CIRCADIAN PHASE-ADJUSTMENT BY DAILY INJECTIONS OF HISTAMINE RECEPTOR ANTAGONIST, KETOTIFEN

AHMAD ALSAWAF

STUDENT ID#31481102

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE

OF

DOCTOR OF SCIENCE

MAJOR COURSE: BIOLOGICAL INFORMATION SYSTEM

SUPERVISED BY

PROFESSOR IKEDA MASAYUKI

GRADUATE SCHOOL OF INNOVATIVE LIFE SCIENCE

UNIVERSITY OF TOYAMA

TOYAMA, JAPAN

2017

UNIVERSITY OF TOYAMA

TABLE OF CONTENTS

Summary

Studies have shown that histaminergic neuronal activity in the brain involves maintenance of arousal, whereas blocking of histamine H_1 receptors (H_1R) either pharmacologically or genetically, enhances sleep. Drowsiness following generic (i.e., over-the-counter) anti-allergic drug administration, which may contain classic H_1R antagonists, may be due to transport of anti-histamines to the brain. In fact, modern H_1R antagonists such as cetirizine, fexofenadine, and mizolastine, which were designed to be less permeable to the blood-brain barrier, produce little or no drowsiness. Conversely, one of the first generation H_1R antagonists, diphenhydramine, is currently sold as a sleeping pill (Drewell® and Neoday®) in Japan. In addition, doxepin (Sinequan®), which is a first generation H_1R antagonist known to induce sleep, has been approved by the FDA to treat insomnia in the USA since 2010. One concern of using these classic H_1R antagonists to treat insomnia is their non-specific actions, including anti-cholinergic actions. An overdose of classic H_1R antagonists produces cocaine-like arousal-inducing actions, resulting in a narrow effective dose-window (i.e., sharp bell-shape dose-response curve) for sleep induction. Because drowsiness is an undesirable property for the general use of antihistamines, the newer and more specific H_1R antagonists have been designed to reduce brain permeability. Therefore, developing brain-permeable specific H_1R antagonists with fewer side effects is still imperative for advancing insomnia treatment. However, development of new drugs requires significant time and effort. Moreover, use of newly patented drugs is costly. Therefore, we have suggested an alternative strategy (i.e., drug re-positioning) to treat insomnia using ketotifen, which is categorized as an early phase second generation H_1R antagonist,

likely to be more specific than the first generation antihistamines, while maintaining its permeability to the brain.

In our previous rat model studies, ketotifen represented a typical S-shape dose-response curve for non-REM sleep induction. Also, repeated administration of ketotifen (3 mg/kg, i.p.) for 5 days at the late active phase (ZT21) consistently facilitated non-REM sleep at the end of the active phase (Unno et al, *Eur J Pharmacol* 2012). To further characterize the effects of ketotifen on circadian clock movements, the present study examined free-running locomotor activity rhythms (Ahmad et al, *Sleep and Biological Rhythms* 2016), core body temperature (Tb) rhythms, and clock gene (*mPer2*) expression rhythms in rats following the repeated injections of ketotifen (3 mg/kg, i.p. at ZT21). The results are described as follows.

(i) Locomotor activities of Sprague-Dawley rats were monitored using infrared sensors under 12:12 h light-dark cycles and the daily intraperitoneal injections of ketotifen (3 mg/kg, i.p.) or vehicle (saline) for 5 days 3-h before light onset time (ZT21) or 3-h before dark onset time (ZT9). Following the final injection day, room light was switched to constant dim red light to monitor free-running rhythms. The eye-fitting activity onset time on the free-running rhythms demonstrated significant advancement of circadian phase of locomotor activity rhythms when ketotifen was injected at ZT21. Since non-REM sleep was enhanced by ZT21 ketotifen injections, we speculated that enhanced sleep might cause circadian phase-shifts in this experimental paradigm.

(ii) Rats Tb were monitored by thermistor-based coin-shape data loggers. The same ketotifen injections (3 mg/kg, i.p., for 5 days) were examined as above for locomotor activity recordings. The results demonstrated that ketotifen did not decrease or increase

of core body temperature immediately after the injections. However, during the injection days, circadian Tb rhythms were amplified. These results suggest, circadian phase-shifts by daily ketotifen injections were not directly caused via temperature modulations.

(iii) To conclude the effects of daily ketotifen injections on the circadian clock, we also evaluated *mPer2* transcriptional rhythms in the hypothalamic suprachiasmatic nucleus (SCN) and hippocampus using a real-time RT-PCR. After 5 day injections of ketotifen (3 mg/kg, i.p., at ZT21), rats were scarified and sampled brain tissues at ZT0, ZT3, ZT6, ZT9 or ZT12 to estimate *mPer2* transcriptional rhythms. The results demonstrated that peak *mPer2* transcription in the SCN was located at ZT6 in the ketotifen injected group and at ZT9 in the saline injected control. In addition, peak *mPer2* transcription in the hippocampus was located at ZT9 in the ketotifen injected group and at ZT12 in the saline injected control. The results indicate advanced *mPer2* transcriptional rhythms both in the SCN and hippocampus in ketotifen-injected group. Hence, we suggest daily sleep inductions at the end of active phase advance the molecular clock movements, resulting in an advancement of behavioral rhythms in rats.

Although further experimental and clinical trials will be needed to conclude the possible benefits of ketotifen to treat insomnia, the present study provides strong evidences that daily ketotifen administrations at the end of active phase will be effective to treat circadian rhythm sleep disorders, such including the delayed sleep phase disorder.

Chapter one:

1.1. Introduction and purpose:

The activity of histamine in the brain involves arousal controls whereas blocking of histamine H1 receptors (H1R) either via pharmacological or genetically methods enhances sleep (1). Drowsiness by generic (i.e., over-the-counter) anti-allergic drug administration, which may contain classic H1R antagonists, may be due to transport of anti-histamines to the brain. Modern H1R antagonists such as cetirizine, fexofenadine, and mizolastine, which were designed to be less permeable to the blood-brain barrier, produce little or no drowsiness (2). On the other hand, one of the first generation H1R antagonists, diphenhydramine, which is currently sold as a sleeping medication (Drewell® and Neoday®) in Japan. Also, doxepin (Sinequan®), which is the first generation H1R antagonist known to induce sleep (3), has been approved by the FDA to treat insomnia in the USA since 2010. One important concern upon using these classic H1R antagonists to treat insomnia is their non-specific actions, including anti-cholinergic actions. An overdose administration of classic H1R antagonist produces cocaine-like arousal-inducing actions (4), resulting in a narrow effective dose-window (i.e., sharp bell-shape dose-response curve) for sleep induction (5,6). Since drowsiness is an undesirable property for the general use of antihistamines, the newer and more specific H1R antagonists have been designed to reduce brain permeability. Therefore, improving brain-permeable specific H1R antagonists with fewer side effects is still imperative for advancing insomnia treatment. However, improvement of new drugs requires significant time and effort. Moreover, use of newly patented drugs is costly. Therefore, we have suggested an alternative strategy (i.e., drug re-positioning) to treat insomnia using ketotifen, which is categorized as an early

phase second generation H1R antagonist, likely to be more specific than the first generation antihistamines, while maintaining its permeability to the brain (7). In our previous rat model studies, ketotifen represented a typical S-shape dose-response curve for non-REM sleep induction (6). Also, repeated administration of ketotifen for 5 days at the late active phase (6) consistently facilitated non-REM sleep at the end of the active phase. Thus, we recommend trials using ketotifen as alternative sleep aid to diphenhydramine or doxepin. Circadian sleep disorders, including delayed sleep phase syndrome and non-24-hour sleep-wake disorder, are due to irregularities in the circadian clock system, whose pacemaker is located within the hypothalamic suprachiasmatic nucleus (SCN). To treat circadian sleep disorders, melatonin and its receptor agonists (Ramelteon or Agomelatine) have been investigated, whereas other pharmacological approaches were limited. Here, to test the possible ketotifen actions on circadian sleep disorders, free-running locomotor activities of rats were monitored under constant dim red light following the repeated injections of ketotifen under the light-dark cycles.

1.2. Materials & Methods:

Animals:

Adult male Sprague-Dawley (SD) rats (300–450 g, 60–70 days old), were maintained on a 12:12 h light: dark cycle at a constant ambient temperature (25 ± 1 °C) and housed with our inbred colony at the Gofuku campus of the University of Toyama. Food and water were available ad libitum. All animal experiments were approved by the Animal Care and Use Committee at the University of Toyama.

Locomotor activity monitoring:

We monitored the locomotor activities of rats by infrared sensors (P4488, Hamamatsu Photonics, Shizuoka, Japan) located 30 cm above the sleep recording cages. Activity Counts were digitized and fed into a laptop computer via TTL input board (PIO-32D,Contec, Tokyo Japan) at 3 min intervals. Day-night or circadian locomotor rhythms were monitored and analyzed by original software written by M.I. (PACR ver. 2.0). Rats were initially kept under LD cycles composed of 12 h full spectrum regular light (125 lux; 08:00-20:00) and 12 h dim red light (illuminated with 630 nm LED arrays; < 0.1 lux) and then under constant dim red light. Intraperitoneal injections of 3 mg/kg ketotifen (Tokyo Chemical Industry, Tokyo, Japan) or its vehicle (0.2 mL saline) were examined during the locomotor activity monitoring. Statistical analysis data are presented as the means ± SEM. One-way analysis of variance (ANOVA).

Statistical analysis:

Data are presented as the means ± SEM. One-way analysis of variance (ANOVA) was used for statistical comparisons. A chi-square period-gram was used to estimate free-running period (τ) of locomotor activity rhythms under dim red light.

1.3. Results :

Following the daily injections of ketotifen 3-h before light onset time, we estimated the phase of the circadian clock by switching the lighting schedule from 12:12 h LD cycles to constant (2 weeks) dim red light (Fig. 1). Under dim red light conditions, free-running locomotor activity rhythms longer than 24-h were observed both in saline-injected control rats (t= 24.8±0.1 h; n = 8) and rats receiving repeated ketotifen injections (t = 24.7±0.1 h; n =8). Notably, significant (~1 h) advancement of activity-onset of free-running locomotor activity rhythms was observed in the repeated ketotifen- injection group ($P < .01$; Fig. 2). In contrast, a single ketotifen injection at activity onset time (CT12) or late during the active phase (CT21) both failed to produce significant phase-shifts of free-running rhythms.

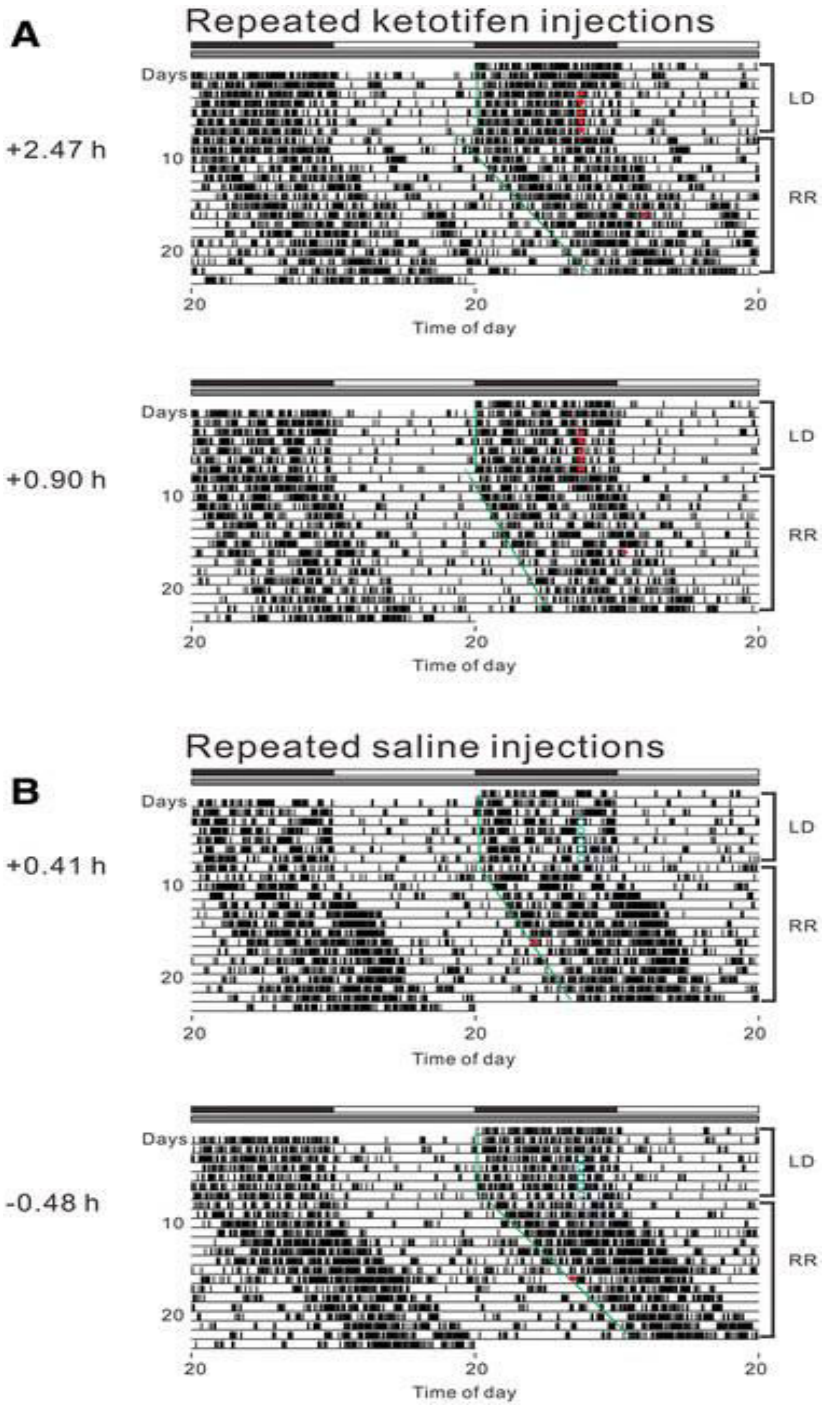


Fig.1. Double-plotted actograms of locomotor activities recorded in rats receiving daily saline injections (open circles; upper) and 3 mg/kg ketotifen injections (asterisks; lower) 3 h before light onset time. On the 9th day of recording, environmental light was switched from 12:12 h light dark (LD) cycle to constant dim red light (RR). Note that

daily activity onset (gray line) is located at the dark onset time under LD cycles regardless of the agent administered. This could be due to the strong masking effect of light. Although there is no rebound increase in activity levels following ketotifen injections, daily activity onset under RR was advanced in ketotifen-injected rats. Single ketotifen injections (single asterisks) in the middle of RR were also examined whereas significant phase-shifts were not induced by single injection.

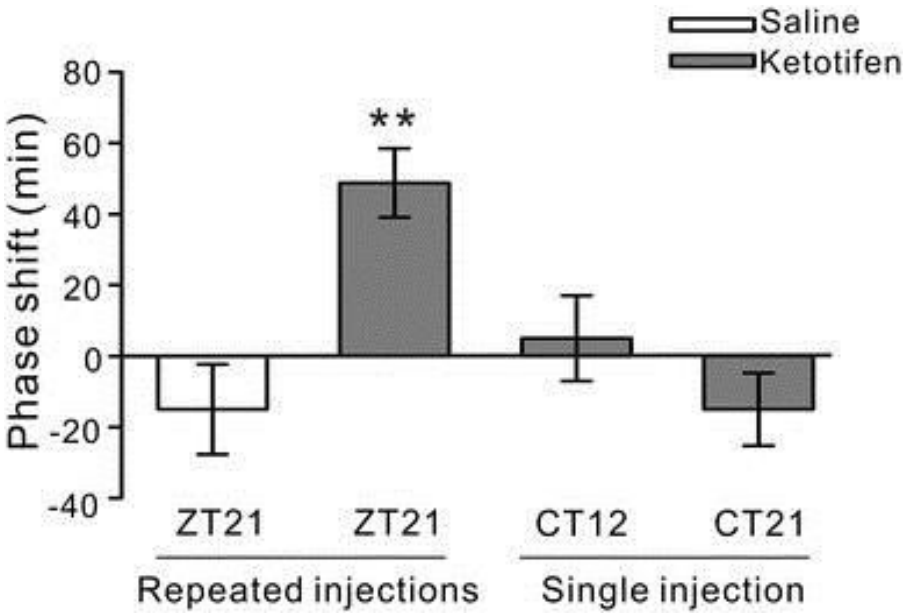


Fig.2. Calculation of the magnitude of the 5 day ketotifen-induced phase shift using the lag time between the activity onset time during LD and RR. In addition, the onset shifts before and after single ketotifen injections in the middle of RR were also calculated. **$F_{3,24}$ = 7.6, P < .01 by one way ANOVA. The zeitgeber time 21 (ZT21) denotes 3 h before light onset time under LD cycles. The circadian time (CT) is defined by the free running rhythms under RR (the activity onset as CT12).

1.4. Discussion :

The rhythms of locomotor activity or of clock gene (*mPer1/2*) expression outside the SCN were significantly reduced in mice lacking histidine decarboxylase (HDC-/-), the histamine synthetic enzyme (8). Nevertheless, rhythmic clock gene (*mPer1/2* and *mBmal1*) expression within the SCN was indistinguishable between the HDC-/- and wild type mice both under LD cycles and constant darkness (8). These former results indicate that the principal site of histamine action, if any, for mouse circadian behaviors, may be located outside the SCN, and that the influence on the SCN clock could be via indirect behavioral feedback pathways. In contrast, circadian action potential firing rhythms in vitro SCN slices were directly phase-shifted by histamine (9). Thus, the impact of the intrinsic histaminergic system for circadian clock control is currently controversial. Here, we demonstrated that repeated ketotifen administration late during the active phase could advance the circadian clock even though ketotifen was administered during the LD cycle. Direction of the circadian phase-shift is mismatched to the histamine-induced phase-shift in SCN slices, because SCN firing rhythms were advanced by histamine (agonist) late at night, similarly following glutamate and photic stimulation (9). Since ketotifen-induced entrainment overcame photic entrainment of the circadian clock, we hypothesize that the effect of repeated H1R blockade exerts itself on the circadian clock via two pathways; (i) attenuating photic input pathways via reducing glutamatergic pathways to the SCN and (ii) new phase-information given to the SCN via behavioral feedback pathways. Behavioral feedback control of the circadian clock has been demonstrated using Triazolam (TRZ) (10). Increased activities via TRZ, dark pulse, or running wheel presentations during the middle of the day (i.e., resting phase for nocturnal rodents) advanced the circadian clock phase. Although it is true that

exercise at a specific time can facilitate human circadian entrainment, the TRZ model may not directly contribute to the treatment of circadian sleep disorders because TRZ is a potent hypnotic in humans. Because daily ketotifen (3 mg/kg) enhances non-REM sleep and reduces REM sleep for 3 hours (6), this monotonic and punctual sleep could be a feedback signal for the SCN. To our knowledge, this may be the first model showing sleep-induced phase-shifts in the circadian clock. However, the underlying neural consequences need to be elucidated in future studies. For future consideration of ketotifen in the treatment of insomnia and circadian sleep disorders, drug safety standards are required for these trials. However, because ketotifen is an over-the-counter drug and is already used for broad applications with minimal side effects, future trials for patients could be easier than those using drugs that have been newly designed to treat insomnia and circadian sleep disorders.

Chapter Two:

2.1. Introduction and purpose:

As we discussed in Chapter 1, ketotifen may advance circadian rhythms by producing sleep at the end of active phase. Beside the "sleep-induced circadian phase-shifts" was hypothesized, alternative interpretation may be available if body temperature levels are dramatically changed by ketotifen administrations because temperature shifts may be a cue to trigger circadian phase-shifts (11) and indeed histaminergic control of thermoregulatory centers has been reported widely (12,13). To address this possibility, we analyzed core body temperature in rats during and after the daily injection of ketotifen (ZT21).

2.2. Materials & Methods:

Animals:

We used male Sprague-Dawley (SD) rats of 60 days old, and maintained on a 12:12 h light: dark cycle at a (25 ± 1 °C) and housed with our laboratories at the Gofuku campus of the University of Toyama. Food and water were available. All animal experiments were approved by the Animal Care and Use Committee at the University of Toyama.

Body temperature recordings:

The micro data logger system for temperature recordings (Thermokuron^{TM} SL type, KN Laboratories) were placed at the abdomen of rats and recorded temperature at 1 min interval.

Drug administration:

After the temperature measurement setup, the rats were back into recording cages with 12:12 light dark cycles for few days. 3 mg/kg ketotifen (Tokyo Chemical Industry, Tokyo, Japan) or its vehicle (0.2 mL saline) were injected intraperitoneally 3-h before light onset time for five days.

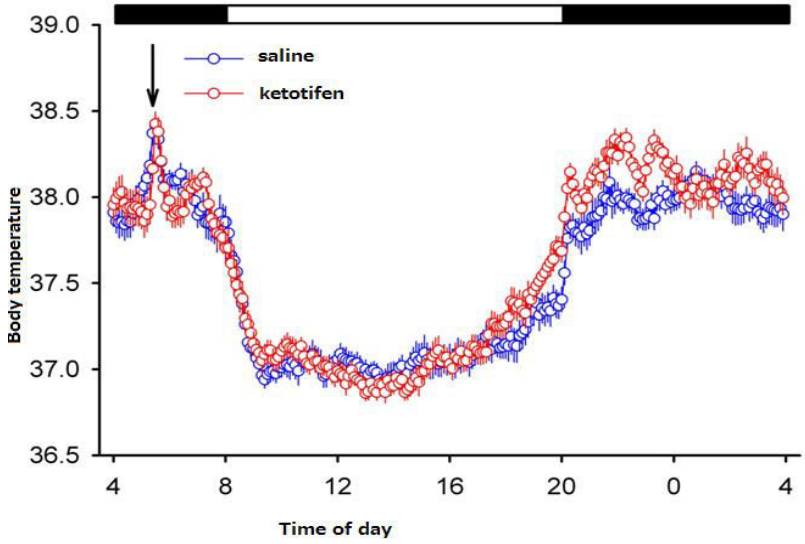
2.3. Results:

Daily injections of ketotifen, 3 hours before ending of activity phase (ZT21) caused a temporal increase in the body temperature levels for both in saline injected controls and ketotifen injected rats due to experimental access to animals, then only small decrease (non dramatic) in body temperature levels upon ketotifen injection. (**Fig.1.A**). Similar to the locomotor activity rhythms, rising phase of temperature rhythms in ketotifen injected group shows that there might be advance in body temperature rhythms although the data are still preliminary. (**Fig.1.B)**

2.4. Discussion:

Administration of ketotifen (3 mg/kg, intraperitoneally), for 5 days before starting the light period failed to cause dramatic changes in body temperature levels. The body temperature kept a strong circadian rhythm, with a variation range of about 1°c. It's well known that body temperature falls after sleep onset, both in entrained and in free-running conditions (13). Since ketotifen seems to have little effects on the body temperature levels, the phase-advance in locomotor activity rhythms following ketotifen injections seems to be dependent on sleep process rather than via temperature changes whereas further investigations are needed to explain the effect of ketotifen on the circadian rhythms.

A



B

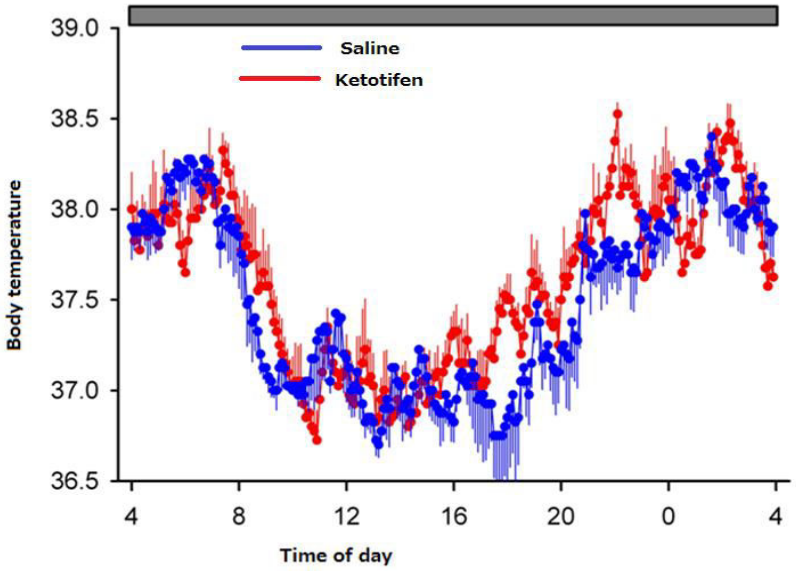


Fig.1. Rat body temperature rhythms after the ketotifen injection (3mg/kg, i.p) at ZT21. **A.** Average body temperature rhythms during the 5 day ketotifen injection session under 12h/12h light/dark (LD) cycles. **B.** Average body temperature rhythms under dim RR immediately after the 5 day ketotifen injection session. N=4 for each group.

Chapter Three:

3.1. Introduction and purpose:

The hypothalamic suprachiasmatic nucleus (SCN) is the central circadian pacemaker in the mammalian brain which regulates diverse circadian activities including sleep, body temperature and locomotor activities (14). The SCN neurons represent action potential firing rhythms and intracellular Ca^{2+} concentration rhythms at approximately 24 hour periodicity (15) and these neuronal activity rhythms could be driven by clock gene transcriptional translational feedback loops (16). The Per2 is one of clock genes rhythmically expressed in the SCN (17). In addition, expressions of mammalian circadian clock genes are found not only in the SCN but also in other brain areas and peripheral tissues, which raised peripheral clock concept (18–21). As we have shown in the first chapter, daily ketotifen injections at the end of active phase may advance circadian clock oscillations. To confirm this possibility, we further analyzed Per2 transcriptional rhythms in rats. We collected SCN and hippocampus (HC) tissues after 5 days of ketotifen injections (3 mg/kg, i.p., at ZT21 under dim red light), and Per2 transcriptional levels were analyzed using the real-time RT-PCR.

3.2. Materials & Methods:

Animals:

We used Sprague-Dawley (SD) 60–70 days old male rats (300–450 g), were maintained on a 12:12 h light: dark cycle at a constant ambient temperature (25 ± 1 °C) and housed in our laboratories at the Gofuku campus of the University of Toyama. The supply of food and water were available ad libitum. The animal experiments were done under approval of the Animal Care and Use Committee at the University of Toyama.

Collection of the tissue of the SCN and Hippocampus:

The suprachiasmatic nucleus (SCN) and hippocampus (HC) samples were collected at 5 different zeitgeber times in the day, .frozen brain sections with a thickness of 99 micrometer including the suprachiasmatic nucleus and hippocampus were prepared using a cryostat slicer, and suprachiasmatic nucleus (SCN) tissues were punched out from the brain section using 23 G (disposable syringe tips) and we cut one side hippocampus (HC) tissues.

Real-time RT-PCR assay:

A- Punching out SCN and cutting out hippocampus:

Preparing the puncher:

We used (0.60X32 mm) needle and sharpened the needle head and made it flat by grindstone. We removed the shaving waste by washing through syringe, and then we washed the needle by ethanol by setting syringe into the sharpened needle. We sterilized the needles by using gas burner, then wrapped with aluminum foil.

Brain Removal:

We anesthetized the rats using 50 mg/kg of pentobarbital, and then we beheaded the rats by scissor and removed the brain. We put it on glass slide and put in the -80 degree freezer for 30 minutes.

Making Frozen Brain Slices:

We put the frozen brain in 15℃ cryostat which makes the brain temperature go up

and make it softer. Slices that contain suprachiasmatic nucleus and hippocampus were collected and under the microscope we confirmed the positions of suprachiasmatic nucleus and hippocampus. We dipped the collected tissues in 350µL RLT buffer in the ice.

B- RNA Extraction:

We homogenized the collected brain tissues and added 350µL of 70% ethanol to melt the deoxyribonucleic acid so the ethanol and the ribonucleic acid will precipitate. We used RNeasy Mini Kit column, eppendorf tubes and a beaker and sterilized them for 10 minutes by using ultra violet light. We put the 700µL samples in columns and centrifuge it for 15 second, and threw the precipitated liquid in the bottom of the columns and added 500µL of RPE buffer and centrifuged it at 25℃ 12000 rpm for 15 second. We threw the precipitated liquid in the bottom of the columns and added 500µL of RPE buffer and centrifuged it at 25℃ 12000 rpm for 2 minutes. We transferred the columns into new UN-capped Eppendorf tubes and centrifuge without adding any buffer for one minute, and then threw the precipitated liquid in the bottom of the columns.

C- Reverse Transcription:

We removed the deoxyribonucleic acid and prepared the reagents of Quanti Tect Reverse Transcription Kit（QIAGEN）with our samples and put them in the ice and transferred them into the clean bench. We added 1mL of gDNA wipeout buffer into 6 ml of the sample and mixed them. We put it in the thermal cycler（Techne PROGENE）at 42℃ for 2 minutes to warm it up , then we put it back directly in the ice. We added

to each sample 2ml of transcription buffer primer mix, 0.5ml of RT buffer (reverse transcriptase enzyme-containing buffer) and 0.5ml of Random primer and put it in the thermal cycler（Techne PROGENE）at 42℃ for 15 minute to reverse transcriptase then inactivate the reverse transcription reaction at 95℃ for 3 minute then cool it at 4℃ for 5 minute then we used the synthesized cDNA in RT-PCR.

D- Real Time PCR:

We used Rotor-Gene SYBR Green RT-PCR Kit（QIAGEN）according to the real time PCR procedures and we used the following primers（3' - 5'）:

Per2 forward, 59-AGCAGTCCCCTACAGCTTAACCT-39;

Per2 reverse, 59-CCGAGATGCGCCAGATGT-39,

GAPDH forward, 59-GGCACAGTCAAGGCTGAGAATG-39;

GAPDH reverse, 59-ATGGTGGTGAAGACGCCAGTA- 39.

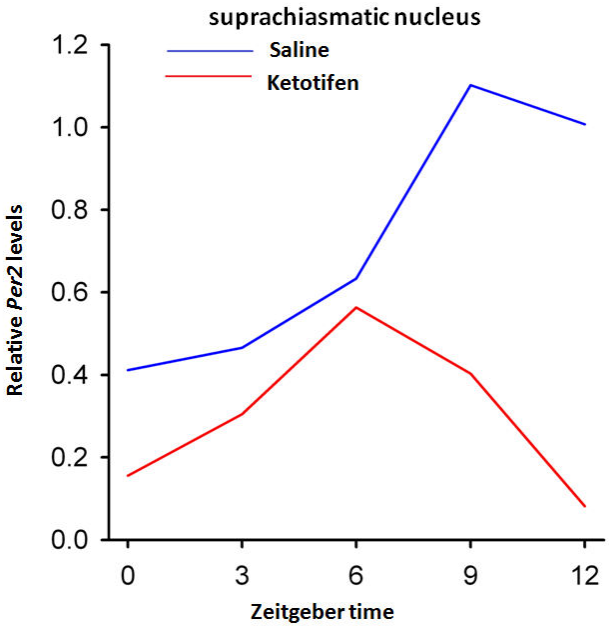
Each primer (50 mM) was used in Rotor-Gene SYBR Green RT-PCR master mix (Qiagen) according to standard methods. Finally, the PCR amplification was monitored in a strip tube (25-ml reaction volume) set in the 72-well rotor of a real-time PCR system GAPDH forward, 59-GGCACAGTCAAGGCTGAGAATG-39; and GAPDH reverse, 59-ATGGTGGTGAAGACGCCAGTA-39. Each primer (50 μM) was used in Rotor-Gene RG 3000-A according to standard methods. Finally, the PCR amplification was monitored in a strip tube (25-ml reaction volume) set in the 72-well rotor of a real-time PCR system. we 7.5 μL 2×Rotor-Gene SYBR Green（Rotor-Gene SYBR Green RT-PCR Kit）,0.3 μL Forward primer（50 μM）0.3 μL Reverse primer（50 μM）0.35 μL template cDNA 6.55μL RNase free water（Rotor-Gene SYBR Green RT-PCR Kit）,the final volume of the PCR reaction solution should be 15 μL. we

dispensed the PCR reaction solution in 0.1 mL Strip Tube（QIAGEN）and we set it up in the Rotor gene 3000A（CORBRTT RESEARCH ） and used Rotor-Gene version 6.0 program、we chose 72 – well rotor of rotor type、and set up the reaction volume15 μL、at 95℃ 5minute 、and rotor speed at normal speed、the denaturation at 95℃、5 second、the annealing / extension step at 60℃、 10second、 the cycling of rotor speed at normal speed、 and the cycles number was 60. In the melting point the starting temperature is 65℃、the ending temperature is 95℃、 the hold of the first importing point is at 60 second 、 we set up the hold of each point at 5 second. We started the measurements with setting up the source at 470 nm and the detection filter at 510 nm, the gain at 9.33. We obtained the cDNA amplification curve depending on the fluorescence intensity of SYBR Green, and compared all the samples.

3.3. Results:

Administration of ketotifen (3 mg/kg at ZT21) for 5 days under LD cycles advanced Per2 transcriptional rhythms for ca. 3 hours in the SCN. The Per2 transcriptional level in the hippocampus was highest at ZT12 and still the peak was not visible during the sampling period (ZT0-12) in the saline injected control. Therefore, it is hard to estimate the net effect of ketotifen on the hippocampus Per2 rhythms. However, since hippocampus Per2 rhythms in ketotifen injected group displayed peak at ZT9, it seems likely that the rhythm was also advanced in the hippocampus. In addition, ketotifen tends to reduce *Per2* expression in the SCN ($P = 0.06$ by two way ANOVA) after repeated injections of ketotifen.

A



B

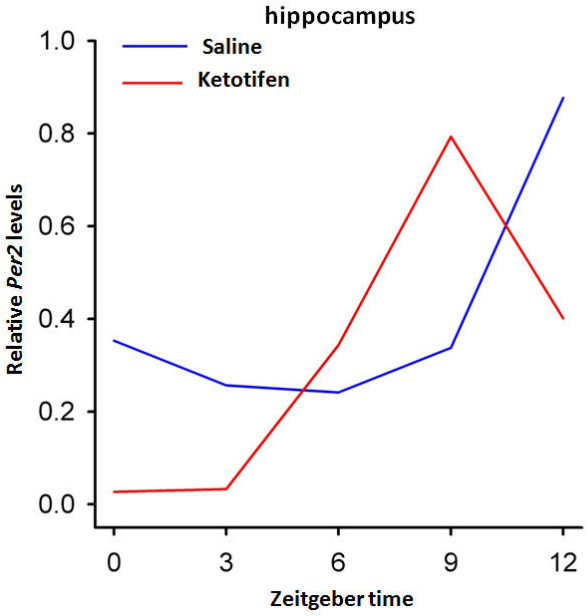


Fig. 1. Per2 transcriptional rhythms in the SCN (**A**) and hippocampus (B) in rats after receiving 5 day ketotifen injections (3 mg/kg, i.p.).

3.4. Discussion:

As we described in chapter 1, ketotifen injections for 5 days under LD cycles have advanced the activity onset time in free-running locomotor activity rhythms under successive constant dim red light. This phase-shift in behavioral rhythm may be caused by pharmacological and steady sleep inductions at the end of active phase of rats because body temperature analyses showed limited effect on body temperature levels by ketotifen. In this chapter, to conclude whether the ketotifen-induced circadian phase-shifts are coincided with molecular clock movements, we investigated the Per2 transcriptional rhythms in the SCN and HC. Although the results are yet preliminary, the current results demonstrated advancement of Per2 rhythms following repeated injections of ketotifen at ZT21, consistent with behavioral phenotype. Interestingly, overall transcriptional levels were reduced in the SCN. This could be explained constitutive activation of SCN neurons via endogenous histaminergic systems. Indeed, histaminergic projections have been identified in mouse and rat SCN (22). Also, recent studies demonstrated direct action of histamine on in vitro SCN neurons and eventually on the clock regulations (23). However, H1R antagonist alone failed to induce circadian phase-shifts in the in vitro SCN (24), different mechanisms may underline between the in vivo and in vitro models. Here, in the present study, we propose the mechanism via steady sleep inductions, which is similar to the activity-induced phase-shifts proposed by Turek and Losee-Olson in nearly 30 years ago, but with apparently different concept. Importantly, since ketotifen is currently available as an OTC drug and thus further trial study for human circadian sleep disorders will not be so difficult. We hope this study will be a scientific trigger for such studies and beneficial for future human health.

References:

1-Thakkar MM. Histamine in the regulation of wakefulness. Sleep Med. Rev. 2011; **15:** 65-74.

2- Hindmarch I, Shamsi Z. Antihistamines: models to assess sedative properties, assessment of sedation, safety and other side-effects. Clin. Exp. Allergy 1999; **29:** 133-142.

3- Richey SM, Krystal AD. Pharmacological advances in the treatment of insomnia. Curr. Pharm. Des. 2011; **17:** 1471-1475.

4- Tanda G, Kopajtic TA, Katz JL. Cocaine-like neurochemical effects of antihistaminic medications. J. Neurochem. 2008; **106:** 147-157.

5- Ikeda-Sagara M, Ozaki T, Shahid M, Morioka E, Wada K, Honda K, Hori A, Matsuya Y, Toyooka N, Ikeda M. Induction of prolonged, continuous slow-wave sleep by blocking cerebral H1 histamine receptors in rats. Br. J. Pharmacol. 2012; **165:** 167-182.

6- Unno K, Ozaki T, Mohammad S, Tuno S, Ikeda-Sagara M, Honda K, Ikeda M. First and second generation H1 histamine receptor antagonists produce different sleep-inducing profiles in rats. Eur. J. Pharmacol. 2012; **683:** 179-185.

7- Tashiro M, Mochizuki H, Sakurada Y, Ishii K, Oda K, Kimura Y, Sasaki T, Ishiwata K, Yanai K. Brain histamine H1 receptor occupancy of orally administered antihistamines measured by positron emission tomography with 11C-doxepin in a placebo-controlled crossover study design in healthy subjects: a comparison of olopatadine and ketotifen. Br. J. Clin. Pharmacol. 2006; **61:** 16-26.

8- Abe H, Honma S, Ohtsu H, Honma K. Circadian rhythms in behavior and clock gene expressions in the brain of mice lacking histidine decarboxylase. Mol. Brain Res. 2004; **124:** 178-187.

9- Biello SM. Circadian clock resetting in the mouse changes with age. Age 2009; **31:** 293-303.

10- Turek FW, Losee-Olson SA. Benzodiazepine used in the treatment of insomnia phase-shifts the mammalian circadian clock. Nature 1986; **321:** 167-168.

11-Tataroglu O, Zhao X, Busza A, Ling J, O'Neill JS, Emery P. Calcium and SOL Protease Mediate Temperature Resetting of Circadian Clocks. Cell. 2015; **163:** 1214-1224.

12-Haas H, Panula P. The role of histamine and the tuberomamillary nucleus in the nervous system. Nat Rev Neurosci. 2003; **4:** 121–130.

13-Haas HL, Sergeeva OA, Selbach O. Histamine in the nervous system. Physiol Rev.

2008; **88:** 1183–1241.

14-Moore RY and Eichler VB: Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res. 1972; **42:** 201–206.

15- Ikeda M, Sugiyama T, Wallace CS, Gompf HS, Yoshioka T, Miyawaki A, Allen CN. Circadian dynamics of cytosolic and nuclear Ca^{2+} in single suprachiasmatic nucleus neurons. Neuron. 2003; **38:** 253-263.

16- Ikeda M, Ikeda M. *Bmal1* is an essential regulator for circadian cytosolic Ca^{2+} rhythms in suprachiasmatic nucleus neurons. J. Neurosci. 2014; **34:** 12029-12038.

17- Zylka MJ, Shearman LP, Weaver DR and Reppert SM: Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. Neuron 1998; **20:** 1103 – 1110.

18-Schibler U, Ripperger JA and Brown SA: Circadian rhythms: Chronobiology – reducing time. Science 2001; **293:** 437 – 438.

19-Sakamoto K, Nagase T, Fukui H, Horikawa K, Okada T, Tanaka H, Sato K, Miyake Y, Ohara O, Kako K and Ishida N: Multitissue circadian expression of Rat period Homolog (rPer2) mRNA is governed by the mammalian circadian clock, the suprachiasmatic nucleus in the brain. J. Biol. Chem. 1998; **273:** 27039 –27042.

20-Oishi K, Sakamoto K, Okada T, Nagase T and Ishida N:Humoral signals mediate the circadian expression of rat period homologue (rPer2) mRNA in peripheral tissues. Neurosci. Lett. 1998; **256:** 117 – 119.

21- Oishi K, Sakamoto K, Okada T, Nagase T and Ishida N: Antiphase circadian expression between Bmal1 and periodhomologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats. Biochem. Biophys. Res. Commun. 1998; **253:** 199 –203.

22- Michelsen KA, Lozada A, Kaslin J, Karlstedt K, Kukko-Lukjanov TK, Holopainen I, Ohtsu H, Panula P. Histamine-immunoreactive neurons in the mouse and rat suprachiasmatic nucleus. Eur. J. Neurosci. 2005; **22:** 1997-2004.

23- Kim YS, Kim YB, Kim WB, Lee SW, Oh SB, Han HC, Lee CJ, Colwell CS, Kim YI. Histamine 1 receptor-Gβγ-cAMP/PKA-CFTR pathway mediates the histamine-induced resetting of the suprachiasmatic circadian clock. Mol Brain. 2016; 9: 49 (doi: 10.1186/s13041-016-0227-1).

23- Kim YS, Kim YB, Kim WB, Yoon BE, Shen FY, Lee SW, Soong TW, Han HC, Colwell CS, Lee CJ, Kim YI. Histamine resets the circadian clock in the suprachiasmatic nucleus through the H1R-CaV 1.3-RyR pathway in the mouse. Eur. J. Neurosci. 2015; **42:** 2467-2477.