling repair and other restorative processes, such systems should promote aging deceleration and longevity. The baseline activity of these systems and their damage-ameliorating capacity typically decrease with age. Accordingly, the mechanisms that suppress the activity of the anti-aging systems and induce diseases (including age-related disorders), cell aging, or cell death, should be a part of the aging program [1]. According to one of the authors of this work (V.P.S.) [1], transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2, transcriptional factor 2 of the NFE family) is involved in one of the most prominent important cell anti-aging programs (Fig. 1). Nrf2 regulates transcription of the antioxidant and detoxifying enzymes, which form an efficient cell defense system [1-6]. However, the role of Nrf2 is not confined to these activities. Nrf2 is also one of the central regulators of cell homeostasis that controls expression of over 1% of human genes implicated in biotransformation, redox homeostasis, energy metabolism, DNA repair, and proteostasis [2]. Nrf2 strongly influences a wide variety of physiological and pathological processes. In turn, Nrf2 is strictly controlled, mostly through the modulation of its stability. It was also found that such defense systems, apart from being directly controlled by regulators (inhibitors involved in the aging program), can be regulated by proteins participating in circadian, ultradian, and other rhythms, i.e., by the Master Clock (Fig. 1) [1].

In this review, we analyze the data on the molecular mechanisms of interaction between the Nrf2-mediated defense system, GSK3 (as an Nrf2 inhibitor), and circadian clock core proteins within the framework of aging and anti-aging programs.

TRANSCRIPTION FACTOR Nrf2

Nrf2 as a factor of the anti-aging programs. Nrf2 is activated by oxidative stressors and electrophilic agents; it enables stress adaptation by upregulating the activity of the cell antioxidant system and other metabolic processes and by controlling expression of over 200 target genes under various types of stress. The products of these genes regulate a large number of defense mechanisms, such as drug detoxification, pentose phosphate pathway, and autophagy [3]. Besides, Nrf2 directly inhibits induced expression of various inflammation-related genes via binding to proximal regulatory regions [10]. Mouse cells with the knocked out Nrf2-encoding gene (*Nfe2l2*) exhibit higher ROS levels [11] and elevated sensitivity to oxidative stress [12]. With aging, the level of Nrf2 decreases, and the ability of this protein to become activated in response to stress is compromised [1, 2, 6, 13]. The activity of Nrf2 positively correlates with lifespan [2]. In light of all the above-mentioned, Nrf2 is considered as a component of special anti-aging program responsible for healthspan prolongation and longevity [2]. Systems suppressing the Nrf2 activity are components of the aging program [1]. The most important of them are regulatory systems that cause proteasomal degradation of Nrf2. They include the Kelch-like ECH-associated protein 1 (Keap1), GSK3, and β-transducin repeats-containing protein (β-TrCP) (see Figs. 1 and 2).

c-Myc is another Nrf2-suppressing protein [14]. Besides, although Bach1 (BTB domain and CNC

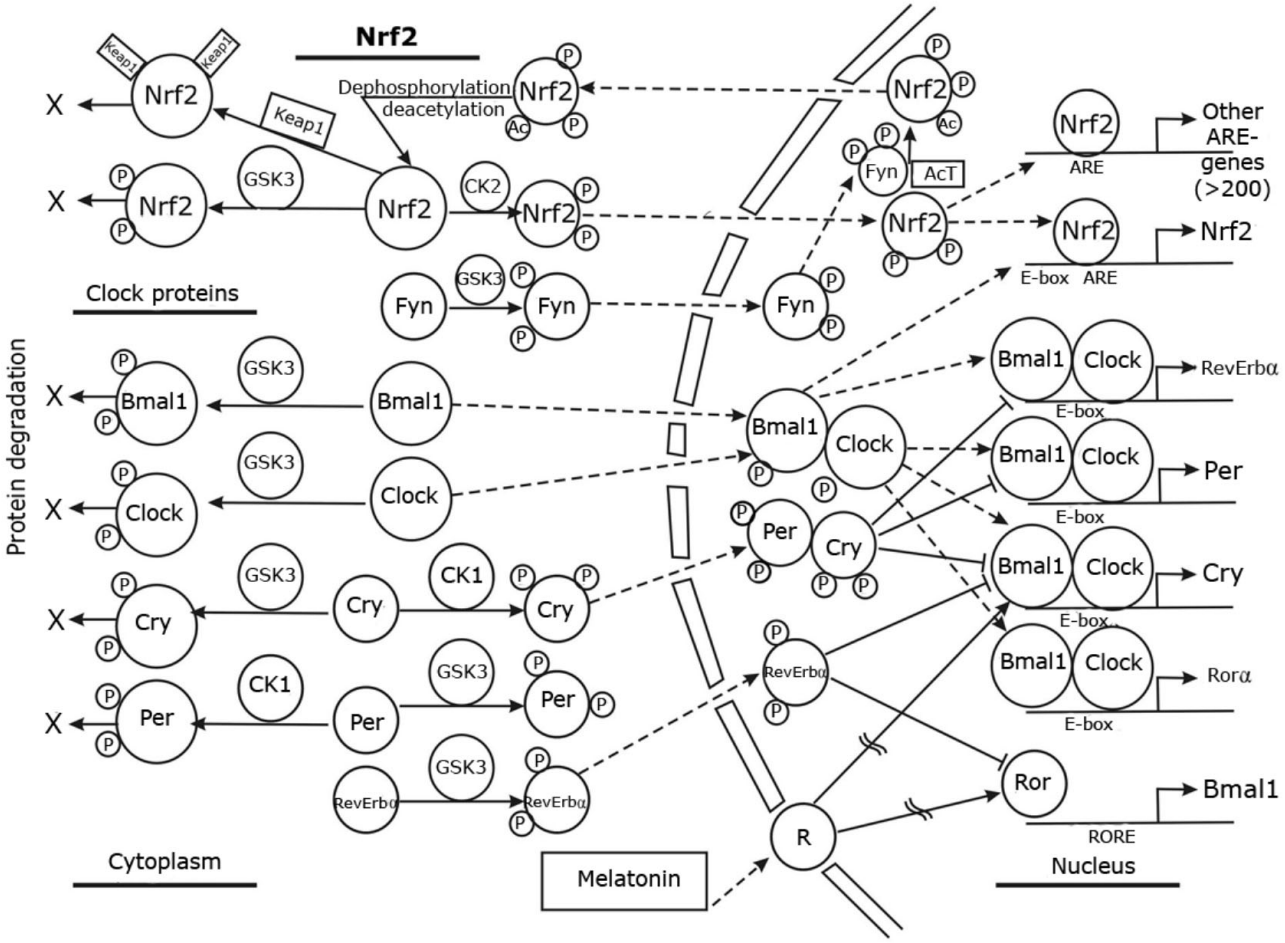

Fig. 2. Molecular mechanisms of circadian rhythms and cytoplasm–nucleus oscillations of the Nrf2 transcription factor controlling expression of cytoprotective enzymes. A characteristic feature of the Nrf2-regulated genes is the presence of ARE sequences in their promoters. At the same time, most circadian clock genes contain the E-box sequence (also present in the *Nfe2l2* gene promoter). The gene *Bmal1* contains the RORE (RAR-related orphan receptor response element) sequence in the promoter. Dashed lines with arrows, translocation of the corresponding proteins into and out of the nucleus; solid lines with arrows, direct effect, including catalysis; solid line with a blunt end, inhibition; solid line with a break, indirect effect (for example, upregulation of expression of clock proteins and Nrf2 by melatonin); circle with letter "p", phosphate groups attached to the proteins; the presence of two phosphate groups after interaction of the modified protein with one or another kinase indicates that the reaction proceeds by the double phosphorylation mechanism (for more details, see the text). AcT, acetyltransferase; Clock, circadian locomotor output cycles kaput protein; Cry, cryptochrome protein; Per, period protein; Rev-erbα, reverse erythroblastosis virus α protein.

homolog 1 protein 1) is an inhibitor of Nrf2, it competes with it for binding to the antioxidant response elements (AREs) [15] (Fig. 1). Apart from GSK3 mentioned above, other kinases, such as Fyn kinase, may inactivate Nrf2 and initiate its export from the nucleus [16, 17] (Fig. 2).

The activity of Nrf2 in the cell is not maintained at a constant level. The content of this protein constantly oscillates and undergoes circadian and ultradian changes. Activators that protect Nrf2 against proteolysis increase its amount in the cytoplasm; subsequent equilibration of its concentration (i.e., increase in the Nrf2 level in the nucleus) activates transcriptional response of AREs. The increase in the content of Nrf2 and induction of the ARE-containing genes leads to the GSK3 activation. In turn, GSK3 phosphorylates Nrf2 and promotes its proteasomal degradation with the involvement of β-TrCP [18].

Biorhythms are of paramount importance for adaptation; they depend on many factors, including the redox status. Disruption of circadian rhythms is a characteristic aging-related problem. Therefore, maintaining proper operation of circadian clock might be a promising lifespan prolongation strategy [19]. The circadian clock mechanism is based on the Clock and Bmal1 transcription factors. These proteins form heterodimers a via their PAS (Per-Arnt-Sim) domains and induce expression of clock-controlled genes by binding to the E-box sequences in their promoters [20, 21]. The periodicity of expression of circadian rhythm genes is secured by the Per1 and Per2 proteins. They translocate to the nucleus and form stable complexes that also include cryptochrome proteins Cry1 and Cry2. These complexes suppress transcription by binding to the upregulating factors Clock/Bmal1. As a result, the levels of Per1 and Per2 mRNAs and the content of the corresponding proteins periodically increase and decrease with the cycle duration of approximately 24 h [22, 23]. Hence, this mechanism is an example of a negative feedback loop [24, 25]. The protein regulators of circadian rhythms participate in many metabolic pathways, e.g., those involving AMPK (AMP-activated protein kinase), an essential systemic regulator of metabolism [26], which phosphorylates Cry1 and promotes its degradation [27].

Nrf2 as an oscillator. *Regulation of Nrf2 activation in the nucleus and the cytoplasm*. Understanding biorhythm regulation presents serious difficulties since both Nrf2 and ROS have their own rhythms. It was established that stimulation of ARE-dependent gene expression resulting from the action of Nrf2 activators is not mediated by the increase in the Nrf2 stability and its accumulation in the cell. Instead, this stimulatory effect results from the increase in the frequency and decrease in the amplitude of the Nrf2 translocation cycles between the cytoplasm and cell nucleus [28]. In terms of the ***oscillatory model*** suggested by Xue et al., GSK3 *performs a regulatory role* by ensuring Nrf2 inactivation and degradation [28]. According to this model, Keap1 and Nrf2 stay in contact because of the threonine protein phosphatase PGAM5 (protein phosphatase 5 of the phosphoglycerate mutase family) that binds Keap1 and Nrf2 to the outer mitochondrial membrane [29]. Under oxidative (more precisely, electrophilic) stress, Nrf2 dissociates from Keap1, gets phosphorylated by casein kinase 2 (CK2) [3], and translocates to the nucleus with the help of importins α5 and β1 [30]. In the nucleus, Nrf2 activates its target genes by binding to AREs in the gene promoter sequences [3, 14]. Next, Nrf2 is phosphorylated by the Fyn kinase [16], acetylated, and then removed from the nucleus via the export channel of the nuclear membrane (exportin-1/crm1) [28]. GSK3β regulates this process by phosphorylating Nrf2 and causing its degradation with the help of β-TrCP [3, 31, 32]. In the same work [28], Xue et al. demonstrated that Nrf2 retention in the cell nucleus with the aid of the crm1 export channel-blocking leptomycin B results in the downregulation of the ARE-dependent transcriptional activity and this cyclic process has a period of approximately 2 h [28]. According to the above model, this can be related to the acetylation and inactivation of Nrf2 (Fig. 2). Xue et al. estimated [28] that in unstimulated cells; the content of Nrf2 oscillates 2-3 times before the protein degradation, while in the cells exposed to the oxidative stress the number of such oscillations is higher [28].

The oscillatory model that involves Nrf2 suggests an alternative regulatory mechanism. The operation of the nuclear Nrf2 may be regulated by deacetylation, circadian control of the *Nfe2l2* gene expression, and conformational changes in the Nrf2/Keap1 complex [28]. It was found that in the absence of oxidative stress, the Keap1/Nrf2 interaction is cyclic. Initially, the complex exists in the *open* conformation, in which Nrf2 binds to one of the two Keap1 subunits, and then transits to the *closed* conformation, in which Nrf2 is bound to both Keap1 subunits [33]. The oscillatory model is based on the Nrf2 activation via its release from the Nrf2/Keap1 complex, since the rate of this process influences the oscillation frequency and the cytoprotective transcriptional response. Under the influence of Nrf2 inducers (oxidants and electrophilic agents), Nrf2/Keap1 complexes are fixed in the closed conformation that prevents Nrf2 release. As a result, no regeneration of free Keap1 takes place, and *de novo* synthesized Nrf2 molecules do not undergo degradation. This model implies the existence of other Nrf2 activation strategies, for example, Nrf2 deacetylation by nuclear deacetylases [28].

Interaction between biorhythms and Nrf2-based systems. Nrf2 expression is characterized by a circadian rhythm with a period of 23.7 h [34]. Circadian variations and gender-dependent differences in the levels of the antioxidant gene transcripts can influence the organism's response to oxidative stress at different times of the day [35]. Early et al. [36] established that deletion of the *Bmal1* gene (as well as knockdown) disrupts Nrf2 activity

in the macrophages, which contributes to the ROS accumulation of ROS and the anti-inflammatory cytokine IL-1β. They also found that Nrf2 knockout reduced lipopolysaccharide (LPS)-induced expression of three primary target genes of Nrf2, namely, *Hmox1* (heme oxygenase), *Gsr* (glutathione reductase), and *Nqo1* (NAD(P)H:quinone oxidoreductase 1) compared to Bmal1$^{+/+}$ macrophages. Like most genes that encode circadian clock core proteins, *Nfe2l2* contains the E-box sequence. The Bmal1/Clock heterodimer binds to this sequence and activates Nrf2 transcription followed by the upregulation of transcription of the target *Hmox1*, *Gsr*, and *Nqo1* genes. The same authors reported that the baseline ROS level in peritoneal myeloid cells considerably increases in the second half of the day, which inversely correlates with the circadian rhythm of Bmal1 and Nrf2 expression.

In contrast to Bmal1$^{+/+}$ macrophages, Bmal1$^{-/-}$ macrophages are characterized by a higher base level of ROS, as well as by a higher ROS content after LPS induction (table). The expression of Nrf2 and its capacity to regulate IL-1β in myeloid cells are controlled by the molecular clock.

Pekovic-Vaughan et al. found the level of the Nrf2 protein to undergo rhythmic changes [34]. The rhythmic pattern of Nrf2 expression was also observed in cell lysates and nuclei of Rat1 fibroblasts (table). These findings provide evidence for autonomous, stable rhythmic Nrf2 expression at the cellular level. A mutation in the core part of the E-box sequence of the *Nfe2l2* gene promoter completely abolishes its induction by the Clock/Bmal1 complex. In the lungs of wild-type (WT) mice, Nrf2 mRNA exhibits a clear rhythmic expression pattern lacking in the Clock$^{\Delta 19}$ mice. The time-dependent binding of Clock/Bmal1 to the E-box in the *Nfe2l2* gene promoter was also demonstrated. These results indicate that the *Nfe2l2* gene is directly regulated by the nuclear clock components (both *in vitro* and *in vivo*) via the conserved E-box sequence in the promoter. The data obtained in embryonic fibroblasts from with the *Nfe2l2*-deficient mice indicate direct, Nrf2-dependent rhythmic control of the downstream targets (table).

In the WT mice, Nrf2 induction by D3T (H3–1,2-dithiole-3-thione) results in the activation of the E-box- and D-box-containing clock genes (*Rev-ErbA*, *Rev-ErbB*, *Dbp*, *Per3*) [37]. Full activation of these genes requires the functioning of the Keap1/Nrf2 signaling pathway, as their activity is significantly attenuated in the Nrf2$^{-/-}$ mice. The loss of Nrf2 causes circadian rhythm disruption in the embryonic fibroblasts of Nrf2$^{-/-}$ mice. This direct effect suggests Nrf2 involvement in the regulation of rhythm amplitude and period length [37] (table). Hence, Nrf2 regulates expression of core proteins and stabilizes circadian rhythms of molecular clock and is responsible for the linking redox potential and general timing [37]. The knockout of the Nrf2 gene in the mouse liver changes the length of the circadian cycle. Nrf2 acts most probably via regulation of the Cry2 and Rev-erbα expression. Nrf2 and clock proteins likely form an inhibitory loop that integrates cell redox signaling within the circadian rhythm [37]. Nrf2 belongs to the CncC (Cap'n'collar) family of transcription factors. It has been revealed recently that constitutive overexpression of CncC proteins in *Drosophila* positively influences neuronal functions by modifying synaptic mechanisms. Suppression of Keap1, a CncC inhibitor, promotes synaptic function and increases the lifespan [38]. The results obtained by Hansen et al. indicate that the ratio between glutathione reduced and oxidized forms (GSH/GSSG) controls Nrf2 in the cytoplasm, but does not affect Nrf2 binding to AREs in the nucleus [39]. Conversely, since thioredoxin 1 (Trx-1) overexpression does not influence Nrf2 dissociation and translocation to the nucleus, nuclear Trx-1 is assumed to be responsible for the Nrf2 regulation at the level of Nrf2–DNA interaction and not for the cytoplasmic events, such as Nrf2 dissociation and translocation to the nucleus [39].

GLYCOGEN SYNTHASE KINASE 3 (GSK3)

Below, we discuss GSK3β protein kinase, its inhibitory effect on Nrf2 and other proteins, and mechanisms of its action in the regulation of cell functions.

GSK3 (ATP:protein phosphotransferase, EC 2.7.1.37) is an intracellular serine/threonine protein kinase (molecular weight, 47 kDa) ubiquitously synthesized in all tissues of an organism [40, 41]. It is represented by two paralogs (α and β), which are routinely referred to as *isoforms* in the literature, even though the term *isoenzymes* would be more precise from the biochemical viewpoint. Apart from the common GSK3β1 form, there is also longer GSK3β2 form, which is expressed in the brain during its development [42].

The unique role of GSK3β in the regulation of cell functions is related to the fact that it affects the activity of more than 100 proteins. In turn, GSK3β itself is influenced by multiple stimuli. For instance, Akt1 activation results in the phosphorylation and inhibition of GSK3β [43].

GSK3 in involved in most cell processes, such as cell growth, differentiation, and death; it also modulates responses to hormonal, nutritional, and stress stimuli. Stress-induced GSK3β translocation may result in its interaction with mitochondrial proteins, including PI3K-Akt, PGC-1α, HKII, PKCε, respiratory chain components, and mPTP subunits. The mitochondrial pool of GSK3β regulates biogenesis, energetics, permeability, and motility of mitochondria, as well as apoptosis [44]. Some of the essential functions of GSK3β are β-catenin inhibition and involvement in the Wnt signaling pathway that plays a major role in embryogenesis, cell growth and

Interaction between genetic and chemical activators of biorhythms and Nrf2 systems

Target	Influence	Object	Effect	References
A. Rhythms of Nrf2 and other markers of stress response				
BR	BR	murine liver	expression of Nrf2-induced genes is higher during the daytime than at nighttime; with the peak of Nrf2, Nqo1, and Dbp expression in the late afternoon and the peak of Keap1, Gclc, and Rev-erbα expression – in the early afternoon	[35]
ROS rhythm	BR	macrophages	basal ROS level is increased by ~25% ($p \leq 0.05$) in the afternoon (compared to the time before the morning) and negatively correlates with the circadian rhythm of Bmal1 and Nrf2 expression	[36]
Nrf2 rhythm	BR	mice	peak of Nrf2 protein at the circadian time 3-7 (CT3-CT7); the lowest content of Nrf2 at CT15-CT19	[34]
Nrf2 rhythm	BR	Rat1 fibroblasts	rhythmic expression of Nrf2 protein in cell lysates ($p < 0.01$, one-way ANOVA to assess the effect of time) and in the nuclei ($p < 0.001$, *t*-test)	[34]
BR	H_2O_2, D3T	MEFs	H_2O_2 (100 μM) and D3T (100 μM) **increase** the amplitude (by ~2.5 and ~5 times, respectively, *t*-test, $p < 0.05$), but not the duration of the period	[37]
B. Genetic activation of biorhythms and Nrf2 systems				
Nrf2 rhythm	BR gene knockout	mice	expression of Nrf2 mRNA in the lungs of WT mice (but not in $Clock^{\Delta 19}$ mice) has a distinct rhythm ($p < 0.05$, one-way ANOVA); knockdown of Bmal1 gene **reduces** the level of Nrf2 mRNA ($p < 0.05$)	[34]
ROS level	BR gene knockout	macrophages	both baseline (fourfold, $p \leq 0.01$) and LPS-induced (~2.3-fold, $p \leq 0.05$) ROS levels are **higher** in $Bmal1^{-/-}$ macrophages compared to $Bmal1^{+/+}$ macrophages	[36]
Nrf2 level	BR gene knockout	MEFs	expression levels of Nrf2 and its target genes are **elevated** in synchronized $Cry1^{-/-}/Cry2^{-/-}$ murine MEFs	[34]
BR	Nrf2 knockout	MEFs	Nrf2 knockout **reduces** the amplitude of the rhythm and the duration of the period (by ~5 times and by ~15%, respectively, *t*-test, $p < 0.05$) in MEFs	[37]
BR	Nrf2 overexpression	murine hepatocytes MMH-D3	Nrf2 overexpression **decreases** the amplitude of the rhythm (by ~2 times) and the duration of the period (by ~2%, *t*-test, $p < 0.05$) compared with the WT mouse cells	[37]
BR gene expression	Nrf2 knockout and knockdown	MEFs	in $Nrf2^{-/-}$ MEFs, the peak of Rev-erbα protein accumulation was delayed by 4 h (but did not disappear) compared to the WT MEFs; Nrf2 knockdown in MEFs **reduces** the level of Rev-erbα expression by ~45-50%	[37]

Note. BR, biorhythms; Dbp, albumin site D-binding protein; D3T, H3-1,2-dithiol-3-thione; Gclc, catalytic subunit of glutamate-cysteine ligase; MEFs, mouse embryonic fibroblasts.

differentiation [41], neurogenesis, and synaptic plasticity [45]. GSK3β regulates the cell cycle by inhibiting cyclin D1, which is necessary for the cell entry to the S phase [46]. It is also implicated in glucose metabolism regulation via inhibition of insulin receptor substrate (IRS) proteins and kinesins [47].

In all likelihood, GSK3 phosphorylation at serine-9 (S9) is especially prone to oscillations. These oscillations may be rapid (e.g., during neuronal depolarization/polarization) or slow, as in the case of changes in the levels of circulating GSK3-regulating hormones and circadian rhythms in the suprachiasmatic nucleus (SCN) and the liver [48].

GSK3β is constitutively activated by autophosphorylation at Y216 and inactivated by phosphorylation at S9. Active, (Y216-phosphorylated) GSK3β accounts for at least a half of the total GSK3β pool in cultured cells [49]. In the mouse brain, GSK3β is predominantly present in

the active form, whereas the portion of its inactive (S9-phosphorylated) form is insignificant [50]. GSK3β is phosphorylated at Y216 by Pyk2 and Fyn kinases or autocatalytically [51]. Phosphorylation at S9 inhibits GSK3β activity and represents the primary mechanism of its regulation. Many kinases phosphorylate GSK3β at S9, including protein kinases A, B, and C, PrkG1, ILK, p70S6K, and p90SRK, while protein phosphatase 2A (PP2A) dephosphorylates it [52]. In the brain, the major mechanism of GSK3β regulation is its inhibition via phosphorylation at S389 by the mitogen-activated protein kinase p38 [53].

GSK3 involvement in the aging program. Indirect evidence for the GSK3β involvement in the aging program is presented by Krishnankutty et al. [49], who studied three GSK3β fractions in the mouse brain and neurons: the Y216-phosphorylated active enzyme, the inactive S9- and Y216-phosphorylated enzyme, and the unphosphorylated (also inactive) GSK3β. Although the total GSK3β level does not change with age, aging is associated with the decrease in the fraction of inactive, S9-phosphorylated isotype (thus, the relative content of this form is two times lower in 1.5-year-old females compared to the 3-week-old ones) [49].

GSK3β and cell aging. The changes in the ratio between the GSK3β-isoforms, with the preservation of the total GSK3β level, were observed in the stationary culture of mouse brain primary neurons. The relative content of the S9-phosphorylated (inactive) GSK3β form was maximal (over 30%) after 3 days of culturing without reinoculation and then gradually decreased to 15% after 12 days of culturing. The activity of the Y216-phosphorylated GSK3β, conversely, gradually increased "with age" [49]. Senescent WI-38 human fibroblasts with the population doubling level [(PDL) of 58-64], in contrast to middle-age (PDL of 38-41) and young cells (PDL of 26-30), manifested traits typical of aging cells, including increased size, flattened shape, and high senescence-associated β-galactosidase activity [54]. The levels of GSK3α and GSK3β were increased in the nuclei of senescent cells. Lithium (a GSK3 inhibitor) decreases the activity of the enzyme and reduced age-dependent p53 accumulation associated with the senescence state, as well as induced cell transition to the reversible quiescent state. These results indicate that a fraction of the p53 pool activated in aging cells is modulated by the p53 binding to GSK3β in the nucleus, which promotes p53 activity and cell aging [54]. Similarly, the baseline level of phosphorylated (inactive) GSK3 in aged (18 months-old) Syrian hamsters (*Mesocricetus auratus*) is much lower than that in the young (1-3 months-old) animals [55]. In aged hamsters, lithium does not influence the period of the locomotor activity rhythm and the level of GSK3 phosphorylation, unlike its effect in younger hamsters [55]. These data provide indirect evidence for GSK3β as a *bona fide* component of the aging program.

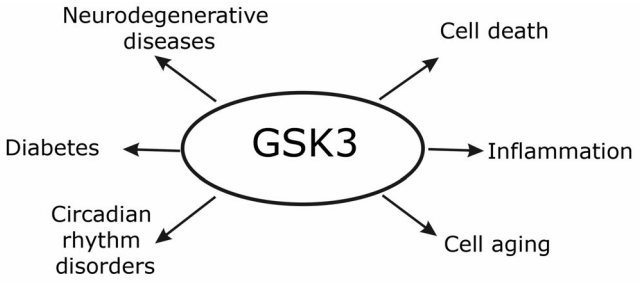

Fig. 3. Negative effects of GSK3 activation.

GSK3β and age-related disorders. The involvement of GSK3β in the aging program is also confirmed by association of changes in its activity with aging-related diseases (Fig. 3). In neurons, GSK3β selectively phosphorylates microtubule-associated tau protein at the sites that are hyperphosphorylated in the in Alzheimer's disease (AD) brain [56]. Hyperphosphorylated tau protein exhibits a decreased affinity for the microtubules. It accumulates in a form of helical filaments representing the main components of neurofibrillary tangles and neuropil threads in the AD brain. Neurofibrillary tangles are also detected in patients with amyotrophic lateral sclerosis, Parkinson's disease, dementia, corticobasal degeneration, trauma-caused brain damage, Down syndrome, post-encephalitis parkinsonism, and Niemann–Pick disease. In the brain tissue of AD patients, the GSK3β level is increased by 50% [56]. GSK3β inhibition improves cognitive symptoms associated with AD and other diseases mentioned above. GSK3β activity is increased in the cellular (growth factor-deprived) and animal (cerebral ischemia-based) models of neurodegeneration [57]. The anti-inflammatory effect of GSK3β is due to the stimulation of IL-1β, IFN-γ, IL-6, and IL-12 production and suppression of IL-10 synthesis [48] via the Toll-like receptors of monocytes [58].

Mechanism of GSK3 action. *Prephosphorylation and GSK3.* Phosphorylation of glycogen synthase and other GSK3 targets requires the prephosphorylation of these substrates by another kinase in position +4 relative to the GSK3 phosphorylation site, which is a widely occurring but not universal consensus S/TXXXS/T sequence [50, 59, 60]. This double modification often results in subsequent ubiquitination and proteasomal degradation mediated by the corresponding adaptor proteins (e.g., F-box proteins).

Protein degradation. Phosphorylation and ubiquitination. F-box proteins are responsible for the substrate recognition, each protein recognizing a specific substrate group [61]. Based on the structure of the substrate-recognizing region, F-box proteins are subdivided into three categories: proteins with WD40 repeats (Fbxw), proteins with leucine-rich repeats (Fbxl), and proteins with other domains. It is assumed that Fbxl3 is responsible for ubiquitin-dependent degradation of the clock protein Cry;

Fbxl3 mutation in mice results in the prolongation of the circadian period to ~26 h [62, 63]. β-TrCP (also known as Fbxw1) recognizes the clock Per protein after its phosphorylation by casein kinase 1 (CK1) (but not by GSK3) [64]. β-TrCP targets frequently contain the DSGXXS degradation motif, the phosphorylation of both serine residues in which significantly promotes target protein recognition by β-TrCP [65]. Since this motif is similar to the consensus sequence for GSK3 (SXXXX(X)S), β-TrCP binds many GSK3 substrates. For example, this sequence was identified in β-catenin and Nrf2 [60].

Below, we describe the role of modifications of Nrf2 and circadian clock proteins by GSK3.

Regulation of Nrf2 activity by GSK3. GSK3 phosphorylates specific serine residues in the Neh6 domain of Nrf2 with the formation of the degradation domain recognized by the ubiquitin ligase adapter protein β-TrCP; ubiquitinated Nrf2 is degraded by the proteasome complex, containing Cullin1 (Cul1) and RING-box 1 (Rbx1) proteins. According to an alternative scenario, GSK3β represses Nrf2 by activating tyrosine kinases in a β-TRCP-independent fashion. GSK3β phosphorylates Fyn kinase at Y213. Activated Fyn accumulates in the nucleus, where it phosphorylates Nrf2 (at Y568 in mice and rats and Y576 in humans), resulting in the export and degradation of Nrf2 [16] (see Fig. 2).

Cuadrado [3] suggested the double flux controller model to explain how Keap1 and β-TrCP regulate the stability of Nrf2. Under oxidative stress or in the presence of thiol-interacting compounds, the Nrf2 level in the nucleus increases, resulting in the stimulation of expression of the ARE-containing genes. In the absence of stress, Nrf2 is predominantly ubiquitinated by Cullin 3 (Cul3) with Keap1 as a substrate adapter protein for the ligase E3 complex (Rbx1/E3/Cul3) [3] (see Fig. 2). It was found that the Neh6 domain of Nrf2 contains two β-TrCP-binding sequences [31]. GSK3-mediated phosphorylation of S338 (and S342) in the Neh6 domain strengthens GSK3 binding to β-TrCP [31]. Nrf2 prephosphorylation is apparently mediated by kinases belonging to the CMGC (CDK/MAPK/GSK3/CLK) family, the catalytic site of which is blocked by the flexible portion (T-loop) of the polypeptide chain, unless this site is phosphorylated by the signal kinase. Unlike most CMGC kinases, the T-loop of GSK3 is mostly phosphorylated at Y279 (GSK3α) or Y216 (GSK3β). Therefore, GSK3 is capable of baseline catalysis in the absence of signaling [50, 66, 67]. This feature enables control of the Nrf2 stability at an additional regulation point [15, 31]. Based on other data, Nrf2 can be prephosphorylated by the DYRK kinase [68]. Inhibition of both GSK3 and prephosphorylating kinase stabilizes Nrf2 [68]. Interestingly, the Nrf2-related transcription factor Nrf1 is also degraded by the proteasome in a β-TrCP-dependent fashion. In this case, the degradation depends on the DSGLS motif, which is recognized and phosphorylated by CK2 and not by GSK3 [68]. β-Catenin is prephosphorylated by the same kinase as glycogen synthase [68]. Hence, the rate of ubiquitination and degradation of each of these substrates partly depends on the regulation of their specific prephosphorylating kinases and GSK3/CK2 (Fig. 2).

In tumor cells, in which Keap1 (and hence, ubiquitin ligase complex Rbx1/E3/Cul3) cannot interact with Nrf2, GSK3 retains its ability to suppress the Nrf2 activity. In these cells, as well as in the embryonic fibroblasts of $Keap1^{-/-}$ mice, GSK3 inhibition by CT99021 increases Nrf2 activity [31].

GSK3 and biorhythms. Circadian rhythms are is based on a conserved biochronometer system required for the adaptation of behavioral and physiological processes to the 24-h environmental cycles [25, 69]. The regulation by the circadian oscillator of the SCN is exerted via its neuronal links with gonadoliberinergic neurons and a humoral pathway involving melatonin (pineal gland hormone) [69, 70]. Melatonin secretion by the pineal gland is stimulated by light [71]. Different light-sensing systems associated with the SCN differ in the representatives of the Bathyergidae family (characterized by decelerated aging and high longevity quotient), depending on the lifespan and sociality [72].

Melatonin acts via membrane receptors MT1 and MT2 or through the receptor-independent mechanisms, including the Nrf2 signaling [73, 74]. Besides, melatonin binds not only to the plasma membrane receptors but also to the receptor proteins on the nucleus surface; it also operates at the chromatin level, directly affecting protein synthesis. It was demonstrated that the genes for the nuclear receptors Rorα, Rorβ, and Rorγ (the so-called orphan nuclear retinoid receptors Ror/Rzr) are expressed in various organs and tissues, including the hypothalamic SCN, retina, and epiphysis [75]. The ligands of these receptors are cholesterol and its derivatives, but not melatonin [75]. Melatonin stimulates expression of the clock genes via the RORE-elements of the Bmal1 gene [25] and via Nrf2 expression through a chain of intermediate links [74, 75] (line with a break in Fig. 2). Nuclear melatonin receptors exist beyond any doubt [75]. For instance, it was established that melatonin is a ligand of the vitamin D receptor (VDR) in the nucleus, with a K_d of 21.2 ± 1.9 μM [76].

GSK3α and GSK3β are expressed in the hypothalamic SCN [48]. In mice, the level of GSK3α mRNA is higher than the level of GSK3β mRNA [77]. GSK3 is responsible for the feedback loop that impacts the functioning of molecular clock in the SCN neurons [78]. The expression of GSK3α and emergence of the phosphorylated form of GSK3β in the SCN are characterized by a circadian rhythm [77].

At the beginning of the nighttime, the GSK activity in rat SCN neurons decreases (the number of cells histochemically stained for the phosphorylated inactive GSK3β form increases and reaches the maximum within

4 h). However, the activity of GSK3β increases towards the end of the night. Immunofluorescent staining of the mouse SCN showed that at the end of the night, light significantly increases GSK3 activity, i.e., decreases the level of phosphorylated GSK3β as early as 30-60 min after the light pulse [79]. In the control system, the content of phosphorylated GSK3 decreases late at night, whereas the GSK3 activity increases. No decrease in the amount of phosphorylated GSK3 (i.e., no increase in the content of the active GSK3 form) was observed in the experimental system. Therefore, light pulse suppresses GSK3 activity, resulting in the attenuation of its oscillations [80]. Even hippocampal extracts from mice permanently kept in the dark for at least 2 weeks were characterized by a distinct endogenous circadian rhythm in the GSK3β (but not GSK3α) phosphorylation [81]. In *Drosophila*, the functioning of Shaggy (Sgg), a GSK3 homolog, in small ventral lateral neurons that play a key role in the regulation of general rhythmic locomotor activity of adult individuals, is crucial for maintaining normal rhythms [82].

GSK3 substrates involved in circadian rhythm regulation. GSK3 interacts with Per2 *in vitro* and *in vivo*, phosphorylates Per2 *in vitro*, and promotes its translocation to the nucleus (see Fig. 2). It also causes proteasomal degradation of its partner protein Cry2 [48, 83, 84] and phosphorylates Cry2 together with the serine protein kinase DYRK1A by S557 and S553 residues, respectively [24, 85]. GSK3 phosphorylates (i) Bmal1 (S17/T21), resulting in its subsequent ubiquitination and degradation [86], and (ii) Clock (S427/S431) [87] (Fig. 2). The kinase assay of GSK3 activity revealed that this enzyme regulates Clock phosphorylation/degradation by modification of a specific cluster of serine residues (phosphodegron) [87].

The studies of GSK3β phosphorylation at S9 (which suppresses kinase activity, as mentioned above) demonstrated that the GSK3β activity is maximal from the end of the night until the early morning. This results in the upregulation of the Cry2 phosphorylation at S557, which facilitates rhythmic degradation of this protein [87]. Besides, GSK3 phosphorylates Rev-erbα (protein suppressing Bmal1 expression and, accordingly, Bmal1-induced expression of clock genes); however, this modification causes activation (not degradation) of Rev-erbα and its translocation to the nucleus [88].

Cry2 and Per2 play a prominent role as suppressors of circadian protein expression. GSK3β interacts with Per2 *in vitro* and *in vivo* (as mentioned above) [48]. These interactions do not result in the degradation of Per2 (unlike Cry2), but promote its translocation to the nucleus (in contrast to the casein kinase-dependent phosphorylation) (Fig. 2). GSK3β overexpression causes a shift in the Per2 phase, which changes the duration of the period by ~15% (3-4 h) or results in a complete loss of circadian rhythms and generation of extreme phenotypes. Some other molecular mechanisms also regulate the cyclic expression of the *Per1* and *Per2* genes [23]. The level of the Per proteins is regulated by several factors that promote protein stability and, presumably, ability to translocate to the nucleus. On the other hand, Per phosphorylation by CKIε is responsible for the cytoplasmic degradation of Cry-unbound Per (unlike its GSK3-dependent phosphorylation), thus preventing premature Per accumulation in the cytoplasm. Per is less stable in the absence of Cry and readily undergoes ubiquitination and proteasomal degradation [22].

The GSK3 ortholog in the *Drosophila* fruit fly, Sgg, plays a central role in determining the length of the circadian period. Its mutation in *Drosophila* causes prolongation of the circadian period, whereas upregulation of its activity shortens the period [89]. Sgg phosphorylates Tim (Timeless, an analog of Cry in *Drosophila*) and regulates nuclear translocation of the Per/Tim heterodimer [89]. It should be noted that Tim and Cry2 form dimers with Per in the clock structures of *Drosophila* and mice, respectively. It is quite possible that GSK3 contributes to the clock functioning by regulating components that operate together with the Per proteins [24]. GSK3 phosphorylates the core circadian clock proteins (Bmal1, Clock, Per, Cry, and Rev-erbα) in mammals and regulates their stability [25] (Fig. 2).

Conserved structure of clock proteins and GSK3 in the evolutionary tree. Using the Ensembl 100 database, we screened for the presence of orthologs of the following genes in vertebrates, *Drosophila melanogaster*, and the nematode *Caenorhabditis elegans*: *GSK3A* (ENSG00000105723), *GSK3B* (ENSG00000082701), *CLOCK* (ENSG00000134852), *CRY1* (ENSG00000008405), *CRY2* (ENSG00000121671), *BHLHE41* (DEC2) (ENSG00000123095), and *NPAS1* (ENSG00000130751) (the human gene identifier according to the Ensembl database is given in parentheses). These genes code for proteins involved in the regulation of biorhythms. The *GSK3B* gene is highly conserved even in invertebrates (71.27% identical positions in the human and nematode genes) and virtually identical in humans and macaques. The *GSK3A* gene has no orthologs in birds, some reptiles, and the elephant shark *Callorhinchus milii*. In particular, no *GSK3A* ortholog was detected in the common snapping turtle *Chelydra serpentina*, three-toed box turtle *Terrapene carolina triunguis*, Agassiz's desert tortoise *Gopherus agassizii*, and Goode's thornscrub tortoise *Gopherus evgoodei*. No *NPAS1* ortholog was found in all tested birds and some reptiles, such as the blue-ringed sea krait *Laticauda laticaudata*, mainland tiger snake *Notechis scutatus*, Eastern brown snake *Pseudonaja textilis*, Australian saltwater crocodile *Crocodylus porosus*, Anole lizard *Anolis carolinensis*, Argentinian black and white tegu *Salvator merianae*, Komodo dragon *Varanus komodoensis*, common snapping turtle *C. serpentina*, Goode's thornscrub tortoise *G. evgoodei*, Chinese soft-shell turtle *Pelodiscus sinensis*, and three-toed box turtle

Terrapene carolina triunguis. No *NPAS1* ortholog was found in the Western clawed frog *Xenopus tropicalis*.

The *GSK3B*, *CLOCK*, *CRY1*, *CRY2*, and *DEC2* genes have orthologs in most vertebrates. Even the *Saccharomyces cerevisiae* yeast contains two *GSK3B* orthologs, but not orthologs for the other tested genes. *C. elegans* lacks the *CRY1*, *CRY2*, and *DEC2* orthologs, and *D. melanogaster* has no *CRY1* and *CRY2* orthologs. Human Clock protein only poorly aligns with the proteins from *C. elegans* and *D. melanogaster* (only 10-20% of positions are identical). Presumably, GSK3α does not play a central role in the circadian rhythm regulation, since it is absent from a large number of species (e.g., birds and many reptiles) and its knockout causes no severe phenotypic disruptions. Instead, this function is fulfilled by GSK3β, which, despite minor variations, is present in animals at all branches of the tree of life. Its knockout results in death at the embryonic stage. Since biorhythm regulation is characteristic of all animals, including reptiles and birds, below we discuss mostly the functions of GSK3β.

GSK3 inhibitors. *Lithium*. Lithium ions prolong the circadian rhythm periods in many species, including unicellular organisms, insects, mice, and humans [77, 90, 91]. Lithium at the concentration of 1-10 mM inhibits GSK3β *in vitro* and *in vivo* in all tested species [92-95]. It decreases the GSK3β/Sgg activity and prolongs the periods of locomotor activity in flies even when they had been permanently kept in the dark (without external light activators) [96]. Lithium at low concentrations (~1 mM) predominantly affects the biorhythm amplitude (presumably, via GSK3β), whereas at high concentrations (~10 mM), it contributes to the prolongation of the period [97].

SB415286. Analogous daytime suppression was documented with another GSK3 inhibitor (SB415286; 1 mM), which decreased the frequency of spontaneous neuronal spikes by 66% relative to the control [98].

Benzofuran-3-yl-(indole-3-yl)maleimides. Recently, a new generation of GSK3β inhibitors [benzofuran-3-yl-(indole-3-yl)maleimides] has been developed with IC_{50} values within the 4-680 nM range toward human GSK3β. One of them (with IC_{50} of 67 ± 6 nM) is characterized by an acceptable selectivity and solubility at the concentrations of 10-29 μg/kg. In mice, such inhibitors exhibit the antipsychotic activity, analogous to lithium and valproate, which are commonly used to treat bipolar disorder and other manic-depressive states [99].

Genetic activation/inactivation of GSK3. The inhibitory phosphorylation of GSK3α and GSK3β in the SCN alternates with a 24-hour period. Transgenic mice with mutations in both GSK3α and GSK3β ($GSK3\alpha^{21A/21A}/\beta^{9A/9A}$) resulting in the permanent activation of the enzymes have disrupted behavioral rhythms, including significantly decreased rhythm amplitude, prolonged active period, and extended daytime activity period [100]. GSK3 overexpression accelerates the onset of the *mPER2* gene expression peak by approximately 2 h [48]. In contrast, genetic inactivation of both GSK3β alleles together with deletion in one of the GSK3α alleles in synchronously oscillating mouse embryonic fibroblasts (3/4 GSK3α/β KO MEFs) results in a considerable delay in the periodicity of endogenous clock mechanism, especially for the cyclic *Per2* expression [83]. Lavoie et al. [101] revealed that the circadian activity period in $GSK3\beta^{+/-}$ mice is extended (23.83 ± 0.05 h) in comparison to the WT mice (23.54 ± 0.10 h). SB216763 (GSK3α/GSK3β inhibitor) at the concentrations of 10 μM and 40 μM reduces the period by 1.8 and 7.3 h, respectively [102]. Suppression of GSK3β expression with small interfering RNA (siRNA) or GSK3 inhibitors (CHIR 99021 and 1-azakenpaullon) shortens the circadian rhythm period [103]. Another GSK3 inhibitor (kenpaullone, 25 μM) causes a phase-long delay in the *Per2* transcription [83].

In synRas transgenic mice with constitutively activated V12-Ha-Ras in the neurons, the level of GSK3β expression is upregulated, and the inhibitory phosphorylation of GSK3β (at S9) in the SCN is decreased. In contrast, suppression of the Ras activity with antibodies in oscillating cultures of human bladder carcinoma cells (the T-24 line) decreases the levels of GSK3β protein, increases GSK3β phosphorylation, and prolongs the Bmal1 promoter activity period [104].

GSK3β activity is a prerequisite for enabling circadian locomotor behavior essential for the correct sleep-wake pattern. Despite the standard total duration of daily periods of wakefulness, non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep, transgenic mice overexpressing GSK3β are characterized by an increased number of repetitions of each of these periods, i.e., their sleep and wakefulness periods are more fragmentary [105]. The sleep structure is of paramount importance in the adaptation and evolution. For instance, sleep in humans is more efficient than in other primates in its length, profundity, and the number of REM phases [106]. Taken together, alterations in the structural organization of the sleep-wake cycles resulting from the GSK3β overexpression are similar to those observed during manic-depressive disorders and some neurodegenerative diseases. Success lithium administration to manic-depressive patients is likely due to its inhibitory effect on GSK3β. It cannot be ruled out that the hyperactivity episodes in such patients are related to the impact of GSK3β on the dopaminergic and serotonergic systems of the brain.

CONCLUSIONS

Long-lived species typically possess more efficient/large/robust damage repair systems, including

antioxidant defense mechanisms. Originally, Frolkis [107] has coined the term *anti-aging (vitauct) systems* for the systems responsible for damage repair and other restorative processes, since they promote longevity. With aging, the activity of such systems usually decreases below its baseline. Accordingly, the aging program involves (i) systems that inhibit/suppress the anti-aging mechanisms and/or (ii) systems the operation of which results in the development of diseases, including aging-associated disorders, cell aging, or cell death [1, 108]. The antagonistic pleiotropy concept assumes the presence of genes that promote reproductive success at a young age, despite their delayed adverse effects later in life [108]. With aging, the activity of such systems may not decrease but even increase. As for GSK3 and Keap1 responsible for ubiquitin-dependent proteasomal degradation of Nrf2, their activity increases with age [2], while activity of Nrf2 decreases. Taken together, the experimental data discussed in this review demonstrate that Nrf2 and GSK3 are components of antagonistic and actively interacting anti-aging and aging programs, respectively [1].

Robust and, nonetheless, plastic circadian rhythms are characteristic not only of proteins involved in the biorhythm regulation (mostly, transcription factors), but also of their regulator, GSK3, as well as the Nrf2-induced antioxidant system that orchestrates a plethora of proteins in the antioxidant defense mechanisms. Molecular clock is the basis of the regulatory mechanism that enables an organism to prepare for and to respond to daily environmental challenges. The Nrf2 system is induced by oxidants (electrophiles), which initiate the synthesis of antioxidant/detoxifying enzymes that prevent cell damage. Even that the biorhythm hormone melatonin possesses no antioxidant properties *per se*, it activates Nrf2 [74], albeit indirectly. This provides an additional evidence for the adaptation-promoting function of the circadian clock system.

The situation with Nrf2 is complicated not only by the circadian rhythms in the Nrf2 activity (i.e., its temporal oscillations), but also by the spatial oscillations represented by the nuclear and cytoplasmic Nrf2 pools. Moreover, by orchestrating the antioxidant defense system, Nrf2 counteracts the effect of toxic substances and oxidants and influences expression of circadian clock proteins (table).

GSK3 can be considered as a characteristic representative of the aging programs: unlike the Nrf2 activity, the activity of GSK3 increases with age, both *in vivo* and *in vitro*, as well as in diseases. GSK3 is involved in various metabolic pathways, including those associated with aging-associated diseases (type 2 diabetes and cancer) and neurodegenerative disorders. GSK3 is also implicated in the cell death and inflammation. GSK3 inhibitors are presently considered as promising therapeutics for treating the problems mentioned above.

Our interest in GSK3 is dictated by its regulatory influence on Nrf2 that is exerted via at least three different pathways: (i) GSK3 is directly involved in the Nrf2 degradation, as it facilitates Nrf2 ubiquitination and proteasomal cleavage (and not merely inactivation, as in the case of other kinases); (ii) GSK3 phosphorylates Fyn kinase that translocates to the nucleus and modifies Nrf2, resulting in the Nrf2 removal from the nucleus; and (iii) GSK3 phosphorylates proteins Bmal1 and Clock of the positive branch of circadian clock and causes their proteasomal degradation, which decreases Nrf2 expression. It should be noted that promoters of the genes negatively regulating the biorhythms (Cry1, Cry2, and Rev-erbα) and of the *Nfe2l2* gene contain the E-box and, therefore, their transcription is upregulated by the Clock/Bmal1 complex [23, 36]. Hence, the GSK3- and β-TrCP-mediated system is a "regulating valve" that controls minor oscillations in the Nrf2 levels and fine-tunes ultradian and circadian (Bmal1-dependent) Nrf2 regulation [3, 36]. Together with the data on circadian and Bmal1-mediated Nrf2 regulation, this indicates that Nrf2 and the clock genes form a regulatory loop that integrates cell redox signals into circadian rhythms [37].

Phosphorylation of various substrates by GSK3 produces a broad spectrum of effects ranging from changes in the enzyme activity to protein translocation, modification of protein-protein interactions, and changes in the protein stability [60]. GSK3-catalyzed phosphorylation of proteins frequently results in their ubiquitination and proteasomal degradation. In the case of Nrf2, Cry2, Clock, and Bmal1, the recognition of the ubiquitinated protein by the proteasome is mediated by adapter proteins, e.g., β-TrCP [60]. These events are often preceded by the phosphorylation of the same protein by another kinase. Protein phosphorylation by GSK3 does not invariably cause protein degradation. On the contrary, phosphorylation of the negative biorhythm regulators Rev-erbα and Per2 by GSK3 increases their stability. Cry is the only negative regulator of circadian biorhythms, whose phosphorylation by GSK3 causes its degradation (similar to the positive regulators Bmal1 and Clock) and not translocation to the nucleus (as is the case with Rev-erbα and Per). Presumably, Cry facilitates adjustment of biological clock to the environmental light rhythm, because it also inhibits *Per* transcription and regulates the levels of Per protein [22]. Therefore, GSK3 interacts (i) with virtually all core clock proteins (Bmal1, Clock, Per, Cry, Rev-erbα) to produce a wide variety of effects, influencing the length of various phases and (ii) with Nrf2 to regulate the cell antioxidant status [3, 15, 18, 25, 29-32, 36, 37, 89].

Genetic and biomolecular data convincingly demonstrate an importance of feedback loops in gene expression. The biorhythms are also linked with the cell bioenergetics via NAD^+ metabolism. It was found that the NAD^+-dependent deacetylase SIRT1 binds to the

Clock/Bmal1 complex with a circadian-type periodicity and regulates circadian transcriptional programs via deacetylating core clock proteins (Bmal1 and Per2) and chromatin-associated proteins [109, 110]. Oxidative stress may reset the molecular clock [111]. Modulation of the pentose phosphate pathway also causes alterations in the rhythmic behavior and affects tissue clocks [112].

Researching proteins located at the intersection of signaling and regulatory pathways (such as clock proteins [20-25, 37, 113] and pineal gland hormone melatonin, its precursors, and metabolites [69-75, 114]) and comparing them in short- and long-lived species using biochemical and bioinformatics tools allows to elucidate molecular mechanisms underlying the processes and phenomena responsible for the timing of ontogeny events and longevity (acute and chronic phenoptosis, neoteny, etc.) [7-9, 115], as well as for the potentially possible suppression of the cytokine storm in COVID-19 [116, 117].

The period of the cell clock rhythm is precisely adjusted by the phosphorylation-based signaling – network that includes multiple protein kinases, with GSK3 being the most universal kinase, at least in terms of the number of substrates involved. Therefore, GSK3 functions as a hub in the cross-regulation network of circadian rhythms and antioxidant defense mechanisms, which constitutes the main subject of this review. Attempts to simultaneously influence both pathways (see pathways directed from the Master Clock to the Nrf2 system in Fig. 1) are exemplified by the development of a preparation representing a hybrid of sulforaphane (well-known Nrf2 activator) and melatonin (circadian clock regulator) [118, 119] and substances that inhibit GSK3 and activate Nrf2 at the same time (2,4-dihydropyrano[2,3-*c*]pyrozoles) [120]. Presumably, such preparations can be used not only for treating neurodegenerative diseases but also for prolonging the healthspan and attaining longevity.

Ethics declarations. The authors declare that they have no conflict of interest. This article does not contain description of studies with the involvement of humans or animal subjects.

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