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REVIEW

Neuroendocrine-immune correlates of circadian physiology: studies in experimental models of arthritis, ethanol feeding, aging, social isolation, and calorie restriction

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Abstract Virtually all neuroendocrine and immunological variables investigated in animals and humans display biological periodicity. Circadian rhythmicity is revealed for every hormone in circulation as well as for circulating immune cells, lymphocyte metabolism and transformability, cytokines, receptors, and adhesion molecules. Clock genes, notably the three Period (Per1/Per2/Per3) genes and two Cryptochrome (Cry1/Cry2) genes, are present in immune and endocrine cells and are expressed in a circadian manner in human cells. This review discusses the circadian disruption of hormone release and immune-related mechanisms in several animal models in which circulating cytokines are modified including rat adjuvant arthritis, social isolation in rats and rabbits and alcoholism, the aging process and calorie restriction in rats. In every case the experimental manipulation used perturbed the temporal organization by affecting the shape and amplitude of a rhythm or by modifying the intrinsic oscillatory mechanism itself.

Keywords Circadian rhythms · Hypothalamic-pituitary axis · Cytokines · Lymph nodes · Spleen · Clock genes · Arthritis · Alcoholism · Social isolation · Calorie restriction · Aging

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The circadian clock

Organisms populating the Earth are under the steady influence of daily and seasonal changes resulting from the planet's rotation and orbit around the sun. This periodic pattern is most prominently manifested by the light–dark cycle and has led to the establishment of endogenous circadian timing systems that synchronize biological functions to the environment. This is the basis of predictive homeostasis [1], evolving as an adaptation to anticipate predictable changes in the environment, such as light and darkness, temperature, food availability, or predator activity. Therefore, the circadian clock is one of the most indispensable biological functions for living organisms that acts like a multifunctional timer to adjust the homeostatic system, including sleep and wakefulness, hormonal secretions, immune function and various other bodily functions, to the 24-h cycle [2, 3] (Fig. 1).

In mammals, the circadian system is composed of many individual, tissue-specific cellular clocks. To generate coherent physiological and behavioral responses, the phases of this multitude of cellular clocks are orchestrated by a master circadian pacemaker residing in the suprachiasmatic nuclei (SCN) of the hypothalamus. At a molecular level, circadian clocks are based on clock genes, some of which encode proteins able to feedback and inhibit their own transcription. These cellular oscillators consist of interlocked transcriptional and post-translational feedback loops that involve a small number of core clock genes (about 12 genes identified currently). The positive drive to the daily clock is constituted by two, basic helix-loop-helix, PAS-

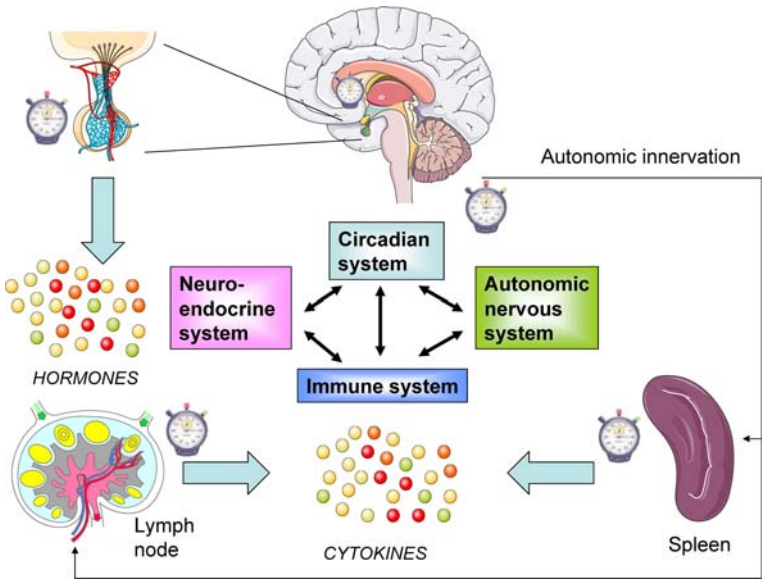

Fig. 1 Temporal interactions among the neuroendocrine system, the autonomic nervous system and the immune system as adaptive mechanisms to the environmental changes

domain containing transcription factor genes, called Clock and Bmal1. The protein products of these genes form heterodimeric complexes that control the transcription of other clock genes, notably three Period (Per1/Per2/Per3) genes and two Cryptochrome (Cry1/Cry2) genes, which in turn provide the negative feedback signal that shuts down the Clock/Bmal drive to complete the circadian cycle [4]. Per and Cry messenger RNAs peak in the SCN in mid-to-late circadian day, regardless of whether an animal is nocturnal or diurnal. Other clock genes provide additional negative and positive transcriptional/translational feedback loops to form the rest of the core clockwork, which has been characterized in rodents by a transgenic gene deletion methodology. Clock gene expression oscillates because of the delay in the feedback loops, regulated in part by phosphorylation of the clock proteins that control their stability, nuclear re-entry, and transcription complex formation [3, 5].

Clock genes are expressed in a tissue-specific fashion, often with unknown function. Although a substantial number of genes are rhythmic (about 10% in the SCN or peripheral tissues), the rhythmic genes tend to be different in the different tissues. For example, in comparisons between heart and liver, or between the SCN and liver, only a 10% coincidence was seen [4]. The phase of the peripheral clock oscillations is delayed by 3–9 h as compared to that of SCN cells, suggesting that the peripheral tissues are receiving timing cues from the master SCN oscillator. Furthermore, oscillations in isolated peripheral tissues dampen rapidly, unlike the persistent rhythms in isolated SCN neurons [4, 6].

Sorting of the cycling transcripts into functional groups has revealed that the major classes of clock-regulated genes are implicated in processes specific to the tissue in which they are found. For example, many cycling transcripts in the liver are involved in nutrient or xenobiotic metabolism. It is also of interest that many of the regulated transcripts correspond to rate limiting steps in their respective pathways, indicating that control is selective and very efficient. Indeed, about 10% of the genome is under control of the circadian clock [7].

As noted, the trillions of cellular clocks in primates are synchronized by a few thousand neurons located in the SCN. It is remarkable that such a small group of neurons display the properties of a central clock. Indeed, these "neuronal oligarchies," like the human ones, control trillions of cells in the body by: (a) taking control of the major communication channels (the endocrine and autonomic nervous systems); (b) concentrating the relevant information in a private way (i.e., light information arriving via the retino-hypothalamic tract). Thus, it is not surprising that anatomical studies have showed that the SCN projects to at least three different neuronal targets: endocrine neurons, autonomic neurons of the paraventricular nucleus (PVN) of the hypothalamus, and other hypothalamic structures that transmit the circadian signal to other brain regions [2]. The SCN projections are generally indirect, via the sub-PVN zone [8]. Through autonomic nervous system projections involving the superior cervical ganglia the SCN controls the release of a major internal synchronizer, the pineal substance melatonin [9].

Recordings from single dispersed SCN neurons have demonstrated that the circadian mechanism is not an emergent property of the SCN neuronal network but it is expressed in each individual cell. Multisynaptic links of SCN occur through the hypothalamic sub-PVN zone outflow to the adrenocorticotropic and other neuroendocrine axes and to autonomic ganglia that innervate the viscera

including all the endocrine and immune system. The innervation of the dorsomedial hypothalamus contributes to circadian control of the orexin/hypocretin system that participates in wakefulness [8]. By projecting to areas outside the hypothalamus, such as the lateral geniculate bodies and the PVN of the thalamus, the SCN neurons can synchronize hypothalamic-induced behavior (e.g., feeding) and locomotor activity. The circadian control of rest/activity cycles involves not only neuronal connections but also SCN paracrine signaling, e.g., the secretion of transforming growth factor-α and prokineticin–2 [10].

In the case of the immune system, our own work concentrated on the role of the autonomic nervous system (parasympathetic and sympathetic) in the circadian control of lymph node function [11, 12]. These studies were the continuation of our former studies on the role of sympathetic and parasympathetic nerves in thyroid follicular and C cell and parathyroid cell regulation [13, 14]. The concept that autonomic nerves are very efficient avenues to convey time of day information to the periphery has since then been generalized to tissues like the adrenal, pancreas, liver, ovaries, and many other organs [2].

Although circadian rhythms are anchored genetically, they are synchronized by and maintain certain phase relationships to external factors [15]. These rhythms will persist with a period different from 24 h when external time cues are suppressed or removed, such as in complete social isolation or in constant light or darkness. Research in animals and humans has shown that only a few such environmental cues are effective entraining agents for the circadian oscillator ("Zeitgebers").

Indeed, temporal organization is an important feature of the biological systems and its main function is to facilitate adaptation of the organism to the environment. The light–dark cycle, food, ambient temperature, scents and social cues have been identified as "Zeitgebers" in rats [6]. Stress is also capable of perturbing temporal organization by affecting the shape and amplitude of a rhythm or by modifying the intrinsic oscillatory mechanism itself. In particular, social stress in rodents has been found to cause disruptions of the body temperature, heart rate, and locomotor activity rhythms (see, e.g., [16–18]).

An entraining agent can actually reset, or phase shift, the internal clock. Depending on when an organism is exposed to such an entraining agent, circadian rhythms can be advanced, delayed, or not shifted at all. Therefore, involved in adjusting the daily activity pattern to the appropriate time of day is a rhythmic variation in the influence of the Zeitgeber as a resetting factor [15]. In humans, light exposure during the first part of the night delays the phase of the cycle; a comparable light change near the end of the night, advances it. At other times during the day light exposure has no phase-shifting influence [19, 20].

Circadian organization of the immune system

Light and daily rhythms have a profound influence on immune function. Many studies have described circadian variations of immune parameters such as lymphocyte proliferation, antigen presentation, and cytokine gene expression. The number of lymphocytes and monocytes in the human blood reach maximal values during the night and are lowest after waking. Natural killer (NK) cells, by contrast, reach their highest level in the afternoon, with a normal decrease in number and activity around midnight [21–24].

Immune cells have been checked for the presence of clock genes [25–29]. In a study aimed to investigate whether circadian clock genes function in human peripheral blood mononuclear cells, circadian clock genes human Per1, Per2, and Per3 were found to be expressed in a circadian manner in human peripheral blood mononuclear cells, with the a peak level occurring during the second part of the active phase [30, 31]. To investigate the presence of molecular clock mechanisms in NK cells as well as the circadian expression of critical factors involved in NK cell function, Arjona and Sarkar [32] measured the circadian changes in the expression of clock genes (Per1, Per2, Bmal1, Clock), Dbp (a clock-controlled output gene), CREB (involved in clock signaling), cytolytic factors (granzyme B and perforin), and cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-α in NK cells enriched from the rat spleen. Thus, the existence of molecular clock machinery is conserved across different lymphocyte subsets and peripheral blood cells. Moreover, they may share common entrainment signals. Emerging data in the human and animal literature suggest that circadian regulation may be crucial for the host defenses against cancer [33].

Both the humoral arm and the delayed (cellular) arm of the immune system function in a rhythmic manner. Indeed, circadian variations in immunocompetent cells in peripheral blood are of a magnitude to require attention in medical diagnostics [34, 35]. Circadian changes in the circulation of T, B, or NK lymphocyte subsets in peripheral blood and in the density of epitope molecules at their surface, which may be related to cell reactivity to antigen exposure, have been reported. Changes in lymphocyte subset populations can depend on time of day-associated changes in cell proliferation in immunocompetent organs and/or on diurnal modifications in lymphocyte release and traffic among lymphoid organs. Circadian rhythmicity is revealed in circulating cells, lymphocyte metabolism and transformability, circulating hormones and other substances that may exert various actions on different targets of the immune system, cytokines, receptors, and adhesion molecules, cell cycle events in health and cancer, reactions

to antigen challenge, and disease etiology and symptoms [25–31, 36, 37].

It must be noted that the role of the SCN, the central circadian pacemaker, in entrainment of lymphocyte function and in coordinating signals by which circadian information is conveyed to the immune cells remains unsettled. Rhythms in the number of circulating T cells persisted in rats with disrupted circadian output [38]. Similarly, SCN ablation did not affect the 24-h rhythms in cell cycle phase distribution in bone marrow cells [39], suggesting that some rhythms in the immune system are SCN-independent. It is known that circadian gene expression can be maintained in vitro [40]. Thus, some peripheral clocks may be able to independently generate circadian oscillations and this could be also the case for lymphocytes. Rather than a mere rhythm generator for the periphery, the SCN should be envisioned as a transducer for light entrainment. However, there are entrainment signals other than light that may be coordinating the rhythm in NK cell function and other immunological parameters. For example, feeding is an important Zeitgeber for peripheral clock gene expression [38], and interestingly enough, internal desynchronization produced by restricted feeding during the light period slowed down tumor progression in mice [41]. Daily activity rhythms are also considered to act as entrainment cues for peripheral tissues [42] and may as well influence the molecular clock in lymphocyte cells. In addition, intrinsic immunological outputs such a cytokine secretion could function as entrainment factors for immune cells. Indeed, interleukin (IL)-6 has been shown to induce Per1 expression in vitro [43].

Several studies have investigated the changes in cytokine levels that occur during the 24 h sleep–wake cycle in humans; however, it is difficult to measure these changes because endogenous cytokine levels are low (for ref. see [44]). Plasma TNF-α levels peak during the dark phase of cycle, and the circadian rhythm of TNF release is disrupted by sleep pathology like obstructive sleep apnea. Plasma IL-1β levels also have a diurnal variation, being highest at the onset of non-REM sleep. The levels of other cytokines (including IL-2, IL-6, IL-10, and IL-12) and the proliferation of T cells in response to mitogens also change during the 24-h cycle. Although the production of macrophage-related cytokines (such as TNF-α) increases during sleep (in response to in vitro stimulation), this occurs in parallel with the rise in monocyte numbers in the blood. The production of T-cell-related cytokines (such as IL-2) increases during sleep, independent of migratory changes in T-cell distribution [44]. All of these observed diurnal changes could be specific to the effects of sleep or associated with the circadian oscillator. To dissociate the effects that result from the sleep–wake cycle from those due to the endogenous circadian oscillator, experimental procedures such as constant routine or forced desynchrony need to be used. At present, there are no reports of studies using these methods to elucidate the effects of sleep on immunity.

Sleep and the immune system share regulatory molecules. These are involved in both physiological sleep and sleep in the acute-phase response to infection or in chronic inflammation. This supports the view that sleep and the immune system are closely interconnected. It is feasible that sleep influences the immune system through the action of centrally produced cytokines that are regulated during sleep. These endogenous cytokines are known to function through the autonomic nervous system and the neuroendocrine axis, although other pathways might be involved.

During the last years, we have examined the regulation of circadian rhythmicity of lymph cell proliferation in a number of experimental models in rat submaxillary lymph nodes. The bilateral anatomical location of submaxillary lymph nodes and their easily manipulable autonomic innervation allowed us to dissect some of humoral and neural mechanisms regulating the lymphoid organs and their interaction. A significant diurnal variation of rat submaxillary lymph node ornithine decarboxylase activity, an index of cell proliferation in immunocompetent organs [45] and endocrine glands [46], was uncovered, displaying maximal activity at early afternoon [47]. Such a maximum coincided with peak mitotic responses to lipopolysaccharide (LPS) and concanavalin A (Con A) in incubated lymph node cells. A purely neural pathway including as a motor leg the autonomic nervous system innervating the lymph nodes was identified [12]. The combined sympathetic-parasympathetic denervation of the lymph node suppressed circadian variation in lymph cell proliferation. In addition, a hormonal pathway involving the circadian secretion of melatonin also plays a role to induce rhythmicity [48].

Changes in circadian rhythms in acute and chronic inflammation

Besides acute inflammation there is a range of other clinical conditions where peripheral cytokine signals might modulate brain function. Numerous studies showed that the therapeutic administration of cytokines for the treatment of hepatitis, cancer, multiple sclerosis or rheumathoid arthritis induces depressive symptomatology which widely overlaps with the syndrome of "sickness behavior" observed in animal models of acute inflammation [49–51]. However, during acute infection and inflammation the amounts of circulating inflammatory cytokines are huge, usually two orders of magnitude or more above baseline levels. In contrast, circulating levels of cytokines are only moderately increased in the most frequent clinical situations in which cytokines play a role in inducing symptoms of

depression, such as chronic infection or inflammation, stress, alcoholism, aging, cancer, cardiovascular disease or autoimmune disorders. Slightly increased TNF-α and possibly also IL-6 levels are often found in patients with these diseases [51–54].

Circadian neuroimmune connections imply that a very important feedback component is provided by the immune cells to the brain. Indeed, there are several mechanisms by which the immune system can modify central clock structures [55–57]. In the case of rheumatoid arthritis, inflammation is characterized by increased local synovial and systemic levels of the pro-inflammatory cytokines IL-1, IL-6, IFN-γ, and TNF-α, which are directly involved in disease's pathophysiology [58]. Such increased cytokine production plays a key role in neuroendocrine activation pathways in arthritis [59]. As large, hydrophilic proteins, cytokines can only cross the blood–brain barrier at leaky points (the circumventricular organs) or via specific active transport mechanisms [60]. Cytokines act at the level of the organum vasculosum laminae terminalis, a circumventricular organ located at the anterior wall of the third ventricle. IL-1 binds to cells located on the vascular side of this circumventricular structure, thereby inducing synthesis and release of second messenger systems, such as nitric oxide (NO) synthase (NOS)/NO and the cyclooxygenase/prostaglandin systems [61]. It must be noted that a central compartment for cytokines exists and that there are data indicating that an increase in peripheral cytokines can evoke a mirror increase in brain levels of cytokines (for ref. see [55]).

Inflammatory stimuli can also induce CNS stress response through afferent peripheral neural signaling. This was shown mainly for cytokines from the peritoneum that can cause early rapid activation of the nucleus tractus solitarius in the brainstem via the vagus nerve [62]. Experimental evidence suggests that symptomatology after antigen administration, like anorexia and depressed activity, is a part of a defense response to antigenic challenge and is mediated by the neural effects of cytokines. These changes are known generally as "sickness behavior," that is, the "nonspecific" symptoms (anorexia, depressed activity, loss of interest in usual activities, disappearance of body care activities) that accompany the response to infection [49–51]. These "nonspecific" symptoms of infection include fever and profound psychological and behavioral changes in circadian structure [56]. Sick individuals experience weakness, malaise, listlessness, and inability to concentrate [57]. They consistently show evidence of decreased amplitude of circadian rhythmicity, like superficial sleep at night and hypersomnia, loss of interest and depressed activity during the day.

The link between immune system and sleep was first identified in the 1970s, when a sleep-inducing factor was isolated and chemically characterized from human urine: Factor S, a muramyl peptide derived from bacterial peptoglycan (for ref. see [63]). Subsequently muramyl dipeptide and factor S-related peptidoglycans were all shown to induce the key immunoregulatory cytokine IL-1. IL-1β is a potent somnogen, as well as a potent pyrogen. In fact, IL-1β is one of the most neurologically active molecules known. Subsequent studies revealed that bacterial LPS, LPS components and viral synthetic dsRNA, as well as killed and living bacteria can induce IL-1, TNF-α, IL-6, and IL-10. The presence of systemic inflammation, characterized by an elevation of certain potent pro-inflammatory cytokines, such as IL-1, IL-6, IL-10, and TNF-α may predispose patients to develop cardiovascular complications.

It is of importance to note that a clinically relevant immune circadian component is the T helper1 (Th1)/T helper 2 (Th2) balance [64]. Both branches support different functions of defense. Th1 responses include cell-mediated reactions that are important for cellular pathogens, whereas Th2 responses regulate production of antibodies in response to extracellular pathogens and mediate allergic processes. Moreover effects of IFN-γ, a major Th1 cytokine, and IL-4, a major Th2 cytokine, are antagonistic. A factor critical for the development of an effective immune response is thus the cytokine balance determining the selection of the effector mechanisms of type 1 or type 2 immunity [64, 65].

Th1 cells releasing mainly INF-γ, aside from other cytokines including IL-2 and TNF-α, become activated in response to intracellular viral and bacterial challenges and support various cellular (type 1) responses, including macrophage activation and antigen presentation. In contrast, the cytokines typical of Th2 immunity, IL-4 as well as IL-5, IL-10, and IL-13 tend to drive humoral (type 2) defense via stimulating mast cells, eosinophils, and B cells against extracellular pathogens. Nocturnal sleep favors a shift toward Th1 mediate immune defense. A circadian peak of the ratio of IFN-γ / IL-10 production in whole blood samples is found during nocturnal sleep. This peak was completely abolished after the administration of cortisone at 21:00 h in the preceding evening, suggesting that the suppression of endogenous cortisol release during early sleep plays a mediating role for the Th1 shift [64, 65]. However, slow-wave sleep not only suppresses the release of glucocorticoids, but also promotes the release of growth hormone (GH) and prolactin which support Th1 cell-mediated immunity.

One of the most studied physiological roles of immune variables on circadian CNS function is the regulation of sleep / wake cycle by pro- and anti-inflammatory cytokines. It is now clear that pro-inflammatory cytokines induce sleep while anti-inflammatory cytokines prevent

sleep induction [63, 66]. LPS injections produce similar results to those of the pro-inflammatory cytokines on sleep regulation and exert differential effects on EEG activity in rats depending on the time of administration [67].

In the last years a number of studies have started to unravel the basis for the circadian modulation by immune factors on the circadian system itself. Several reports indicate a possible immune feedback regulation of the circadian clock. For example, immunosuppressant drugs such as cyclosporine affect the phase of locomotor activity [68] and of hormone secretion [69, 70]. Moreover, immune-related transcription factors are present and active in the SCN and its activity is partially necessary for light-induced phase shifts [68].

Introduction of gram-negative bacteria into the body causes the liberation of toxic, soluble products of the bacterial cell wall, such as LPS, also known as endotoxin. Peripheral administration of LPS exerts profound effects on the sleep–wake cycle and sleep architecture and may produce, at higher doses, fever and a characteristic "sickness behavior" observed during inflammatory diseases, including sleep pattern changes and fever oscillations along the day [63, 66]. In mice, susceptibility to lethal doses of endotoxin increase dramatically during the resting period [71] and a similar temporal pattern of induced mortality has also been established for TNF-α [72].

Results in hamsters indicate that LPS treatment induces changes in the phase of locomotor activity rhythms in a manner similar to light-induced phase delays [73]. The phase-shifting response to LPS was reduced when the activation of NF-κB, a transcription factor reported to play a role in the photic input of the circadian system [68] was prevented. LPS treatment stimulates the dorsal area of the SCN as assessed by c-Fos activation [73]. Astrocytes have been shown to be mediators of immune mechanisms in several experimental models. Indeed, these cells express cytokines and their receptors in diverse cerebral structures, as well as subunits of the immune-related transcription (NF-κB), and they respond to stimulation of LPS and pro-inflammatory cytokines [74–76].

Data from our laboratory indicate that melatonin, administered in the drinking water, has the capacity to counteract the effect of LPS on body temperature in hamsters, when injected at "Zeitgeber" time (ZT) 0 (ZT12 defined as the time of light off) [77]. Evidence that melatonin improves survival from endotoxin shock has also been published [78, 79].

Therefore, one possible mechanism through which infection-related changes in circadian rhythms can occur is by modifying directly the activity of cells in the SCN. Cytokine receptors, e.g., IFN-γ receptors, have been detected in neuronal elements of ventrolateral SCN [80]. Expression of SCN IFN-γ receptors followed a 24-h rhythm, coinciding with the expression of Janus kinase 1 and 2 as well as the signal transducer and activator of transcription factor 1, the main intracellular signaling pathway for IFN-γ. In an ontogeny study, SCN IFN-γ receptors were found to reach their adult pattern between postnatal day 11 and 20, at a time when capacity for photic entrainment of the pacemaker became established [81]. Indeed, high doses of an IFN-γ / TNF-α cocktail disrupt electrical activity of SCN neurons [82, 83].

The capacity of intracerebroventricular administration of IFN-γ to modify 24-h wheel running activity was assessed in golden hamsters [84]. Animals received IFN-γ or saline at ZT 6 or ZT 18. Intracerebroventricular administration of IFN-γ at ZT 6 produced a significant phase advance in acrophase of rhythm, an effect not seen with injection at ZT 18. IFN-γ depressed mesor value of rhythm significantly; the effect was seen both with ZT 6 and ZT 18 injections [84]. IFN-γ was very effective to disrupt circadian rhythmicity of pituitary hormone release [85]. The results supported the view that the circadian sequels arising during the immune reaction can rely partly on central effects of IFN-γ [84]. A disruptive effect of systemic administration of IFN-α on the circadian rhythm of locomotor activity, body temperature and clock-gene mRNA expression in SCN has also been documented in mice [86]. Moreover, LPS incubation modified the circadian arginine-vasopressin release from SCN cultures [87]. Motzkus et al. [43] demonstrated that IL-6 induced murine Per1 expression in SCN cell cultures.

In a recent study day/night variations of transcripts encoding cytokine receptors and suppressors of cytokine signaling were correlated in groups of mice of different ages with Fos induction elicited by intracerebroventricular injections of TNF-α and IFN-γ [88]. Cytokine-elicited Fos induction was high at early night, when suppressors of cytokine signaling levels were low. Such Fos induction was significantly reduced in the older SCN at early night, and paralleled by reduced expression of IFN-γ receptor transcripts as compared to the younger SCN.

Most of the neuroendocrine effects of cytokines have been examined at single time point in the day–night cycle, thus overseeing the intricacies of significant daily variation in pituitary hormone release. Due to this we measured the circadian pattern of plasma ACTH, GH, prolactin, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) at six different time points within a 24-h cycle in adult male Wistar rats that received five daily injections i.p. of human IFN-γ (105 U.I./kg b.w.) or saline at 08:30 h [85]. A factorial ANOVA for main effects indicated a significant 43% increase of circulating prolactin in IFN-γ-treated rats. Time of day changes were significant for the five hormones examined and these diurnal variations became altered by IFN-γ administration, with a

phase-advance of ACTH peak, a suppression of the rest phase peak of GH, the appearance of a second peak of prolactin at an early phase of daily photoperiod, and the blunting of the 24-h variations of plasma FSH [85]. The data further pointed out to an effect of IFN-γ on the mechanisms responsible for the circadian organization of pituitary hormone release. Indeed, severe immune challenges such as animal models of sepsis [89] or infection with blood-borne parasites such as Trypanosoma cruzi or Trypanosoma brucei [90] or HIV-infected animals or patients [91] display different levels of circadian disruption, including complete arrhythmicity, suggesting that circadian rhythms can be considered a good quality-of-health indicator.

In recent years we examined the circadian disruption of hormone release and immune-related mechanisms in several animal models in which circulating cytokines are increased including rat adjuvant arthritis, alcoholism, calorie restriction, social isolation in rats and rabbits, and the aging process. Basic rationale for the experimental approach used was that most published studies dealing with hormone or immune changes in the above-mentioned situations were performed at single time-points in the 24-h span, an important drawback in view of the circadian nature of hormone release and immune function and on the fact that most manipulations employed disrupt circadian rhythmicity. The results obtained are reviewed below.

Rat adjuvant arthritis

Rheumatoid arthritis is a T-cell-driven autoimmune process associated with the production of autoantibodies. Rheumatoid arthritis is initiated by $CD4^+$ T cells, which amplify the immune response by stimulating other mononuclear cells, synovial fibroblasts, chondrocytes and osteoclasts. The release of cytokines, especially of TNF-α, IL-1, and IL-6, causes synovial inflammation. In rheumatoid arthritis the inflammatory process, usually tightly regulated by mediators that initiate and maintain inflammation and mediators that shut the process down, becomes imbalanced leaving inflammation unchecked and resulting in the destruction of cartilage and bone.

Efforts to develop safer and more effective treatments for rheumatoid arthritis rely heavily on the availability of suitable animal models [92]. Among these models, the rat's adjuvant arthritis is widely employed [93]. Hallmarks of this rat model are a reliable onset and progression of easily measurable, polyarticular inflammation, marked bone resorption and periosteal bone proliferation. Induction of adjuvant disease can be done with either Freund's complete adjuvant (FCA) supplemented with mycobacterium or by injecting synthetic adjuvants. The pathogenesis for development of adjuvant disease following injection of mycobacterial preparations is not fully understood, although a cross-reactivity of mycobacterial wall antigens with cartilage proteoglycans exists [93].

After FCA injection to rats, the inflammatory disease of the joints shows four stages in its time-course: preclinical (first week), acute (weeks 2–4), post-acute (weeks 5–8), and recovery (weeks 9–11) [94]. The preclinical stage of FCA arthritis (first week) is characterized by discrete radiological lesions of the forepaws and slight increase in the threshold for struggle triggered by foot pressure, presumably due to an impending, initially painless, stiffness.

The acute stage or arthritis (weeks 2–4) is defined by signs of hyperalgesia, lack of mobility and a pause in body weight gain; during the acute period, hindpaw and forepaw joint diameters increase [94]. In the later, acute, stages of disease (day 12+), adjuvant arthritis rats are often relatively immobile due to severity of paw swelling. At day 18, an increase in scratching behavior and signs of hyperalgesia are clearly established as compared to the adjuvant's vehicle-injected group [95, 96].

We have examined a number of immune and neuroendocrine circadian rhythms in FCA-injected rats by looking for changes in the preclinical phase of arthritis (2–3 days after FCA injection) as well as in the acute phase of the disease (18 days after FCA injection). Generally, changes in circadian rhythms in lymph node immune function tended to be more profound at the preclinical phase of the disease. For example, B-cell- and T-cell-mediated mitogenic activity of LPS and Con A, respectively, were modified in amplitude or acrophase during the preclinical phase [97] while exhibiting few or none changes during the acute phase of experimental arthritis [98]. Similarly, 24-h variations of B and T cells, as well as of $CD4^+$ (T helper) and $CD8^+$ (T cytolytic) cells became significantly changed during the preclinical phase [99], with absence of changes during the acute phase [98]. In the case of lymph node cell proliferation and local autonomic nerve activity, the increase in amplitude and mesor of rhythms found in the preclinical phase of arthritis was higher than that observed as the disease progressed [100]. Therefore, the results suggested that some sort of homeostatic compensation of initial changes in circadian rhythmicity of immune changes might occur with the development of arthritis.

As far as the changes in neuroendocrine rhythmicity during rat's arthritis, early data had indicated in FCA-injected rats that the 24-h organization of the biologic responses was altered. For example, morning-evening differences in circulating ACTH and corticosterone disappeared by days 7–21, and between days 6 and 8 after FCA injection a loss of the circadian rhythm in adrenocortical ornithine decarbxylase activity was found [101]. In our own studies conducted during the preclinical phase of

arthritis, a significant effect of immune-mediated inflammatory response on diurnal rhythmicity of circulating ACTH, GH, prolactin and thyrotropin (TSH) release was found, and was partially sensitive to immunosuppression by cyclosporine [69]. Interestingly, immunosuppressant treatment with cyclosporin or its analog tacrolimus also affects the phase of circadian locomotor activity rhythms, by inducing non photic phase shifts and blocking the effect of light pulses on the clock [68].

Further experiments indicated that hypothalamic levels of corticotrophin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), and somatostatin were altered in the preclinical phase of arthritis [70]. In the median eminence, adjuvant's vehicle-injected rats exhibited significant 24-h variations for the four hypophysiotropic hormones examined, with maxima at noon. These 24-h rhythms were inhibited or suppressed 3 days after FCA injection. Again immunosuppression by cyclosporine impaired the depressing effect of FCA injection on CRH, TRH, and somatostatin content in median eminence, but not that on GnRH. The activity of cyclosporine was less evident in other hypothalamic regions examined [70]. Generally, a decrease amplitude or mesor of transmitter rhythms were detectable, mainly in anterior and medial hypothalamic regions [102].

We also examined the changes in circadian rhythms of CNS and hypophyseal hormones and neurotransmitters during the acute phase of Freund's adjuvant arthritis (i.e., 18 days after FCA administration). Differing from the relative compensation of circadian immune changes seen at this time of arthritis, changes in 24-h rhythms of neuroendocrine parameters persisted during the clinical phase of the disease [103]. Daily rhythms in plasma LH, testosterone and TSH became suppressed or disrupted in arthritic rats. Concerning GH, the depressed mean values found in the preclinical phase of arthritis also persisted during the acute phase, as it was the case for the changes in catecholamine transmitter activity [104]. A 24-h variation in dopamine (DA) content were blunted in the anterior hypophyseal lobe, but remained unaltered in the neurointermediate lobe [104]. Disruption of endocrine circadian rhythms of plasma prolactin, insulin-like growth factor-1, LH and testosterone and of pituitary prolactin mRNA was reported in male Long Evans rats injected with FCA 23 days earlier [105].

Alcohol

Under ethanol feeding basal plasma levels of several hormones are altered. Among them, the hormones of the hypothalamic pituitary gonadal axis have been extensively studied [106]. Both acute and chronic (25–70 days) ethanol exposure are associated with low levels of hypothalamic GnRH and pituitary LH in adult and peripubertal male rats [107, 108]. A direct effect of ethanol on testosterone secretion in male rats has also been described [106, 107, 109].

It must be noted that most published studies were performed at single time-points in the 24-h span, an important drawback in view of the demonstrated disruption of circadian rhythms caused by ethanol intake, e.g., access to ethanol shortens the period of rhythms under constant conditions (free-running) in rats [110].

In our laboratory a series of studies was undertaken to analyze the effect of chronic ethanol feeding on circadian variations in the activity of the hypothalamic-pituitary axis and immune response in peripubertal male rats. In a first experiment chronic ethanol feeding of peripubertal male rats brought about significant modifications in FSH, LH, testosterone, prolactin and TSH release [111]. The secretion of prolactin augmented and that of FSH, LH, testosterone and TSH decreased, in ethanol-fed rats. Significant changes in the 24-h secretory pattern of circulating hormones in rats receiving ethanol were also apparent. They included the appearance of two rather than one peak of FSH during the inactive phase of daily cycle, the suppression of the maximum of plasma LH during the first part of inactive phase, and the appearance of a second peak of testosterone and prolactin during the second part of the inactive phase and of a second peak of plasma TSH during the first part of the activity phase. The significant positive correlation between individual LH or prolactin concentration and circulating testosterone levels found in controls was lost after ethanol administration [111]. The distorted prolactin rhythm found in ethanol-treated rats correlated with a significant disruption of 24-h rhythms in median eminence DA, serotonin (5-HT), γ-aminobutyric acid (GABA) and taurine levels as well as in median eminence DA and 5-HT turnover, all suspected modulators of prolactin release [112]. Thus chronic exposure of rats to ethanol results in significant changes in pituitary hormone secretion but identification of the site(s) of action of ethanol to induce these effects remains elusive.

Recently an analysis was published on the role of free radical damage at the adenohypophyseal level on the decline in serum LH and FSH levels in rats fed with ethanol for 5–60 days [113]. There were increases in pituitary 8-oxo-deoxyguanosine immunoreactivity, a marker of oxidative damage to nucleic acids, and an overall increase in malondialdehyde and 4-hydroxynonenal, markers of lipid peroxidation. Pituitary protein carbonyl formation, a marker of protein oxidation, and tyrosine nitration of proteins, an index of nitrosative stress, increased significantly after 30–60 days of ethanol consumption, respectively

[113]. The data provided evidence for ethanol-induced oxidative damage at the pituitary level, presumably contributing to pituitary dysfunction. Since the above-mentioned study on adenohypophyseal oxidative damage was performed at single time points, presumably at morning hours, and in view that 24-h changes in redox state occurred in a number of tissues [114], we considered it worthwhile to analyze the 24-h changes in pituitary gene expression of the prooxidant enzymes NOS 1 and 2 and of heme oxygenase-1 (HO-1), as well as in plasma NO_2^- and NO_3^- (NO_x) levels in ethanol and control rats [115]. Animals were killed at 6 time intervals during a 24-h cycle. Anterior pituitary mRNA levels encoding NOS1, NOS2, and HO-1 were measured. Ethanol feeding of prepubertal rats changed significantly the 24-h pattern of expression of NOS1, NOS2, and HO-1 in the adenohypophysis and augmented NOS2 and HO-1 mRNA levels. Peak values for the three enzymes in ethanol-fed rats occurred at the beginning of the scotophase (i.e., at 21:00 h). Ethanol feeding augmented mean values plasma NO_x levels with a maximum at 13:00 h while in controls a biphasic pattern was observed, with peaks at 09:00 h and 17:00–21:00 h [115]. Therefore one of the mechanisms by which ethanol augments oxidative damage in the adenohypophysis may include overproduction of NO and carbon monoxide.

At a neuronal level, ethanol alters the circadian expression patterns of per clock genes in various brain regions, including the SCN. Notably, circadian functions of β-endorphin-containing neurons that participate in the control of alcohol reinforcement become disturbed after chronic alcohol intake. In rats, prenatal [116] or postnatal [117] ethanol exposure alters the circadian expression of proopiomelanocortin (POMC) mRNA encoding the peptide β-endorphin and of the clock governing genes rPer1, rPer2 and rPer3, in the arcuate nucleus, and rPer1 and rPer2 mRNA levels in the SCN during the adult period. As alcohol intake alters the expression of clock genes, as a consequence, a variety of neurochemical and neuroendocrine functions become disturbed. Further steps in this pathologic chain are alterations in physiological and immune functions that are under circadian control, and, as a final consequence, addictive behavior might be triggered or sustained by this cascade. This has been postulated as a pathologic conditions that contributes to the negative health consequences of chronic alcohol intake [118].

A bidirectional interaction between alcohol and the clock genes occurs [118]. At the behavioral level, both adult and perinatal ethanol treatment after the free-running period and light response of the circadian clock in rodents; genetic ethanol preference in alcohol-preferring rat lines is also associated with alterations in circadian pacemaker function [119]. In this respect, it has been shown that per2 gene activity regulates alcohol intake through its effects on the glutamatergic system by affecting glutamate reuptake mechanisms and thereby a variety of physiological processes that are governed by the circadian clock. The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption [120]. Per2 (Brdm1) mutant mice, which have a deletion in the PAS domain of the Per2 protein, show alterations in the glutamatergic system. Lowered expression of the glutamate transporter Eaat1 is in these animals, leading to reduced uptake of glutamate by astrocytes. As a consequence, glutamate levels increase in the extracellular space of Per2 (Brdm1) mutant mouse brains. This is accompanied by increased alcohol intake. In humans, variations of the PER2 gene are associated with regulation of alcohol consumption [120]. It is interesting that acamprosate, a drug used to prevent craving and relapse in alcoholic patients acting through a dampening of the hyper-glutamatergic state [121, 122] reduced augmented glutamate levels and normalized increased alcohol consumption in Per2 (Brdm1) mutant mice [120]. Therefore, the data point out glutamate as a link between dysfunction of the circadian clock gene Per2 and enhanced alcohol intake.

There is considerable evidence indicating that ethanol consumption alters immune system function and leads to increased infection (e.g., pneumonia and tuberculosis) and susceptibility to different neoplastic diseases. The mechanisms through which ethanol affects immune reactions are not entirely understood. Several reports have shown that ethanol intake changes the total number of lymphocytes and their subsets [123]. Long-term ethanol administration lowered the number of B lymphocytes in both mice [124] and rats [125] and a rise in the relative number of T lymphocytes with no change in the ratio of $CD4^+$ to $CD8^+$ cells, A drop in the absolute number of $CD4^+$ cells in rats was also noted [125, 126]. However, most of the results in experimental animals have been obtained at single time-points in a 24-h span.

In a study carried out to analyze the effect of chronic ethanol feeding on 24-h variations in T and B cell number and effects in thymus and spleen of peripubertal male rats, the ethanol diet brought about significant modifications in the 24 h pattern of several immune parameters [127]. Ethanol decreased thymic and splenic weight and modified 24 h rhythmicity of thymic and splenic T, B and $CD4^+$–$CD8^+$ cells, thymic $CD4^+$ and splenic $CD8^+$ cells, thymic and splenic T/B and $CD4^+/CD8^+$ ratios, as well as of mitogenic responses to Con A and LPS in both tissues. After ethanol administration, mean values of thymic T cell number decreased, and mean values of thymic and splenic $CD8^+$ and $CD4^+$–$CD8^+$ number augmented. Consequently, thymic T/B ratio and thymic and splenic $CD4^+/CD8^+$ ratio decreased in ethanol-fed rats [127]. These findings coexisted with a significant increase in thymic cells' response to

LPS. Hence, chronic ethanol administration effectively disrupted circadian organization of immune responsiveness in growing rats.

Arjona et al. explored daily rhythms of cytotoxic factors (granzyme B and perforin), IFN-γ, and NK cell cytotoxic activity in the spleens of adult male rats whose mothers were fed during pregnancy with chow food or an ethanol-containing liquid diet or pair-fed an isocaloric liquid diet [128]. When adult rats exposed to ethanol during their fetal life showed a significant alteration in the physiological rhythms of granzyme B and IFN-γ associated with decreased NK cell cytotoxic activity. The same authors [37] reported the NK cell function followed over a 24-h period, mRNA and protein levels of granzyme B, perforin, and the cytokine IFN-γ, as well as NK cell activity, in the splenocytes of ad libitum-fed, pair-fed, and ethanol-fed Sprague Dawley male rats. Circadian rhythms occurred in mRNA and protein levels of granzyme B, perforin, and IFN-γ. A circadian pattern was also detected in NK cell cytolytic activity. Chronic ethanol suppressed NK cell activity by directly disrupting the circadian rhythms of granzyme B, perforin, and IFN-γ. These findings identify the circadian functions of splenic NK cells and show the vulnerability of these rhythms to chronic ethanol administration [37]. To investigate whether these oscillations were under a genuine circadian control the daily expression of clock genes (Per1, Per2, Clock, and Bmal1), a clock-controlled gene (Dbp), cytolytic factors (granzyme B and perforin), and cytokines (IFN-γ and TNF-α) were measured in NK cells enriched from rats maintained in constant darkness. In addition, it was assessed whether the disruption of the NK cell clock by RNA interference affected the expression of cytolytic factors and cytokines. Persistent 24-h oscillations were found in the expression levels of clock genes, cytolytic factors, and cytokines in NK cells enriched from DD rats. In addition, RNAi-mediated Per2 knockdown caused a significant decrease of granzyme B and perforin levels in the rat-derived NK cell line RNK16 [29].

The effects of ethanol and central administration of β-endorphin on perforin, granzyme B, and IFNγ that modulate NK cell cytolytic activity were monitored to understand the mechanism involved in ethanol's suppression of NK cell activity [129]. A group of male Fischer-344 rats was fed an ethanol-containing diet (8.7% v/v), and a control group was pair-fed an isocaloric diet. At the end of 2 weeks, both groups were infused with β-endorphin into the PVN of the hypothalamus for 18 h, and the spleen was removed for analysis of perforin, granzyme B, and IFN-γ mRNA and protein levels. PVN administration of β-endorphin increased the mRNA and protein expression of granzyme B and mRNA expression of IFN-γ in pair-fed animals. Ethanol significantly reduced both basal and β-endorphin-induced levels of granzyme B and IFN-γ. Therefore chronic ethanol consumption suppresses β-endorphin-induced NK cytolytic activity, granzyme B, and IFN-γ in male Fischer-344 rats [129].

β-Endorphin neuronal function is known to be regulated by various proinflammatory and anti-inflammatory cytokines. To evaluate the effects of ethanol on the proinflammatory and anti-inflammatory cytokines known to control β-endorphin neuronal and NK cell functions during immune challenges the effects of chronic ethanol consumption on the basal and LPS-activated NK cells' functions in the spleen, the β-endorphin peptide precursor POMC gene expression in the arcuate nucleus of the hypothalamus, and mRNA levels of pro-inflammatory cytokines IL-1beta, TNF-α and anti-inflammatory cytokines IL-6 and IL-10 were measured in the spleen and in the ARC [130]. Ethanol feeding via a liquid diet for 2 weeks suppressed both basal and LPS-stimulated NK cell cytolytic functions and the levels of cytotoxicity-regulatory perforin and granzyme B mRNAs in the spleen. Ethanol feeding reduced the basal and LPS-stimulated levels of POMC mRNA in the arcuate nucleus. Ethanol also impaired LPS-induced levels of IL-1beta and TNF-alpha mRNAs both in the spleen and in the arcuate nucleus. In contrast, ethanol feeding did not cause any significant changes in basal and the LPS-stimulated expression of IL-6 and IL-10 mRNAs in the spleen and of IL-6 mRNA levels in the arcuate nucleus. The results indicate that ethanol suppression of hypothalamic POMC levels and splenic NK cell functions is associated with a reduced expression of pro-inflammatory cytokines in neuroendocrine and immune cells [130].

As mentioned, chronic ethanol administration influences various circadian rhythms (sleep, motor activity and food intake). For example, ethanol intake or withdrawal can disrupt the period of circadian rhythms, access to ethanol shortening the free-running period in rats [110]. In addition, circadian rhythms modulate the response to ethanol and many other pharmacological substances. Relevant to this, the central pacemaker of the mammalian circadian system, the SCN, are composed mainly of GABAergic neurons [6] and the role of these neurons in circadian rhythmicity has been demonstrated [131]. Indeed, they can be a substrate for the effect of ethanol on 24-h rhythms.

Aging

Aging is characterized by changes in neuroendocrine function which are manifested in rodents in reproductive physiology and, less perceptible, in other functions such as thyroid, adrenal, or growth/metabolic functions (for ref. see [132–134]). The contribution of each level of the axis (hypothalamus, adenohypophysis or peripheral tissues) is

not yet clearly established. In man, aging is associated with primary testicular dysfunction, as indicated by a decreased testosterone response to exogenous LH [135] and a diminished Leydig cell mass [136]. As a result of reduced testosterone negative feedback, serum gonadotropin levels increase but the degree of this increase is inappropriately low when compared to that seen in younger men with low serum testosterone levels [136] indicating that hypothalamic/pituitary control of testicular function is also altered [137].

In contrast, most of the studies in male rat models of aging have demonstrated a primary hypothalamic/pituitary dysfunction with a secondary testicular failure, e.g., testosterone production in response to human chorionic gonadotropin stimulation is unchanged with aging whereas serum LH levels at baseline and in response to exogenous GnRH are decreased in old compared to younger rats [138, 139]. In old rats, multineuronal dysfunctions are demonstrable in the hypothalamus, with a decline in the activity of monoamine-containing, e.g., DA, and neuropeptide-containing neurons, e.g., GnRH, GH-releasing and inhibiting hormones, and alterations in the regulatory mechanisms of these neurons. Changes in other neurons that project to hypophysiotropic hormone neurons, i.e., norepinephrine (NE) or 5-HT containing neurons, were also thought to contribute to the decline of hypophysiotropic hormone secretion with aging. In addition, changes in hypothalamic hormone processing are known to occur in the adenohypophysis of old rats [132, 134, 139–141].

It is interesting that a number of similarities exist between ethanol intoxication and the aging process. A reduced amplitude, shorter free-running periods and desynchronization of circadian rhythms are associated with advanced age in both rodents and humans (for ref. see [142]). Aged rodents showed, among other, an altered response to the phase-shifting effects of light pulses and changes in the time it takes to re-entrain to a new light–dark cycle. In healthy elderly humans, although circadian rhythmicity persists, a number of 24-hour rhythms are dampened and/or advanced. As after ethanol administration, the constitutive release of prolactin becomes disrupted in aging rodents, a situation known to be associated with involutive morphological changes in the adenohypophysis [132]. In addition, age-related alterations in the amounts or turnover of hypothalamic hormones or neurotransmitters acting on the adenohypophysis have been described.

Virtually all pituitary hormones are secreted in a circadian manner and exhibit diurnal rhythmicity [143]. Hence, age-related changes may occur not only in the average concentration of a hormone, but also in its 24-h pattern of secretion. These changes in rhythmicity presumably play an important role in neuroendocrine aging. However, most studies on the effect of aging on hypothalamic neurotransmitters and neuropeptides in rats have been obtained as single time points (generally between 09:00 and 13:00 h) in a 24-h cycle. In our laboratory a study was designed to examine the 24-h rhythmicity of serum prolactin and median eminence and anterior pituitary content of DA, 5-HT, GABA, taurine and somatostatin in 2 months-old and 18–20 months-old Wistar male rats [144]. The concentration of prolactin was higher in aged rats, with peaks in both groups of rats at the early phase of the activity span. Median eminence DA content of young rats attained its maximum at the middle of rest span and decreased as prolactin levels augmented while the lowest values of adenohypophyseal DA were observed at the time of prolactin peak. DA rhythmicity disappeared in aged rats. GABA content of median eminence and adenohypophysis was lower in aged rats, with maximal values of median eminence GABA at light–dark transition in young rats and at the second half of activity span in aged rats. Serum prolactin correlated positively with median eminence GABA in young rats and negatively with pituitary GABA in young and aged rats. Median eminence somatostatin was lower, and adenohypophyseal somatostatin higher, in aged rats. Median eminence somatostatin peaked at the beginning of the activity phase (young rats) or at the end of the rest phase (aged rats). Prolactin levels and somatostatin content correlated significantly in young rats only. Median eminence and pituitary 5-HT and taurine content did not change with age [144]. The results indicate a disruption of prolactin regulatory mechanisms in aging that resembled that caused by ethanol in rats.

Aging is characterized by a significant alteration in circadian rhythmicity including a reduced amplitude, shorter free-running periods and desynchronization of the rhythms (for ref. see [145]. Therefore, the occurrence of age-related circadian changes of central neuroendocrine mechanisms must be taken into account when trying to define their relative contribution to the regulation of adenohypophyseal hormone release in aged subjects.

We previously reported the effect of aging on circadian organization of plasma prolactin, GH, FSH, LH, TSH, insulin and testosterone [103], and of hypothalamic and hypophyseal NE and DA turnover and content [104], in an experimental model for rheumatoid arthritis in rats (Freund's adjuvant-induced arthritis). More recently we reported the changes in 24-h organization of hypothalamic and hypophyseal 5-HT turnover, and somatostatin and excitatory and inhibitory amino acid concentration, in independent group of young (2 months) and aged (18–20 months) male rats [146]. DA turnover was also measured to assess the reproducibility of findings in this group of rats as compared to our previous results in arthritic rats.

How the single time-point approach can be misleading to give a full picture of the studied phenomena is illustrated

by the results of our study that indicate that aging rats had suppressed or disrupted 24-h variations in several neuroendocrine parameters in the hypothalamic-pituitary unit, including 5-HT and DA turnover and content of somatostatin and of excitatory and inhibitory amino acids [146]. By looking at morning time intervals only, one could wrongly conclude that 2 months-old and 18–20 months-old rats had essentially similar 5-HT and DA turnover in their anterior hypothalamus, as well as similar concentrations of somatostatin and excitatory and inhibitory amino acids. However, mean values of most parameters decreased with age, except for DA content in the anterior pituitary lobe and aspartate content in the neurointermediate lobe, which increased with age.

Indeed, several of the discrepancies found in the literature on the direction of changes to analyze aging activity on neurotransmitters and neuropeptides in the hypothalamic-hypophyseal unit can be attributed to the fact that most studies have been obtained as single time points in the daily cycle. For example, many studies have reported that 5-HT levels in the hypothalamus are unchanged in aging male rats [147–149], although others have found increased [150, 151] or decreased [152] levels with aging. Hypothalamic 5-HT turnover has been reported to increase with aging [147, 151, 152] when assayed at single time-points in morning hours. Our results, analyzing the whole 24-h period, clearly demonstrated that 5-HT turnover in anterior and medial hypothalamic blocks indeed decreased with age in rats [146].

Another controversial subject is to what extent somatostatin activity changes with age in the hypothalamic-hypophyseal unit. The pattern of GH secretion undergoes significant changes in the aging rat, resulting in decreased daily secretion of GH [133]. This has been attributed to an increased somatostatin tone [153], since hypothalamic somatostatin content was reported to augment in old animals [154]. However, rat hypothalamic somatostatin content was also found to diminish [155] or to remain unchanged [156] with aging. As a further confusion on this matter, discrepancies exist as far as somatostatin mRNA levels in the hypothalamus that were reported to increase [157], to decrease [158], or to remain unchanged [159]. Our results demonstrated that somatostatin levels decreased in the hypothalamus of aged rats, but showed a peak of increased amplitude in the anterior pituitary lobe during the rest phase of the 24-h cycle [146]. Indeed, in at least one study pituitary somatostatin content increased by about 2-fold in 20 month-old rats as compared to younger counterparts [155]. It remains to be identified whether the decrease in hypothalamic somatostatin content represents either a decrease in somatostatin synthesis or an increase in somatostatin release (as suggested by the higher peak levels of adenohypophyseal somatostatin). In any event, the results suggest that the alterations in hypothalamic somatostatin content may contribute to changes in GH release with age in rats [103].

Excitatory amino acids are thought to have an important role in the regulation of hypophysiotropic hormone secretion. For example, the in vitro and in vivo effects of the glutamate agonist NMDA on LH release were less in old rats than in younger rats [160, 161]. In addition, glutamate content of mediobasal hypothalamus/preoptic area fragments decreased in old as compared to younger rats. Such a decrease, documented at single time points (at morning hours), is also supported by our study on 24-h changes of hypothalamic glutamate content [146].

Alterations in inhibitory amino acids may also play a role in mediating age-related changes in hypophysiotropic hormone secretion. In studies in vitro, GABA-A and GABA-B receptor agonists decreased GnRH and glutamate release from hypothalamic perfusates, whereas GABA-A and GABA-B receptor antagonists enhanced neuropeptide and glutamate release [162]. With aging, the concentrations of GABA in the rat hypothalamus decreased significantly [161, 163] and a reduction in binding to GABA-A receptors was observed in all brain areas examined in male rats [164]. Our study [146] documented the depressive effect of aging on the hypothalamic content of the three inhibitory amino acids studied, i.e., GABA, taurine and glycine, as well as a general disruption of 24-h variations in concentration.

Hypothalamic DA turnover rate [147, 165] and in vitro DA release [166, 167] decrease in aged rats (assayed at a single time point in the morning). In vivo, secretion of DA into the hypophyseal portal blood has been found to decrease [168] or to increase [169] with aging, when assessed at single time-points in the morning. Our results on DA turnover and hypophyseal DA content throughout the 24-h cycle supported the existence of a lower DA turnover in medial and posterior hypothalamus, and increased levels of anterior pituitary DA, in aged rats [146] similarly to that described when the effects of aging on hypothalamic and pituitary DA turnover or content were assessed in an experimental model of rheumatoid arthritis in rats [104].

Summarizing, the discussed studies documented the existence of significant disruption of circadian organization of components of the hypothalamic-hypophyseal unit in aged rats. Both the efficacy of input and output pathways from the central nervous system circadian pacemaker (the hypothalamic SCN) and the functioning of the central pacemaker itself, change with advancing age [145]. In addition, some of the decline in overt circadian rhythmicity can be due to deteriorating function of the effector systems [145].

Social isolation

Increased cytokine levels have been reported as a consequence of exposure to psychosocial stressors like social conflict, social isolation or overcrowding. The most profound change that occurs with individual housing is an increase in aggression of males seen in both mice and rats following even relatively brief periods of individual housing [170, 171]. Individually housed animals are also hyperresponsive to stressors [172]. For example, in one study it was found that group size per se had limited long-term effects on pathophysiological measures of social stress, although it had a significant influence on many aspects of behavior when rats were first introduced into their groups [173]. Over weeks 1–8, single housed rats continued to spend much more time apparently attempting to escape (sniffing and chewing at the bars and suddenly dashing around their cage) while those housed in groups spent more time sleeping and feeding [173].

Solitary housing of usually social animals like rats or mice causes complex neurobiological changes. Socially isolated animals exhibited a decrease in the electrical activity of neurons within the hypothalamus and had lower basal plasma corticosterone levels than did animals raised in social conditions [170]. Although this could be interpreted as indicating less psychosocial stress in isolation, individual housing of animals is associated with an increase in aggression of males [170, 174], hyperresponsiveness to several stressors [172] and a behavior comprising more time spent attempting to escape and less time sleeping and feeding [173]. Decreases in plasma levels of prolactin were found in subordinate hamsters after exposure to social conflict [175] and in isolated male hamsters as compared to hamsters with a family [176]. This indicates that isolation can be considered as a mild stress for rats.

To examine whether social isolation in growing male rats affected 24-h variations of activity of the hypophyseal gonadal axis the circadian pattern of plasma prolactin, LH, FSH, and testosterone levels at six different time points within a 24-h cycle in growing male rats kept in isolation or group-caged for 30 days [177]. Isolation brought about a decrease in prolactin, LH and testosterone secretion and an increase of FSH secretion. In isolated rats the 24-h secretory pattern of prolactin and testosterone became modified, i.e., the maximum in prolactin seen in control animals at the beginning of the activity span was not longer detected while the maximum in circulating testosterone taking place at 17:00 h in controls was phase-delayed to 21:00 h in isolated rats [177].

Since the changes induced by isolation on the pituitary testicular axis could be due to an effect of this mild stress on the endogenous clock that modulates the circadian variation of hormone secretion the mean levels and 24-h variations of plasma prolactin, growth hormone, ACTH, corticosterone, and leptin were measured in a second study [178]. That isolation of young male rats for 4 weeks is a stressful signal, which was indicated by the elevated corticosterone secretion and modified 24-h pattern found. Although no substantial changes in 24-hour profile or mean value of plasma ACTH were observed, adrenocortical function clearly augmented as a consequence of social isolation. Plasma corticosterone levels correlated significantly with plasma ACTH and adrenal gland corticosterone concentration in control rats only. On the other hand, the daily secretory patterns of two stress-related pituitary hormones, prolactin and GH, became distorted and their total output decreased after isolation. Four weeks of social isolation in growing rats induced a mild hyperleptinemia and disruption of the 24-h pattern of plasma leptin by phase-advancing its nocturnal peak about 4 h [178].

Examination of social isolation effects on circadian rhythmicity of pituitary hormone release was extended to another species, the rabbit. The rabbit exhibits an unusual form of maternal care, with a single and very short visit (3–5 min) every day to nurse [179]. This daily nursing visit of the doe is extremely regular, with some individuals showing a day-to-day variability of only a few minutes. In contrast with the elaborate nest-building process seen e.g., in rodents, maternal care in rabbits is restricted to those single, brief nursing bout per day, an activity that is displayed with circadian periodicity during the dark phase of daily photoperiod (around 02:00 h) [180]. Despite the short duration of each nursing bout, the altricial rabbit pups (which are blind for the first 10 days of life) can locate the mother's nipples and suckle milk due to the perception of an olfactory signal that is emitted from the mother's ventrum. In a series of studies we demonstrated that (a) Both in control and in does separated from their litter, plasma level of prolactin changed in a similar way throughout the day showing two maxima, at 05:00–09:00 h and at 17:00–21:00 h, respectively, litter separation significantly augmenting FSH and LH release and disrupting their 24-h rhythmicity in the mother [181]. (b) separation of newborn pups from their mother augmented circulating gonadotropin and PRL levels and disrupted 24-h rhythmicity of their release in pups and to a similar way in both sexes [181–184].

Available information indicates that psychosocial events like social isolation affect primarily T lymphocytes, and less B lymphocytes [185–188]. For example, social isolation of pigs in the first days of life caused a significant decrease in immune reactivity at day 12, as assessed by T-cell mitogenesis (Con A response) without affecting B-lymphocyte proliferation induced by LPS [189]. Again a major pitfall in these studies is the absence of information

on circadian rhythmicity of the immune response after manipulation. To address this point we examined the effect of social isolation on submaxillary lymph node lymphocyte subset populations, IFN-γ release and mitogenic responses to Con A and LPS in groups of rats that were killed at 6 time intervals during the 24-h span [190]. After isolation lymph node T, B, non T-non B, $CD8^+$ and $CD4^+$–$CD8^+$ cells augmented, whereas lymph node $CD4^+/CD8^+$ ratio, IFN-γ release, and mitogenic responses decreased. Social isolation resulted in disruption of 24 h rhythmicity of every immune parameter tested. $CD4^+/CD8^+$ ratio, IFN-γ release, and Con A and LPS responses correlated significantly with plasma prolactin or GH levels while T/B ratio correlated with plasma prolactin levels only. B, non T-non B and $CD4^+$–$CD8^+$ cells correlated negatively with plasma prolactin. It seemed feasible that modifications in mean value and 24-h rhythmicity of plasma prolactin and GH levels were involved in the effect of social isolation on immune responsiveness [190].

In another study isolated rats showed increased splenic Con A response with peak activity during the activity span [191]. Mean 24 h values of splenic LPS response decreased in isolated rats as compared to grouped rats. Mean values of splenic $CD4^+$ and $CD8^+$ cells augmented in isolated rats. The highest in vitro IFN-γ production occurred in the isolated group and the lowest in the grouped rats, the differences among groups being significant [191]. The results indicate that social isolation augmented cell-mediated immunity in rat spleen.

When the effects of social isolation/individual housing of adult mice on behavioral and immune responses were examined [192], the only difference detected between individually housed adult mice and those grouped was a reduction in cell proliferation and in the production of the Th1 cytokine IL-2, thus resembling the decrease in Con A and LPS response and IFN-γ release (another Th1 cytokine) found in lymph nodes. When individually housed mice were exposed to a mild psychological stress like a forced exposure to a novel environment, they showed a lesser type 1 (IL-2) and type 2 (IL-4) cytokine production and splenocyte proliferation than grouped male mice [192]. Individually housed mice were more susceptible than group housed mice to experimentally induced illness such as tumors [193–197] and virus infection [198].

Caloric restriction

Homeostasis defines the mechanisms that react to maintain a constant, fixed set point of a physiological variable (reactive homeostasis) as well as those that are active in advance to maintain a set point that itself is rhythmic (predictive homeostasis) [1]. This last type of homeostasis evolves as an adaptation to anticipate predictable changes in the environment, such as light and darkness, food availability, temperature or predator activity, and is the basis of the circadian clock as discussed above [6].

One of those environmental predictable changes, the scarcity of food, occurs rhythmically in nature every the year and can be reproduced in laboratory conditions by calorie restriction. Experimental calorie restriction (e.g., 25–50% reduction of caloric intake), without deficiency in essential nutrients have been widely employed in this respect [199–203].

Indeed, the effect of calorie restriction has been explained from the evolutionary view that organisms have evolved neuroendocrine and metabolic response systems to maximize survival during periods of food shortage [204]. When environmental energy resources are plentiful, organisms grow, reproduce, and store excess energy in adipocytes for later use as fuel. Once organisms encounter a period of food shortage, seasonally or hazardously occurring in nature, they suspend growth and reproduction, induce defense molecules such as glucocorticoids and shift whole-body fuel utilization from both carbohydrate and fat to almost exclusively fat. The effect of calorie restriction might derive from these adaptive responses [200, 204].

Under calorie restriction the use of energy is greatly reduced and the basal plasma levels of several hormones are altered [205–207]. Among them, the hormones of the hypothalamic pituitary gonadal axis have been extensively examined [208–210]. In a study analyzing the effect of calorie restriction on the 24-h variation of pituitary-testicular function in young male Wistar rat animals were submitted to a calorie restriction equivalent to a 66% of food restriction for 4 weeks starting on day 35 of life [211]. Rats were killed at 6 time intervals around the clock. Mean secretion of prolactin augmented and that of LH and testosterone decreased in calorie restricted rats, whereas FSH release remained unchanged. Significant changes in the 24-h secretory pattern of circulating prolactin, LH and testosterone levels occurred in calorie restricted rats. These include the appearance of a second maximum of plasma prolactin, the blunting of LH peak and a phase-delayed of testosterone peak. The significant positive correlation between individual LH and testosterone levels found in controls was not longer observed in calorie restricted rats [211]. Availability of nutrients presumably affects the mechanisms that modulate the circadian variation of pituitary-gonadal axis in growing male rats.

Presentation of the restricted calorie diet was at 09:00–10:00 h daily. Since calorie restricted rats received only 7 g of food / 24 h, this amount was consumed over a short time, followed by a starvation span. Therefore, the possibility that food presentation and availability would serve as a synchronizer for the circadian system must be considered.

Food availability acts as a Zeitgeber resulting in e.g., "food anticipatory activity" among other phenomena [212]. Rats anticipate a scheduled daily meal by entrainment of a circadian pacemaker separate from the light-entrainable circadian pacemaker located in the SCN. These rhythms exhibit the properties of an entrained, circadian oscillatory process, including circadian limits-to-entrainment (i.e., food-anticipatory activity occurs only if feeding intervals are within the circadian range [213]; there is a gradual resetting in response to shifts of the presumed entraining stimulus like mealtime [214, 215]; free-running occurs during total food deprivation or at the entrainment limits [214–217]).

With the aim to assess whether the chronobiological sequels of calorie restriction could be the consequence of stress, we examined the 24-h variations of plasma ACTH, corticosterone, GH and leptin, and of adrenal corticosterone content [218]. Significantly lower ACTH levels were detected in calorie restricted rats. Plasma corticosterone levels during the light phase of daily cycle (but not in the whole set of 6 time points throughout the 24-h cycle) were significantly higher in calorie restricted rats. Time of day variations of plasma ACTH and corticosterone attained significance in calorie restricted rats only, with a maximum toward the end of the resting phase. The daily pattern of adrenal gland corticosterone mirrored that of circulating corticosterone, calorie restriction reducing its levels. Plasma ACTH and corticosterone correlated significantly in controls only. Calorie restriction decreased plasma GH and leptin and distorted their 24-h rhythmicity. Plasma ACTH levels in calorie restricted rats were lower, and plasma corticosterone levels were higher than those of pair fed, isolated controls and grouped caged controls [218]. The results indicate that a reduced availability of nutrients is a stressor that affects the circadian variation of pituitary hormones and leptin.

Malnutrition produced by low or absent proteins in diet is linked to increased susceptibility to infection, often associated with severe marasmus or kwashiorkor. In contrast, calorie restriction of adult rats by a diet enriched in proteins and low in fat and carbohydrates only partially affected weight and significantly increased immune responses [219, 220]. The immunological status of adult rodents fed a calorie restricted diet is superior to the immunological status of the non-restricted animals and through this mechanism caloric restriction may retard immunosenescence. Indeed, experimental calorie restriction (e.g., 25–50% reduction of caloric intake), without deficiency in essential nutrients, is a very unique manipulation in slowing the aging process in rodents [199–203]. To examine this subject, mitogenic responses, lymphocyte subset populations and IFN-γ release were determined in submaxillary lymph nodes at 6 time intervals during the 24-h span [190]. After caloric restriction, mean values of Con A response, lymph node T and $CD4^+$ cell number and $CD4^+/CD8^+$ ratio augmented, whereas those of B cell number, IFN-γ release and glutamine and glutamate concentration decreased. Calorie restriction modified 24 h rhythmicity of lymph node mitogenic responses to Con A and LPS, and of T, T-B, $CD4^+$, and $CD4^+$–$CD8^+$ lymph node cell subsets. It also changed the 24 h pattern of lymph node IFN-γ release. Availability of nutrients presumably affects the mechanisms that modulate the circadian variation of immune responsiveness in growing rats.

In a subsequent experiment the effect of calorie restriction on splenic immune responses was studied [191]. Calorie restricted rats showed increased splenic Con A response with peak activity during the activity span. The highest values of T cells occurred in calorie restricted rats and mean values of splenic $CD4^+$ and $CD8^+$ cells augmented in these animals. It is of interest that these immunological changes correlate with the efficacy of calorie restriction to prevent experimental allergic encephalomyelitis in rats [221, 222].

Conclusions

Temporal organization is an important feature of the biological systems and its main function is to facilitate adaptation of the organism to the environment. The daily variation of biological variables arises from an internal time-keeping system and the major action of the environment is to synchronize this internal clock to a period of exactly 24 h. The light–dark cycle, food, ambient temperature, scents and social cues have been identified as environmental synchronizers or "Zeitgebers" in rats.

This review discusses the circadian disruption of hormone release and immune-related mechanisms in several animal models in which circulating cytokines are modified including rat adjuvant arthritis, social isolation in rats and rabbits and alcoholism, the aging process and calorie restriction in rats. In every case the experimental manipulation used perturbed the temporal organization by affecting the shape and amplitude of the rhythm. Further experiments are needed to assess whether the changes in amplitude as well in timing of 24-h rhythms discussed herein can be attributed to an effect on the SCN or to a masking effect on some output(s) of the clock.

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