# Toward overcoming neurodegenerative disease by the circadian molecular clock study

## — My 30 year history in a national institute —

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The mammalian clock gene, *Period2*, was discovered by my research group studying clock genes in 1998. I summarize the progress of understanding the circadian clock molecular mechanism after this discovery. Our group has demonstrated the importance of glycogen synthase kinase 3-dependent phosphorylation of *Period2* and its nuclear transfer and E4BP4 (*vrille*) negative transcriptional regulation, as well as *Clock/Bmal*, *Period/Cry* E-box dependent negative feedback loop. A role of myo-inositol for elongation of the circadian clock was uncovered through collaboration on ice plant projects with Tsujiko Co., Ltd, Shiga prefecture. When we started the molecular study of the circadian clock, we only considered the daily rhythm. Fortuitously, our research on the peripheral clock mechanism (PPAR*α*) revealed a new mechanism of seasonal clocks, which can count photoperiods to adapt to winter (torpor). Our generation of researchers entered Japanese national institutes during a period called "the basic research shift era." But, basic research grants were cut significantly during the 24 to 25 year period after we joined the institutes, and our research mission was abruptly changed to applied science. After several years of frustration and contemplation, we gave up studies using mice and concentrated on using *Drosophila* to reduce costs and save time. Consequently, we found a causative role of sleep abnormality around a young age in two neurodegenerative (Gaucher's and Parkinson's) diseases by using fly models. I summarize an application for the molecular mechanism of neurodegenerative disease. I am greatly thankful that I was able to spend more than 30 years on the study of molecular circadian clocks with the people who have been involved, from when I started as a researcher in 1986 at the Fermentation Research Institute of the Agency of Industrial Science and Technology to the present day at AIST.

*Keywords*: Circadian clock, clock gene, transcriptional factor, sleep, neurodegenerative disease, Gaucher's disease, Parkinson's disease

## 1 Beginning of circadian clock gene research

The author joined the Fermentation Research Institute, the Agency of Industrial Science and Technology in 1986, after strong insistence and recommendation by Group leader Youji Mitsui of the Animal Cell Research Laboratory. At his lab, they were looking for a person with skills for animal gene cloning since they wanted to investigate the vascular endothelial cell growth factor. I had a couple of offers from national universities at that time, but I decided on this lab because it seemed they would allow me to do whatever I wanted to do. Immediately after I joined, I worked like a worker ant from early morning to midnight for isolating and sequencing various clones, but I could not pick out the target gene. Unable to just watch our hard work, the Group leader recruited a student, Shinichiro Nishimatsu (currently, Kawasaki Medical School), from Dr. Kazuo Murakami's lab at the University of Tsukuba. However, in less than three months, we received reports of the successful FGF cloning from two places, one in Japan and the other in USA, and my first project was terminated. While I was filled with a sense of defeat, there was a paper published in *Nature*[1] by Mike Young's group that a specific RNA sequence similar to a *Drosophila* clock gene, *Period*, was detected in birds, mice, and humans. This paper got me excited. That is because the research of genes involved in behavior was the reason I jumped into this field. Furthermore, it was my dream to study behavior from genes ever since I became interested in psychology and behavior science in high school. At that time, for circadian clock genes, the *Period* gene of *Drosophila* was cloned for the first time in 1984, but it was a total mystery why such a gene could affect complex behavior. Therefore, I immediately shifted the target to clone circadian clock genes in mammals, and embarked on the labor of isolating and sequencing candidate clones every day. However, the sequences I obtained were all an RNA repetitive sequence (ACNGGC) of a specific RNA region partially similar to the *Period* gene, and I was unable to obtain the targeted *Period* homologous gene composed of 1,100 or more amino acids from mice. Looking back, this seemed to be the noncoding RNA that is the current megatrend in molecular biology. Excellent colleagues who joined the Biological Clock Group during this time included the following people: Miwa Matsui (*in situ* hybridization); Masae Kurama (diurnal expression of endothelin); Kaname Saida (cloning of endothelin family); Koichiro Kako [establishment of EMSA method and a role

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of CRE for clock ; currently, lecturer at Life Science Center of Tsukuba Advanced Research Alliance (TARA)], a post-doc from Dr. Eisuke Munekata's lab at the Department of Applied Biology, University of Tsukuba; Motoo Watanabe from Mitsubishi Chemical Corporation; and Satoru Suzuki who graduated from the open university of Japan while he was a technical support staff in my lab.

## 2 Background of the biological clock research

When we started circadian rhythm research, most of the study was done in the field of physiology and anatomy, and the study at molecular and gene levels was very much behind. The master clock that governs the 24-hour rhythm in mammals is located in the brain region called the suprachiasmatic nucleus (SCN) in the hypothalamus. This nomenclature was given because it is situated directly above the optic chiasm. It is located right above the region where the left and right optical nerves cross each other in the brain. The SCN tissue consisting of about $10^4$ cells receives input from the optical nerves. (This is why light can entrain the phase of the clock to an environmental phase.) The output of SCN includes the pineal body (main producer of melatonin), the satiety center, the feeding center, the thermoregulatory center, and the autonomous nervous system. The firing frequency of individual nerve cells in the SCN is high during the day and low at night. It has been gradually discovered that there is a 24-hour rhythm in the hormone secretion from the SCN, but it was totally unknown which molecule (gene product) is required for the generation of such a 24-hour rhythm in mammals.

As mentioned above, this mystery has been unraveled through the advances in *Drosophila* molecular biology. It has now been discovered that in *Drosophila*, various gene products are involved in the generation of the 24-hour rhythm, as shown in Table 1, including the *Period* gene, using the forward genetic approach where genes of mutations of *Drosophila* that show rhythm abnormality are analyzed.

The clock gene is defined as the gene that affects the behavioral circadian rhythm (phenotype includes arrythmic, long period, short period, or all of the above) when there is a mutation in one gene. Please refer to the original paper for the description of phenotypes of individual clock genes (Table1). One of the important characteristics of such clock genes is 24 hours-rhythmic expression of the gene products (mRNA or proteins) in our body. I present as an example the rat *per2* gene that we isolated for the first time in the world.[2] Surprisingly, we found that the circadian clock gene, rat *per2* gene, expressed not only in the central pacemaker clock (SCN) in the brain, but also in other tissue such as stomach, liver, kidneys, as well as blood cells, hair cells, and nail cells. (We called these present in places other than the brain peripheral clocks.)[3] It is obvious that such peripheral clocks are controlled by the SCN, because

**Table 1. Circadian Clock gene in *Drosophila* (fly) and humans**
Most of the biological clock genes except for the *Clock* gene, surprisingly, were discovered from the fly.

| *Drosophila* gene | Human genes |
|---|---|
| *Period* | *Period1* |
| | *Period2* |
| | *Period3* |
| *Timeless* | Not applicable |
| *Timeout/Timeless 2* | *Timeless* |
| *Cryptochrome* | *Cryptochrome 1* |
| | *Cryptochrome 2* |
| *Clock (Jerk)* | *Clock* |
| | *NPAS2/MOP4* |
| *Cycle* | *Bmal1/MOP3* |
| | *Bmal2/MOP9/CLIF* |
| *Doubletime* | Casein kinase 1 epsilon |
| | Casein kinase 1 delta |
| *Shaggy* | Glycogen synthase kinase 3$\beta$ |
| Phosphatase 2A | Not applicable |
| *Slim* | *FWD1/β-TrCP* |
| *Brill* | *E4BP4* |
| *Pearl Domain 1* | *CBP* |
| *Ultra Spiracle* | *Coup-TF* |
| *Clockwork Orange* | *Dec1, Dec2* |
| *Twin of Eyeless* | *PAX6* |
| Not applicable | Protein kinase G type 2 |
| *Ataxin 2* | *Ataxin 2* |
| Not applicable | *FBXL3, 21* |
| *Jetlag* | *FBXL15* |
| *Kissmet* | *CHP7 (Chromatin helicase DNA-binding protein 7)* |
| *Unfulfilled* | *NR2E3* |
| Not applicable | *RORα* |
| *E75* | *Rev-erbα, Rev-erbβ* |

the rhythmic expression of peripheral clock genes is abolished after the lesion of SCN and the fact that when the organs are cultured, the peripheral organs such as liver, kidneys, and heart lose their rhythmic gene expression in a few days while the SCN cells possess automaticity and the 24-hour rhythm is not lost for a long time. In our bodies, not only every cell, but also the expression of circadian clock genes showed a 24-hour rotation.

## 3 Circadian clock gene *Period2* is a period determining factor for mammalian behavior

The molecular mechanism of the circadian clock in mammals is shown by the transcription-translation feedback loop of clock gene products including *Period* (Fig. 1b). The main idea of this molecular mechanism is based on the *Drosophila* model (Fig. 1a). The *Drosophila* clock mutant *Period* was isolated by Ronald Konopka and Seymour Benzer in 1971.[4] Dr. Benzer unfortunately passed away in November 2007. He was the strongest candidate for the Nobel Prize in the field of circadian clocks. He can be considered the true founder of this field, as he moved from the field of physics and created the basic notion of the first generation of molecular biology along with Francis Crick and Sydney Brenner. That is because at that time when the behavioral trait was thought to

be determined by multiple factors, he proposed the one gene-one behavior theory which continues to shine. Such a unique bold hypothesis had emerged from his background in physics as a first generation of molecular biologist applying phage molecular genetics. In the early days, Dr. Benzer discovered the linearity of genes and the qualitative difference (cistron) of point mutation using the T4 phage. Benzer was one of the first scientists to rise to prominence in the field of behavioral molecular biology. Later, the *Period* gene was cloned in 1984 by the J Hall group and the M Young group (2017Nobel Prize), and it was found that one amino acid mutation in a different position produced phenotypes for short period, long period, and arrhythmic periods (stop codon).[5]

After a long period of dark ages, the existence of period homologous genes in mammals came into light again in the latter half of the 1990s after the Genome Project of human and rodents was completed. During this time, we won a NEDO grant, and many post-docs worked in my Group [Tomoko Kuroiwa, Marek Banasik, Toshiyuki Hamada (currently, Associate Professor, Hokkaido University), Yutaka Sadakane (currently, Professor, Suzuka University of Medical Science), Zhi-Qiang Qu (currently, Professor, Qingdao University), Naoto Hayasaka, and Norihiko Yamazaki]. At last we identified the rat *Period2* (*rper2*) gene with the Kazusa DNA Research Institute and discovered its 24-hour oscillatory expression in peripheral organs.[2] The greatest contributors to that paper were Takahiro Nagase (Kazusa DNA Research Institute) and Katsuhiko Sakamoto (currently, Associate Professor of Insect Function Studies, Faculty of Agriculture, Kobe University), who was a post-doc in my laboratory. In the competition with the United States, the paper almost made it to *Science* but was turned down right before publication. Quite unwillingly, we sent our paper to the *Journal of Biological Chemistry*, and surprisingly enough it was accepted in Rapid Communication in one week. Because of the collaboration with the Kazusa DNA Research Institute, we were able to take lead in the *Period* gene cloning for humans and rats, but for mice *period* gene, we were secondary to Dr. Yoshiyuki Sakaki of the University of Tokyo and Dr. CC Lee of the University of Texas.[5] There were three homologs (*per1*, *per2*, and *per3*) of the *Drosophila Period* gene in mammals including humans, and it was later revealed that *per2* was deeply involved in the behavioral clock function. Accidentally, we were very lucky enough to start on the analysis of *per2*. Mice lacking these genes were created, and it was found that individually, *per1* was for short period rhythms, and *per3* had hardly any phenotypes [later, it was found that *per3* was involved in delayed sleep phase syndrome (DSPS)[6] and in peripheral clocks of lungs and adrenal glands]. However, *per2 gene knock out* mice or overexpressed *per2* transgenic mice showed a drastic phenotype of arrhythmic behavior in the conditions of constant dark or constant light, respectively. Moreover, while not observed in *per1*-destroyed mice, in *per2 gene knock out* mice, the oscillatory expression of other important clock genes *Bmal1* was lost in the SCN. From this fact, *per2* was considered to be an important factor that controls the oscillatory expression of other clock genes. Moreover, we discovered the bipolar nuclear localization sequence[Term 1] in *rper2* amino acids sequence.[7] Therefore, Koyomi Miyazaki, who was then employed as a member of staff scientists in our lab after working as a post-doc, and others prepared the *rper2* gene that deletes this nuclear localization sequence (NLS). When the NLS deleted *rper2* was overexpressed in COS1 cells,[Term 2] we found that the *Cry1*[Term 4] and *per2* nuclear localization was inhibited by trapping in the cytoplasm.[7] Therefore, we attempted to create a transgenic (TG) mouse with normal *rper2 overexpressors*, and another with NLS deleted *rper2 overexpressors*.[8] As a result, the NLS deleted *rper2 overexpressor* mouse showed long period, while normal *rper2 overexpressor* mouse showed short period in the circadian behavior rhythm. This work was the first example in my laboratory where observation *in vitro* was reproduced *in vivo*.

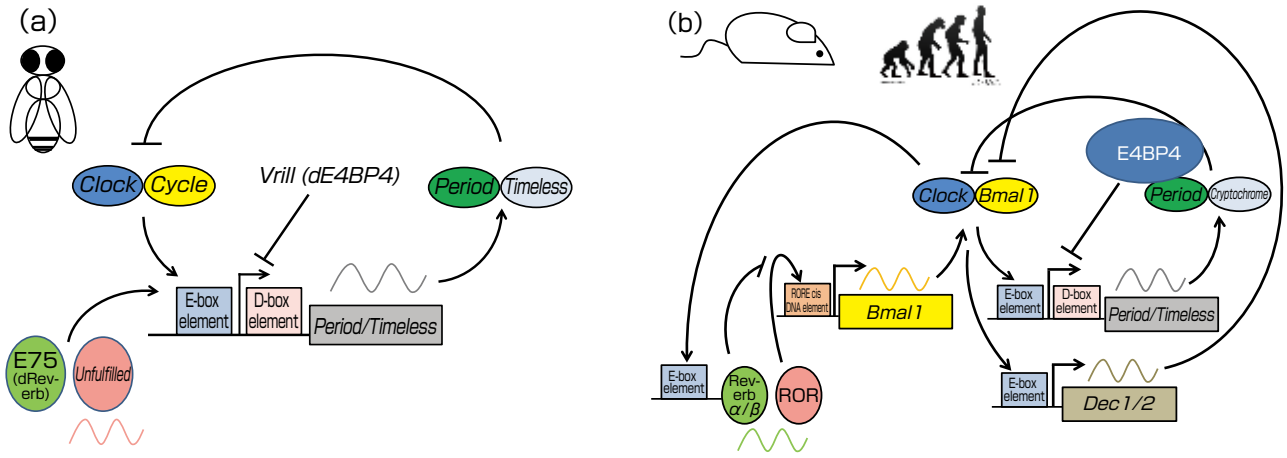


**Fig. 1 Compared biological clock molecular model for *Drosophila* (a) and mammals (b)**
In both species, a negative feedback model of clock gene transcription with extremely similar sequences occurs.

Immuno-staining the nuclear translocation suggests ion of *per2* was delayed in the long period type. We believe that the period was extended due to the delay of nuclear localization of regulating proteins such as *Period* and *Cry*, as shown in Fig. 1b. Moreover, it was observed that the *rper2*-overexpressed mice might have high body temperature and high wakefulness in active phase which suggests that the brain region other than the SCN might be involved in these phenomenon. Later, Chisato Iidaka (current name Chisato Kinoshita; Department of Pharmacology, Teikyo University School of Medicine), who joined our Group as a graduate student at the Tokyo Institute of Technology, discovered that glycogen synthase kinase-3$\beta$ (GSK-3$\beta$) was a factor that promoted the nuclear localization of *per2*.[9] The GSK-3$\beta$ directly binds to *per2*, causes phosphorylation, and promotes nuclear localization. This pathway explained well the action mechanism of LiCl that was effective against depression. That is, when LiCl causes the self-phosphorylation of GSK-3$\beta$, it becomes inactive and delays the nuclear localization of *per2*, and this extends the period of activity. This pathway is attracting attention of researchers developing anti-depressant drugs as a target for new drug discovery. Recently, Kazuki Sakata, a graduate student at the University of Tsukuba, *et al.* found that high concentration of inositol extended the period of courtship rhythm and locomotor rhythm in *Drosophila*.[10] Myoinositol, an ingredient derived from ice plants (*Mesembryanthemum crystallinum*) grown in a plant factory of Tsujiko Co. Ltd. of Shiga Prefecture, was discovered originally by us during the screening for a substance that affects Drosophila mating rhythm. Myoisitol is highly expected to be an antidepressant in the future.

The mystery of GSK-3$\beta$ was found in *Drosophila*. ATP-mediated protein kinase B (AKT, also called PKB), which was known in the signal pathway of nutrition stimulation, inhibited the nuclear localization of clock gene product *Timeless* through the target of rapamycin (TOR) pathway.[11] In AKT-overexpressed flies, TOR-S6 kinase in the central pacemaker of the fly brain was activated, then the glycogen synthase kinase-3$\beta$ (SGG) was phosphorylated, and inhibited the nuclear localization of clock gene product *Timeless*. In summary, nutrients signal from food delayed the peripheral clock, then the peripheral clock affected the central pacemaker in the brain by a feedback mechanism. This feedback mechanism is also common in mammals.

## 4 Rhythm disorder and clock gene mutation

Sleep-wake rhythm syndrome in humans include the advanced sleep phase syndrome (ASPS), the delayed sleep phase disorder (DSPS), and the non-24-hour sleep-wake syndrome. These syndromes occur on a familial basis, but the causal gene was totally unknown. However, in 2001, linkage analysis was carried out of a family of ASPS in Utah, USA, and the responsible gene was mapped to Chromosome 2. Finally, it was identified that the 662nd serine of *hper2* gene product was replaced by glycine[12] (Fig. 2). This is the binding region of casein kinase I$\varepsilon$ (CKI$\varepsilon$), and particularly the first serine on the N-terminal side is thought to be an important amino acid in initiating phosphorylation. The main function of *per2* is thought to be phase delay, and it is assumed that the mutation at the phosphorylation site causes the loss of the function of *per2* protein and the phase of circadian rhythm is advanced. At the same time as this discovery, Ebisawa and our group found an example where valine mutated into glycine in the *hper3* region of an amino acid very close to the casein kinase I$\varepsilon$ phosphorylation site, in the DSPS family line.[13] In both family lines, the change in one amino acid of a sequence of a clock gene product affects the entrain function. Recently, it is becoming clear that clock gene sequence polymorphism is involved in the nocturnal/diurnal tendencies in humans and mice. Also, we succeeded in making a model mouse with nocturnal tendencies from the mutant strain of the *Clock* gene. As the age of personalized medicine arrives in the future, the importance of the genetic background of an individual's sleep rhythm will be increasing.

## 5 Factors affecting the *Period2* rhythmic expression

As mentioned in this paper, the 24-hour rhythmic expression of *per2* is very important in maintaining the biological rhythm in mammals, and the main regulatory point is transcription. Until now, CACGTT, a non-canonical E-box,[Term 6] was known as the cis sequence[Term 5] involved in the *per2* mRNA rhythmic expression. Tomoya Ohno, who joined our Group as a graduate student at Life Science and Technology, Tokyo Institute of Technology(TIT) because I was a visiting professor at TIT, biochemically identified that the bZIP transcription factor[Term 7] E4BP4 (*Drosophila vrille* homolog) had an inhibitory effect on *per2* rhythmic expression (Fig. 1b).[14] As a result of detailed analysis, he found there were two E4BP4 binding sites, the A-site[Term 9] and the B-site,[Term 10] near the *mper2* promoter[Term 8] DNA region. When a mutation was made into these two sequence sites, the *per2* rhythmic

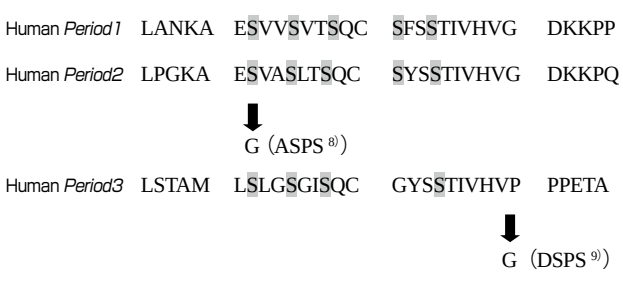


**Fig. 2 Mutated amino acids in hPERIOD protein in human abnormal +rhythm syndrome**

Serines (S) in the region are target amino acids of phosphorylation by casein kinase I, and this region is important for the phase regulation of circadian rhythm. The arrows point to amino acid mutation in advanced sleep phase syndrome (ASPD) and delayed sleep phase syndrome(DSPS).

expression was inhibited specific to the B-site. Moreover, circadian time-specific binding was confirmed at the B-site by both a gel retardation assay and the ChIP method. Finally, the *per2* promoter rhythmic expression activity was compared with the mutation to the A-site, the B-site, and the E2-box by the luciferase activity using the real-time monitoring system. Interestingly, the 24-hour oscillation was not lost in the single mutation of E2, but it was proven at a cellular level that the 24-hour oscillation disappeared with the mutation to both the E2-box and the B-site. Conventionally, the importance of the A-site only was reported *in vitro*,[14] but in a complex system such as oscillatory expression, it is important to conduct analysis by introducing to a cellular or an *in vivo* level system. Since the oscillatory expression was maintained in the single mutation of E2-box, it became clear that the *per2* oscillation could not be explained only by the negative feedback model of *Clock/Bmal1* and *per/Cry*. Moreover, we found that the E4BP4 bound with *per2* and *Cry2* in the cell.[15] From these facts, we proposed a new model for a negative transcription-regulating factor complex for biological clocks (Fig. 1b). In this new model, these complexes go through nuclear localization in circadian time specific manner, and *per2* and *Cry2* complex target the *Clock/Bmal*, while E4BP4 complex targets the B-site (D-box) to negatively regulate the transcription. There are several reports that E4BP4 is important in the 24-hour oscillatory expression of phosphatidylcholine transporter Mdr2,[16] cytochrome P4503A4 (CYP3A4) involved in drug catabolism, and cholesterol 7$\alpha$-hydroxylase (Cyp7$\alpha$) that is involved in bile acid synthesis. Therefore, it is thought that the transcription factor E4BP4 acting negatively on the 24-hour rhythm transcript is important in peripheral circadian clocks, particularly in liver rhythm formation.

## 6 Biological clock regulation by chromatin

This chromatin topic was tackled by Yoshiaki Onishi (currently, AIST Kansai), who specializes in chromatin biochemistry and who moved from a different group in AIST. The *Bmal1* which formed the heterodimer with a circadian clock gene product CLOCK protein is an extremely important protein that causes the 24-hour oscillation of the biological rhythm, and consists of the negative feedback loop. However, why this gene is transcribed through 24-hour oscillation remained a mystery. First, Transcriptional rhythmic regulation of *Bmal1* gene was accomplished by the binding of clock transcription factor REV-ERB$\alpha$ and ROR$\alpha$ to upstream region of *Bmal1* gene, using various deleted DNA constructs. Onishi further found that the intranuclear matrix protein SAF-A (also called hnRNPU) bind rhythmically at the downstream of the cis element RORE, to control the transcription.[17] That is, the state of chromatin around *Bmal1* gene was widely open or closed. As SAF-A has been reported to involve in the regulation of the c-Mycc-Max complex, *Bmal1* will become very important in controlling cancer cells in the future.

## 7 Different functions of peripheral clock (Haradokei) and central clock in *Drosophila* and mammals

Until recently, most of the relationships between the peripheral clock (Haradokei) and the central clock was studied in mammals, but an interesting study has been published by Sehgal *et al*. using *Drosophila*.[18] In *Drosophila*, the tissue that has both the functions of liver and fat tissues of humans is called the fat body (Fig. 3). Using genetic engineering methods to transfer the dominant negative type *Clock* gene so that the biological clock function in the fat body was lost, a fly that was very weak against fasting (i.e. quick to starve to death) was created because its glycogen storage was significantly decreased. Therefore, this fly would feed frequently during the night. Interestingly, such abnormality was not seen in the $CLK^{JRK}$ fly that had *Clock* gene mutation throughout the body. Therefore, they hypothesized that there must be different effects of clock genes on the central clock and peripheral clock, and created a fly in which the *Clock* gene function was lost specifically in the central pacemaker. As a result, the glycogen storage increased in the fat body, and the fly became stronger against fasting compared to a normal fly. Through this experiment, it was shown that the functions of the peripheral clock (Haradokei) and the central pacemaker were different, as observed in mammals. Unfortunately, in this paper, the direct relationship between the clock gene and glycogen storage was not demonstrated, but this point was proven using mammals by Ryosuke Doi (currently, Astellas Tsukuba Research Center), who was a graduate student at the University of Tsukuba of which I was a professor. I shall describe the glycogen study.[19]

The most important organ for glycogen storage and for maintaining blood glucose levels is liver in mammals. It was known in the 1970s that there was a circadian rhythm in the glycogen synthase that was the rate-limiting enzyme in glycogen synthesis. In later molecular biology analysis, it was

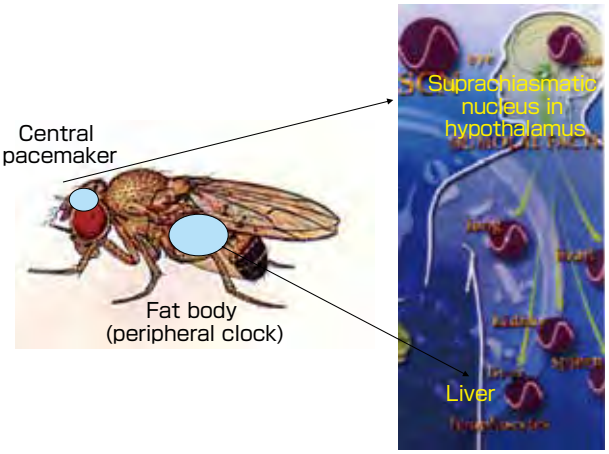


**Fig. 3 Fat body, the organ corresponding to human liver in *Drosophila* (fly)**
The central pacemakers in *Drosophila* are located in the lateral neurons in the brain.

clarified that the glycogen synthase 2 (Gys2) was expressed mainly in the liver, and its family glycogen synthase 1 was expressed in muscles, nerves, and fat. Doi *et al.* looked at the rhythmic expression regulating the mechanism of Gys2 in the liver. First, the rhythmic expression of genes involved in glucose metabolism was investigated in the normal mouse and the mouse with mutation in the *Clock* gene (Cl/Cl) using the real-time PCR method. As a result, abnormalities were seen in the *Clock*-mutant mouse for the rhythm expression of phosphoenolpyruvate carboxykinase (Pepck), glucose-6-phosphate, glucose transporter 2, and others. The most interesting difference was the almost complete disappearance of the expression rhythm of Gys2 mRNA in Cl/Cl mice. On the other hand, there was no significant difference in glycogen phosphorylase, which was the rate-limiting enzyme of glycogen breakdown, between the normal and the *Clock*-mutant mice. The data indicate that the glycogen storage rhythm in the liver was under the rate limitation of glycogen synthesis but not in the breakdown. Moreover, arrythmicity was detected not only in Gys2 mRNA but also in the Gys2 protein level in *Clock*-mutant mice. Furthermore, the data suggests that this glycogen rhythm was not dependent on insulin or glucagon levels that changed depending on food. From these facts, it was considered that the Gys2 rhythm was an endogenous[Term 3] regulatory expression of mRNA. We hypothesized that it might be directly controlled by clock gene products, and searched the genome sequence for the cis element binding to clock gene products. Since we could not find typical rhythm cis elements in about 3 kb of the 5' upstream region, after trial-and-error, we found two E-box elements in tandem in the first intron of the Gys2 gene. By making mutations into these two E-box elements, it was shown that both elements contributed equally by using the *in vitro* reporter assay and the chromatin immune-precipitation method from the liver extract. It was thought that these elements might function *in vivo*. Therefore, we addressed this point by investigating the effect on rhythm oscillation using the real-time reporter assay method. Using the *mper2*-dLuc as a positive control, E1, E2, and both were mutated and compared. As a result, both of E1 and E2 mutations were most attenuated. From this experiment, we showed that glycogen synthesis rate-limiting enzyme Gys2 was regulated rhythmically at a transcription level through the two E-box elements recognized by *Clock* and *Bmal*. We think the Gys2 gene was one of the output from the peripheral clock. This is the first data in the world that showed that the glycogen metabolism was under the direct control of the molecular clock. Haruhisa Kawasaki (Institute for Chronobiology, Foundation for Advancement of International Science), who joined our group after returning from studying in the United States, advanced this study, and found that C/EBP$\alpha$, an important transcription factor involved in glycogen storage in fetal liver, was also regulated by core feedback loop through the E-box element in the upstream region of C/EBP$\alpha$ gene.[20]

## 8 Three cis elements that comprise the circadian clock

As input of the circadian clock system, various elements other than light are being found. That is because the concept of peripheral clocks in all tissues has become established because of the discovery of the rhythmic clock gene expression in all tissues. The most recent peripheral clock model is shown in Fig. 4.[21] The cholesterol in food and HEME (hemoglobin) supplied from blood are becoming very important, as they have been considered to be important factors that synchronize with the circadian clock in liver and other internal organs. Cholesterol positively controls clock proteins *Clock* and *Bmal* through the transcription factor ROR dependent RORE binding, while HEME negatively controls *Clock* and *Bmal gene* through the transcription factor REV-ERB (Figs. 1 and 4).[1] A recent paper showed

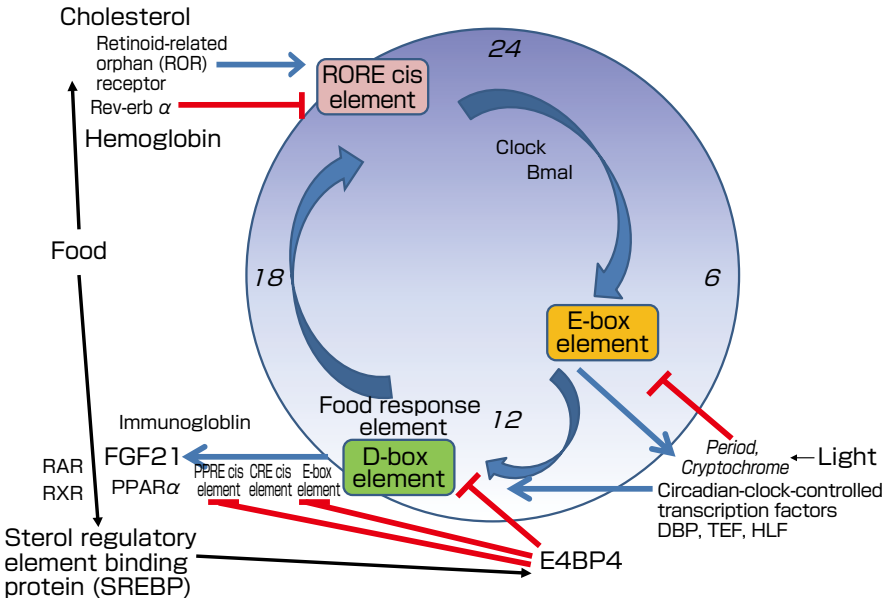


**Fig. 4 Circadian clock synchronization with various environmental factors such as light, food, and fat**
ROR, Rev-erb, *Clock*, *Bmal*, *Period*, and *Cryptochrome* (*Cry*) are clock genes. DBP, TEF, HLF, E4BP4, PPAR, RAR, RXR, and SREBP are circadian-clock-controlled transcription factors. RORE, E, D, CRE, and PPRE are target DNA sequences (cis element) for the transcription factor binding.

that when a ligand-binding site of ROR$\alpha$ is expressed in an insect cell, cholesterol coprecipitates with ROR$\alpha$. Moreover, from X-ray crystal analysis, the possibility of ROR$\alpha$ ligand being cholesterol is indicated, because cholesterol sulfate and 25-hydroxycholesterol bind to this ligand-binding site of ROR$\alpha$. On the other hand, in chronobiology, what is in the upstream of clock proteins *Clock* and *Bmal* is the big issue, and this is being solved. These clock proteins *Clock* and *Bmal*, other than regulating *Period* and *Cry* in the core loop, regulate the transcription factors DBP, TEF, and HLF as output, bind with the nine-base sequence called the D-box, and create a large 24-hour rhythm in the peripheral cells (Fig. 4). Recently, fasting-induced hormone, FGF21, was reported as the factor that had this D-box in its regulatory sequence.

Katsutaka Oishi, who was employed as a research scientist from research technician of the Biological Clock Group, previously conducted genome-wide expression analysis using clock-mutant mice. He discovered the peroxisome proliferator-activated receptor $\alpha$ (PPAR$\alpha$) that was a nuclear receptor and fatty acid as ligand, among the genes that was rhythmic expression regulated by clock gene products in the liver, and also discovered that fatty acid breakdown ($\beta$-oxidation) occurred specifically during the night via this PPAR$\alpha$.[22][23] Moreover, It was found that FGF21 was expressed with a large circadian rhythm, when fibrate, a ligand of peroxisome proliferator receptor α, was administered to the abdominal cavity of the mouse.[24] We also reported that fasting by the fibrate (particularly the catabolization of fatty acid) advanced the circadian rhythm in the body and enhanced the fluctuation of the FGF21 rhythm.

Since this phenomenon is not observed in PPAR$\alpha$KO mice, it was considered to be dependent on the peroxisome proliferator response element (PPRE). However, when a ketone diet (a diet method of ingesting high fat and low carbohydrates) was carried out, FGF21 was increased without depending on PPAR$\alpha$. That suggests a pathway for inducing FGF21 without depending on PPRE. Recently, it was reported that not only the known PPRE but also new D-boxes and E-boxes were discovered through the detailed analysis of the mouse FGF21 promoter (Fig. 4). More interesting is the fact that the transcription factor E4BP4 suppressed both the *Bmal/Clock*-dependent transcription and the PPARα-dependent transcription at the same time. That the E4BP4 regulated the *Bmal/Clock*-dependent transcription was easily predictable, because we had already made a complex with E-box (cis element consisting of CACCTG) dependent negative regulators, *Period2* and *Cry* with oE4BP4.[8] However, it is very interesting that E4BP4 suppressed the PPRE-dependent transcription with PPAR$\alpha$ at the same time. This indicates that the suppression of rhythmic transcription of E4BP4 is much more powerful than we initially thought, acts on E-boxes and PPRE as well as D-boxes, and plays the role of a major brake on the peripheral clock. It is known that E4BP4 negatively controls the transcription targeting genes of the D-box with an insulin dependent manner after a meal. As mentioned in Chapter 5, the mechanism in which transcription factor E4BP4 acts negatively on the formation of 24-hour rhythm transcription is very important particularly in liver function. There is a report that E4BP4 is activated on time-specific light stimuli and induced at transcription factor SREBP in bird pineal body. The effects of light and food are very complex even in one gene expression. This is a field that needs further attention.

In humans, there was an interesting report that blood FGF21 was expressed in circadian rhythm following fatty acid rhythms.[25] In the same paper, they showed that FGF21 was induced by unsaturated fatty acid like linoleic acid rather than saturated acid like palmitic acid, using human hepatocarcinoma-derived cell HepG2. More surprisingly, when the circadian rhythm of FGF21 was compared in normal and obese (having BMI 25 or over) humans, the amplitude of rhythm was very small in obese humans[25] (Fig. 5). In the future, by studying the diurnal dynamics of such fasting-induced hormone FGF21, it may become possible to prevent obesity and aging by increasing the amplitude of rhythm. Recently, it was reported that FGF21 was induced from brown adipose cells when Swiss mice were kept in low temperature. FGF21 is studied as the factor that induces hibernation and diurnal torpor, and please refer to other reviews for details.[26] In summary, nuclear receptor PPARα binds with PPRE in the liver during fasting, and transcription factor ATF2 binds to CRE in brown adipose cells during low temperature to induce FGF21. However, in the case of a ketone diet when FGF21 is induced without PPAR$\alpha$ pathway, the pathways through D-boxes and CRE are important for this induction. (Fig. 4). By analyzing FGF21 induction comprehensively, the relationship between fasting and fat metabolism will become clearer in the future.[26][27]

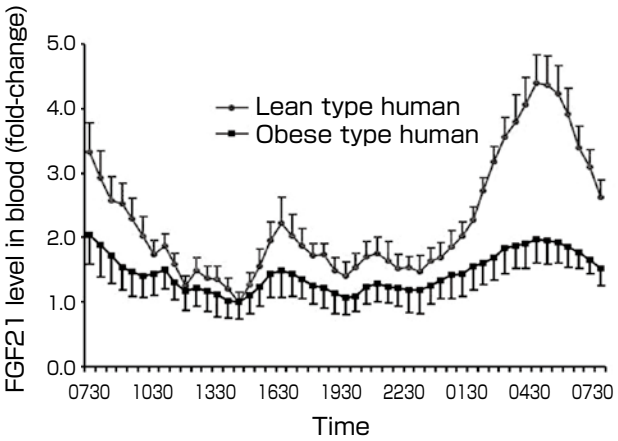


**Fig. 5 Three peaks of circadian rhythm of the fasting-induced hormone, FGF21 in humans[25]**
Clear circadian rhythm is detected in lean humans compared to obese humans.

## 9 Basic model of biological clock learned from *Drosophila*

As mentioned above, we learned of almost all the actors (molecules) and the relationships (pathways) from *Drosophila* (Table 1). Though quite late in the game, I decided to study the *Drosophila* system. Earlier, every time I ran into Dr. Sumihare Noji (currently, President, Tokushima University), at the Molecular Biology Society of Japan, he recommended me to introduce *Drosophila* system studies. Coincidentally, Director Tatsuo Katsura of the National Institute of Bioscience and Human Technology (currently, AIST) recommended a co-professorship of the University of Tsukuba to me, and it became possible to take in more students at the lab. At that time, Tetsuya Okada joined our Ishida Group of Clock Gene, AIST with the recommendation of Dr. Michiko Ohtomi of Toho University. Dr. Ohtomi had been studying the biological clock for a long time, and was a student under Dr. Takeo Deguchi (Tokyo Metropolitan Institute of Gerontology), who studied diurnal rhythm of melatonin in the pineal body. Later, Okada moved and received tutelage of Dr. Osamu Hayaishi, who was the President of the Osaka Bioscience Institute famous for sleep and prostaglandin study, but Dr. Hayaishi passed away at 2015. Here, I was in trouble, as I had never done *Drosophila* genetics before. I looked for someone who was experienced in *Drosophila* genetics and found Takehide Murata in Riken Tsukuba. He had directly learned molecular genetics from Dr. Teiichi Tanimura (currently, School of Science, Kyushu University) who identified the *Timeless* (named Ritsu) gene in *Drosophila* in Japan. After Okada studied at Riken for a while, he discovered the E-box, as well as the *vrille*/E4BP4 gene product binding element responsible for the rhythmic expression in the upstream region of *Drosophila* clock gene *Timeless* in our laboratory[28] Later, Takaomi Sakai (currently, School of Science and Engineering, Tokyo Metropolitan University) joined our group as a post-doc from Dr. Yuzuru Oguma's lab at the College of Biological Sciences, University of Tsukuba, and the *Drosophila* clock gene research was rapidly accelerated in my laboratory. Sakai was interested in the female mating behavior because he worked on it during graduate school, and found that this female mating behavior showed circadian rhythm depending on clock genes.[29] More interestingly, he discovered a diversity of female mating rhythms in different fly species. Therefore, he set up a hypothesis that such time specific mating is effective in species differentiation driven by reproductive isolation. To test this hypothesis, Izumi Nishinokubi, who was employed as a technical support member from Dr. Kenji Tomioka's lab at Okayama University, carried on the experiment. The clock gene *Timeless* was isolated from *Drosophila ananassae*, transferred to the *Timeless* null mutant fly of *Drosophila melanogaster*, and this *ananassae timeless gene*, was regulated by a heat shock promoter. The result was not so simple as to reproduce the mating circadian behavior of *D. ananassae* with a replacement of one clock gene (we got an intermediate of *D. ananassae* and *D. melanogaster*), but we obtained an unexpected by-product where the locomotor rhythm of diurnal fly could be changed to nocturnal by shifting the expression period of the *Timeless* protein by 12 hours.[30] This result supports a recent ecological experiment where mice could be changed from nocturnal to diurnal by environmental adaptation. It is my pleasant memory that I wrote a review, "Time, love and species," where I discussed these findings.[31] As mentioned earlier, the discoverer of the Period gene, Dr. Benzer passed away, but his book "*Time, Love, Memory*" became a bestseller in the United States. During the age from Sakai to Nishinokubi, we mainly studied the time specificity of female mating receptivity in circadian behavior. Then, Yasutaka Hamasaka, who got his degree in Sweden, joined our Ishida Group of Clock Gene. When I talked with him, he was interested in the brain neural connection of close-proximity (CP) rhythm, in which the male pursued the female fly. An American group started a new CP rhythm system using a CCD camera, after seeing our first paper in the *Proceedings of the National Academy of Science*.[32] This system had much better throughput than the arranged marriage method used by Sakai, and we incorporated this method. Hamasaka created various *Drosophila* with destroyed biological clock region in the brain, by utilizing a molecular genetic method (such as causing cell death in certain nerves only). As a result, we identified the center for close-proximity rhythm in *Drosophila*, and clarified that males stopped pursuing females when the brain area called evening oscillator was deleted.[33] Since this center became active during summer, it is most likely related to seasonal rhythms. Later, we attempted to develop an automating machine for close-proximity rhythm, locomotor and sleep rhythm. Jointly with Taisei Co., Ltd., we introduced moving image analysis of IR camera, and Takahiro Suzuki, a post-doc at the graduate school of the University of Tsukuba, created a program in C language. The device was named Automated Circadian System (AutoCircaS) (Fig. 6). The prototype of AutoCircaS was assembled uniting many parts that lay in the corner of our lab as junk, and Taisei and Suzuki (who set up a venture company Shigray Inc.) brushed up AutoCircaS as a selling product. For this accomplishment, AutoCircaS was awarded one of the Chiba Prefecture Monozukuri big prize in 2016.

One day, I received a call from Masami Shimoda (National Institute of Agrobiological Sciences), an old friend of mine, and he asked for our help to figure out why a *Drosophila* fly that lacked a gene for a certain mental disease was showing abnormal behavior. According to him, there was a *Drosophila* family gene (*dfmr1*) of fragile X syndrome[Term 11] that was accompanied by mental retardation in humans. Furthermore, there were three family genes in mammals, but only one homologous gene in flies. I thought this would be very easy to analyze, and conducted behavioral analysis, and found that the locomotor rhythm was strikingly arrythmic. Shimoda rescued the normal *dfmr1* genes of the mutant fly (Fig. 7), and found that the behavior of the fly became normal, the core clock

gene oscillation was maintained, and the eclosion rhythm showed normal.[34] This experiment indicated that the *dfmr1* gene possessed output functions from central pacemaker tolocomotor behavior. Later, it was clarified that poor growth of nerve cells was a common cause of disease in humans and *Drosophila*. As mentioned here, the *Drosophila disease* model contributes to the research of neurodegenerative disease with unknown mechanisms.

Schizophrenia is a disease that is most difficult among the psychiatric disease. Recently, through genetic analysis of a family with frequent occurrences of schizophrenia in Scotland, the disrupted-in-schizophrenia1 (DISC1) gene was reported to show frequent mutual translocation of Chromosome 1 and Chromosome 11. For DISC1, we worked jointly with Masami Shimoda mentioned earlier, Katsuo Furukubo-Tokunaga of the University of Tsukuba, and Akira Sawa of Johns Hopkins School of Medicine, to analyze behavior for expressing human DISC1 in *Drosophila*.[35] As a result, the human DSIC1 transgenic *Drosophila* showed tendency to have longer sleep time.

## 10 Essence of dementia learned from *Drosophila* genetics

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer disease, accompanied by so-called dementia symptoms, and is a major social issue confronting the current longevity society. As the *Drosophila* model for familial Parkinson disease, transgenic fly expressing human mutant $\alpha$-synuclein[Term 12] is famous and published in *Nature* and other journals. Using this *Drosophila* model for Parkinson disease, we found that sleep disorder preceded behavioral abnormality such as tremors. Recently, there was a report that patients with a hetero variant of Gaucher disease[Term 13] causal gene had 28 times more risk for Parkinson disease.[36] That is, the Gaucher disease causative gene is the most risk factor for Parkinson disease. However, the molecular mechanism is currently unknown. If this mechanism can be solved from the clock and sleep gene pathway, we can develop new treatment methods from new perspectives.

Takahiro Suzuki (currently, Shigray Inc.), who was a graduate student ( the University of Tsukuba) in Ishida Group of Clock Gene, AIST, succeeded in creating a model animal by transforming a variant human Gaucher disease gene in a *Drosophila* compound eye.[37] Gaucher disease is a lysosome disease[Term 14] that is specified as one of diseases in the Research on Measures for Intractable Diseases Project of the Ministry of Health, Labour and Welfare. Due to a genetic mutation, the activity of enzymes called glucocerebrosidase is lost before birth. Therefore, glucocerebroside which is a substrate of this enzyme cannot be transferred into ceramides, and the substrate accumulates in the liver, spleen, bones, nerves, and others. The gene of glucocerebrosidase was firstly cloned in USA by Dr. Shoji Tsuji (currently, Faculty of Medicine, the University of Tokyo) *et al.* in 1986. Many patients with human Gaucher disease die in childhood, and a complete cure of this disease is extremely difficult even today. Suzuki *et al.* set out to create a model *Drosophila* for Gaucher disease, and expressed this human variant glucocerebrosidase gene in *Drosophila* using a compound-eye-specific driver, and found a formation abnormality in the compound eye. With further detailed molecular biological analysis, we clarified that this eye morphological abnormality was caused by endoplasmic reticulum (ER) stress. Therefore, ambroxol, a chaperone drug thought to reduce endoplasmic


**Fig. 6 New automated behavior analyzer for small animals**
AutoCircaS (Automated Circadian System) enables measurement of rhythms for sleep, locomotor, and courtship (mating) behavior in *Drosophila*. The software was created by Takahiro Suzuki of Shigray Inc.

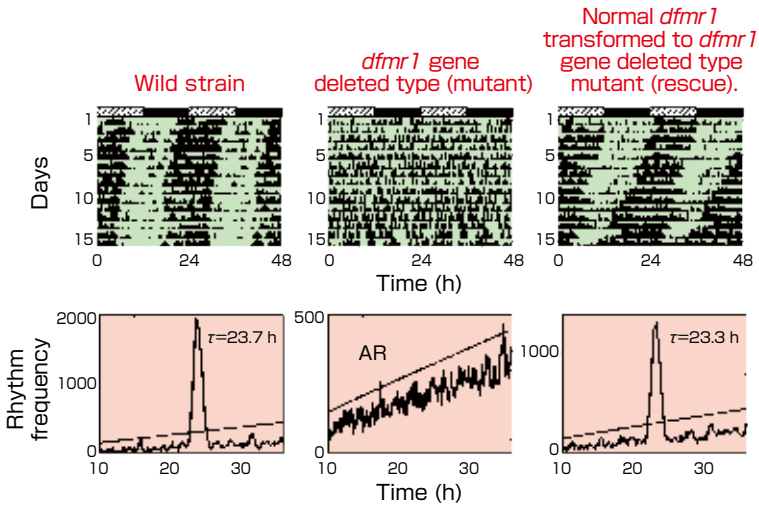


**Fig. 7 Restored circadian rhythm is detected after transformation of a normal *dfmr1* gene in *Drosophila* mental retardation gene, *dfmr1* B55 mutant.**

reticulum (ER) stress[Term 15] was administered, and the above eye abnormality and ER stress were reduced (Fig. 8). From these data, we concluded that human Gaucher disease involved not only the accumulation of enzyme substrates as explained conventionally, but was a molecular mechanism of ER stress caused by mutant proteins themselves.

To link such neurodegenerative molecular mechanism to Parkinson disease and to propose a treatment method, we recently succeeded in creating a Gaucher disease model *Drosophila* where human mutant glucocerebrosidase was expressed throughout the body, as well as in specific sites. We recently succeeded in creating a disease model *Drosophila* that had both Parkinson disease and Gaucher disease because *Drosophila* allowed creation of models faster and easier than with mice.

We recently developed the second generation of the Garcher disease model *Drosophila*. This was a model in which a Minos-insertion mutation[Term 17] was introduced into a GBA homologous gene[Term 16] in *Drosophila*. This mutant showed accumulation of hydroxyl glucocerebroside, an enzyme substrate, as observed in the Gaucher patients. By analyzing the gene expression in this second generation of Garucher disease model *Drosophila*, the increased expression of several genes (factors) for autophagy[Term 18] related Parkinson disease was observed. It was shown that this Gaucher disease model *Drosophila* had a short lifespan, and showed motor dysfunction as well as sleep disorder.[38] Interestingly enough, in the new Gaucher model *Drosophila* and the Parkinson disease model *Drosophila*, sleep disorder was found in the young age (corresponding to about the late 20s to 30s in humans). From this fact, in neurodegenerative disease accompanying dementia, it is important to prevent sleep disorder in the young generation. These results may lead to the development of new treatments, early diagnosis, and prevention of neurodegenerative disease accompanying dementia including Parkinson disease and Gaucher disease. We are still continuing drug screening using the dementia model *Drosophila* mentioned above.

## 11 Conclusion

The clock gene research at AIST was started by using mice and cell strains, but in the mice system, two years were necessary to manipulate one gene by genetic engineering, and the maintenance of mice and cultivation of the cell line were expensive. In the latter phase at AIST, we ran out of budget, and therefore, changed our research system to flies and mammalian cell cultures only. The merit of the *Drosophila* system is that it has a short life-span of about 60 days, the stock facilities for normal and all genetic variants are available all over the world, and the researchers and organizations in fly society are very friendly and any mutants are supplied readily by an e-mail request. We have succeeded in creating useful mutant fly lines for the development of new treatments for neurodegenerative disease such as dementia at AIST. Using such new mutants, primary screening of dementia drugs is still going on and we are receiving small funding from companies. Looking back at the 30 years of scientist life at a national research institute(AIST), I am very grateful that I can continue my work with many collaborators, participants including young students. and with the cooperation of many company people. I was unable to mention the names of all the collaborators, however, I express my deepest thanks here for the 30 years.

Now, a big stream of the science funding in Japan tends toward application, and there is an economical demand that science be immediately useful. It is becoming difficult to study basic science in national research institutes and universities. However, real innovation in science and technology comes from pure basic research, particularly for antibody medicine in Japan. In the future, I hope national

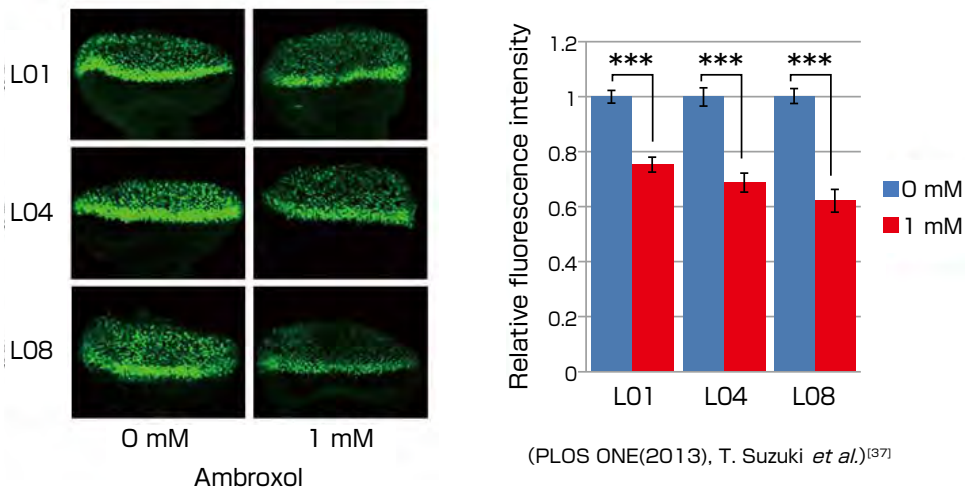


**Fig. 8 Ambroxol is effective for Gaucher disease model *Drosophila*.**
After ambroxol treatment in food, the protein level of ER stress maker, Xbp-1 (green), decreased significantly.

agencies and ministries nurture real organizers with good judgement (mekiki in Japanese) over cross-sectional basic research so that bioscience in Japan does not decline.

The author established the Institute for Chronobiology in the Foundation for Advancement of International Science (FAIS) (3-24-16 Kasuga, Tsukuba) in April 2016. Here, we are continuing our basic research on clock genes, sleep, and neurodegenerative disease. I hope you might drop in and talk to us at my new laboratory if you stop by at Tsukuba Science City. While proofreading this article, the 2017 Nobel Prize in Physiology or Medicine was awarded to "Discoveries of Molecular Mechanisms Controlling the Circadian Rhythm." I would like to say congratulations to Drs. Jeffrey C. Hall, Michael Rosbash, Michael W. Young who are my good old friends and teachers in this field.

## Terminologies

Term 1. Bipolar nuclear localization sequence: a special amino acid sequence region within a protein to transport a specific protein from the cytoplasm to the nucleus..

Term 2. COS cell: A cell line name, which comes from SV40 transformed renal fibroblast cells of African Green Monkey. Since it shows good expression efficiency of foreign genes, it is often used in transient expression of proteins.

Term 3. Endogenous: In general, a situation where the root or cause of a phenomenon exists within itself.

Term 4. *Cry*: Blue-light photoreceptor protein that was originally discovered in plants. Later, it was found in many animals including humans.

Term 5. Cis element: Also called cis-acting element. Existing in the vicinity of the gene, gene transcription (reading of RNA from DNA) is modulated when a regulating protein binds to this site. This region itself does not synthesize proteins, and it is solely for binding with other proteins (a transcription factor).

Term 6. TE2-box: When an E-box sequence is categorized, it can be divided into the canonical E-box CAGNTG sequence and the second E-box. CACGTT sequence was named E2-box. An E-box is a DNA sequence to which transcription factors such as *Clock* and *Bmal* can bind and activate transcription. In Fig. 1, it is referred to as E cis element.

Term 7. bZIP transcription factor: bZIP (basic zipper protein) is one of the motifs of a secondary protein structure, and has the ability to bind with DNA.

Term 8. *mper2* promoter: Transcription factor binding region in the upstream of mouse *Period2* gene.

Term 9. A-site: DNA sequence for the binding with E4BP4 transcription factors on the upstream region of *Period2* gene.

Term 10. B-site: DNA sequence for the binding with E4BP4 transcription factors on the downstream region of *Period2* gene.

Term 11. Fragile X syndrome: Mental retardation disease that is accompanied by low IQ. This syndrome has been confirmed to be genetic. The branching of nerve cells is defective.

Term 12. Variant $\alpha$-synuclein: Missense mutation of a protein comprised of 140 amino acid residues coded by the SNCA gene. This protein mutation is the cause of familial Parkinson's disease.

Term 13. Variant Gaucher disease: Disease discovered by a French physician, Philippe Gaucher. Due to a genetic factor, the activity of enzymes called glucocerebrosidase (GBA) is low, since it is deficient or lacking from birth. Glucocerebroside (glucolipid) cannot be broken down into ceramides and Glucocerebroside accumulates in the liver, spleen, or bone. In severe cases like type III, patients die by age 2.

Term 14. Lysosome disease: A general name for congenital metabolic disorder where enzyme substances accumulate in the body as waste products, because enzymes related to lysosome are lacking.

Term 15. Endoplasmic reticulum (ER) stress: Proteins that were not folded into a normal higher-order structure (unfolded protein) accumulate in the endoplasmic reticulum, and this causes stress on the cells. Since ER stress inhibits normal physiological functions of the cells, the cells have a mechanism to avoid such stress and to maintain normal conditions called homeostasis.

Term 16. GBA homologous gene: GBA stands for glucocerebrosidase, the causal gene for Gaucher disease in humans. The gene has similar DNA sequence as well as similar function as human's. This DNA was found in *Drosophila*, and this is called the GBA homologous gene.

Term 17. Minos insertion mutation: The genetic engineering technique used frequently in *Drosophila*. Insertion mutation using movable genetic element can affect specific gene function. The property of transposons can move over chromosomal DNA and insert genomes at random.

Term 18. Autophagy: One of the mechanisms of the cell to break down junk proteins within the cell. It is also called, self-eating. Dr. Yoshinori Ohsumi of the Tokyo Institute of Technology received the Nobel Prize for Physiology and Medicine in 2016 for discovering this mechanism.

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