Review

Chronopharmacology Focused on Biological Clock

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Summary: The mammalians circadian pacemaker resides in the paired suprachiasmatic nuclei (SCN) and influences a multitude of biological processes, including the sleep-wake rhythm. Clock genes are the genes that control the circadian rhythms in physiology and behavior. The effectiveness and toxicity of many drugs vary depending on dosing time associated with 24 hr rhythms of biochemical, physiological and behavioral processes under the control of circadian clock. Such chronopharmacological phenomena are influenced by not only the pharmacokinetics but also pharmacodynamics of medications. Identification of a rhythmic marker for selecting dosing time will lead to improved progress and diffusion of chronopharmacotherapy. The mechanisms underlying chronopharmacological findings should be clarified from the viewpoint of clock genes. On the other hand, several drugs have an effect on circadian clock. The knowledge of interactions between circadian clock and drugs should be very useful for clinical practice. Therefore, I introduce the regulatory system of biological rhythm from viewpoints of clock genes and the possibility of pharmacotherapy based on clock genes.

Key words: chronopharmacology; chronotherapy; chronopharmacokinetics; circadian rhythm; biological clock; clock gene

Introduction

The study on the individualization of pharmacotherapy has been carried out aiming at further improvement of pharmacotherapy. The individualization of pharmacotherapy has been performed mainly by monitoring drug concentrations; however, pharmacogenetic approach such as genetic diagnosis has become a very attractive field by the rapid progress of molecular biology. Consequently, the dosage adjustment has been based on the interindividual differences of drug pharmacokinetics. However, intraindividual variability as well as interindividual variability should be considered to aim at further improvement of rational pharmacotherapy, because many drugs vary in potency and/or toxicity associated with the rhythmicity of biochemical, physiological and behavioral processes.1-5) Theoretically, it has been argued that drug administration at certain times of the day should improve the outcome of pharmacotherapy. This has been accepted by the medical community and/or described in interview form for the treatment of nocturnal asthma, allergic rhinitis, arthritis, myocardial infarction, congestive heart failure, stroke, and peptic ulcer disease; however, many drugs are still given without regard to the time of day. Identification of a rhythmic marker for selecting dosing time will lead to improved progress and diffusion of chronopharmacotherapy. In contrast, several drugs cause alterations in the 24 hr rhythms of biochemical, physiological and behavioral processes.6) The alteration of rhythmicity is sometimes associated with therapeutic effects (i.e. antidepressant drugs), or may lead to illness and altered homeostatic regulation.

In all living organisms, one of the most indispensable biological functions is the circadian clock (suprachiasmatic nuclei; SCN), which acts like a multifunction timer to regulate homeostatic systems such as sleep and activity, hormone levels, appetite, and other bodily functions with 24 hr cycles.7) Recently, clock genes were identified as the genes that ultimately control a vast array of 24 hr rhythms in physiology and behavior.8) The knowledge of clock genes should be applied in clinical practice. Therefore, I introduce the regulatory system of biological rhythm from viewpoints of clock genes and the possibility of pharmacotherapy based on clock genes.
Fig. 1. Molecular clock mechanisms of a core feedback loop and clock-controlled genes. Heterodimers of CLK (CLOCK) and BMAL1 (MOP3) activate transcription of clock genes and clock-controlled genes. The CRY (cryptochrome) proteins shut down CLOCK-BMAL1 transcription in the nucleus (circle), forming a negative feedback loop (small square). PER2 stimulates the transcription of Bmal1, forming a positive feedback loop (middle square). The phosphorylation of PER1 (period) and PER2 by CKIe (casein kinase I epsilon) may regulate their cellular location and stability. Clock-controlled gene products, which include PER3, DBP (D-element binding protein), and AVP (arginine vasopressin), transduce the core oscillation to downstream output systems.

Biological Clock

The SCN of the anterior hypothalamus are the site of the circadian pacemaker in mammals. Like any timing system, the circadian clock is made up of three components: an input pathway adjusting the time, a central oscillator generating the circadian signal, and an output pathway manifesting itself in circadian physiology and behavior. The daily changes in light intensities are thought to be the major environmental cue involved in circadian entrainment. Light signals are perceived by photoreceptor cells in the retina and transmitted to neurons of the SCN via the retinohypothalamic tract. A great deal of research shows that the inherited period of the human pacemaker clock is not precisely 24 hr. In fact, in most people, it is somewhat longer, closer to 25 hr. Environmental time cues, termed synchronizers or zeitgebers, the strongest one being the daily light-dark cycle occurring in conjunction with the wake-sleep routine, set the inherited pacemaker circadian timekeeping systems to 24 hr each day.

Clock genes are the genes that control the circadian rhythms in physiology and behavior. Three mammalian clock genes (Per1, Per2 and Per3) are rhythmically expressed in the SCN. Per1 and Per2 are induced in response to light. In particular, Per1 induction is considered to be an initial event in light-induced resetting and entrainment of the circadian biological clock. The transcriptional machinery of the core clockwork regulates a clock-controlled output rhythm as shown in Fig. 1. Namely, CLOCK-BMAL1 heterodimers act through an E box enhancer to activate the transcription of Pers, vasopressin and Dhp mRNA showing a specific output function from the SCN to periphery. This activation can be inhibited by the PER and CRY proteins. A circadian rhythm of Pers mRNA expression is discovered not only in the SCN but also in other tissues. The circadian rhythm in the periphery is governed by that in the SCN, since the circadian rhythm in physiological function and Pers mRNA expression are abolished in SCN-lesioned rats and Clock mutant mice. Such a cascade of clock genes may contribute to the organization of biological rhythms in the whole body; however, the mechanisms employed by circadian output pathways are poorly understood but are likely to involve both nervous and humoral signals. Plasma glucocorticoid levels show a circadian rhythm via the hypothalamus-pituitary-adrenal (HPA) axis under the control of the SCN. Glucocorticoids regulate various physiological responses and developmental processes by binding to and modulating the transcriptional activity of their cognate nuclear receptor. A transit induction of Per1 and Dhp mRNA levels is observed by a single administration of dexamethasone. Glucocorticoid hormones are particularly attractive candidates, since they are endogenous substances and play an important role in the entrainment of peripheral oscillators but not SCN. The regulatory system of biological rhythm should be clarified in detail from the viewpoint of clock genes.
The most effective and approach to increase the efficiency of pharmacotherapy (chronotherapy). One rationale for pharmacotherapy is based on the presence of 24 hr rhythm dependencies of drug pharmacokinetics, effects, and safety constitutes the rationale for pharmacotherapy (chronotherapy). One approach to increase the efficiency of pharmacotherapy is the administration of drugs at times at which they are most effective and/or best tolerated. The chronotherapy of a medication may be accomplished by the appropriate timing of conventionally formulated tablets and capsules, and a special drug delivery system to synchronize drug concentrations to rhythms in disease activity. Chronotherapy is especially relevant in the following cases. The risk and/or intensity of the symptoms of disease vary predictably over time as exemplified by allergic rhinitis, arthritis, asthma, myocardial infarction, congestive heart failure, stroke, and peptic ulcer disease. The therapeutic-to-toxicity ratio of a medication varies predictably according to chronobiological determinants as exemplified by antitumor medications. The pharmacokinetics and pharmacodynamics of a medication vary depending on biological rhythms. The goal of pharmacotherapy is hormonal substitution to mimic the rhythmic variation of hormone levels in healthy individuals. Also on the horizon are drugs to fix broken biological clocks, perhaps a factor in all illness in the opinion of some physicians. Table 1 shows several examples of chronopharmacotherapy.

**Chronotherapeutic Approach**

The knowledge of 24 hr rhythm in the risk of disease plus evidence of 24 hr rhythm dependencies of drug pharmacokinetics, effects, and safety constitutes the rationale for pharmacotherapy (chronotherapy). One approach to increase the efficiency of pharmacotherapy is the administration of drugs at times at which they are most effective and/or best tolerated. The chronotherapy of a medication may be accomplished by the appropriate timing of conventionally formulated tablets and capsules, and a special drug delivery system to synchronize drug concentrations to rhythms in disease activity. Chronotherapy is especially relevant in the following cases. The risk and/or intensity of the symptoms of disease vary predictably over time as exemplified by allergic rhinitis, arthritis, asthma, myocardial infarction, congestive heart failure, stroke, and peptic ulcer disease. The therapeutic-to-toxicity ratio of a medication varies predictably according to chronobiological determinants as exemplified by antitumor medications. The pharmacokinetics and pharmacodynamics of a medication vary depending on biological rhythms. The goal of pharmacotherapy is hormonal substitution to mimic the rhythmic variation of hormone levels in healthy individuals. Also on the horizon are drugs to fix broken biological clocks, perhaps a factor in all illness in the opinion of some physicians. Table 1 shows several examples of chronopharmacotherapy.

**Influence of Biological Rhythms on Physiological Function and Diseases**

Chronotherapeutic approach is based on the presence of 24 hr rhythms in physiological functions and diseases. Examples are described below. Figure 2 shows the approximate peak time of 24 hr rhythms relative to diurnally active human beings. The peak in serum cortisol, aldosterone, testosterone, platelet adhesive ness, blood viscosity and NK-cell activity is observed during the initial hours of daytime. Hematocrit is greatest and airway caliber (FEV1) best around the middle and afternoon hours, respectively. Insulin, cholesterol, triglycerides, platelet numbers, and uric acid peak later during the day and evening. The rhythms of basal gastric acid secretion, white blood cells (WBC), lymphocytes, pro lactin, melatonin, eosinophils, adrenal corticotrophic hormone (ACTH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) shows a peak at specific times during the nighttime.

24 hr rhythms in the processes that make up the pathophysiology of diseases cause prominent day-night patterns in the manifestation and severity of many medical conditions as depicted in Figure 3. The onset of migraine headache is most frequent in the morning around the time of awakening from nighttime. The sneezing, runny nose, and stuffy nose in allergic and infectious rhinitis are worst in the morning upon arising from nighttime. The symptoms of rheumatoid arthritis are worst when awakening from nighttime, while those of osteoarthritis are worst later in the day. The morbidity and mortality events of myocardial infarction are greatest during the initial hours of daytime. The incidence of thrombotic and hemorrhagic stroke is greatest in the morning around the time of commencing diurnal activity. Ischemic events, chest pain, and ST-segment depression of angina are strongest during the initial three to five hours of daytime. Pain and gastric distress at the onset and acute exacerbation of peptic ulcer disease are most likely in the late evening and early morning. Epilepsy seizures are common around sleep onset at night and offset in the morning. The symptoms of congestive heart failure are worse nocturnally. The manifestation of ST-segment elevation in Prinzmetal’s angina is most frequent during the middle to latter half of the nighttime. The risk of asthma attack is greatest during nighttime. The regulatory mechanisms underlying 24 hr rhythm of physiological function and diseases should be clarified from the viewpoint of clock genes.

**Table 1. Chronotherapy to be incorporated into clinical practice**

- The morning daily or alternate-day dosing strategy for methylprednisolone that was introduced during the 1960s constitutes the first chronotherapy to be incorporated into clinical practice.
- Evening, once-daily dosing of specially formulated theophylline tablets for treatment of nocturnal asthma.
- Before-bedtime administration of verapamil HCL as a unique controlled onset extended-release 24 hr dosage form to optimize the treatment of patients with ischemic heart disease and/or essential hypertension.
- Evening administration of HMG-CoA-reductase antagonists for the management of hyperlipidemia.
- Evening, once-daily dosing of conventional H2-receptor antagonist or morning once-daily administration of protonpump antagonist tablet medications for the management of peptic ulcer disease.
- Before-bedtime administration of hypnotics for sleep induction and maintenance.
- Morning application of testosterone drug-delivery patch systems to achieve physiologic androgen-replacement therapy.
- Programmed-in-time infusion of antitumor medications according to biological rhythms to moderate toxicity and enhance dose intensity in cancer treatment.
- Programmed-in-time administration of tocolytic medication relative to the circadian rhythm in uterine contractility to avert preterm labor and birth.
The onset of myocardial infarction occurs frequently in the early morning, and it may partly result from 24 hr rhythm of fibrinolytic activity. Plasminogen activator inhibitor-1 (PAI-1) activity shows a 24 hr rhythm.\textsuperscript{22} Basic helix-loop-helix (bHLH)/PAS domain transcription factors play a crucial role in controlling the biological clock. A novel bHLH/PAS protein, cycle-like factor (CLIF) is expressed in endothelial cells and neurons in the brain, including SCN. In endothelial cells, CLIF forms a heterodimer with CLOCK and up-regulates the \textit{Pai-1} gene through E-box sites. Furthermore, PER2 and CRY1 inhibit the \textit{Pai-1} promoter activation by the CLOCK:CLIF heterodimer. Namely, CLIF regulates the 24 hr rhythm of \textit{Pai-1} gene in endothelial cells. In addition, the results potentially provide a molecular basis for the morning onset of myocardial infarction.

\textit{Bmal1} is a transcription factor controlling circadian rhythm and contributes to the control of adipogenesis and lipid metabolism activity in mature adipocytes.\textsuperscript{23} The level of \textit{Bmal1} mRNA increases during adipose
differentiation in 3T3-L1 cells. In white adipose tissues isolated from mice, BMAL1 is more highly expressed in the adipocytes fraction than the stromal-vascular fraction. Bmal1 knockout mice embryonic fibroblast cells fail to be differentiated into adipocytes. BMAL1 induces several factors involved in lipogenesis in 3T3-L1 adipocytes. The promoter activity of these genes is stimulated by BMAL1. These factors shows a 24 hr rhythm in mice adipose tissue. Furthermore, overexpression of BMAL1 in adipocytes increases lipid synthesis activity. Thus, BMAL1 plays important roles in the regulation of adipose differentiation and lipogenesis in mature adipocytes.

A sleep disorder in humans is associated with a genetic mutation affecting circadian clock function. Familiar advanced sleep-phase syndrome (FASPS) is documented in three families. Affected individuals experience early evening sleepiness and early morning awakening. Individuals with FASPS have a circadian period about an hour shorter than normal. Taking one of the FASPS families, Toh uses multiple sets of dense genomic markers to map the mutation and clarifies that the mutant gene is hPer2. The hPer2 mutation changes serine 662 to a glycine (S662G). This occurs in a region of hPER2 homologous to the casein kinase I epsilon (CKIε) binding region of mPER1 and mPER2. Serine 662 is in fact part of a consensus CKIε phosphorylation site, and the S662G substitution renders the mutant protein less readily phosphorylated by CKIε than the wild-type hPER2 in vitro. Thus, a variant in human sleep behavior can be caused by a missense mutation in a clock component, hPER2, which alters the circadian period.

**Influence of Biological Rhythms on Pharmacokinetics and Pharmacodynamics**

Biological rhythms not only impact the pathophysiology of diseases, but the pharmacokinetics and pharmacodynamics of medications. Chronopharmacology is the investigative science that elucidates the biological rhythm dependencies of medications.

**Chronopharmacokinetics**

Chronopharmacokinetic studies have been reported for many drugs in an attempt to explain chronopharmacological phenomena and demonstrate that the time of administration is a possible factor of variation in the pharmacokinetics of a drug. Time-dependent changes in pharmacokinetics may proceed from 24 hr rhythms in each process, e.g. absorption, distribution, metabolism and elimination. Thus, 24 hr rhythms in gastric acid secretion and pH, motility, gastric emptying time, gastrointestinal blood flow, drug protein binding, liver enzyme activity and/or hepatic blood flow, glomerular filtration, renal blood flow, urinary pH and tubular resorption may play a role in such pharmacokinetic variations.

The clock genes are expressed not only in the SCN, but also in other brain regions and various peripheral tissues. A microarray analysis experiment has revealed that there are many genes expressing a circadian rhythm in the liver. The liver is a biological clock capable of generating its own circadian rhythms. Since the liver is a major organ of metabolism and detoxification, knowledge of circadian effects on transcriptional activities that govern daily biochemical and physiological processes in the liver may play a key role in toxicology. Relative levels of gene expression in the liver of rats is investigated from the viewpoint of time of day. Expression levels are determined for 3906 genes using high-density oligonucleotide microarrays. Of them 30% are clearly expressed while 70% are not expressed or the expression is very low. The maximum estimated changes observed for most genes (90%) of rhythmic genes are less than 1.5-fold. 67 genes show significant rhythmic expression. These altered genes include DNA binding and regulation of transcription, drug metabolism, ion transport, signal transduction and immune response.

A circadian rhythm is demonstrated for six genes involved in regulation of gene transcription. The retinoic acid receptor-alpha and the retinoid X receptors (alpha and gamma) play an important role in regulation of gene expression by forming transcriptionally active complexes on DNA. Aryl hydrocarbon receptor nuclear translocator (Arnt) works as a transcription factor in diverse signaling events including response to xenobiotics. A circadian expression is also demonstrated for Pitx2 and Pitx3 genes. These genes encode paired-like homeodomain transcription factors 2 and 3, the members of homebox gene family. Drug metabolism is the main function of the liver. There is a significant circadian rhythm in cytochrome P-450 4a3 (Cyp4a3) and putative N-acetyltransferase camello 4 (Cml4) of phase I and phase II of drug metabolism. Liver cytochrome P450 4a isoforms play an important role in regulation of renal function by catalyzing the formation of 20-hydroxyeicosatetraenoic acid. This may be one of mechanisms underlying circadian rhythm of renal function and blood pressure. In mouse liver, circadian regulation of transcripts is demonstrated for the cytochrome P450 such as Cyp17, Cyp2a4, Cyp2e1, Cyp2c22 and so on. Cml4 encodes the protein catalyzing acetylation of aromatic amines and hydrazines. The rhythmic pattern nicely corresponds to the pattern of Cml2 in mouse liver. Circadian rhythm is demonstrated for other members of the phase II of drug metabolism such as glutathione S-transferases (GST) and carboxylesterase. The liver is the major organ of metabolism and endures a flux of metabolites across membranes. A significant circadian rhythm is demonstrated for genes involved in ion transport.
of the solute carrier transporter such as \textit{Slc34a1} and \textit{Slc2a8}. A rhythmic gene expression is demonstrated for solute carriers such as \textit{Slc12a2}, \textit{Slc16a1}, \textit{Slc19a1}, and \textit{Slc25a1}. In addition to the anion and solute transporters \textit{Abcc2} and \textit{Aqp9}, expressions of \textit{Slc10a1}, \textit{Slc22a1}, \textit{Slc27a1}, \textit{Slc2a2}, and \textit{Slc7a2} show circadian rhythm. Furthermore, there is a significant circadian expression of ion transporter genes such as \textit{Hcn4}, \textit{Trpc4}, \textit{Scn2b}, \textit{Scn4a}, \textit{Chrnb2}, \textit{Atp9a}, \textit{Atp7b}, \textit{Timm10}, and \textit{Nrip}. Since one of the important defense mechanisms includes the active extrusion of xenobiotics by transporter, genes involved in ion or solute transport activity may have significant implications in toxicology studies. Coordinated rhythmic oscillations in phase I and phase II components of drug metabolism during the day may explain differential responses to drugs in toxicology.

DBP is able to activate the promoter of a putative clock oscillating gene, \textit{Per1}, by directly binding to the \textit{Per1} promoter \cite{13,28,29}. The \textit{Per1} promoter is cooperatively activated by DBP and CLOCK-BMAL1. On the other hand, \textit{Dbp} transcription is activated by CLOCK-BMAL1 through E-boxes and inhibited by the PER and CRY proteins, as is case for \textit{Per1}. Thus, \textit{Dbp}, a clock-controlled gene whose expression oscillates with a very high circadian amplitude, may play an important role in central clock oscillation. \textit{Dbp} participates in the regulation of several clock outputs, including locomotor activity, sleep distribution, and liver gene expression. Also, DBP is a major factor controlling circadian expression of the steroid 15 \textit{\alpha}‑hydroxylase (\textit{Cyp3a4}) and coumarin 7‑hydroxylase (\textit{Cyp2a5}) genes in mouse liver \cite{30}. Thus, the mechanisms underlying 24 hr rhythm of drug metabolism have been gradually clarified.

A significant portion of the transcriptome in mammals, including the PAR-domain basic leucine zipper (PAR bZip) transcription factors DBP, HLF, and TEF, is controlled by circadian clock. Triple mutant mice are epilepsy prone, age at an accelerated rate, and die prematurely \cite{31}. The PAR bZip transcription factors DBP, TEF, and HLF accumulate in a highly circadian manner in several peripheral tissues such as liver and kidney. To identify PAR bZip target genes whose altered expression will contribute to the high morbidity and mortality of PAR bZip triple knockout mice, the liver and kidney transcriptomes of these animals are compared with those of wild-type or heterozygous mutant mice. The disruption of these three genes in mice alters gene expression patterns of many proteins involved in drug metabolism and transporter. The various levels at which PAR bZip transcription factors might intervene in the coordination of xenobiotic detoxification are described in Fig. 4. The PAR bZip proteins control the expression of many enzymes and regulators involved in detoxification and drug metabolism, such as cytochrome P450 enzymes, carboxylesterases, aminolevulinic acid synthase (ALAS1), P450-oxidoreductase (POR), sulfotransferases, GST, aldehyde dehydrogenases, UDP-glucuronosyltransferases, members of drug transporter families, and constitutive androstane receptor (CAR). Some genes encoding detoxification enzymes such as CYP2A5, CYP2C50, CES3 may be direct PAR bZip target genes. The expression of other detoxification enzymes, mostly regulated by CAR, whose circadian transcription is governed by PAR bZip proteins. Other enzymes in the xenobiotic defence appear to be controlled by both CAR and PAR bZip proteins.
clarify the mechanism underlying neural control of liver clock systems, evolution of this mechanism will be useful to the understanding of liver clock functions such as drug metabolism and energy metabolism.

—Chronopharmacodynamics—

Biological rhythms at the cellular and subcellular level can give rise to significant dosing-time differences in the pharmacodynamics of medications that are unrelated to their pharmacokinetics. This phenomenon is termed chronesthesia. Rhythms in receptor number or conformation, second messengers, metabolic pathways, and/or free-to-bound fraction of medications help explain this phenomenon. For example, the antitumor effect of IFN-β and the antiviral effect and lymphocyte stimulating effect of IFN-α in mice are more efficient during the early rest phase than during the early active phase. The dosing schedule-dependent effect of IFN-β or IFN-α is also closely related to that of IFNs receptors and ISGF expression in tumor cells or lymphocytes.

The term chronotoxicity refers specifically to predictable-in-time variation in patient vulnerability to the side effects of medications due to biological rhythm determinants. Chronotoxicities are known especially with antitumor agents. For example, the body weight loss with irinotecan hydrochloride (CPT-11) of nocturnally active mice is more serious in the late active phase and the early rest phase and milder in the late rest phase and the early active phase. CPT-11-induced leukopenia is more serious in the late active phase and milder in the late rest phase. The lower toxicity of CPT-11 is observed when DNA synthesis and type I DNA topoisomerase activity in bone marrow cells decrease and higher toxicity is observed when these activities begin to increase. The finding indicates that the choice of dosing time associated with the 24 hr rhythm of DNA synthesis may help to achieve a rational chronotherapeutic strategy, reducing the toxic effects of CPT-11 and/or increasing its therapeutic effects. Cell division in many mammalian tissues is associated with specific times of day. In the regenerating liver of mice, the circadian clock controls the expression of cell cycle-related genes that in turn modulate the expression of active Cyclin B1-Cdc2 kinase, a key regulator of mitosis. Among these genes, expression of wee1 is directly regulated by the molecular components of the circadian clockwork. On the other hand, the circadian clockwork oscillates independently of the cell cycle in single cells. The intracellular circadian clockwork can control the cell-division cycle directly and unidirectionally in proliferating cells. Thus, the regulatory mechanisms underlying 24 hr rhythm of pharmacodynamics should be also clarified from viewpoints of clock genes.

Angiogenesis is important for tumor growth and metastasis. Hypoxia-induced expression of vascular endothelial growth factor (VEGF) plays an important role in tumor-induced angiogenesis. The levels of VEGF mRNA in tumor cells implanted in mice rise substantially in response to hypoxia, but the levels show a 24 hr rhythm. Luciferase reporter gene analysis reveals that PER2 and CRY1, whose expression in the implanted tumor cells shows a 24 hr rhythm, inhibit the hypoxia-induced VEGF promoter activity. Namely, the negative limbs of the molecular loop periodically inhibit the hypoxic induction of VEGF transcription, resulting in the 24 hr fluctuation of its mRNA expression. Furthermore, the antitumor efficacy of antiangiogenic agents is enhanced by administering the drugs at the time when VEGF production increases.

Methionine aminopeptidase2 (MetAP2) plays an important role in the growth of endothelial cells during the tumor angiogenesis stage. MetAPs show a 24 hr rhythm in implanted tumor masses. The mechanism underlying the 24 hr rhythm of MetAP2 activity is investigated in tumor-bearing mice. The 5′ flanking region of MetAP2 includes eight E-boxes. The transcription of the MetAP2 promoter is enhanced by the CLOCK:BMAL1 heterodimer, and its activation is inhibited by PER2 or CRY1. Deletion and mutation of the E-boxes in the region indicates that the E-box nearest to the initiation start site plays an important role in the transcriptional regulation by clock genes. In sarcoma180-bearing mice, the pattern of binding of CLOCK and BMAL1 to the E-box and transcription of the MetAP2 promoter shows a 24 hr rhythm with higher levels from the mid-light to early dark phase. MetAP2 protein expression varies with higher levels from the late-dark to early-light phase. Namely, the 24 hr rhythm of MetAP2 activity is regulated by the transcription of clock genes within the clock feedback loops. Furthermore, the antitumor efficacy of MetAP2 inhibitor is enhanced by administering the drugs at the time when MetAP2 activity increases.

—Chro-No Drug Delivery System (chrono-DDS)—

The effectiveness and toxicity of many drugs vary depending on the 24 hr rhythms of biochemical, physiological and behavioral processes. Also, several drugs can cause alterations to the 24 hr rhythms leading to illness and altered homeostatic regulation. The alteration of biological rhythm is a new concept of adverse effects. It can be minimized by optimizing the dosing schedule. Many researches demonstrate the rationale behind chronotherapy; however, drug delivery research has focused on a constant drug release rate. The reason why the majority of DDS is designed without emphasis on proven oscillatory phenomena may be in drug delivery limitations. Advances in chronobiology and global market constraints changes the traditional goal of pharmaceutics such as a constant drug release rate. The increasing research interest on Chrono-DDS may create a new sub-discipline in
chronopharmaceutics.

The technologies in chronopharmaceutics includes: CONTIN®, physicochemical modification of the active pharmaceutical ingredient, OROS®, CODAS®, CEFORM®, DIFFUCAPS®, chronomodulating infusion pumps, TIMERx®, three-dimensional printing, controlled-release erodible polymer and controlled-release microchip strategies. As examples of ChronoDDS on the market, there are compounds such as theophylline (Uniphyl®), famotidine (Pepcid®), simvastatin (Zocor®), COER-verapamil (Covera-HS®, Verelan® PM), diltiazem (Cardizem® LA) and propranolol (InnoPran® XL). Most data have been compiled from the FDA electronic orange book, specific product package inserts and United States patents and specific pharmaceutical company websites. Future development in chronopharmaceutics may be performed by the new technology such as system biology and nanomedicine.

**Influence of Drugs and Environmental Factors on Biological Rhythms: Adjustment and Manipulation of Rhythms**

The 24 hr rhythms of physiology and behavior are influenced by various environmental factors such as feeding schedules, genetic factors and social interactions as well as lighting conditions and several drugs. Since the period of the central circadian pacemaker in humans is slightly longer than 24 hr described above, synchronization of the circadian system with the light-dark cycle occurs by daily phase-advances of the circadian clock. In humans, the time-of-day-dependent phase-shifting effects of light are summarized in a phase-response curve (PRC). Morning light advances the central circadian pacemaker, late afternoon and evening light delays the pacemaker, and light at midday is without phase-shifting effects. On the other hand, the phase-shifting agents (zeitgebers) such as melatonin, 5-hydroxytryptamine (5-HT, serotonin), and behavioral arousal have a PRC distinct from light. Phase advances occur between midday and early evening. Phase delays occur between late night and midday. As a group, phase shifts produced by nonphotic zeitgebers are similar to phase shifts produced by dark pulses presented to animals housed in constant light. Photic and nonphotic (i.e., extrinsic timekeeping) effects on intrinsic timekeeping may be important components of disordered timekeeping in depressive illness.

SCN neurons receive information about light intensity in the environment via direct synaptic connections with the retina, which adapts the phase of SCN oscillator to the photoperiod. The SCN clock then synchronizes overt rhythms in physiology and behavior. *Per1* and *Per2* transcription is rapidly induced by light in a time-of-day-dependent manner. The responsiveness of *Per1* mRNA to light is closely related to behavioral phase delays induced by light. Light-induced phase delays in locomotor activity during subjective night are significantly inhibited when mice are pretreated with *Per1* antisense phosphorothioate oligodeoxynucleotide (ODN). Therefore, the gated expression of *Per1* may be an important step in causing photic entrainment.

It is well known that not only photic but also nonphotic stimuli can entrain the SCN clock and several drugs have been investigated to modulate the circadian rhythm by causing a phase shift in the rhythm in the peripheral or central nervous system. The acute and circadian time-dependent reduction of *Per1* and/or *Per2* mRNA in the hamster SCN by 5-HT1A/7 receptor agonists is strongly correlated with phase resetting in response to the drug. Therefore, nonphotic shifts may involve changes in *Per1* and/or *Per2* mRNA levels in the SCN; however, with the exception of chronic treatment with antidepressant drugs which are given over the course of many months, most studies report normal temporal response of the clock to an acute treatment and the response to repetitive administration is generally unknown.

A variety of physiological rhythmic variables are influenced by the cyclic variation of environmental factors. One of those factors is feeding schedule. The change in glucocorticoid rhythmicity appears to play an important role in physiological rhythmicity by the manipulation of the feeding schedule, because plasma corticosterone levels show anticipatory increases preceding the time of feeding, and the continuous administration of corticosterone disturbed the rhythmicity of behavior, physiological function and cyclic gene expression. Such effects are not influenced by SCN lesions. Also a time-restricted feeding schedule can change the rhythmic phase of locomotor activity, physiological function including corticosterone and clock genes in periphery by up to 12 hr while leaving the rhythmic phase of clock genes in the SCN unaffected. Ventromedial hypothalamic lesions abolish food-shifted circadian adrenal rhythmicity. The paraventricular nucleus appears to be the site where the feeding-associated circadian oscillation is connected to the HPA axis. On the other hand, the manipulation of the feeding schedule can modify the chronopharmacological action and chronopharmacokinetics of drugs. In humans, the pattern of diet intake substantially modifies plasma cortisol levels in addition to body temperature rhythm. Namely, the rhythmicity of the plasma cortisol levels can be kept normal only when the feeding pattern is diurnal, but is reversed or disturbed under a nocturnal or continuous feeding pattern. Producing new rhythmicity by manipulating the conditions of living organs by the rhythmic administration of altered feeding schedules or several drugs appears to lead to the new concept of chronopharmacotherapy.
Influence of Drugs on Biological Rhythms:
Disruption of Rhythms

Several drugs cause alterations in the 24 hr rhythms of biochemical, physiological and behavioral processes. The alteration of rhythmicity is sometimes associated with therapeutic effects, or may lead to illness and altered homeostatic regulation. Interferons (IFNs) have been widely used as antiviral and antitumor agents; however, IFNs cause adverse neuropsychiatric effects such as depression and neurosis and they are reported to sometimes lead to suicide. When IFNs are administered during the early active phase in diurnally active humans, alterations in the 24 hr rhythm are suggested by the changes in the lymphocyte counts and cortisol levels, however, the mechanism has not been clarified from the viewpoint of the disruptive effect of the drug on the clock genes.

Figure 5 shows the disruptive effect of interferon-α (IFN-α) on the rhythm of Per genes mRNA expression in the SCN. These findings are supported by the inhibitory effect of IFN-α on the mRNA expression of Clock and Bmal1, which are important factors in activating the transcription of Pers, vasopressin and the Dbp gene showing specific output function from SCN to the periphery. Also, the rhythmicity of locomotor activity and body temperature are severely blunted by the repetitive administration of IFN-α. Since IFN-α influenced both the SCN and periphery, it is difficult to clarify whether the IFN-α effects on clock genes are secondarily related to the IFN-α effect on locomotor activity. However, IFN-α acts on the SCN as shown in the expression of ISGF and the rhythmicity that SCN controls in the periphery. The rhythmicity of locomotor activity is severely altered by the continuous administration of corticosterone or a time-restricted feeding schedule while leaving the rhythmic phase of clock genes in the SCN unaffected. Thus, the possibility that altered locomotor activity could in turn lead to changed clock gene expression in the SCN is low in the case of IFN-α. The photic induction of the Per1 gene in SCN is also completely disturbed by daily administration of IFN-α at the early active phase, which may have caused a functional disorder in the resetting and entrainment of SCN. Therefore, IFN-α effects at the SCN clock gene level may be responsible for some of the adverse behavioral and physiological effects. IFN-α sometimes causes ocular adverse effects associated with retinal or optic neuropathy, although the mechanism is not clear at present. Such ocular adverse effects caused by IFN-α may decrease the photic information from the retina to SCN and the stimulation of the light responsive element of the per gene.

Interestingly, an inhibitory effect of mRNA expression of each clock gene in the SCN is observed by the repetitive administration of IFN-α during the early active phase, but not the early rest phase. Similar dosing schedule-dependent inhibition of Per1 mRNA expression is demonstrated during the repetitive administration of IFN-γ, which can be induced by IFN-α or IFN-β.

Fig. 5. Influence of IFN-α dosing schedule on mRNA expression of clock genes in the SCN. In panels (a)-(c), RNA levels for the Per1 (a), Bmal1 (b) or Clock (c) in the SCN of mice after a single dose of IFN-α (2 MIU/kg, sc) at ZT0 (zeitgeber time 0) (closed square) or ZT12 (closed triangle), or saline (open circle) daily for 6 days. Each point represents the mean ± SEM of 6 observations. All mRNAs except for Clock in all groups and Bmal1 in groups injected with IFN-α at ZT12 show significant 24 hr rhythms (Per1 in groups injected with IFN-α at ZT12; p < 0.05, respectively, others; p < 0.01, respectively, ANOVA). **p < 0.01, *p < 0.05, compared with the value of controls at corresponding ZTs (Bonferroni’s test). White bars indicate the light period, and grey bars indicate the dark period.
in combination with other cytokines.\textsuperscript{58} The expression of IFN-\(\gamma\) receptor in SCN follows a 24 hr rhythm with a peak at the early active phase.\textsuperscript{59} This may be why the administration of IFN-\(\alpha\) during the early rest phase can reduce its side effects. The observations for humans described above correspond well to the findings indicating that alteration of the clock genes is induced by IFN-\(\alpha\) administration during the early active phase in nocturnally active rodents. Furthermore, the 24 hr dependency of the disruptive effect of IFN-\(\alpha\) on clock genes in SCN may be applicable to other drugs as shown in the case of IFN-\(\gamma\). Thus, alteration of the clock function, a new concept of adverse effects, can be overcome by devising a dosing regimen that minimizes adverse drug effects on clock function.

**Conclusions**

Many drugs are still given without regard to the time of day. Identification of a rhythmic marker for selecting dosing time will lead to improved progress and diffusion of chronopharmacotherapy. To monitor rhythmic markers such as clock genes it may be useful to choose the most appropriate time of day for administration of drugs that may increase their therapeutic effects and/or reduce their side effects. Furthermore, to produce new rhythmicity by manipulating the conditions of living organisms by using rhythmic administration of altered feeding schedules or several drugs appears to lead to a new concept of chronopharmacotherapy. Attention should be paid to the alteration of clock gene expression and to consider it an adverse effect when it leads to altered regulation of the circadian system, which is a serious problem affecting basic functioning of living organisms. One approach to increasing the efficiency of pharmacotherapy is administering drugs at times during which they are best tolerated.

**References**

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