Studies on Circadian Oscillation of Melatonin Release in Avian Pineal Gland

鳥類松果体のメラトニン概日リズムに関する研究

Contents

General introduction

Almost all living organisms exhibit rhythmic phenomena with various period lengths, ranging from seconds to years. However, the circadian rhythm, with a period length of about one day, is the most common rhythm. Typical examples of the circadian rhythm are observed in body temperature, blood pressure, metabolism, sleep-wake patterns, pineal and adrenal hormone secretion and so on. All of these rhythms are regulated by the individual's biological clock (circadian oscillator). As the approximately 24-h period of the circadian rhythm is entrained by the daily light-dark cycles of day and night, it has been assumed that the circadian clock is one of the strategies by which living organisms have adapted to the daily rotation of the earth. In other words, the circadian rhythm system is considered to be an essential genetic function, which has been acquired during the process of evolution by all living organisms from prokaryotes to eukaryotes, and which is indispensable in maintaining the homeostasis of physiological functions.

In modern society, there are many situations in which humans must over-ride regular day-night cycles, such as overseas travel, shift work. These situations may result in various syndromes, such as jet lag, sleep delay, periodic gastric ulceration, depression and so on. If artificial manipulation of biological rhythms becomes possible in the future, it may contribute towards therapies for such time-related syndromes. With this possibility in mind, much research has been carried out in the field of biological rhythms.

The common characteristics of endogenous circadian rhythms are as follows. 1) Circadian rhythms persist under constant conditions, and reflect the individual's circadian period. 2) Circadian rhythms are entrained to environmental cycles, such as light dark. 3) The period of a circadian rhythm resists changes in temperature

(so-called temperature compensation. 4) Circadian rhythms are generated by an endogenous circadian oscillator.

The site of the endogenous circadian oscillator differs between species. In mammals, the suprachiasmatic nucleus (SCN), which is located at the bottom of the hypothalamus, has been proposed as a candidate circadian oscillator based on the following pieces of experimental evidence. Firstly, lesions of the SCN erased all circadian rhythms, including those in body temperature, corticosterone levels and locomotor activity [Turek, 1985]. Secondly, the implantation of fetal SCN tissue into the third ventricles of rats with SCN lesions restored the circadian rhythm in locomotor activity [Drucker-Colin et al., 1984]. Thirdly, the only brain area in which a circadian rhythm in ^{14}C-deoxyglucose uptakes was observed in rats was the SCN. Fourthly, a circadian rhythm in the electrical firing rate was recorded in SCN neurons in brain-slice cultures [Shibata and Moore, 1988]. A circadian rhythm in vasopressin release was also observed in an SCN neuron culture [Murakami et al., 1991]. On the other hand, many other reports suggest the possibility that multiple oscillators may be present in rats and hamsters. For example, a circadian rhythm in melatonin release has been recorded in a retinal culture system [Tosini and Menaker, 1996]. Furthermore, daily administration of methamphetamine restored the circadian rhythm in locomotor activity in rats with SCN lesions [Honma et al., 1988]. Similarly, restriction of the feeding time restored anticipatory feeding activity in such rats [Krieger and Hauser,1997]. In addition, it is likely that many peripheral organs, such as the liver, adrenal gland and muscle, may have a circadian rhythm in the expression of *per* mRNA, one of the clock-related genes [Damiola et al., 2000; Stokkan et al., 2001]. Taken together, these results indicate that there are multiple oscillators, including the SCN, in mammals. The most commonly proposed hypothesis is that the SCN is the predominant circadian oscillator and that other sites act as sub-oscillators

under its control [Stokkan et al., 2001].

In contrast to mammals, the pineal gland is well known to be involved in circadian oscillator systems in avian species. For example, pinealectomy abolished the circadian rhythm in locomotor activity in the house-sparrow, while re-implantation of pineal gland tissue into pinealectomized house-sparrows restored this rhythm [Zimmerman and Menaker, 1979]. However, pinealectomy did not erase the circadian rhythm in pigeons, Japanese quails or chicks [Simpson and Follett, 1981, Ebihara et al., 1984]. In these latter birds, the SCN, retina and pineal gland are all components of the circadian oscillator. Therefore, although the avian circadian system is very complicated and varies greatly between species, the pineal gland appears to be the key component.

Melatonin release increases during the night-time and decreases during the daytime, irrespective of whether an animal is diurnal or nocturnal. During experiments on this hormone, Deguchi (1979a) was the first to show directly that the chick pineal gland has a circadian oscillator function. He demonstrated that the activity of N-acetyl-transferase (NAT), a melatonin-synthesizing enzyme, showed a circadian rhythm in a chick pineal gland culture. He also showed that this NAT activity rhythm was entrained to a light-dark cycle, even if the light and dark periods were reversed. He therefore hypothesized that the chick pineal gland possesses a photoreceptor [Deguchi, 1979b]. Subsequently, he proved the existence of a rhodopsin-like substance in the chick pineal gland [Deguchi, 1981]. In 1994, one of the photoreceptors in the chick retina was identified and named *pinopsin* [Okano et al. 1994]. These findings all indicate that the chick pineal gland has circadian clock, photoreceptor and melatonin synthetic functions. However, it has still to be determined whether the photoreceptor, circadian clock and mechanism for melatonin synthesis reside within separate pineal cells, or whether a single pineal cell can in

itself generate the circadian rhythm in melatonin secretion. In order to answer this fundamental question, continuous measurement of melatonin levels in individual pineal cell cultures is necessary. As described in the first chapter, I therefore serially diluted dissociated pineal cells and cultured the individual cells with 15 μl of medium in a Terasaki plate under light-dark cycle, reversed light-dark cycle and constant dark conditions, and measured melatonin release at 12-hour intervals. My hypothesis was that if individual pineal cells showed increased melatonin release during the dark period, they must each possess a photoreceptor. Furthermore, if the rhythm was maintained under constant darkness, individual pineal cells must also contain a circadian clock.

The regulation of rhythmic melatonin secretion from the pineal gland also differs between mammalian and avian species. The rhythm of melatonin production by the mammalian pineal gland is generated by various circadian timing systems, comprising visual projection, the circadian pacemaker (i.e., the SCN), and the projection of sympathetic fibers to the pineal gland. The norepinephrine released from mammalian sympathetic fiber terminals stimulates pineal melatonin release via cAMP production [Klein, 1985; Zatz, 1982], but conversely, it inhibits melatonin synthesis in avian pineal glands by reducing cAMP levels [Deguchi, 1979a; Takahashi et al., 1989; Zatz and Mullen, 1988a]. This difference in the action of norepinephrine on melatonin release between mammalian and avian species results from a difference in the norepinephrine receptor types present; whereas mammalian pineal glands contain $\alpha 1$ receptors, avian glands contain $\alpha 2$ receptors [Takahashi et al., 1989]. Another major difference between mammalian and avian pineal glands is that, as mentioned above, the circadian rhythms of the latter persist *in vitro* as well as *in vivo* [Deguchi, 1979b; Kasal et al., 1979]. However, applying a pulse of light during the dark period rapidly decreases melatonin release from the pineal glands of

both mammalian and avian species. Many studies have been conducted in an attempt to analyze the mechanisms responsible for melatonin production by chick pineal cells. These found that reducing intracellular cAMP levels by norepinephrine treatment and exposure to light reduced melatonin synthesis [Zatz and Mullen, 1988a; Zatz et al., 1988; Takahashi et al., 1989], whereas treatment with vasointestinal polypeptide (VIP), forskolin (a specific adenylate cyclase activator) and 8-bromo cAMP (a cAMP analogue) stimulated melatonin release [Zatz and Mullen, 1988b; Nikaido and Takahashi, 1989]. Furthermore, Bay K 8644 (a dihydropyridine calcium channel agonist) stimulated melatonin release by chick pineal cells, but nitrendipine (a dihydropyridine antagonist of L-type calcium channels), cobalt and manganese ions (inorganic calcium channel blockers) and a low external calcium ion concentration reduced melatonin release [Zatz and Mullen, 1988c; Takahashi et al., 1989]. Although these experimental findings show that cAMP and Ca^{2+} influx are involved in the production of melatonin by chick pineal cells, there is no evidence to show whether pineal cellular cAMP and Ca^{2+} levels are regulated directly by the circadian oscillator. In addition, it is questionable whether endogenous cAMP plays a role in the subjective nocturnal increase in melatonin release under constant conditions. If the circadian oscillator drives the melatonin rhythm via alterations in cAMP levels, then cAMP-dependent protein kinase A may also be involved in controlling the melatonin rhythm. Therefore, in the second chapter, I investigated whether pulse treatment with protein kinase A inhibitors suppressed the subjective nocturnal increase in melatonin release by cultured chick pineal cells maintained in constant darkness. I used H89 as a specific protein kinase A inhibitor [Chijiwa et al., 1990; Maggi et al., 1996], calphostin C as a specific protein kinase C inhibitor [Kobayashi et al., 1989; Tamaoki and Nakano, 1990] and H8 as an inhibitor of both protein kinases A and C [Inagaki et al., 1985; Maggi et al., 1996].

In 1996, D'Souza and Dryer discovered unusual 40-pS cationic channels in chick pineal cells; these were permeable to Ca^{2+} and were active during the subjective night but not during the subjective day. Their observations indicate that the circadian oscillator drives the Ca^{2+} influx rhythm by regulating these cationic channels, and that Ca^{2+} influx through these channels may play a major role in the circadian oscillation of melatonin release under both light-dark cycle and constant dark conditions. Therefore, I also investigated whether intra- and extracellular Ca^{2+} are involved in the subjective nocturnal increase in melatonin release related to adenylate cyclase activation using the intra- and extracellular Ca^{2+} chelators BAPTA-AM, BAPTA and EGTA [Neugulescu et al., 1989; Michelangeli et al., 1989; Li and Mardh, 1996].

During the early 1990s, it was demonstrated that pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates melatonin release via increased cAMP levels in rat pineal cell cultures [Chik and Ho, 1995; Simonneaux et al., 1990; Simonneaux et al., 1993]. PACAP concentrations in the rat pineal gland show a circadian rhythm with increases and decreases during the dark and light periods, respectively [Fukuhara et al., 1998]. In addition, PACAP is localized within the retino-hypothalamic tract, and pulsed PACAP treatment causes phase shifts in the circadian rhythm in a manner similar to light in rats [Hannibal et al., 1997; Hannibal et al., 1998; Harrington et al., 1999]. On the other hand, when PACAP is coadministered with glutamate (the neurotransmitter that conveys light signals to the SCN), it blocks the phase advance induced by glutamate during the late night; conversely, blockade of PACAP neurotransmission with a PACAP antagonist or antibody augments the glutamate-induced phase advance [Chen et al., 1999; Hannibal et al., 1997]. These observations indicate that PACAP may be involved in melatonin release in the rat pineal gland, and that it may be a pivotal modulator of

glutamatergic regulation of the suprachiasmatic circadian oscillator in rats. Furthermore, as mentioned above, VIP stimulates melatonin release from chick pineal cells [Pratt and Takahashi, 1989]. PACAP is a member of the secretin/gastrin/VIP superfamily of peptides, and three types of receptors for VIP and PACAP have been characterized: VIP_1/PACAP and VIP_2/PACAP receptors, which show similarly high affinity for both VIP and PACAP, and the $PACAP_1$ receptor (PACAP-r1), which shows 100–1000-fold higher affinity for PACAP [Arimura, 1992; Arimura, 1994; Chatterjee et al., 1997; Shivers et al., 1991; Usdin et al., 1994]. Therefore, as VIP and cAMP agonists stimulate melatonin release from chick pineal cells, treatment with PACAP may also stimulate melatonin release through common VIP/PACAP receptors. In the third chapter, therefore, I investigated whether treatment with PACAP stimulates melatonin release in chick pineal cell cultures, and if so, which types of PACAP receptors are involved.

Light has two distinct effects on the melatonin rhythm in chick pineal cells. One is to produce acute inhibition of melatonin release by acting on the cAMP cascade via mechanisms other than the circadian clock, while the other involves acting on the circadian clock itself to produce entrainment [Robertson and Takahashi, 1989a,b; Zatz and Mullen, 1988a; Takahashi et al., 1989]. Although many studies concerning the mechanisms responsible for melatonin production and its acute inhibition by light have been conducted in chick pineal cells, the mechanism underlying light entrainment has not been clarified. However, it is known that, while cyclic nucleotides (cAMP or cGMP) do not cause phase shifts in chick pineal cells, they are involved in melatonin synthesis and its acute inhibition by light [Zatz and Mullen, 1988b; Nikaido and Takahashi, 1989; Takahashi et al., 1989]. Moreover, pertussis toxin blocks the acute reduction in melatonin release caused by light pulses, but do not block the phase-shifting effect [Zatz and Mullen, 1988c; Takahashi et al., 1989].

In addition, although the specific photoreceptive substance *pinopsin* [Okano et al., 1994; Max et al., 1995], non-selective cation channels [D'Souza and Dryer, 1996] and MAP kinase [Sanada et al., 2000] may be involved in photo-entrainment, and have been identified in chick pineal cells, the signal transduction pathways related to these components have not been elucidated. Zatz and his colleagues have examined the effects of intra- and extracellular Ca^{2+}-related agents on the rhythm of melatonin synthesis and light-induced effects on this rhythm (phase shifts and acute reductions in melatonin release) [Zatz and Mullen, 1988d; Zatz, 1989; Zatz and Heath, 1995]. Extracellular Ca^{2+}-related agents that stimulate Ca^{2+} influx through the plasma membrane (such as Bay K 8644; a dihydropyridine calcium channel agonist) or retard this process (such as nitrendipine; a dihydropyridine antagonist of L-type calcium channels) increase or decrease melatonin release, respectively, but do not affect the phasing or the effect of light [Zatz and Mullen, 1988d; Takahashi et al., 1989]. In contrast, intracellular Ca^{2+} related agents such as caffeine cause a phase shift in the same manner as a light pulse [Zatz and Heath, 1995]. Furthermore, both thapsigargin, a specific inhibitor of intracellular Ca^{2+} ATPase, and EGTA block light-induced phase advances. These findings suggest that intracellular Ca^{2+} mediates light-induced phase shifts [Zatz and Heath, 1995]. The fact that the phase-response curve for caffeine pulses is almost identical to that for light pulses suggests that intracellular Ca^{2+} is involved in both light-induced phase advances and delays; however, there is no evidence as to whether an intracellular Ca^{2+} antagonist would block a light-induced phase delay in the chick pineal gland. In the fourth chapter, therefore, I compared the effects of various Ca^{2+}antagonists on light-induced phase advances and delays.

The pineal gland is composed of endocrine tissue, which secretes melatonin as a hormone. Although the action of melatonin is considered to be associated with

reproduction in mammals, its function is unclear in avian species. The most interesting question is how a circadian rhythm driven by a pineal circadian oscillator can regulate many physiological rhythms. As there is no anatomically obvious neuronal output from the pineal gland, it is speculated that hormonal substances are involved [Takahashi et al., 1989]. The most likely candidate is melatonin, which increases during periods of darkness and decreases during periods of light, thus reflecting the circadian rhythm of the circadian oscillator. In several species of birds, such as the starling and the pigeon, daily injections or infusions of melatonin entrain the circadian activity rhythm such that the interval during which activity occurs precedes the time of the injection [Cassone, 1990; Chabot and Menaker, 1992]. In addition, daily administration of melatonin in the drinking water was found to correct the arrhythmicity seen in pinealectomized house-sparrows and restore the normal rhythm [Lu and Cassone, 1993]. One explanation of the entraining effects of melatonin may involve direct resetting of the master clock. Another possible explanation may be that melatonin has a masking effect, caused by direct inhibition of locomotor activity at brain sites other than the circadian oscillator or in the muscles [Chabot and Menaker, 1992]. Melatonin is well known to affect thermoregulation in both avian species (such as the chick and house-sparrow) and in mammals [Binkley et al., 1971; Cagnacci et al., 1992; Krause and Dubocovich, 1990; Padmavathmma and Joshi, 1994; Rozenboim et al., 1998; Simpson and Follett, 1981]. Changes in body temperature may also be involved in entrainment by daily melatonin injections, since, like light pulses, changes in temperature have been shown to cause phase-dependent phase shifts in circadian rhythms in a chick pineal cell culture system [Barrett and Takahashi, 1995; Takahashi et al., 1989]. In rats, melatonin entrains the circadian rhythm by resetting the circadian clock in the SCN [Cassone et al., 1986; Gillette and MacArthur, 1996; Krause and Dubocovich, 1990;

McArthur et al., 1991]. In the latter case, the entraining effects of daily melatonin injections were the same in intact and pinealectomized rats [Marumoto et al., 1996; Redman et al., 1983], suggesting that if the entraining effect of melatonin is due to resetting of the circadian clock in the SCN, daily melatonin injections could entrain the circadian rhythm in intact birds as well as pinealectomized ones. Although there are many papers concerning the effects of melatonin on thermoregulation, entrainment facilitation of synchronization to light [Gwinner, 1994 and the studies cited above], there is little information concerning any possible relationships between them. In the fifth chapter, therefore, I compared the effects of single or daily peripheral injections of melatonin on the circadian rhythm, body temperature and locomotor activity in intact house sparrows, Japanese quails and owls. I then, in chapter sixth, investigated whether central administration of melatonin also affected body temperature and/or locomotor activity in Japanese quails (as does peripheral administration) and, if so, whether these two effects were dependent upon each other or independent. I achieved this by direct microinjection of melatonin into various brain regions of the Japanese quail.

Chapter I

Individual pineal cells in chick possess photoreceptive, circadian clock and melatonin synthesizing capacities *in vitro*

Abstract

Chick pineal cells express a circadian rhythm of melatonin release under light-dark cycles (LD), with an increase during the dark period and a decrease during the light period, and this rhythm persists under constant darkness (DD). These results suggest that chick pineal cells possess photoreceptor, circadian oscillator and melatonin synthetic capacity. However, it is unknown whether the photoreceptor, circadian clock and mechanism for melatonin synthesis all reside within individual pineal cells, or whether a single pineal cell alone can generate the circadian rhythm of melatonin secretion. In this chapter, to address these questions, I cultured individual single pineal cells with 15 μl medium per well in a Terasaki plate and measured melatonin secretion every 12 hours under LD, DL and DD. Individual cells secreted more melatonin during the dark period than during the light period under both LD and DL conditions, and those rhythmic secretions persisted under DD. These results suggest that individual pineal cells in chick have photoreceptive, circadian clock and melatonin synthesizing capacities.

Introduction

It is well known that in some avian species the pineal gland plays an important role as one of the oscillators in circadian systems. The melatonin release by cultured chick, pigeon and house sparrow pineal cells increases and decreases during the dark and light periods of LD respectively, and this rhythmic secretion is maintained under constant condition, with a period close to 24 h [Deguchi, 1979; Zatz et al., 1988; Pickard and Tang, 1993; Murakami et al., 1994]. These results suggest that the avian pineal gland possesses photoreceptive, circadian clock and melatonin synthesizing functions. Therefore, cultured chick pineal cells, in particular, have been used extensively as a model system for examining the cellular regulation of circadian rhythm [Nikaido and Takahshi, 1989; Takahashi et al.,1989; Zatz and Mullen,1988a 1988b; Zatz, 1989,1992a; Zatz and Heath, 1995]. Many important findings have been made recently during the study of chick pineal cells, including demonstration of temperature-compensation of the circadian clock [Barrett and Takahashi, 1995], identification of the rhythms of non selective cationic channels [D'Souza and Dryer,1996] and N-acetyltransferase mRNA [Bernard et al.,1997] and identification of photoreceptor molecule [Max et al.,1995; Okano et al.,1994]. Anatomical evidence from avian tissue suggests that the photoreceptors in the pineal gland are situated in the pinealocytes [Collin and Oksche, 1981]. However, it has still to be determined whether the photoreceptor, circadian clock and mechanism for melatonin synthesis all reside within individual pineal cells, or whether a single pineal cell alone can generate the circadian rhythm of melatonin secretion. In order to answer this fundamental question, continuous measurement of melatonin levels in individual pineal cell culture may be necessary. In this chapter, I serially diluted dissociated pineal cells and cultured individual pineal cells with 15 μl of medium in

a Terasaki plate under LD, DL and DD conditions, and measured melatonin release at 12-hour intervals.

Materials and Methods

Pineal cell culture

Chicks were raised from hatching under LD 12:12 (lights on 07.00 h) photoperiod conditions in our laboratory until they were 2-4 weeks old. The pineal gland was removed and collected in cold Hank's salt solution. The tissue was washed with Hank's salt solution containing 100μg/ml gentamicin (Funakoshi, Tokyo), 1000 U/ml penicillin G and 1000 μg/ml streptomycin (Sigma). The tissue was transferred to 10 ml Hank's salt solution containing 2 mg/ml collagenase (Sigma, Type IV). It was minced and incubated for 30 min in a 37 °C shaking water bath, and centrifuged for 2 min at 100 x g. The supernatant was decanted and 10 ml trypsin solution (0.5 mg/ml, Sigma) was added. The tissue was then incubated for 8 min at 37 °C, and 5 ml trypsin inhibitor solution (0.2 mg/ml , Sigma) was added. The tissue was triturated repeatedly through a series of gradually narrowing Pasteur pipettes and centrifuged for 5 min at 500 x g. The resulting cell pellet was resuspended in culture medium with the following composition; Medium 199 with Hank's salt and L-glutamine (Gibco) supplemented with 10 mM HEPES buffer (Gibco), 15% fetal calf serum (Gibco), 100 U/ml penicillin, 100μg/ml streptomycin and 0.9 mg/ml $NaHCO_3$. The cells were then fractionated by iso-osmotic Percoll gradient centrifugation at 450 x g for 20 min, and those at the interface between 1.02 g/ml and 1.07 g/ml were collected and washed with culture medium. The dissociated cells were serially diluted and placed in a collagen-coated Terasaki plate (Falcon) at one cell per well, and maintained in an incubator at 37 °C with 95% air and 5% CO_2 under 12 h light: 12 h dark (LD: lights on at 07:00 h; DL: lights on at 19:00 h) conditions for 4 days and then DD conditions for 2 days. In order to prevent evaporation of the culture medium, extra water was placed in the incubator. All equipment (for example, portable clean bench, CO_2 incubator, microscope) was kept

at 37 °C in a controlled humidity (85%) chamber, and all treatments (for example, medium change) were performed in the chamber in order to prevent any change in temperature. The cultured wells with single pineal cells and without fibroblasts were selected by observation with a microscope on day 3 of culture.

Sampling and radioimmunoassay

Samples were collected at 12 hour intervals for two or four days starting from day 3 or day 5 of culture. Infrared scope was used for sampling under DD. The melatonin content of 10 μl culture medium from each sample was determined by melatonin radioimmunoassay [Murakami, 1995] with modification. Radioiodinated melatonin and non-radioactive melatonin were purchased from New England Nuclear and Sigma, respectively. A rabbit anti-melatonin antibody (HAC-AA92-03RBP86) was supplied by Dr. K. Wakabayashi (Gunma University, Japan), and the final dilution was 1: 288,000. The radioiodinated melatonin (specific activity 2200 Ci/mmol) was used at 5000 dpm per tube. The lower and upper detection limits were 0.25 and 25 pg per tube, respectively. Displacement of 50% was produced by 2.75 pg. Inter- and intra-assay coefficients of variation were 18.7 % and 7.5% at the 5 pg reference, respectively. The significance of differences was analyzed by Student's *t* test.

Results

Single pineal cell culture was successful in 49 wells of 684 wells under LD conditions (LD group) and 20 wells of 360 wells under DL conditions (DL group). The typical example of single pineal cell culture in Terasaki plate is shown in Fig.I-1A.

Figures I-1B and I-2 show the several examples of melatonin secretion patterns from individual cells cultured under LD-DD and DL-DD, respectively. The consecutive data are presented in a normalized form relative to the mean value for each record. This was calculated by dividing the melatonin value of each individual well by the mean value of the time series for that well and multiplying by 100. Thus, the mean value for the time series of each well corresponded to 100%. Under both LD and DL conditions, in all wells examined, individual cells expressed day-night differences in the melatonin secretion pattern, with a relatively high level at night and a low levels during the day. These day-night differences were also observed under DD. Sample No. 34 and 40 (Fig.I-1B) and No. 114 and 122 (Fig.I-2) are examples of wells, which ran successfully after continuous sampling for four days. The net mean value of melatonin secretion from individual pineal cells is shown in Fig.I-3. Melatonin levels were high during the dark period, and low during the light period, and the differences between daytime and nighttime values were significant ($P<0.05$). These day-night differences were also observed between melatonin levels under DD conditions ($P<0.05$, subjective day *vs* subjective night).

Discussion

The findings that individual pineal cells secrete more melatonin during the dark period than during the light period, and that these day-night differences persist under DD strongly suggest that individual pineal cells in the chick have photoreceptive, circadian clock and melatonin synthesizing capacities, and that these functions are linked each other. Although there are at least three cell types in general pineal cell cultures; pinealocytes, fibroblasts and interstitial cells, it seems likely that generation of the circadian rhythm of melatonin does not require cellular interaction of any cells other than pinealocytes. In preliminary experiments, Takahashi demonstrated that single pineal cells in chick pineal cell microculture expressed circadian rhythm of melatonin release under LD conditions [Takahashi,1987]. However, as he discussed in his paper, because there was no evidence of melatonin synthesis under constant condition, these data were insufficient to determine whether photoreceptor, circadian clock and melatonin synthesis activity all take place in individual pineal cell, i.e. it was not known whether melatonin rhythm was endogenous or driven by light cycles. There has been some evidence that individual cells dissociated from the circadian clock-located organ express the circadian oscillation *in vitro*: basal retinal single neurons from the eye of the mollusc *Bulla gouldiana* express circadian rhythm of membrane conductance [Michel,1993]; single pineal cells from the lizard *Anolis carolinensis* secrete melatonin over a circadian period [Pickard and Tang, 1993]; individual neurons in rat suprachiasmatic nucleus cell culture express circadian rhythm in firing rate [Welsh et al.,1995]. These results, together with those from the present study, indicate that generation of circadian rhythm may be a cellular property of all known circadian structures.

I was unable to determine from this study, however, whether or not the phase and period of circadian oscillator in each of the individual cells from a pineal gland are the same. To elucidate this problem, more short-interval sampling is required.

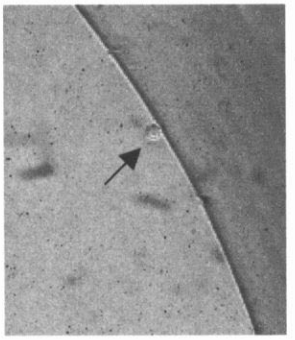


Fig. I-1A

Fig. I-1B

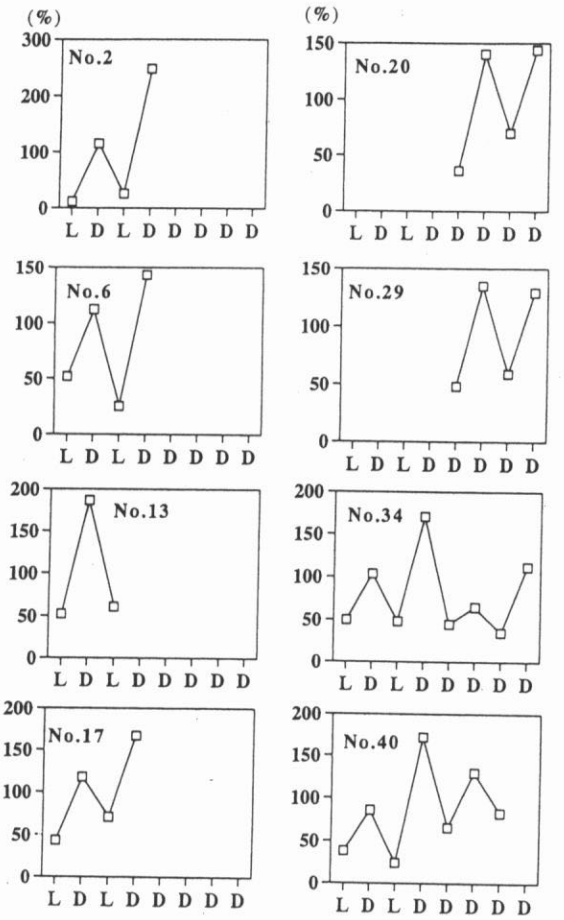


Fig.I-1. A: Example of single pineal cell cultured in Terasaki plate. B: Examples of melatonin secretion pattern in individual pineal cells. Individual pineal cell cultures were kept under LD for 4 days then under DD. Samples were collected at 12 hour intervals starting on day 3 of culture (LD sampling group; No. 2, 6, 13, 17) , or starting on day 5 of culture (DD sampling group; No. 20, 29). In No 34, 40, samples were collected through LD to DD. All data are shown as percentage values relative to mean values.

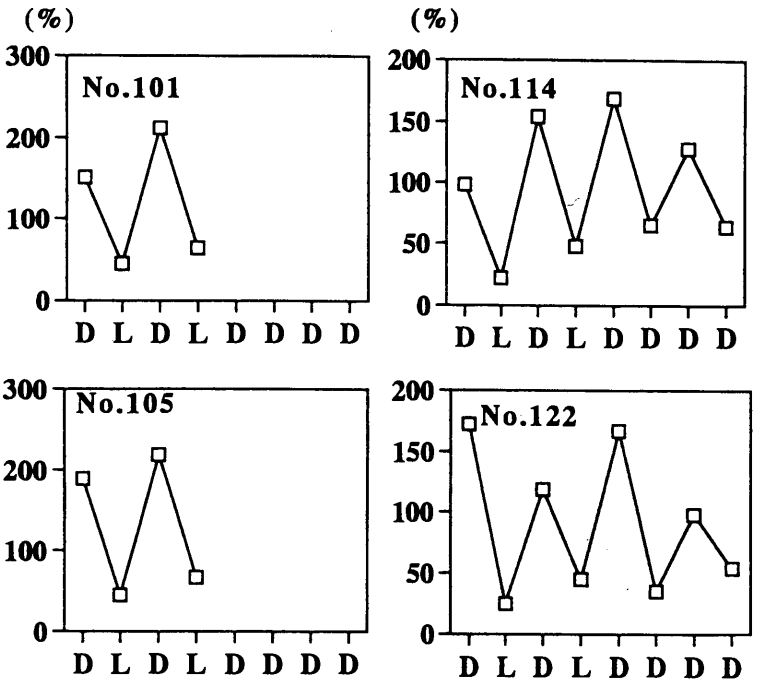


Fig.I-2. Examples of melatonin secretion pattern in individual pineal cells. Individual pineal cell cultures were kept under DL for 4 days then under DD. Samples were collected at 12 hour intervals for 2 days (No. 101, 105), or for 4 days (No. 114, 122) starting on day 3 of culture. All data are shown as percentage values relative to mean values.

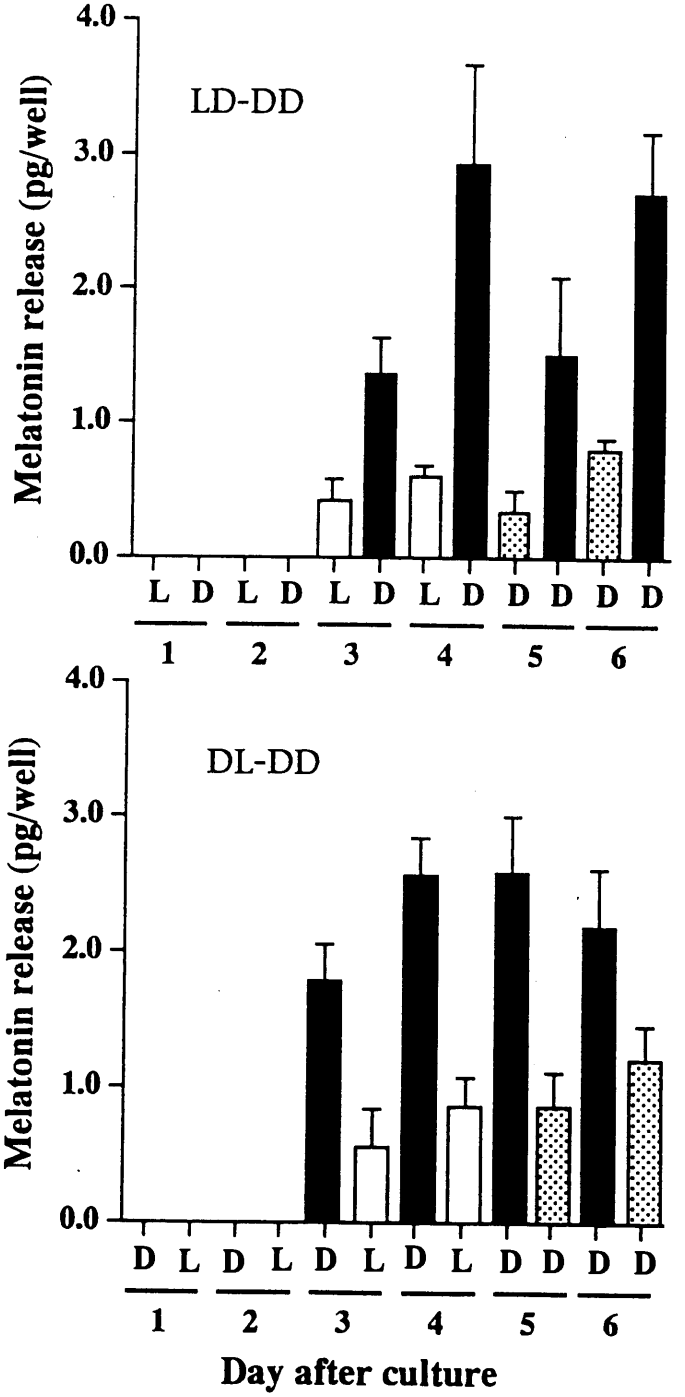


Fig.I-3. Mean levels of melatonin secreted from individual cells. Each bar and vertical line indicate mean ± SEM. The upper numbers indicate total number of samples. Dotted bar represents the melatonin value during the subjective day.

Chapter II

Involvement of protein kinase A in the subjective nocturnal rise of melatonin release by chick pineal cells in constant darkness

Abstract:

In chapter I, I showed that chick individual pineal cells possess the photoreceptor, circadian oscillator and melatonin synthetic capacity. Melatonin release from individual pineal cells increased during the dark periods and decreases during the light periods under light-dark cycles, and this rhythmic secretion was maintained under constant conditions. The mechanisms how circadian oscillator drives the melatonin increase under constant conditions have not been elucidated enough. Therefore, in this chapter, I examined the possibility that cyclic AMP and cAMP-dependent protein kinase A are involved in the subjective nocturnal increase in melatonin release by chick pineal cells cultured under constant darkness. The subjective nocturnal increase of melatonin release was suppressed dose-dependently by H8 (protein kinase inhibitor) and H89 (specific protein kinase A inhibitor), but not by calphostin C (specific protein kinase C inhibitor) in static cell cultures. In a cell perfusion experiment, 9-hour pulses of H8 and H-89 starting at ZT 9 (CT 11.2) h suppressed the subjective nocturnal increase in melatonin rhythm in dose-dependent manner without causing a phase shift. An intracellular Ca^{2+} chelator, BAPTA-AM, and extracellular Ca^{2+} chelators, BAPTA and EGTA, suppressed both the subjective nocturnal increases in melatonin release and cAMP levels dose-dependently. These direct evidences strongly support the hypothesis that camp and cAMP-dependent protein kinase A may be involved in the subjective nocturnal increase in melatonin release by chick pineal cells, and that intracellular Ca^{2+} plays an important role in pineal adenylate cyclase activation.

Introduction

In many vertebrates, melatonin release by the pineal gland shows circadian rhythm with the peak at night and trough in the daytime. The regulation of the rhythmic melatonin secretion varies with species. The rhythm of melatonin production by the mammalian pineal gland is generated by the circadian timing systems comprising visual projection, the circadian pacemaker (suprachiasmatic nucleus), and projection of sympathetic fibers to the pineal. Norepinephrine released from mammalian sympathetic fiber terminals stimulates pineal melatonin release through cAMP production [Klein, 1985; Zatz, 1982], but conversely, it inhibits melatonin synthesis in avian pineal glands by reducing cAMP levels [Deguchi, 1979a; Takahashi et al., 1989; Zatz and Mullen, 1988a]. The major difference between mammalian and avian pineal gland is that the circadian rhythms of the latter persist in vitro as well as in vivo [Deguchi, 1979b; Kasal et al., 1979]. The release of melatonin by cultured pineal cells of the chick, sparrow and pigeon increases during the dark periods and declines during the light periods of light-dark cycles, and their rhythms persist with a period close to 24 h when the cells were then cultured in constant darkness [Robertson and Takahashi, 1988a; Zatz et al., 1988; Murakami et al., 1994]. These observations demonstrate clearly that the circadian oscillator, photoreceptor and melatonin synthetic systems all reside within the pineal cells, and that their functions are coupled each other. In the previous chapter, I proved that circadian oscillator, photoreceptor and melatonin synthetic systems all reside within the individual pineal cells.

Many studies attempting to analyze the mechanisms responsible for melatonin production by chick pineal cells have been conducted. Reducing intracellular cAMP levels by norepinephrine treatment and exposure to light reduced melatonin synthesis [Zatz and Mullen1988a; Zatz et al., 1988; Takahashi et al, 1989]. Whereas forskolin (a specific adenylate cyclase activator) and 8-bromo cAMP (a cAMP analogue) stimulated

melatonin release [Zatz and Mullen, 1988b; Nikaido and Takahashi, 1989]. Furthermore, Bay K 8644 (a dihydropyridine calcium channel agonist) stimulated, but nitrendipine (a dihydropyridine antagonist of L-type calcium channels), cobalt and manganese ions (inorganic calcium channel blockers) and low external calcium ion reduced melatonin release by chick pineal cells [Zatz and Mullen, 1988c; Takahashi et al., 1989]. Although these experimental evidences show that cAMP and Ca^{2+} influx are involved in the synthesis and production of melatonin by chick pineal cells, there is no evidence showing whether pineal cellular cAMP and Ca^{2+} levels are regulated directly by the circadian oscillator or not. Nikaido and Takahashi (1989) observed clear circadian rhythms of both cAMP secretion and cellular cAMP content in cultured pineal cells, but they only studied cells cultured under light-dark cycle conditions. Zatz (1992a) suggested that cAMP may not be the predominant factor involved in control of the melatonin rhythm by the circadian oscillator, because no-oscillation of cAMP levels occurred in constant darkness (data not shown). I also observed no circadian rhythm of cAMP levels in constant darkness in perfusion experiments eight times and static culture experiments three times using various culture conditions (unpublished observations). These observations raise the question does endogenous cAMP really play a role in the subjective nocturnal increase in melatonin release under constant conditions. If the circadian oscillator drives the melatonin rhythm via alterations in cAMP levels, then cAMP-dependent protein kinase A may also be involved in controlling the melatonin rhythm. Therefore, in this study, I examined whether pulse treatment with protein kinase A inhibitors suppressed the subjective nocturnal increase in melatonin release by chick cultured pineal cells maintained in constant darkness. I used H89 as a specific protein kinase A inhibitor [Chijiwa et al., 1990; Maggi et al., 1996], calphostin C as a specific protein kinase C inhibitor [Kobayashi et al. 1989; Tamaoki and Nakano,

1990] and H8 as an inhibitor of both protein kinases A and C [Inagaki et al., 1985; Maggi et al., 1996].

Recently, D'Souza and Dryer (1996) found that an unusual 40-pS cationic channels in chick pineal cells that is permeable to Ca^{2+} and active in the subjective night but not during the subjective day. Their results indicate that circadian oscillator drives the rhythm of Ca^{2+} influx through regulating those cationic channels, and that their Ca^{2+} influx may play predominate role for circadian oscillation of melatonin release under both LD and DD conditions.

Therefore, I also examined whether intra- and extracellular Ca^{2+} are involved in the subjective nocturnal increase in melatonin release related to adenylate cyclase activation using intra- and extracellular Ca^{2+} chelators, BAPTA-AM, BAPTA and EGTA [Neugulescu et al., 1989; Michelangeli et al., 1989; Li and Mardh, 1996].

Materials and Methods

Chemicals

Medium 199, HEPES, calf serum and Hank's solution with and without calcium were purchased from GIBCO (Grand Island, NY). Penicillin-streptomycin, human transferrin, putrescine, sodium selenite, progesterone, melatonin, insulin, 3-isobutyl-1-methyl-xanthine (IBMX), collagen (type VII), trypsin, collagenase, trypsin inhibitor, gentamicin and 8-bromo adenosine 3', 5'-cyclic monophosphate (8-bromo-cAMP) were purchased from Sigma Chemical Co. (St Louis, MO). Percoll was purchased from Pharmacia (Uppsala, Sweden). The protein kinase inhibitors N-[2-(methylamino) ethyl]-5-iso-quinolinesulfonamide dihydrochloride (H8) and N-[2-(p-bromocinn- amylamino) ethyl]-5-iso-quinolinesulfonamide (H89) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan) and the protein kinase C inhibitor, calphostin C was from Kyowa Hakko (Tokyo, Japan). The intracellular Ca^{2+} chelator, *O,O´*-bis (2-aminophenoxy) ethyleneglycol-*N, N,N',N'*-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) and extracellular Ca^{2+} chelator, *O,O´*-bis (2-aminophenoxy) ethyl- eneglycol-*N,N,N',N'*-tetraacetic acid tetrapotassium salt hydrate (BAPTA), were purchased from Dojindo Co. (Kumamoto, Japan). All the other chemicals used were of reagent grade and obtained from commercial sources.

Cell culture

Chicks were raised from hatching until they were 2-4 weeks old under 12 h light: dark cycle (12L: 12D) conditions (lights on at 07.00 h). Pineal cell cultures were prepared as described previously [Murakami et al., 1995] using collagenase and trypsin digestion with the following modifications. The pineal glands were washed with Hank's

salt solution containing 100 μg/ml gentamicin, 1000 U/ml penicillin G and 1000 μg/ml streptomycin, then transferred to 10 ml Hank's salt solution containing 2 mg/ml collagenase, in which they were minced. They were incubated for 30 min in a 37 °C shaking water bath, and then centrifuged for 2 min at 100 ×g. The supernatant was decanted; 10 ml trypsin solution (0.5 mg/ml) and DNAse (300 U/ml) were added. The mixture was incubated for 5 min at 37°C, and 5 ml trypsin inhibitor solution (0.2 mg/ml) was added. The tissues were triturated repeatedly through a series of gradually narrower Pasteur pipettes, and then centrifuged for 5 min at 500 ×g. The resulting cell pellet was resuspended in a culture medium with the following composition: medium 199 with Hank's salt and L-glutamine supplemented with 10 mM HEPES buffer, 15% calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.9 mg/ml $NaHCO_3$. The cells were fractionated by iso-osmotic Percoll gradient centrifugation for 20 min at 450 ×g and the cells at the interface between 1.02 and 1.07 g/ml were collected and washed with a fresh culture medium. The majority of the cells at this dense interface have been demonstrated to be pinealocytes [Nikaido and Takahashi, 1989]. Finally, the cells were suspended in culture medium and placed in collagen-coated 96-well dishes at densities of 2.5×10^4 and 5×10^4 cells /well for the static cell culture and perfusion experiments, respectively, and maintained in an incubator at 37°C in an atmosphere of 95% air and 5% CO_2 under 12L: 12D conditions.

Cell culture for cAMP level measurement

Chick pineal cells were prepared as described above, suspended in serum-free culture medium with the following composition: medium 199 supplemented with 100 mg/ml human transferrin, 5 mg/ml insulin, 30 nM sodium selenite, 20 nM progesterone, 100 mM putrescine, 0.5 mg/ml sodium pyruvate, 10 ng/ml epidermal growth factor, 2 mM

glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.9 mg/ml $NaHCO_3$ and placed in collagen-coated 96-well dishes at a density of 5 x 10^4 cells per well. IBMX (1 mM) dissolved in dimethylsulfoxide (DMSO) was added immediately before each experiment.

Perfusion

The perfusion experiment was performed using the method described by Murakami et al. (1995), with minor modifications. Briefly, on the second day of culture, the culture dish was fitted with a silastic stopper connected to silastic input (0.02 in. ID) and output (0.04 in. ID) tubing that were connected to a 50-ml syringe and a fraction collector (Touyo Corp, Tokyo), respectively. The 50-ml syringe was loaded into a syringe pump (Harvard, 55-4299) and the wells were perfused with medium at a flow rate of 0.25 ml/h and the culture dish was housed in a light-controlled box and illuminated using a glass fiber rod. The cell perfusion apparatus comprised 20 parallel channels and samples were collected at 2-h intervals. Air-equilibrated culture medium with 15% calf serum was used for the cell perfusion experiment, and the entire apparatus was kept at 38 °C in a controlled humidity (85%) chamber.

In order to investigate the effects of H8 and H89 pulse treatment on the melatonin rhythm, medium containing H8 or H89 was perfused at 0.5 ml/h using a 10 ml syringe pump via a double 3-way valves for 9 h, starting at the zeitgeber time (ZT) 9 h, with ZT 12 h defined as the onset of the dark phase of the 12L:12D cycle (Fig. 1C), after which, normal medium was perfused at 0.7 ml/h for 3 h to wash the cells and tubes rapidly. The control cultures were perfused with normal medium according to the schedule for H8 and H89 treatment. After the pulse treatment, the perfusion rate was returned to 0.25 ml/h.

Radioimmunoassay

The melatonin contents of appropriate volumes (5- 25 μl) of culture medium samples were determined by radioimmunoassay [Taniguchi et al., 1993]. The radioiodinated and non-radioactive melatonin used as standards were purchased from New England Nuclear and Sigma, respectively. The rabbit anti-melatonin antibody (HAC-AA92-03RBP86) was supplied by Dr. K. Wakabayashi (Gunma University, Japan). The assay was validated for the culture medium without sample extraction, and no significant interference from the perfusion medium was detectable in the standard assay. The lower and upper detection limits of this radioimmunoassay were 7.5 and 2,000 pg/tube, respectively and the inter- (n=20) and intraassay (n=20) variation were 5.7 and 13.5%, respectively.

The samples used for cAMP level measurement were boiled at 95°C for 3 min immediately after collection, and stored -20°C until assayed. The cAMP concentrations of appropriate volumes (20 - 50 μl) of culture medium samples were measured using a cAMP radioimmunoassay kit (Amersham, Buckinghamshire, England), the upper and lower detection limits of which were 12.5 and 1600 fmol/tube, respectively. The assay was validated for the culture medium without extraction and the inter- (n=9) and intraassay (n=9) variations were 7.2 and 16.6%, respectively.

Experimental schedules

In the static cell culture experiment, the effects of the protein kinase inhibitors H8, H89 and calphostin C on the subjective nocturnal increase in melatonin release were examined using the schedule shown in Fig. II − 1A. Effect of H8 on 8-bromo-cAMP-induced melatonin release was examined using the schedule shown in Fig. II − 1B, and the effects of BAPTA, BAPTA-AM and EGTA on the subjective nocturnal increase in melatonin release and cAMP levels were examined using the schedule

shown in Fig. II −1A. In the perfusion experiment, the effects of pulses of H8 and H89 on the melatonin rhythm were examined using the schedule shown in Fig. II −1C. All the agents, except H8, which was directly dissolved in culture medium, were dissolved in DMSO, and then diluted for use with culture medium. The final concentrations of DMSO did not exceed 0.05%.

Data analysis

All the data obtained in the static cell culture experiment are expressed as percentages relative to the mean melatonin release or cAMP efflux values of the control. Normalized means were compared using analysis of variance followed by Student's *t* -test for unpaired data, and differences at $P<0.05$ were considered significant. In order to reduce the differences between the absolute melatonin rhythm amplitudes among the cell culture dishes in the cell perfusion experiment, the data are presented in normalized forms relative to the mean values of the records [Murakami et al., 1995]. This was performed by dividing the melatonin values from each channel by the mean value of the time series of that channel and multiplying by 100. Thus, the mean value for the time series of each channel corresponded to 100%. The phase of the melatonin oscillations was analyzed using the midpoint of the cycle as the phase reference point, according to the method of Robertson and Takahashi (1988b) and the midpoint phase reference was defined as the time midway between the half-rise and half-fall of each melatonin peak. The significance of differences between the time points was determined using Student's *t*-test. The period length was defined as the difference between 2 consecutive reference points, and the average period length for an individual cell dish was estimated by calculating the average of 3 periods derived from 4 cycles, and the variance about this mean was the inter individual variance.

RESULTS

Effects of protein kinase inhibitors on the subjective nocturnal increase in melatonin release in static cell culture.

In a preliminary study, I measured melatonin levels of static cell culture at 3-h intervals for 48 h from the initiation of constant darkness and found that the subjective nocturnal melatonin increase began at ZT 9-12 h [Akasaka et al., 1994]. Calculations using our perfusion data for the circadian period of 21.75 h of the melatonin rhythm during constant darkness indicated that ZT 9-12 h corresponded to the circadian time 11.5－14.4 h (although the reasons for this short period of free-running rhythm are unknown, I assume that it may be due to chick colony). Therefore, the effects of protein kinase inhibitor on the subjective nocturnal increase in melatonin release were investigated during ZT 9-18 h. The net control melatonin release values during this period was 12.32± 0.25 ng/2.5 x 10^4 cells (mean ± SE, n=18). Administration of H8 inhibited this subjective nocturnal melatonin release by chick pineal cells in a dose-dependent manner, and the minimum significant inhibition and >50% inhibitions were observed with a 10 and 250 μM H8, respectively (Fig. II－2). Administration of calphostin C at dose known to inhibit the protein kinase C and that were at least three orders of magnitude less effective at inhibiting protein kinase A [Kobayashi et al. 1989; Tamaoki and Nakano, 1990] had no effect on melatonin release. At dose over 24 mM, calphostin C induced morphological cell damages (data not shown).

Pulse treatment with H89, a specific protein kinase A inhibitor, also inhibited the subjective nocturnal increase in melatonin release in a dose dependent manner (Fig. II－3), and the minimum and >50% inhibition were observed with 5 and 40 μM, respectively.

It has been reported that at 5-30 μM H8 specifically inhibited protein kinase A stimulated by 1 mM of dibutyryl cAMP in PC12D cells [Chijiwa et al., 1990]. As H8 inhibited protein kinase A and C dose-dependently, I tested whether 100 μM H8 inhibited the 8-bromo-cAMP induced melatonin release by administering 100 μM of H8 together with 0.1, 0.5 or 1.0 mM 8-bromo-cAMP to cultured pineal cells according to the schedule shown in Fig. 1B. Treatment with 8- bromo-cAMP for 6 h increased melatonin release in a dose-dependent manner. As H8 already suppressed the melatonin release without 8- bromo-cAMP, net effect of H8 on the 8-bromo-cAMP induced melatonin release were observed against doses of 0.5 and 1 mM 8-bromo-cAMP ($P< 0.05$) (Fig. II −4).

Effects of H8 and H89 pulses on the circadian rhythm of melatonin release by chick perfused pineal cells.

In order to ensure that the inhibition of the subjective nocturnal increase in melatonin release by H8 and H89 was not due to an acute phase shift of the circadian oscillator itself, H8 and H89 pulses were given at ZT 9-18 h (Fig. II −1C). As shown in Fig. II −5, control chick pineal cells expressed circadian rhythm of melatonin release under light-dark cycle conditions and the rhythm persisted in constant darkness despite the amplitude declining progressively. Nine-hour pulses of H8 (Fig. II −5C) and H89 (Fig. II −6) suppressed the subjective nocturnal increase in melatonin release dose dependently and the magnitudes of the phase shifts induced by H8 (75 mM) and H89 (40 mM) relative to the control were 0.59 (advance) ± 0.54 h and 0.37 (advance) ± 0. 23 h (mean ± 95% confidence limits for 5 cell dishes, n=6). Statistical analysis revealed that there were no significant phase differences between the control and H8 or H89 groups. Furthermore, the effects of H8 and H89 on melatonin release were reversible and no rebound effects after cessation of exposure to these agents were observed.

Effects of intra- and extra cellular calcium chelators on the subjective nocturnal increase in melatonin release and cAMP levels.

The intracellular calcium chelator BAPTA-AM inhibited the subjective nocturnal increase in melatonin release by chick pineal cells in a dose-dependent manner with minimum significant inhibition at 5 mM (Fig. II −7A), and it reduced cAMP levels dose dependently (Fig. II −7A). The net control cAMP efflux value was 329.6 ± 28.4 fmol/10^4 cells/9 hr (n=24). I observed the condition of the cells after incubation with BAPTA-AM, and almost all of them were healthy after exposure to all the doses used.

The extracellular calcium chelators EGTA (Fig. II −7B) and BAPTA (Fig. II −7C) at doses over 1 mM also inhibited the subjective nocturnal increase in melatonin release (Fig. II −7B,C), and at 1 mM EGTA, but not BAPTA, inhibited melatonin release only significantly. Pineal cells cultured with 5 and 10 mM EGTA and BAPTA did not look healthy, and were almost all fibroblasts probably including pineal cells cell that had become detached from the bottoms of the wells.

Discussion

It has been hypothesized that cAMP is involved in the regulation of melatonin production and its rhythm in chick pineal cells [Zatz and Mullen, 1988b; Zatz, 1989; Nikaido and Takahashi, 1989; Pratt and Takahashi, 1989]. However, those observations don't necessarily mean that circadian oscillator drives the circadian rhythm of melatonin via cAMP under constant conditions. There is no evidence to show that the circadian oscillator drives the robust circadian rhythm of cAMP levels under constant as well as light-dark conditions, nor any showing that blockade of cAMP production erases the melatonin rhythm of chick pineal cells maintained under constant conditions. In this study, H8 and H89 both suppressed dose dependently the subjective nocturnal increase in melatonin release in both the static cell culture and perfusion experiments conducted in constant darkness, inhibitory effects were not due to an acute phase shift of the oscillator. H8 and H89 have been demonstrated to inhibit protein kinase C and Ca^{2+}/calmodulin-dependent kinase II, as well as cAMP-dependent protein kinase, which one they inhibit depends on the concentrations used [Inagaki et al., 1985; Chijiwa et al., 1990; Maggi et al., 1996]. Inhibition of protein kinase C and Ca^{2+}/calmodulin kinase II required 10-100 times higher concentrations than did inhibition of cAMP-dependent protein kinase. In fact, the concentrations I used in this study have been shown to inhibit protein kinase A in cultured cells [Inagaki et al., 1985; Chijiwa et al., 1990; Maggi et al., 1996]. Furthermore the specific protein kinase C inhibitor, calphostin C, did not suppress melatonin release by chick cultured pineal cells. Therefore, these results suggest that the inhibition of the subjective nocturnal increase in melatonin release by H8 and H89 was due to inhibition of protein kinase A, and, cAMP and cAMP-dependent protein kinase seem to be a key factors in this increase under conditions of constant darkness.

In order to produce cAMP during subjective night, an adenylate cyclase must be activated by appropriate signals from the circadian oscillator, and the most likely candidate signal is Ca^{2+}/calmodulin, as it has been demonstrated to activate some types of adenylate cyclase [Tang et al., 1991; Impey et al, 1994]. In additions, there are several reports concerning the relationships between Ca^{2+} and melatonin and cAMP levels in chick pineal cells [Takahashi, et al., 1989; Zatz, 1992b]. The nocturnal increase in melatonin was suppressed by inorganic calcium channel blockers and dihydropyridine antagonists [Harrison and Zatz, 1989; Zatz and Mullen, 1988c]. Both reduction of extracellular Ca^{2+} levels with EGTA and retardation of Ca^{2+} influx by nitrendipine, and cobalt ions, also blocked melatonin synthesis [Zatz and Mullen, 1988c; Zatz and Heath, 1995]. In the present study, I demonstrated that intra- and extracellular Ca^{2+} was required for the subjective nocturnal increase in melatonin by chick pineal cells. In addition, both types of chelators reduced both cAMP levels and melatonin release dose- dependently, suggesting that intracellular Ca^{2+}, at least, play an important role in activation of adenylate cyclase in chick pineal cells. These results indicate the following stream ; increase of Ca^{2+} influx activate the adenylate cyclase, then increased cAMP activate the protein kinase A, and activated protein kinase A stimulate melatonin synthesis. If the subjective nocturnal rise of melatonin is caused along that stream, Ca^{2+} influx must be regulated by circadian clock. Recently, D'Souza and Dryer (1996) found that an unusual 40-pS cationic channels in chick pineal cells that is permeable to Ca^{2+} and active in the subjective night but not during the subjective day. Their result indicate that circadian oscillator drive the rhythm of Ca^{2+} influx through regulating those cationic channels. The present study strongly supports their hypothesis that circadian oscillator regulates this non-selective cation channel and that their Ca^{2+} influx regulate cAMP production and melatonin synthesis.

Zatz (1992a) demonstrated that circadian rhythm of melatonin release persisted even when cAMP levels were saturated. In order to explain this observation by Zatz, it may be necessary to consider another intracellular factors than Ca^{2+} influx regulation. Sun et al. (1994) reported that calmodulin kinase IV activated the cAMP responsible elements binding protein (CREB), whereas calmodulin kinase II suppressed them in vitro. If calmodulin kinase IV in chick pineal cells is activated during subjective day, then melatonin release may be reduced even when the cAMP levels is high. On the other hand, Bernard et al., (1997) demonstrated recently that circadian clock regulate the circadian rhythm of melatonin at the N-acetyltransferase (NAT) mRNA levels, whereas cAMP regulate NAT activity without change in mRNA levels. Theses results suggest that circadian rhythms of melatonin reflect NAT mRNA rhythm regulated by circadian clock, and cAMP need only to enhance the activation of NAT. However, further studies are required to elucidate how circadian clock regulate both the cation channels and the NAT mRNA.

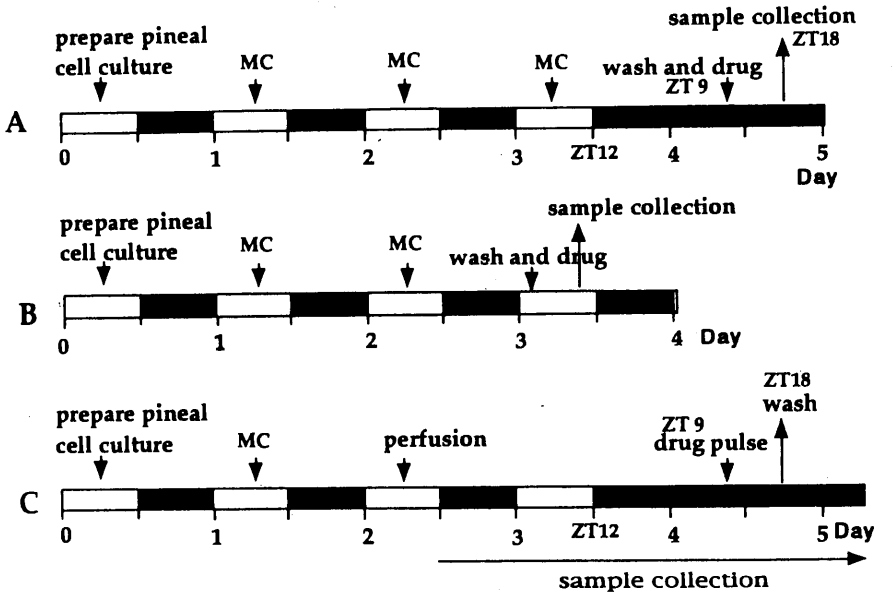


Fig. II − 1. Experimental protocols. Cultured pineal cells were maintained under conditions of a 12 h light: dark cycle, indicated as white and black bars, respectively, and then in constant darkness. The culture medium was changed every day, shown by MC (medium change). A and B are static cell culture experimental protocols and C is the perfusion experimental protocol. Experimental drugs were administered just after washing the cells, indicated by arrows.

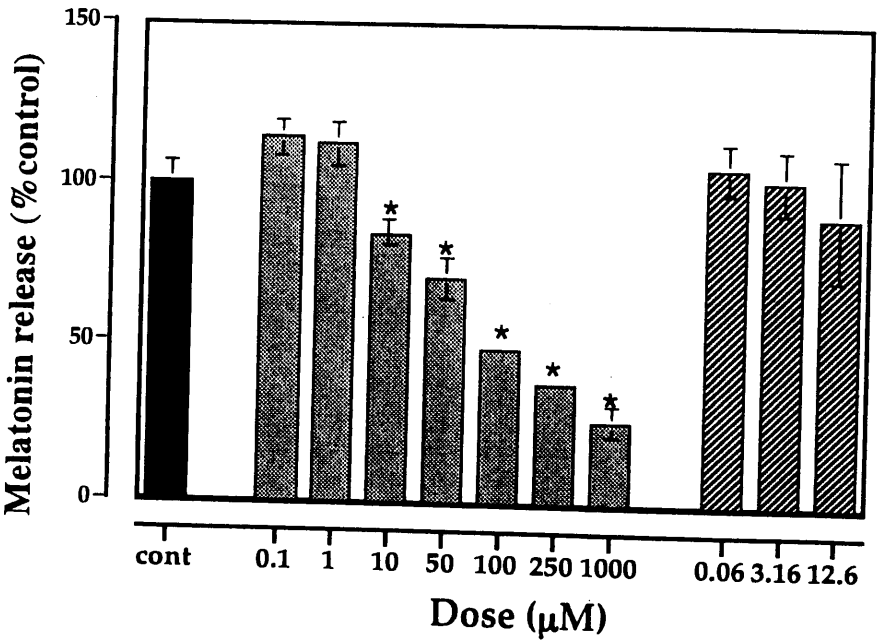


Fig. II－2. Effects of H8 and calphostin C on the subjective nocturnal increase in melatonin release by chick pineal cells. This experiment was performed according to the schedule shown in Fig. II－1A. Dotted and dashed bars represent H8 and calphostin C, respectively. The results are presented as percentages of the control values, and means ± SEM of 8 wells per group are shown. *P<0.05 compared with the control value.

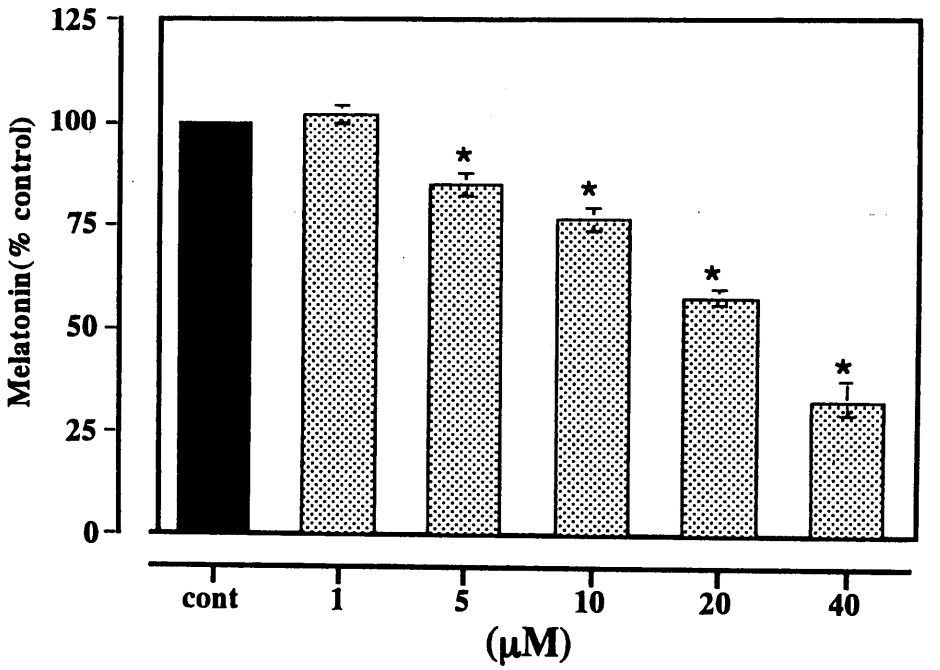


Fig. II－3. Effect of the H89 on subjective nocturnal increase of melatonin release by chick pineal cells. This experiments was performed according to the schedule shown in Fig. II－1A. The results are presented as percentages of the control values, and means ± SEM of 8 wells per group are shown. *P<0.05 compared with the control value.

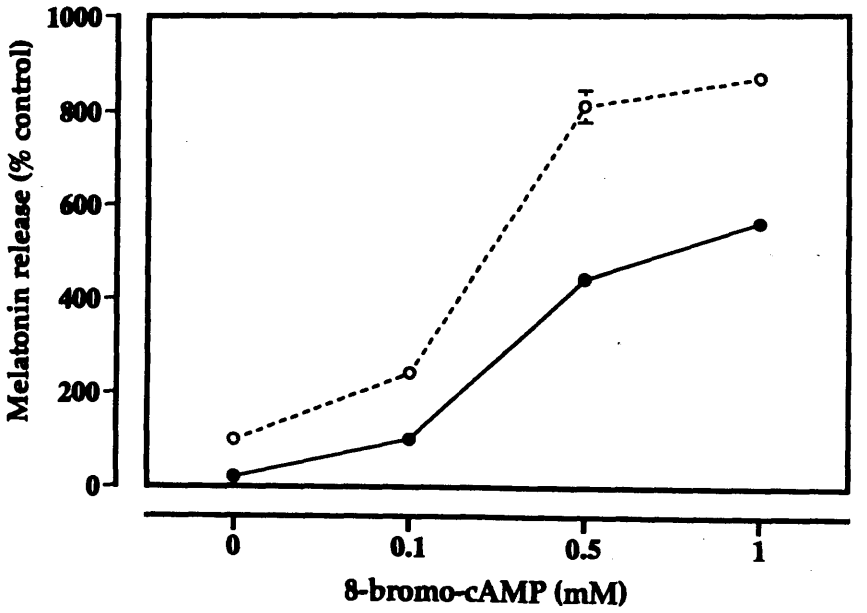


Fig. II－4. Inhibition by H8 of 8-bromo-cAMP-induced melatonin release by chick pineal cells. This experiment was performed according to the schedule shown in Fig. II －1B. The open circles connected with dotted lines represent melatonin release by pineal cells cultured with various doses of 8-bromo-cAMP, and the black circles represent that by pineal cells cultured with 8-bromo-cAMP and 100 μM H8. The results are presented as percentages of the control values, and means ± SEM of 6 wells per group are shown. H8 inhibited melatonin release induced by 8-bromo-cAMP in the all groups significantly.

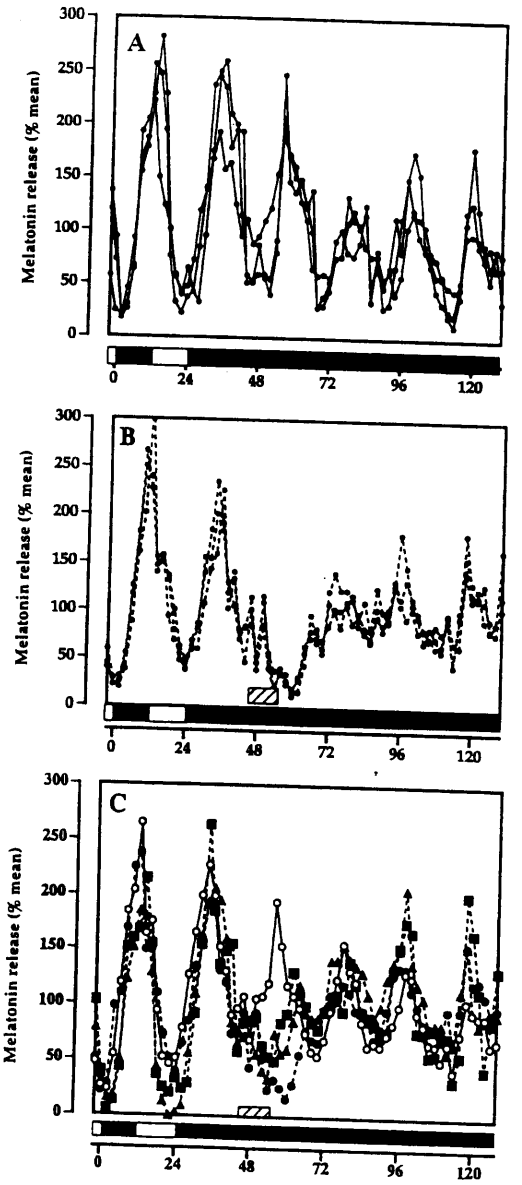


Fig. II−5. Effect of H8 pulse treatment on the circadian rhythm of melatonin release by chick pineal cells. This experiment was performed according to the schedule shown in Fig, II−1C. A and B: Superimposed individual records from 3 replicate wells that received 9 h pulses (indicated as a dashed box) of control medium (A) and 75 μM H8 (B) beginning at ZT 9h. C: Dose dependent inhibition of the subjective nocturnal increase in melatonin release by pulses of 0 (open circle) , 10 (black square), 25 (black triangle) and 75 (black circle) μM H8. Each symbol represents the mean value of 3-6 wells.

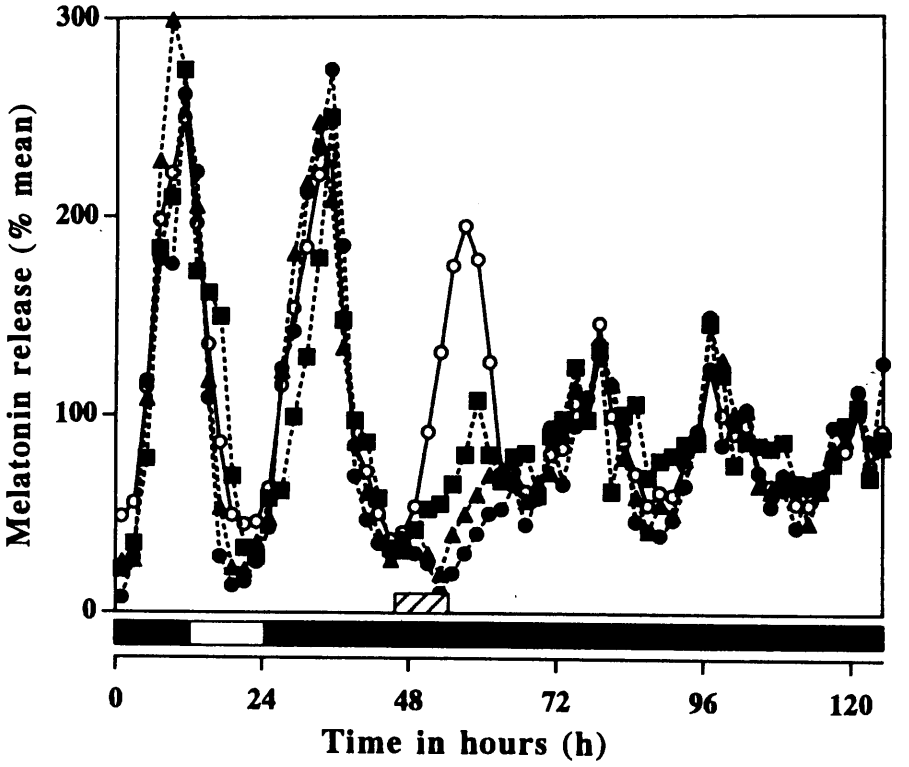


Fig. II — 6. Effects of H89 pulse treatment on the circadian rhythm of melatonin release by chick pineal cells. Dose dependent inhibition of the subjective nocturnal increase in melatonin release by pulses of 0 (open circle), 10 (black square), 20 (black triangle) and 30 (black circle) μM H89. Each symbol represents the mean value of 3 wells.

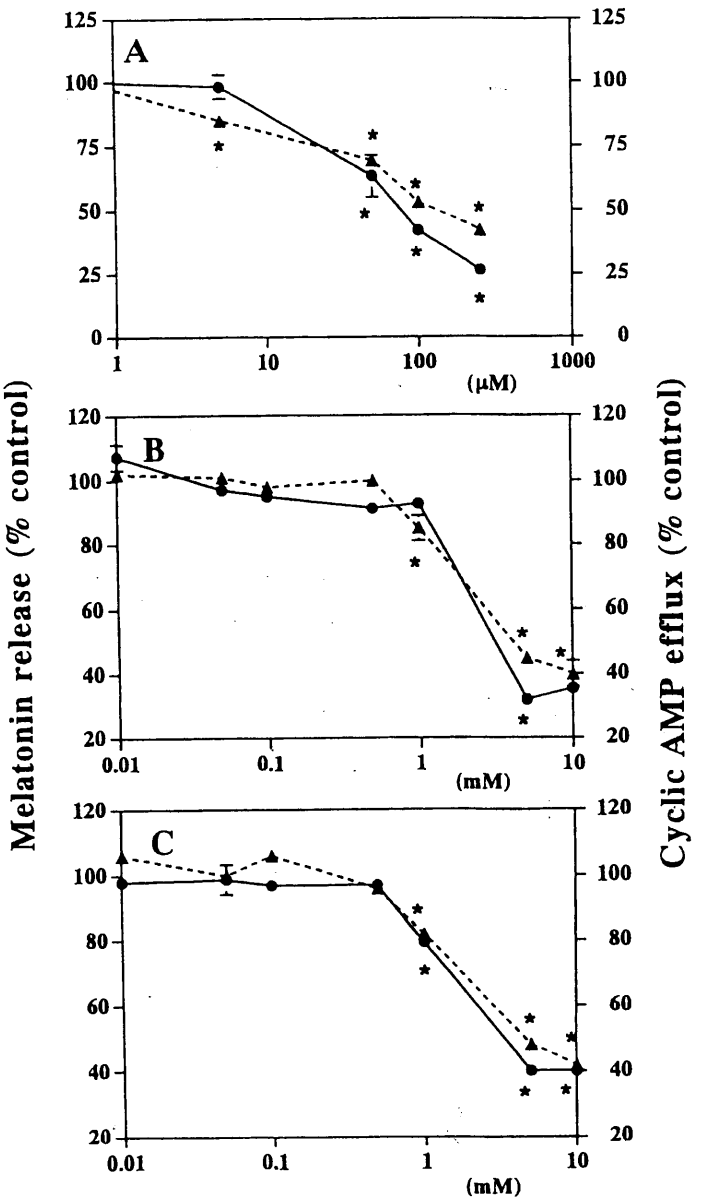


Fig. II－7. Dose-response curves for the effect of BAPTA-AM (A), EGTA (B) and BAPTA (C) on the melatonin release and cAMP efflux. This experiment was performed according to the schedule shown in Fig. II－1A. The black triangles connected with dashed lines and black circles connected with unbroken lines represent the melatonin and cAMP levels, respectively. Results are presented as percentages of the corresponding control (n=24) values, and means ± SEM of 8 wells per drug group are shown.

Chapter Ⅲ

Pituitary adenylate cyclase-activating polypeptide (PACAP) is involved in melatonin release via the specific receptor PACAP-r1, but not in the circadian oscillator, in chick pineal cells

Abstract

In the chapter I and II, I showed that individual chick pineal cells have photoreceptor, circadian clock and melatonin synthetic capacity, and these components link each other. Under constant light condition, circadian clock initiates the subjective increase of melatonin via activation of adenylate cyclase. However, *in vivo*, the regulatory mechanism of melatonin release has not been clarified yet. Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates melatonin release from pineal cells and modulates glutamatergic regulation of the suprachiasmatic circadian clock in rats. In this chapter, therefore, I investigated whether PACAP is involved in melatonin release and the circadian oscillation system in chick pineal cells, and if so, whether its effects are mediated by the PACAP-specific receptor (PACAP-r1) or the vasoactive intestinal polypeptide (VIP) receptor. Chick pineal cells were maintained for 4 days under a 12 h light: 12 h dark cycle, and thereafter-constant darkness. In the dose-range 10^{-10} to 10^{-6}M, PACAP increased melatonin release dose-dependently during the 12-h light period on day 3 of culture, and the degree of stimulation was greater than that produced by VIP. VIP receptor antagonists only slightly inhibited PACAP-stimulated melatonin release. Simultaneous addition of VIP and PACAP produced almost additive melatonin release. Under constant dark conditions, 6-h pulses of PACAP started at zeitogaber times (ZT) 15, 21, 3 and 9 h in separate groups of pineal cells did not cause any phase shift in their melatonin rhythm. In addition, PACAP did not affect the light-induced phase advance (ZT 15 h) and delay (ZT 9 h) in melatonin rhythms. The expression of mRNA for the PACAP-r1 (including its splicing variant with a hop cassette) was observed in chick pineal cells. These results suggest that PACAP participates in melatonin release, but not in the circadian oscillator system, via the specific receptor PACAP-r1 in chick pineal cells.

Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the secretin/gastrin/VIP superfamily of peptides, and three types of receptors for VIP and PACAP have been characterized: VIP_1/PACAP and VIP_2/PACAP receptors, which show similarly high affinity for both VIP and PACAP, and the $PACAP_1$ receptor (PACAP-r1), which shows 100–1000-fold higher affinity for PACAP [Arimura,1992; Arimura,1994; Chatterjee et al.1997; Shivers et al.1991; Usdin et al.1994]. Recently, it has been demonstrated that PACAP stimulates melatonin release via increased cAMP levels in rat pineal cell cultures [Chik and Ho,1995; Simonneaux et al.1990, Simonneaux et al.1993]. PACAP concentrations in the rat pineal gland show a circadian rhythm with increases and decreases during the dark and light periods, respectively [Fukuhara et al.1998]. In addition, PACAP is localized within the retino-hypothalamic tract, and pulsed PACAP treatment causes phase shifts of the circadian rhythm in a manner similar to light in rats [Hannibal et al.1997; Hannibal et al, 1998; Harrington et al.1999]. On the other hand, when PACAP is coadministered with glutamate, the neurotransmitter that conveys light signals to the suprachiasmatic nucleus, PACAP blocks the phase advance induced by glutamate during the late night, while blockade of PACAP neurotransmission with a PACAP antagonist or antibody augments the glutamate-induced phase advance [Chen et al.1999; Hannibal et al, 1997]. These results indicate that 1) PACAP may be involved in melatonin release in the rat pineal gland, 2) PACAP may be a pivotal modulator of glutamatergic regulation of the suprachiasmatic circadian oscillator in rats.

Chick pineal cells, unlike those in the rat, express a circadian rhythm of melatonin release under light-dark cycles (LD), with an increase during the dark period and a decrease during the light period, and this rhythm persists under constant darkness (DD) *in vitro* [Takahashi et al, 1989; Zatz and Mullen 1988c]. In chapter I,

I have demonstrated that individual chick pineal cells have photoreceptor, circadian clock and melatonin synthetic capacities, and that these components are linked to each other. The regulation of melatonin release has been well-documented in chick pineal cell cultures. Briefly, direct light exposure and norepinephrine inhibit melatonin release by reducing cAMP levels, whereas forskolin (a specific adenylate cyclase activator) and 8-bromo-cAMP (a cAMP analogue) stimulate release [Takahashi et al. 1989; Zatz and Mullen,1988a,b]. Bay K 8644 (a dihydropyridine calcium channel agonist) also stimulates melatonin release, but nitrendipine (a dihydropyridine antagonist of L-type calcium channels), cobalt and manganese ions (inorganic calcium channel blockers) and low external calcium ion concentrations reduce release [Zatz and Mullen, 1988c]. Vasoactive intestinal polypeptide (VIP) has also been shown to increase melatonin release in chick pineal cells [Pratt and Takahashi, 1989; Zatz, 1989]. Therefore, as VIP or cAMP agonists stimulate melatonin release in chick pineal cells, treatment with PACAP may also stimulate melatonin release through common VIP/PACAP receptors. In this chapter, therefore, I investigated whether treatment with PACAP stimulates melatonin release in chick pineal cell cultures, and if so, which type of PACAP receptors are involved. To determine the receptor type, two experiments were performed. One was a pharmacological experiment using VIP receptor antagonist, and the other examined the expression of chick PACAP-r1 mRNA in the pineal gland. I also investigated whether PACAP is involved in the circadian oscillator system or light transmission in chick pineal cells as well as the rat circadian systems.

Materials and Methods

Cell culture

Chicks were raised from hatching under 12:12 h LD (lights on at 07.00 h) photoperiod conditions in our laboratory until they were 2–4 weeks old, and then killed by decapitation. All procedures were done in accordance with the Japanese Physiological Society's guidelines for animal care. Their pineal glands were then removed and placed in cold Hank's salt solution (GIBCO, Grand Island, NY). The tissue was washed with Hank's salt solution containing 100 μg/ml gentamicin (Funakoshi, Tokyo), 1000 U/ml penicillin G and 1000 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO), transferred to 10 ml Hank's salt solution containing 2 mg/ml collagenase (Type IV; Sigma), minced and incubated for 30 min in a water bath at 37 °C with shaking. After centrifugation for 2 min at 100-x *g*, the supernatant was decanted and 10 ml papain solution (0.5 mg/ml; Sigma) was added. The tissue was incubated for a further 10 min at 37 °C, triturated repeatedly through a series of gradually narrowing Pasteur pipettes, and then centrifuged for 5 min at 500-x *g*. The resulting cell pellet was resuspended in culture medium containing Medium 199 with Hank's salt and L-glutamine, supplemented with 10 mM HEPES buffer, 15% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.9 mg/ml $NaHCO_3$. The cells were then fractionated by iso-osmotic Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation at 450-x *g* for 20 min, and those at the interface between the 1.02 g/ml and 1.07 g/ml layers were collected and washed with culture medium. Finally, the cells were suspended in culture medium, placed in collagen-coated 96-well dishes at a cell density of 2.5×10^4, and maintained in an incubator at 38 °C with a 95% air and 5% CO_2 atmosphere under a 12:12 h LD cycle (lights on at 07:00 h; light was supplied through glass fibers) for four days, and then under DD conditions for three days. All equipment (for example, the portable

cleanbench, CO_2 incubator and microscope) was kept at 37 °C in a controlled humidity (85%) chamber, and all treatments (for example, sampling and medium changes) were performed in the chamber in order to prevent any change in temperature.

Experimental schedule

In the first experiment, to examine the effects of PACAP and VIP on melatonin release, the cells were incubated with or without various doses of rat, human or bovine PACAP27 (Alex Co., U.S.A) and porcine VIP (Peptide Inc. Co., Osaka Japan) or a combination of VIP+PACAP. In addition, various doses of PACAP and VIP were added in the presence of 100 and 1000 nM of a VIP receptor antagonist. The following two VIP receptor antagonists were tested: porcine VIP-[pC- D-Phe^6, Leu1^7] and VIP-[Ac-Tyr^1, D-Phe^2]GRF (8-29).

In the second experiment, we investigated whether 6-h pulses of PACAP cause phase-dependent phase shifting of the circadian melatonin rhythm under constant darkness. The 10^{-7}, $10^{-8}$and 10^{-9} M PACAP pulses were administered at zeitogaber times (ZT) 15, 21, 3 and 9 h, to separate groups of cells, where ZT 12 marks the initiation of DD. The first samples were collected at 19:00 h just before the initiation of DD; further samples were collected and replaced with fresh medium at 3-h intervals for three days. An infrared microscope was used for sampling under DD.

In the third experiment, I determined whether PACAP blocked or augmented the light-induced phase shift. Five-hour light pulses are administered using light fibers in another CO_2 incubator, starting at ZT 15 for the phase advance and ZT 9 for the phase delay. Light pulses that were not at the maximum intensity of illumination for the magnitude of the phase shift (30 lux) were used. The 10^{-7}M PACAP pulses were initiated 1 h before the start of the light pulses. The start and end times for the pulses

were the same as the sampling times. After the 6-h pulse period, the cells were washed very gently three times with fresh medium, then placed in normal medium. The control wells (containing cells given light pulses only) were also washed with fresh medium as above. For each experiment, each treatment was administered to 5-6 wells, and three independent repeats of each experiment were performed.

Expression of chick PACAP-r1 mRNA

Five pineal glands and one complete brain were collected from chicks, and from rats as a negative control. The tissues were immediately prepared for total RNA extraction using Trizol (Life Technologies Inc., Grand Island, NY). Two micrograms of total RNA was reverse-transcribed using 200 U SuperscriptII reverse-transcriptase (Gibco/BRL, Rockville, MD) with 500 ng oligo (dT) primers in a 20-. l reaction volume containing 20 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each dNTP and 40 units of RNAse inhibitor. For polymerase chain reaction (PCR) amplification, 1 . l of the reverse transcription product was incubated with 25 pmol of the 5' and 3' primers and 0.5 U Taq DNA polymerase (Promega) in a 50-. l reaction mixture including 1 x reaction buffer, 1.5 mM $MgCl_2$ and 0.2 mM each dNTP. The oligonucleotide primers for the chick PACAP1 and VIP receptors were made according to previously published data [Peeters et al.1999] or the GeneBank AB029895 database, and were purchased from Funakoshi (Tokyo, Japan). The sequences were as follows: chick PACAP1 receptor: forward, 5'-TCCAACT-ACTTCTGGCTGTTC-3'; reverse, 5'AGTGT-AGAGCG-AGCCAAT -CTC-3'; chick VIP receptor: forward, 5'-CACCCTGTTG-GTCATCT-CCT-3'; reverse, 5'-ATCAGCAGCA-AGGTGGACTT-3'. The PCR cycle parameters were typically 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C for 30 cycles. The PCR products were separated on a 2% agarose/Tris borate EGTA gel and visualized under

ultraviolet illumination after ethidium bromide staining.

Melatonin assay

The melatonin contents of appropriate volumes (10-50 μl) of the culture medium samples were determined by radioimmunoassay [Murakami et al.1994]. The radioiodinated and nonradioactive melatonin used as standards were purchased from New England Nuclear and Sigma, respectively. The rabbit anti-melatonin serum (HAC-AA92-03RBP86) was supplied by Dr. K. Wakabayashi (Gunma University, Japan). The assay was validated for the culture medium without sample extraction, and no significant interference by the perfusion medium was detectable in the standard assay. The lower and upper detection limits of the radioimmunoassay were 15 and 2,000 pg/tube, respectively, and the inter- (n=20) and intra-assay (n=20) variations were 3.6% and 7.4%, respectively.

Data analysis

In order to reduce differences between the absolute melatonin rhythm amplitudes within each well or dish, the data were normalized relative to the mean values by dividing the melatonin values from each well (n=4-6/single experiment, multiplied by 3 or 4 for repeated experiments) by the mean value obtained over the entire time series for that well and multiplying by 100 [Murakami et al.1994]. Thus, the mean value for the time series of each well corresponded to 100%. The phases of the melatonin oscillations were analyzed using the midpoint of the cycle as the phase reference point, according to a previously described method [Murakami et al.1995] with the additional use of a computer program for interpretation. The midpoint phase reference is defined as the time midway between the half-rise and half-fall of each melatonin peak. To analyze phase shifts, at first, the midpoint phase reference of each

well was determined during the first or second cycle following the pulse treatment. Then, to obtain the magnitude of the phase shift by PACAP or light pulse, the phase difference between wells that received PACAP or light treatment and untreated medium was calculated using the difference between each of the midpoint references. The significance for each time point was determined using Student's *t* test. The data for melatonin release or magnitude of the phase shift were analyzed by ANOVA followed by Fisher's PLSD test *post hoc*. Differences at $P < 0.05$ were considered significant.

Results

PACAP27 produced a dose-dependent increase in melatonin release during the light period in chick pineal cells (Fig. III-1A open circles). A significant increase was observed with doses of 10^{-10} M upwards and the maximum was observed at 10^{-7}M (EC_{50}, 2.24 nM). As in previous reports [Pratt and Takahashi,1989; Zatz et al.1990], VIP also dose-dependently stimulated melatonin release in chick pineal cells (EC_{50}, 5.6 nM). However, the stimulatory effect of VIP was smaller than that of PACAP at the same dose (Fig. III-1. A open circles vs B open circles). When PACAP was added to the pineal cell culture medium in the presence of two types of the VIP antagonists at 100 and 1000 nM (triangles: VIP-[pC- D-Phe^6, Leu1^7] and squares: VIP-[Ac-Tyr^1, D-Phe^2]GRF [8-29]), PACAP-stimulated melatonin release was significantly inhibited by both types of antagonists at 1000 nM ($P<0.05$ PACAP vs PACAP+ VIP-[pC- D-Phe^6, Leu1^7], $P<0.05$ PACAP vs PACAP+ VIP-[Ac-Tyr^1, D-Phe^2]GRF [8-29]), but not at 100 nM (Fig. III-1A). On the other hand, the effect of VIP on melatonin release was significantly inhibited by the two types of antagonists at both 100 nM and 1000 nM ($P<0.05$ VIP vs VIP+100 nM VIP-[pC-D-Phe^6, Leu1^7]or VIP-[Ac-Tyr^1, D-Phe^2]GRF [8-29] , $P<0.01$ VIP vs VIP+ 1000 nM VIP-[pC- D-Phe^6, Leu1^7] or VIP-[Ac-Tyr^1, D-Phe^2]GRF [8-29]) (Fig. III-1B). When VIP and PACAP were added simultaneously, their effects on melatonin release were almost additive until maximum levels were reached (Fig. III-1. C).

The 6-h pulse treatment with PACAP at ZT 15, 21, 3, and 9 h did not cause any phase shifting in the melatonin rhythm in chick pineal cells under constant darkness (Fig. III-2). However, PACAP increased melatonin release in a dose-dependent manner. This stimulatory effect was consistently observed, even when the pulse was administered during the trough of the melatonin rhythm. PACAP pulses did not affect the light-induced phase advance (ZT 15 h) or delay (ZT 9 h) in the melatonin

rhythms. However, the acute reduction of melatonin release by light was inhibited by PACAP (Fig. III-3).

Amplification of complementary DNA from the chick pineal gland and brain with specific primers for the chick VIP and PACAP-r1 receptors confirmed the specificity of the assay for chick tissue. The receptors were detected in the chick tissue, but not the rat tissue (Fig. III-4). The PACAP-r1 receptor was expressed as two splicing forms: PACAP1s, which is a short splicing variant devoid of any hip or hop cassette, and PACAP1/hop, which is a splicing variant with a hop cassette. The latter was expressed at a lower level than PACAP1s.

Discussion

The present study demonstrates that, like VIP [Pratt and Takahashi,1989; Zatz et al.1990], PACAP stimulates melatonin release in chick pineal cells. However, its mechanism of action appears to be different in chicks from that in rats. In rats, there is pharmacological evidence that the effects of VIP and PACAP on melatonin release are not additive, and that PACAP-stimulated melatonin release is completely inhibited by VIP1/PACAP receptor antagonists [Simonneaux et al.1998]. Although 1000 nM of VIP receptor antagonists blocked VIP-induced melatonin release in chicks, they had only a slight inhibitory effect on PACAP-stimulated melatonin release. These results suggest that, in chick pineal cells, PACAP stimulates melatonin release via the PACAP receptor and not the VIP receptor. Furthermore, the effects of VIP and PACAP were additive in chicks. This additive effect on melatonin release may be due to the different receptors in chick pineal cells. The fact that mRNA for the PACAP-r1 receptor with a hop cassette, a specific splicing form, was expressed during the chick pineal complementary DNA amplification experiment supports this hypothesis. On the other hand, the possibility remains that PACAP may stimulate melatonin release via not only cAMP but also an increase of cytosolic Ca^{2+} in the chick pineal gland, as it does in rat pinealocytes [Olcese et al.1996], since our preliminary experiment showed that although melatonin levels did not reach maximum at 10^{-8} M PACAP, cAMP levels reached a plateau at a dose of 10^{-8}M PACAP (data not shown). Further study may be required to elucidate the mechanism of PACAP action on melatonin release.

Although the PACAP-r1 receptor has recently been identified in the chick brain, its role remains unclear [Peeters et al.1999]. In rats, several lines of evidence indicate that PACAP plays a pivotal role in the circadian oscillator systems. PACAP receptors are localized within the retinohypothalamic tract, and PACAP pulse treatment causes phase shifts in the circadian rhythm in a manner similar to light [Hannibal et al.1997;

Hannibal et al.1998]. On the other hand, when PACAP is coadministered with glutamate, the neurotransmitter that conveys light signals to the suprachiasmatic nucleus, it blocks the phase advance induced by glutamate during the late night [Chen et al.1999, Hannibal et al.1997]. Furthermore, blocking of PACAP neurotransmission with a PACAP antagonist or antibody augments the glutamate-induced phase advance [Chen et al.1999; Hannibal et al.1997]. These results indicate that PACAP may be a pivotal modulator of glutamatergic regulation in the suprachiasmatic circadian oscillator. In the present study, however, PACAP affected neither the circadian rhythm of melatonin release nor the light-induced phase shift in chick pineal cells. Although cAMP is involved in the circadian oscillator in the rat suprachiasmatic nucleus [Gillette and Prosser, 1988], it is not involved in the chick pineal gland circadian system [Takahashi et al, 1989; Zatz and Mullen, 1988b]. Thus, pulsed treatment with cAMP or cAMP analogues causes phase-shifting of the circadian rhythm in the rat suprachiasmatic nucleus, but not in the chick pineal gland, *in vitro* [Gillette and Prosser, 1988; Takahashi et al, 1989; Zatz and Mullen, 1988b].

When PACAP was coadministered with light pulses, the light-induced acute reduction of melatonin release was almost blocked by PACAP. This acute reduction of melatonin by light pulses is dependent on the acute reduction of cAMP levels in chick pineal cells [Zatz, 1989]. Therefore, PACAP-induced increases in cAMP levels may overcome the reduction of cAMP caused by light. Alternatively, light and PACAP may produce different forms of activated adenylate cyclase.

In conclusion, the physiological roles of PACAP in melatonin release and the circadian oscillator in the chick pineal gland differ greatly from those in the rat pineal gland and suprachiasmatic nucleus. In the chick pineal gland, PACAP probably regulates melatonin release via the PACAP-r1 receptor, and is not involved in the circadian oscillatory system.

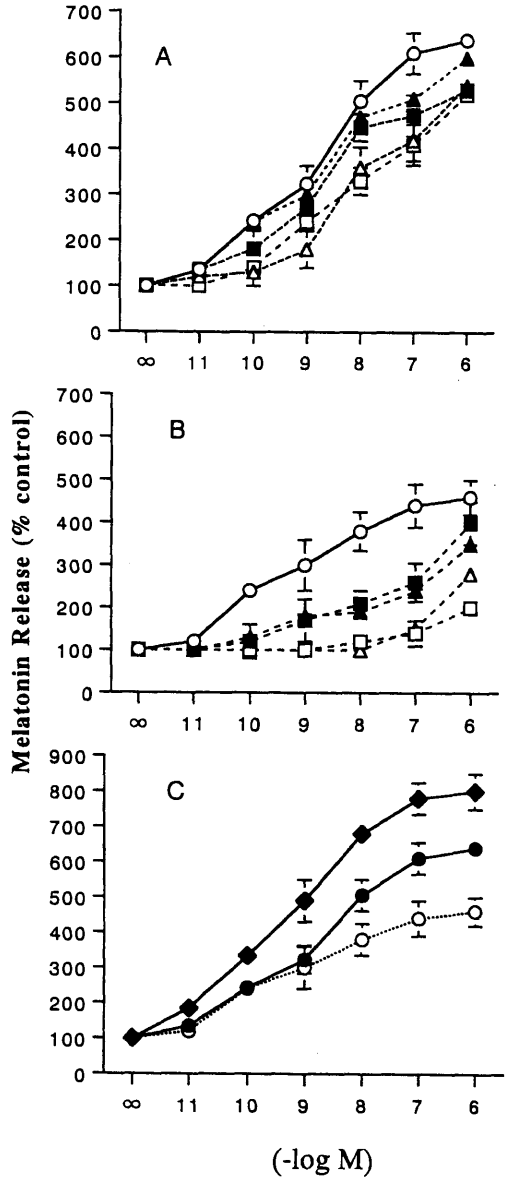


Figure III-1. Dose-response curves of PACAP (A), VIP (B) or their combination (C) on melatonin release, and effect of VIP receptor antagonist on the PACAP-(A) or VIP-induced (B) melatonin release in chick pineal cells. Various doses of PACAP (A) and VIP (B) were administered alone (open circles) or with 100 nM (solid triangles and squares) or 1000 nM (open triangles and squares) of two kinds of VIP receptor antagonists (triangles: VIP-[pC- D-Phe^6, Leu1^7] and squares: VIP-[Ac-Tyr^1, D-Phe^2]GRF [8-29]), respectively. C) Dose response curve of PACAP (solid circles), VIP (open circles) alone and PACAP + VIP (solid diamonds) on melatonin release in chick pineal cells. All symbols and vertical lines represent mean values ± SEM (n=8).

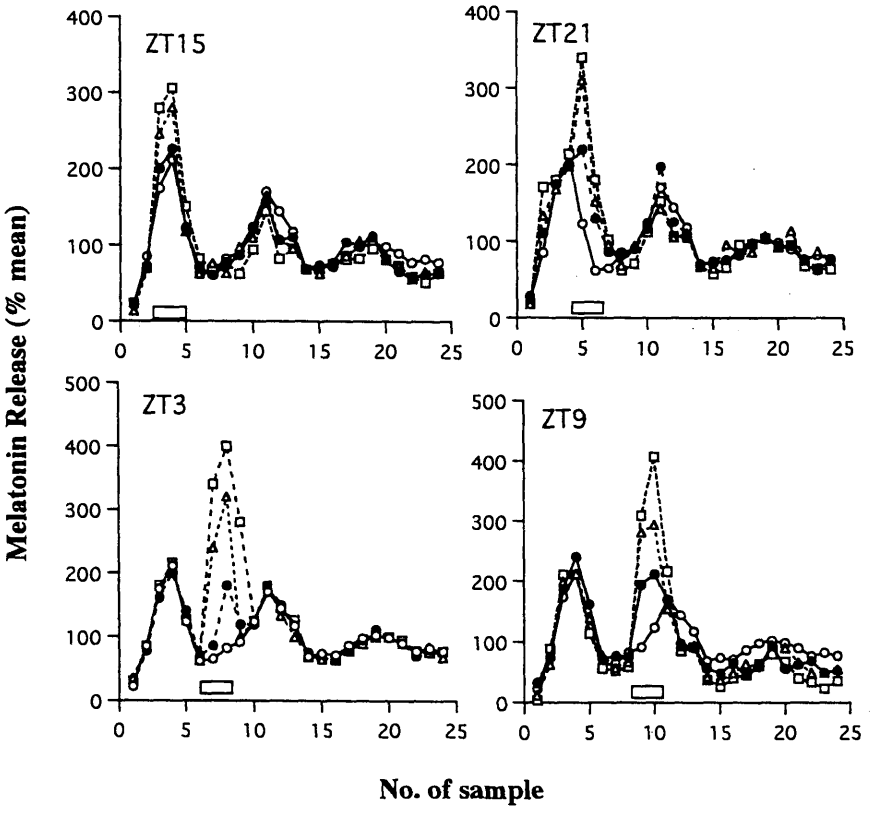


Figure III-2. Effect of PACAP pulse treatment on the circadian melatonin rhythm in chick pineal cells. Six-hour pulses of PACAP (indicated by white boxes) were started at 6-h intervals from ZT 15 in separate groups of cells. Clear squares, clear triangles and solid circles represent the 10^{-7}, 10^{-8} and 10^{-9} M PACAP pulses, respectively. Control is represented by open circles. Each symbol represents the mean melatonin level in 5–6 wells. No phase shifting was observed in any of the cell groups tested.

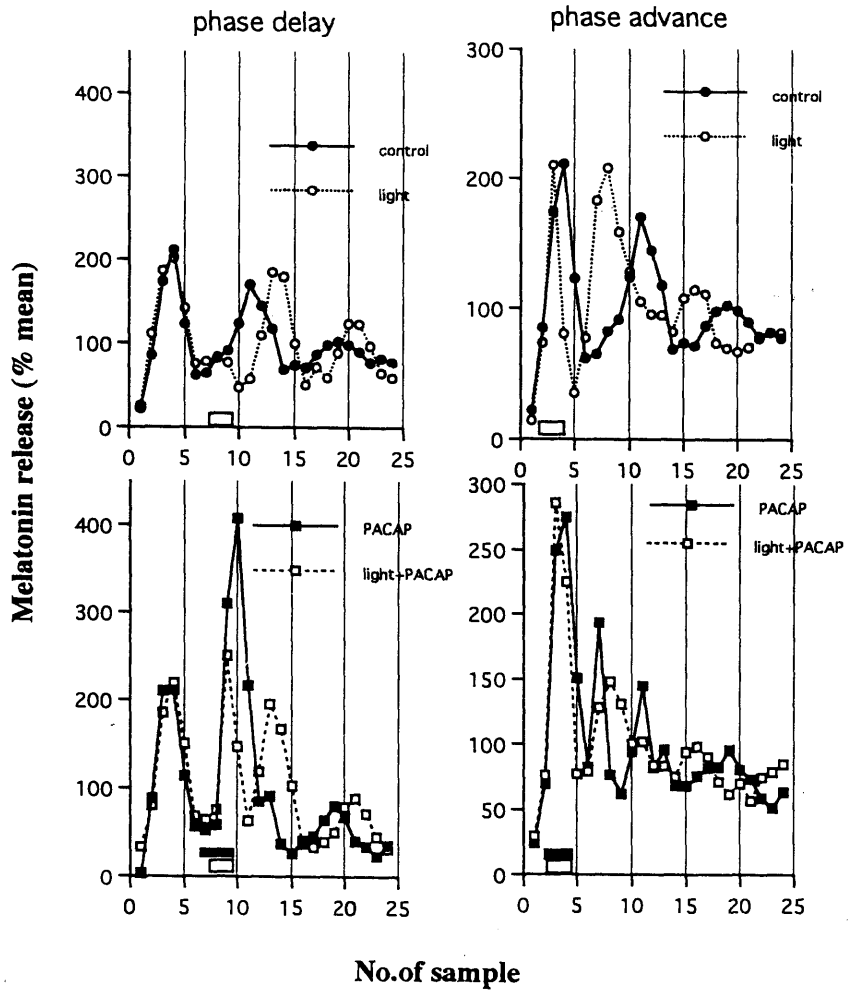


Figure III-3. Effect of PACAP on light-induced phase shifting of the circadian melatonin rhythm in chick pineal cells. Six-hour pulses of 10^{7}M PACAP (indicated by black bars) were started 1 h before the light pulses. Five-hour light pulses (indicated by white boxes) were started at ZT 15 to induce a phase advance and at ZT 9 to induce a phase delay. Each symbol represents the mean value for 6–8 wells.

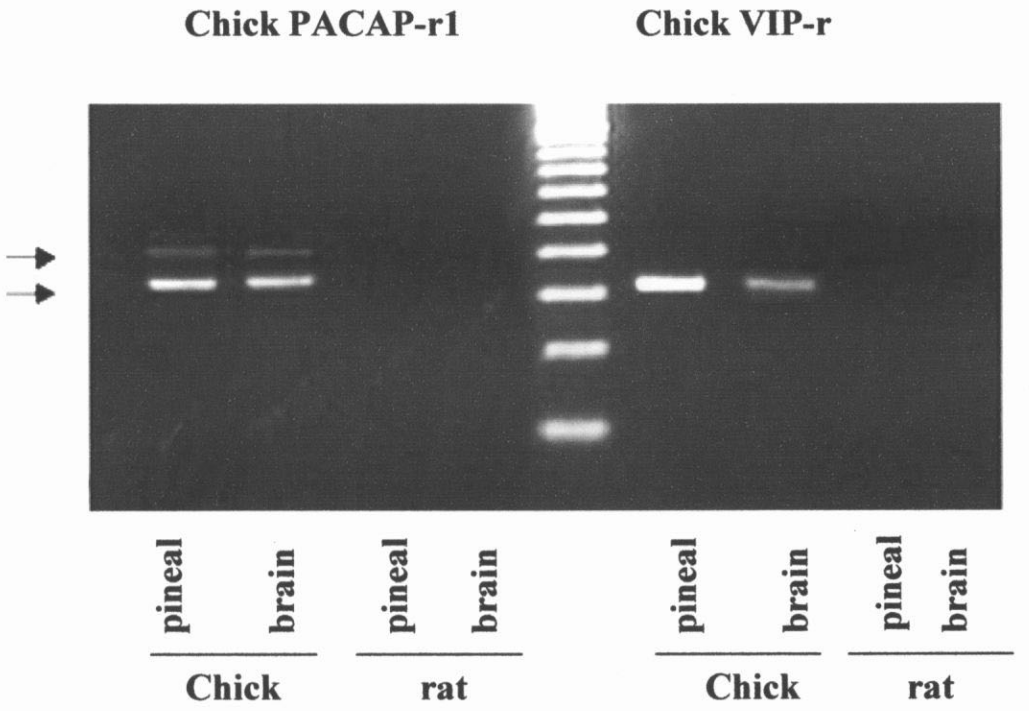


Figure III-4. Chick pineal gland and brain cDNA fragments amplified by PCR in the presence of oligonucleotide primers specific for chick PACAP-r1 and VIP receptors. Rat pineal gland and brain was used as a negative control. The gel exhibits DNA bands of the expected size for PACAP-r1 and its splicing variant with a hop cassette indicated by lower and upper arrows, respectively.

Chapter Ⅳ

Intracellular Ca^{2+}signaling pathway is involved in light-induced phase advance, but may not be in phase delay, of the circadian melatonin rhythm in chick pineal cell

Abstract

Chick pineal cells have photoreceptive, circadian clock and melatonin synthetic capacities, and express circadian oscillation of melatonin release *in vitro*. Light pulses cause phase-dependent phase shift of the melatonin rhythm. The purpose of the chapter Ⅳ was to address the questions whether intracellular calcium is involved in both light-induced phase advance and delay. Thapsigargin and cyclopiazonic acid, which deplete the intracellular calcium stores, blocked the light-induced phase advance in dose-dependent manner. The pulses of ryanodine receptor antagonist (dantrolene sodium or ruthenium red) also blocked the light-induced phase advance. Most agents did not cause a significant phase shift by themselves. On the other hand, all agents used failed to block the light-induced phase delay, even if the magnitude of phase delay was decreased using low intensity light. An antagonist of nitric oxide synthase blocked neither light-induced phase advance nor phase delay. These results indicate the following possibilities: 1) the mechanism of light-induced phase advance and delay may be different in chick pineal cells, or 2) if intracellular calcium is involved in both light-induced phase advance and delay, the sensitivity to light and / or agents used in this study may differ according to Zeitgeber time.

Introduction

Chick pineal cells express a circadian rhythm of melatonin release under light-dark cycles (LD), with an increase during the dark period and a decrease during the light period; this rhythm persists under constant darkness (DD) *in vitro* [Robertson and Takahashi, 1988a; Zatz and Mullen, 1988a,b;Takahashi et al, 1989]. I demonstrated that, using single cell culture method, individual pineal cells in chick possess photoreceptor, circadian clock and melatonin synthetic capacities, and these component are linked to each other [Nakahara et al, 1997a]. Light has two distinct effects on the melatonin rhythm in chick pineal cells. One is acute inhibition of melatonin release by acting on cAMP cascade not via the circadian clock. The other is entrainment of circadian clock by acting on the circadian clock itself [Robertson and Takahashi, 1988b; Zatz and Mullen, 1988a; Takahashi et al, 1989]. Although many studies concerning the mechanisms responsible for melatonin production or acute inhibition by light have been conducted in chick pineal cells, the mechanism for light entrainment has not been clarified. Cyclic nucleotides (cAMP or cGMP) do not cause phase shifts in chick pineal cells, but they are involved in melatonin synthesis or acute inhibition by light [Zatz and Mullen, 1988b; Nikaido and Takahashi, 1989; Takahashi et al, 1989]. Pertussis toxin blocks the acute reduction of a melatonin release by light pulse, but does not block the phase shifting effect [Zatz and Mullen, 1988c; Takahashi et al, 1989]. In addition, although the specific photoreceptive substance (pinopsin) [Okano et al, 1994; Max et al, 1995] and non-selective cation channel [D'Souza and Dryer, 1996] and MAP kinase [Sanada et al, 2000] may be involved in photo-entrainment have been identified in chick pineal cell, the signal transduction pathways related to these components has not been elucidated.

On the other hand, in the mammalian circadian system, light acts on a circadian

clock via glutamic acid, which is released from terminals of the retino-hypothalamic tract; this causes the phase-dependent phase shift (photo-entrainment). Recently, Ding et al (1998) demonstrated that phase advance and delay took two respective signal pathways from the glutamate receptor. Glutamate-induced phase advance, but not phase delay, was blocked by specific protein kinase G inhibitor. Conversely, glutamate-induced phase delay, but not advance, was blocked by inhibitor of ryanodine receptor. These results suggest that light-glutamate signal cause phase advance and delay by means of cGMP dependent pathway and by means of ryanodine receptor activation and release of Ca^{2+}, respectively.

Zatz and his colleagues have examined the effects of intra- and extracellular Ca^{2+} related agents on the rhythm, melatonin synthesis and on the light-induced effects (phase shift and acute reduction of melatonin release) [Zatz and Mullen, 1988d; Zatz, 1989a; Zatz and Heath, 1995]. Extracellular Ca^{2+} related agents that stimulate (such as Bay K 8644; a dihydropyridine calcium channel agonist) and retard (such as nitrendipine; a dihydropyridine antagonist of L-type calcium channels) Ca^{2+} influx through plasma membrane increase and decrease melatonin release, but do not affect the phase and effect of light [Zatz and Mullen, 1988d; Takahashi et al, 1989]. An intracellular Ca^{2+} related agent such as caffeine causes a phase shift in the same manner as a light pulse [Zatz and Heath, 1995]. Thapsigargin, a specific inhibitor of intracellular Ca^{2+} ATPase, or EGTA block the light-induced phase advance. These results suggest that intracellular Ca^{2+} mediate light-induced phase shifts [Zatz and Heath, 1995].

The fact that the phase response curve for caffeine pulses is almost identical with light pulses suggest that the intracellular Ca^{2+} is involved in both the light-induced phase advance and delay; however, there is no evidence as to whether an intracellular Ca^{2+} antagonist would block the light-induced phase delay in chick

pineal gland. In this study, therefore, I compared the effect of various Ca^{2+} antagonists on the light-induced phase advance and those on the phase delay. Here, I found that Ca^{2+} antagonists blocked only the light-induced phase advance, but not the phase delay.

Materials and Methods

Chemicals

Medium 199, HEPES, calf serum and Hank's solution with and without calcium were purchased from GIBCO (Grand Island, NY). Penicillin-streptomycin, collagen (type VII), trypsin, collagenase, trypsin inhibitor, gentamicin and cyclopiazonic acid (Cyc) were purchased from Sigma Chemical Co. (St Louis, MO). Percoll was purchased from Pharmacia (Uppsala, Sweden). Thapsigargin (TPS), NG-nitro-L-rginine methyl ester hydrochloride (LNAME) was from RBI (Natick, MA). Dantrolene sodium (Dan) and ruthenium red (Rut) were purchased from Latoxan (Rosans France). All the other chemicals used were of reagent grade and obtained from commercial sources.

TPS (0.5, 1, 5 μM) and Cyc (30, 60 μM) were used to deplete intracellular Ca^{2+}, by blocking specifically the Ca^{2+} ATPase [Thastrup et al, 1990]. Dan (20,40, 80 μM) and Rut (10, 50 μM) were used as ryanodine receptor antagonist [Luthra and Olson, 1977; Brillantes et al, 1994; Parness and Palnikar, 1995]. LNAME (10, 50,100 μM) was used as inhibitor upon NO synthase [Belvisi et al, 1991]. Water insoluble agents were dissolved in ethanol (Dan) or DMSO (TPS, Cyc), thereafter diluted into culture medium so that the final concentration of ethanol or DMSO was less than 0.05%. The other chemicals were dissolved directly in culture medium. Each medium was pre-warmed for 3 h in CO_2 incubator before replacement.

Cell culture and experimental schedule

Chicks were raised from hatching under LD 12:12 (lights on 07.00 h) photoperiod in our laboratory until they were 2-4 weeks old. The pineal gland was removed and collected in cold Hank's salt solution. The tissue was washed with Hank's salt solution containing 100 μg/ml gentamicin (Funakoshi, Tokyo), 1000 U/ml penicillin

G and 1000 μg/ml streptomycin (Sigma). The tissue was transferred to 10 ml Hank's salt solution containing 2 mg/ml collagenase (Sigma, Type IV). It was minced and incubated for 30 min in a 37 °C shaking water bath, and centrifuged for 2 min at 100 ×g. The supernatant was removed and 10 ml trypsin solution was added. The tissue was then incubated for 3-5 min at 37 °C, and 5 ml trypsin inhibitor solution (0.2 mg/ml, Sigma) was added. The tissue was triturated repeatedly through a series of gradually narrowing Pasteur pipettes and centrifuged for 5 min at 500 ×g . The resulting cell pellet was re-suspended in culture medium with the following composition: medium 199 with Hank's salt and L-glutamine (Gibco) supplemented with 10 mM HEPES buffer (Gibco), 15% fetal calf serum (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.9 mg/ml $NaHCO_3$ The cells were then fractionated by iso-osmotic Percoll gradient centrifugation at 450 ×g for 20 min, and those at the interface between 1.02 g/ml and 1.07 g/ml were collected and washed with culture medium [Nakahara et al, 1997b]. Finally, the cells were suspended in culture medium and placed in collagen-coated 96-well dishes at densities of 5 x 10^4, maintained in an incubator at 38 °C with 95% air and 5% CO_2 under 12 h light: 12 h dark (LD: lights on at 07:00 h; light was supplied through glass fibers) conditions for 4 days and then DD conditions for 3 days. All equipment (for example, portable cleanbench, CO_2 incubator, microscope) was kept at 37 °C in a controlled humidity (85%) chamber, and all treatments (for example, sampling and medium change) were performed in the chamber in order to prevent any change in temperature [Nakahara et al, 1997a].

Experimental schedule

Cells were maintained under a 12:12 h light-dark cycle (lights on 07.00-19.00 h) for 4 days, thereafter under constant darkness for 3 days. Light was supplied to the

culture dish through light fibers from outside the incubator. Culture medium was changed at 16:00 h every day. The first samples was collected at 19:00 h just before the initiation of constant darkness (DD), thereafter collected and replaced with fresh medium at 3 hour intervals for 3 days. Infraredscope was used for sampling under DD. Five-hour light pulse was given through a light fiber using another CO_2 incubator, starting at Zeitgeber time (ZT) 16 h (23.00 h) for phase advance and ZT 10 h (17.00 h) for phase delay [Murakami et al, 1995], with ZT12 h defined as the onset of the dark phase. The light pulses with high (70 lux) and low (8 lux) intensity illumination were used for large and small phase shifts, respectively. In order to determine whether agents block the light-induced phase shift, a 6-h pulse of agents was initiated from 1h before light pulse. The starting and end time of the drug pulses were the same as the sampling time. After 6 h pulse with drugs, cells were washed gently with fresh medium three times, then placed into normal medium. The control well or those given a light pulse alone were also washed with fresh medium as above. In a single experiment, each treatment consisted of 4-6wells. The same experiment was repeated independently three or four times.

Melatonin assay

The melatonin content of appropriate volumes (10-50μl) of culture medium samples were determined by radioimmunoassay [Murakami et al, 1994]. The radioiodinated and non-radioactive melatonin used as standards were purchased from New England Nuclear and Sigma, respectively. The rabbit anti-melatonin serum (HAC-AA92-03RBP86) was supplied by Dr. K. Wakabayashi (Gunma University, Japan). The assay was validated for the culture medium without sample extraction, and no significant interference from the perfusion medium was detectable in the standard assay. The lower and upper detection limits of this radioimmunoassay were

15 and 2,000 pg/tube, respectively and the inter- (n=20) and intra-assay (n=20) variation were 6.2 and 11.4%, respectively.

Data analysis

In order to reduce the differences between the absolute melatonin rhythm amplitudes among the well or dish, the data are presented in normalized forms relative to the mean values of the data [Robertson and Takahashi, 1988b; Murakami et al, 1995]. This was performed by dividing the melatonin values from each well (n=4-6/single experiment × 3 or 4 times repeated experiments) by the mean value of the time series of that well and multiplying by 100. Thus, the mean value for the time series of each well corresponded to 100%. The phase of the melatonin oscillations was analyzed using the midpoint of the cycle as the phase reference point, according to the method described previously [Murakami et al, 1995], except using a computer program. The midpoint phase reference was defined as the time midway between the half-rise and half-fall of each melatonin peak. To analyze the phase shift, the phase of each well was analyzed during the first or second cycle following the pulse treatment and the phase difference between wells that received agents or light and drug-free medium was calculated using midpoint references. The significance for each time point was determined using Student's *t* test. Differences at $P< 0.05$ were considered significant. Significant differences between the magnitude of phase shifts were analyzed by ANOVA.

Results

I measured the circadian rhythm by a continuous sampling at 3-h intervals in static cell culture of chick pineal gland, and compared the effect of various agents on the light-induced phase shift at the same time. All manipulations were performed inside the chamber kept at a constant temperature of 37°C and 85% humidity [Nakahara et al, 1997a]. Figure Ⅳ-1A shows the individual data from 4 wells at the same time in a single experiment and figure Ⅳ-1B shows the average from 6 independent experiment (each composed of 4-6 wells). The circadian oscillation of melatonin release persisted under constant dark with damping of amplitude. The average period of the melatonin rhythm was 21.75h.

A light pulse with high illumination at ZT 16 h (23.00 h) caused both the acute inhibition of melatonin release and large phase advance as shown in figure Ⅳ-2A. The magnitude of phase advance was about 8.3 h. TPS or Cyc reduced significantly the magnitude of the light-induced phase advance. The pulse of TPS or Cyc themselves did not cause significant phase shift (Fig. Ⅳ-2B, C), but reduced melatonin release during the pulse. This blocking effect by TPS and Cyc on the light-induced phase advance was dose-dependent (Fig. Ⅳ-4A). The almost complete blockade of the light-induced phase advance was observed in 5μM TPS. The light pulse with high illumination at ZT 10 h (17.00 h) caused a phase delay with a magnitude of 4.2 h together with acute inhibition of melatonin release (Fig. Ⅳ-4B). TPS did not reduce the magnitude of this phase delay. The pulses of TPS itself given at ZT 10 h did not cause a significant phase shift, but inhibited the melatonin peak during the pulse. Cyc also did not block the light-induced phase delay (Fig. Ⅳ-4B). The magnitude of acute reduction of melatonin by TPS or Cyc themselves was dependent on time and dose (Fig. Ⅳ-2B, Fig. Ⅳ-3B). The dose over 1μM of TPS or 30μM of Cyc caused acute reduction of melatonin with same magnitude as light

pulse. Since the failure of blockade of light-induced phase delay by TPS or Cyc may be due to saturation of phase shift by high intensity light pulse, I re-examined this using the smaller phase delay induced by low intensity light pulse. As shown in figure Ⅳ-3A, low intensity light pulse caused the smaller phase delay of less than 3 h. The average of magnitude was 2.42 h. Nevertheless, neither TPS nor Cyc reduced this light-induced phase delay (Fig. Ⅳ-3B, and Fig. Ⅳ-4C).

The pulses of ryanodine receptor antagonist, dantrolene (Dan) or ruthenium red (Rut) also blocked the light-induced phase advance (Fig. Ⅳ-5B, Fig. Ⅳ-7A). When compared with TPS, these agents did not cause the acute inhibition of melatonin release, or block the light-induced acute inhibition of melatonin release. However, like TPS, Dan and Rut did not block the light-induced phase delay, even if the small phase delay was induced by low intensity light pulse (Fig. Ⅳ-6, Fig. Ⅳ-7C). These results suggest that intracellular Ca^{2+} may not be involved in light-induced phase delay in chick pineal cell. The NO-guanylate cyclase-cGMP-pathway is known to be involved in light-induced phase advance in mammals [Ding et al., 1998]. However, it has been already shown that 8-bromo-cGMP does not cause the phase shift in chick pineal cells [Zatz and Mullen, 1988b]. To confirm the no involvement of NO-cGMP-pathway in light-induced phase delay in chick pineal cells, the effect of an inhibitor of NO synthase (L-NAME) on the light-induced phase advance and delay was examined. L-NAME itself did not cause any change in melatonin release or phase of the rhythm. In addition, neither inhibition of the acute effect of light on melatonin release nor for phase delay by light pulse was observed in co-treatment with L-NAME and light pulse (Fig. Ⅳ-7).

Discussion

In agreement with the previous observations [Zatz and Heath, 1995] TPS blocked the light-induced phase advance. In this study, Cyc also blocked it. These effects were dose-dependent. The pulses of TPS and Cyc themselves did not affect circadian phase, but reduced melatonin release. These results by TPS were consistent with the observation reported by Zatz and Heath (1995). The TPS and Cyc are known to increase intracellular Ca^{2+} and thereafter to deplete intracellular Ca^{2+}, by blocking specifically the Ca^{2+} ATPase [Thastrup et al, 1990]. Indeed, the effect of TPS on the intracellular Ca^{2+} mobilization was shown in chick pineal cell using fura-2 technique by D'Souza and Dryer (1994). Therefore, it is likely that intracellular Ca^{2+} is required for the light-induced phase advance. In addition, the pulses of ryanodine receptor antagonist, Dan or Rut also blocked the light-induced phase advance. This result indicates that light-induced phase advance may depend on an increase of cytoplasmic Ca^{2+} levels through an action involving ryanodine receptor.

The present study also showed that TPS, Cyc, Dan and Rut did not block the light-induced phase delay. The failure of blockade is not due to saturation of magnitude in light-induced phase delay. Even if the magnitude of phase delay was decreased by low intensity light, no blockade was observed. Therefore, these results suggest that the intracellular signaling pathway for the phase advance and delay by light may be different, and intracellular Ca^{2+} is involved in light-induced phase advance, but may not be in the phase delay.

Although agents affecting cyclic AMP or cGMP cause the phase-dependent phase shift in other circadian oscillator, they did not have a phase-shifting effect in chick pineal cells [Eskin et al, 1982]. Recently, Ding et al (1998) demonstrated that the phase advance and delay took two respective signal pathways from the glutamine

receptor. Glutamic acid-induced phase advance, but not phase delay, was blocked by specific protein kinase G inhibitor. However, the glutamic acid-induced phase delay, but not advance, was blocked by the inhibitor of the ryanodine receptor. Theses results suggest that a light-glutamate signal cause the phase advance and delay by means of cGMP dependent pathway and by means of ryanodine receptor activation and release of Ca^{2+}, respectively. As the inhibitor for NO synthesis also block the light (or glutamic acid)-induced phase advance, NO-guanylate cyclase-cGMP -pathway is likely involved in the light-induced phase advance in mammals. In chick pineal cells, however, inhibition of NO synthesis blocked neither the light-induced phase advance nor delay. Theses results together with previous findings [Zatz and Mullen, 1988b] indicate that the NO-guanylate cyclase-cGMP-pathway is not involved in light-induced phase shift in chick pineal cells.

There is a large discrepancy between our speculation and the phase response curve with the caffeine pulse shown by Zatz and Heath (1995). The pulses of caffeine caused both a phase advance and delay time-dependently. The phase response curve due to caffeine is almost identical with those for the light pulse [Zatz and Heath, 1995]. If the caffeine-induced phase delay depends on an increased intracellular Ca^{2+}, why did TPS not block the light-induced phase delay in this study? The reason for this remains unknown. However, there are three possibilities: 1) there may be problems about specificity and/or dose of the antagonist used in this study, 2) there may be differences in sensitivity to light or antagonist between light-induced phase advance and delay, 3) the phase-delay induced by a large dose of caffeine is due to the other actions than the increase of intracellular Ca^{2+} levels [Daly, 1993; Nohmi et al, 1992]. In addition, the reasons why TPS and Cyc pulse alone caused acute reduction of melatonin, and did not cause the phase shift, (although its initial effect is to increase cytoplasmic Ca^{2+} levels) were also unknown. If it is due to the toxic

effects, TPS and Cyc also should block the light-induced phase delay as well as phase advance.

I could not identify the mechanism for light-induced phase delay in this study. In chick pineal cells, however, there are many distinctive mechanisms for in possible light-signal transduction, including involvement of a photoreceptor substance, ion channel or MAP kinase [Okano et al, 1994; D'Souza and Dryer, 1996. Sanada et al, 2000]. D'Souza and Dryer (1996) discovered that cultured chick pineal cells have unusual cationic channels (I_{LOT}) that are permeable to Ca^{2+} and active in the night but not during the day. This channel activity rhythm persisted under constant darkness and was low at expected lights on (measured at CT4-6 h) and high at expected light off (CT16-18 h). Although it is not yet shown whether this channel is involved in light-induced phase shift, the fact that time of high activity of this channel is almost identical with the time of light-inducible phase advance indicates the possible relationship between this channel and light-induced phase advance.

In conclusion, individual chick pineal cells receive directly a light signal via a photoreceptor, and this light signal causes the phase-dependent phase shift. The mechanism of signal pathway from photoreceptor may be different. Intracellular Ca^{2+} is required for the light-induced phase advance, but may not be required for the phase delay. Further studies are required for elucidate the mechanism of the light-induced phase delay.

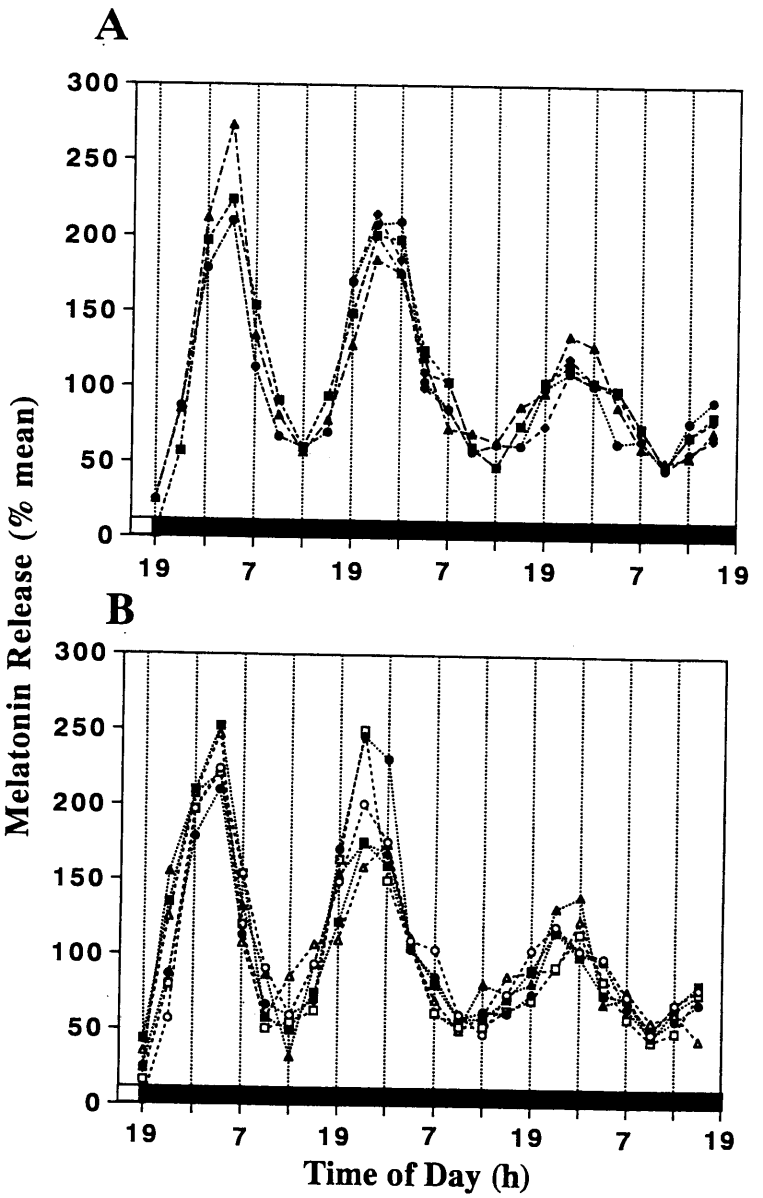


Fig. Ⅳ-1. Circadian oscillation of melatonin release in chick pineal cells. The bar at the bottom of each panel indicates the light-dark cycle. Upper panel (A) demonstrates the variation observed among replicate well (n=4) in a single experiment. Lower panel (B) demonstrates the variation among the average from 6 independent experiments. All samples were collected at 3-hour intervals starting from constant darkness. The data was normalized by the method described in Materials and Methods.

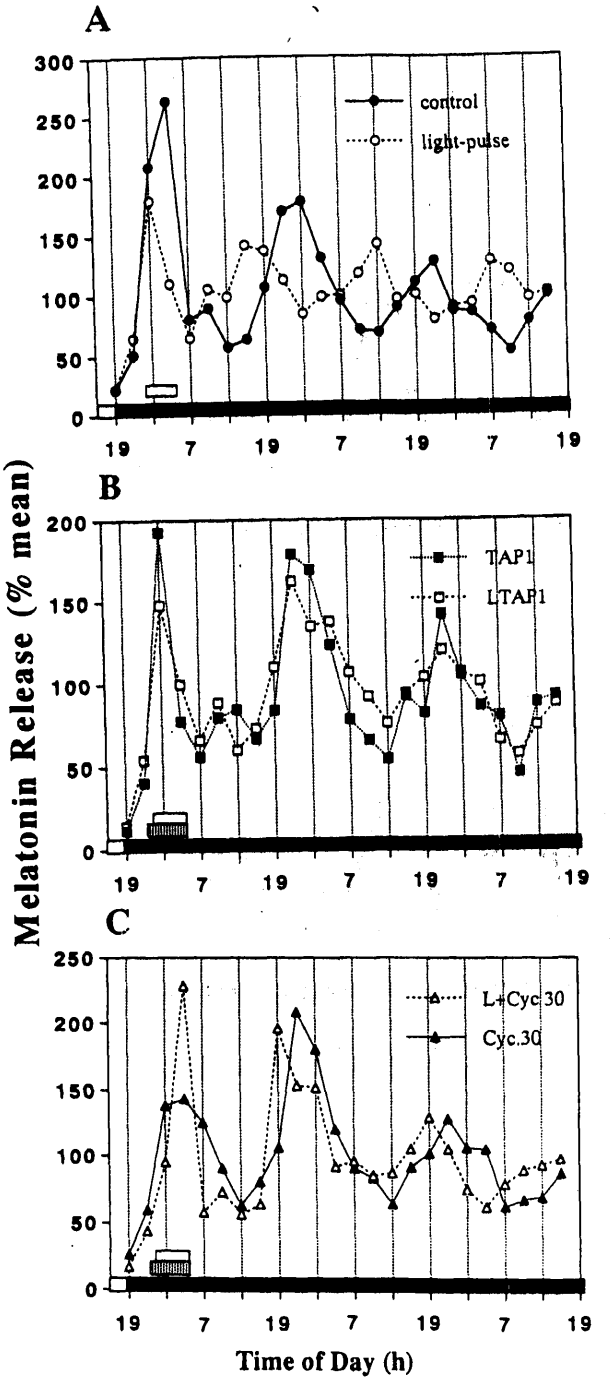


Fig. Ⅳ-2. Effect of TPS and Cyc on the light-induced phase advance of melatonin rhythm in chick pineal cells. The bar at the bottom of each panel indicates the light-dark cycle. A five-hour light pulse with high illumination was given at ZT 16 indicated by white bar (A). Six-hour pulses of 1 μM TPS (B) and 30 μM Cyc (C) were given at ZT15 indicated by dotted bars. All symbols represent the normalized mean levels of melatonin of three independent experiments.

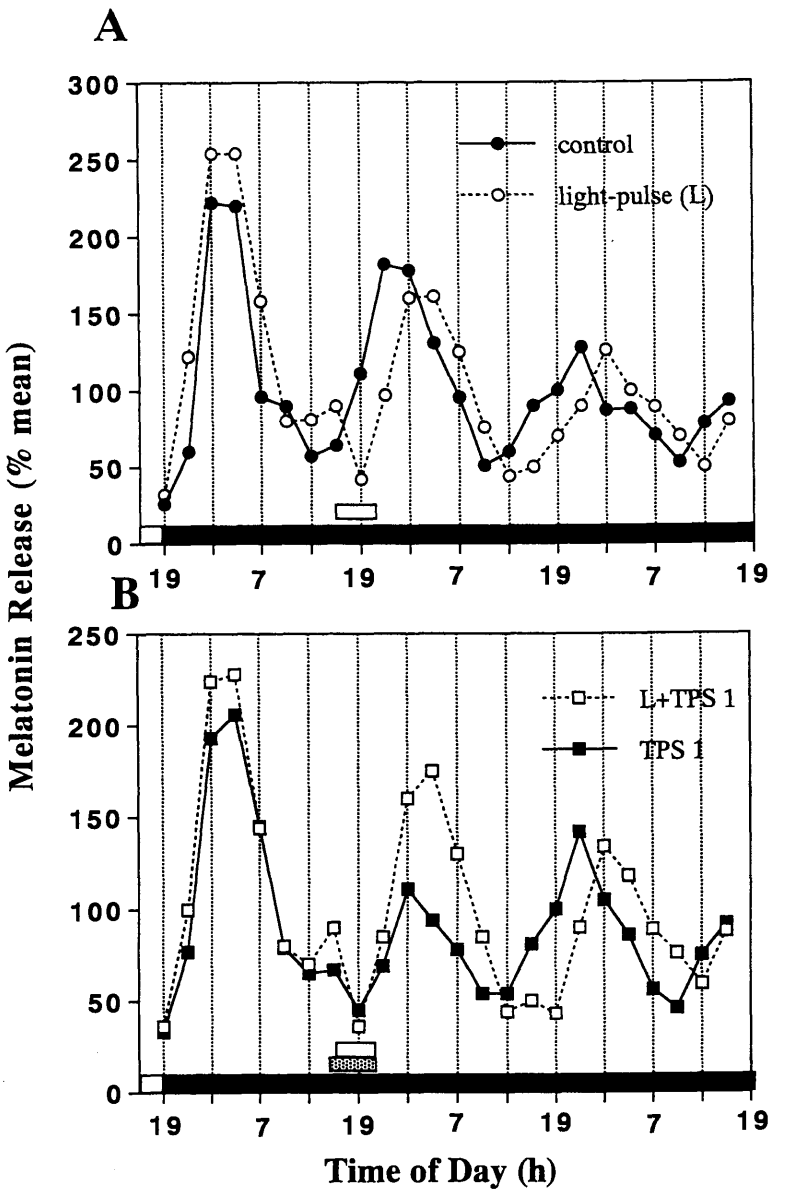


Fig. Ⅳ-3. Effect of TPS on the light-induced phase delay of melatonin rhythm in chick pineal cells. The bar at the bottom of each panel indicates the light-dark cycle. A five-hour light pulse with low illumination was given at ZT 10 indicated by white bar (A). Six-hour pulses of 1 μM TPS (B) were given at ZT 9 indicated by dotted bars. All symbols represent the normalized mean levels of melatonin of three independent experiments.

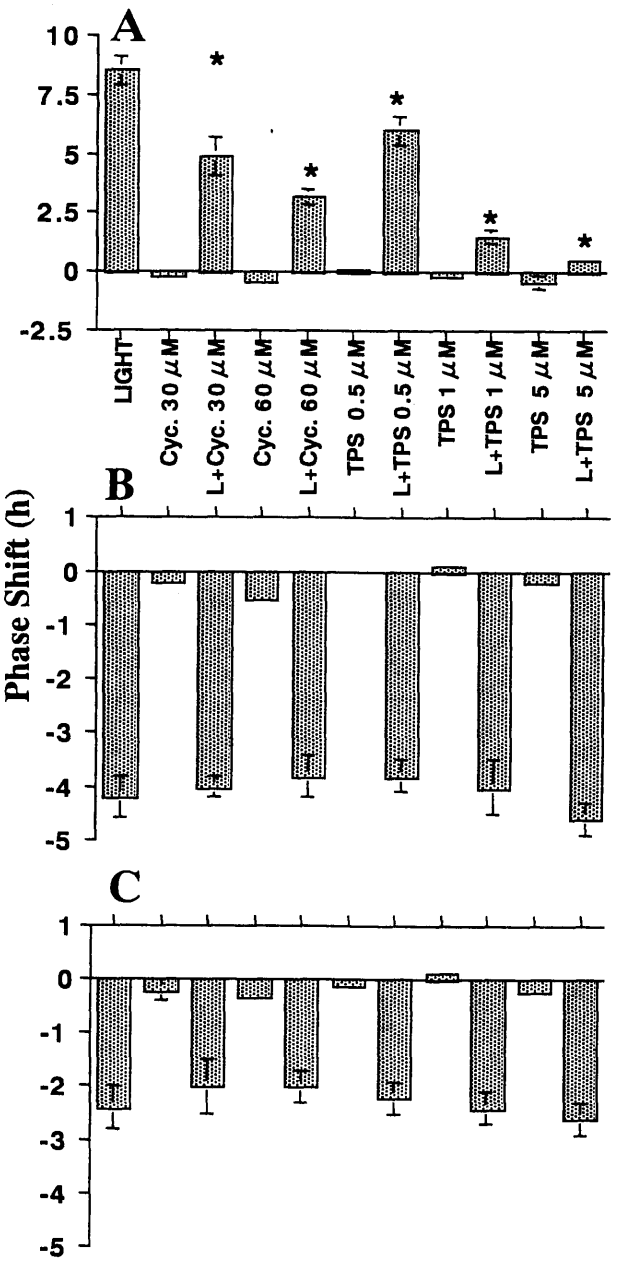


Fig. Ⅳ-4. Effect of various doses of TPS and Cyc on the light-induced phase advance and delay. Vertical axis represents the magnitude of phase advance (A) and phase delay (B, C). The dotted bar and vertical line represent the mean ± SEM of each 3 to 4 independent experiments. The time of pulses is same as those in figures 2-4. The asterisks indicate the significant difference ($P<0.05$) vs light pulse only. B and C showed the effect of TPS and Cycl on the phase delay caused by high intensity and low intensity light pulse, respectively.

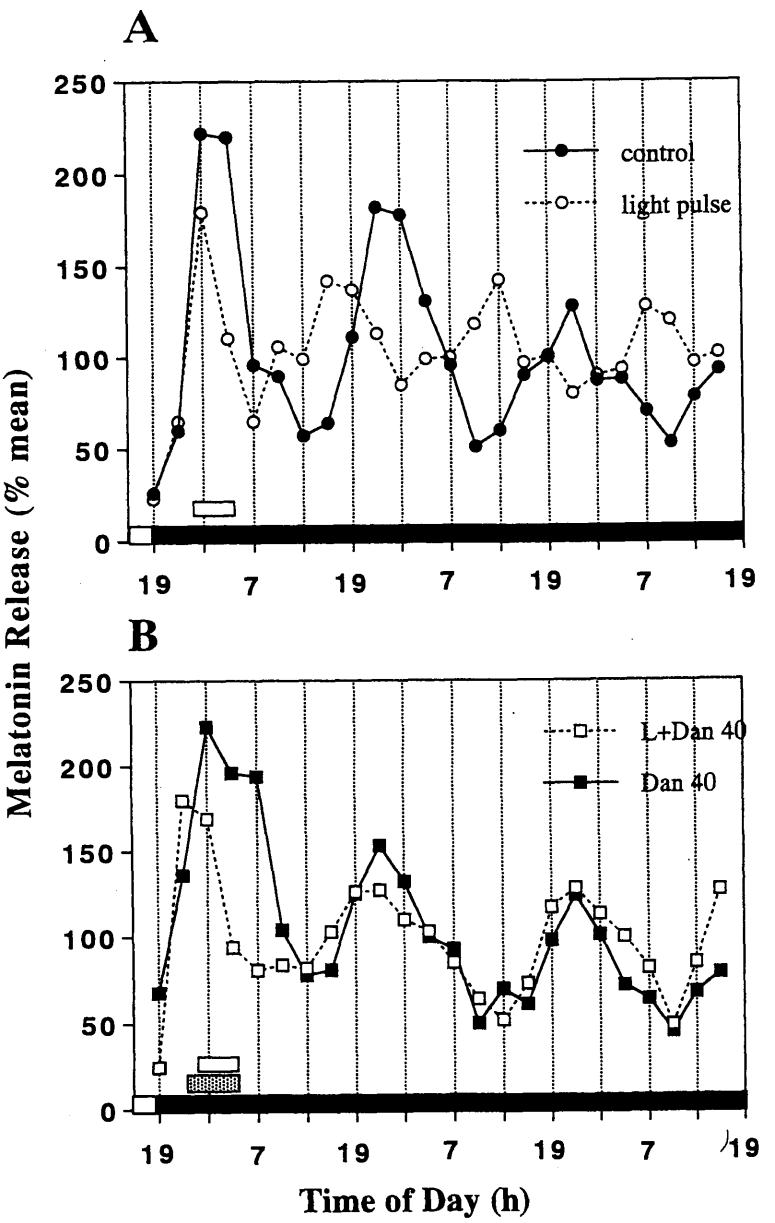


Fig. Ⅳ-5. Effect of Dan on the light-induced phase advance of melatonin rhythm in chick pineal cells. The bar at the bottom of each panel indicates the light-dark cycle. A five-hour light pulse with high illumination was given at ZT 16 indicated by white bar (A). Six-hour pulses of 40 μM Dan was given at ZT15 indicated by dotted bars. All symbols represent the normalized mean levels of melatonin of three independent experiments.

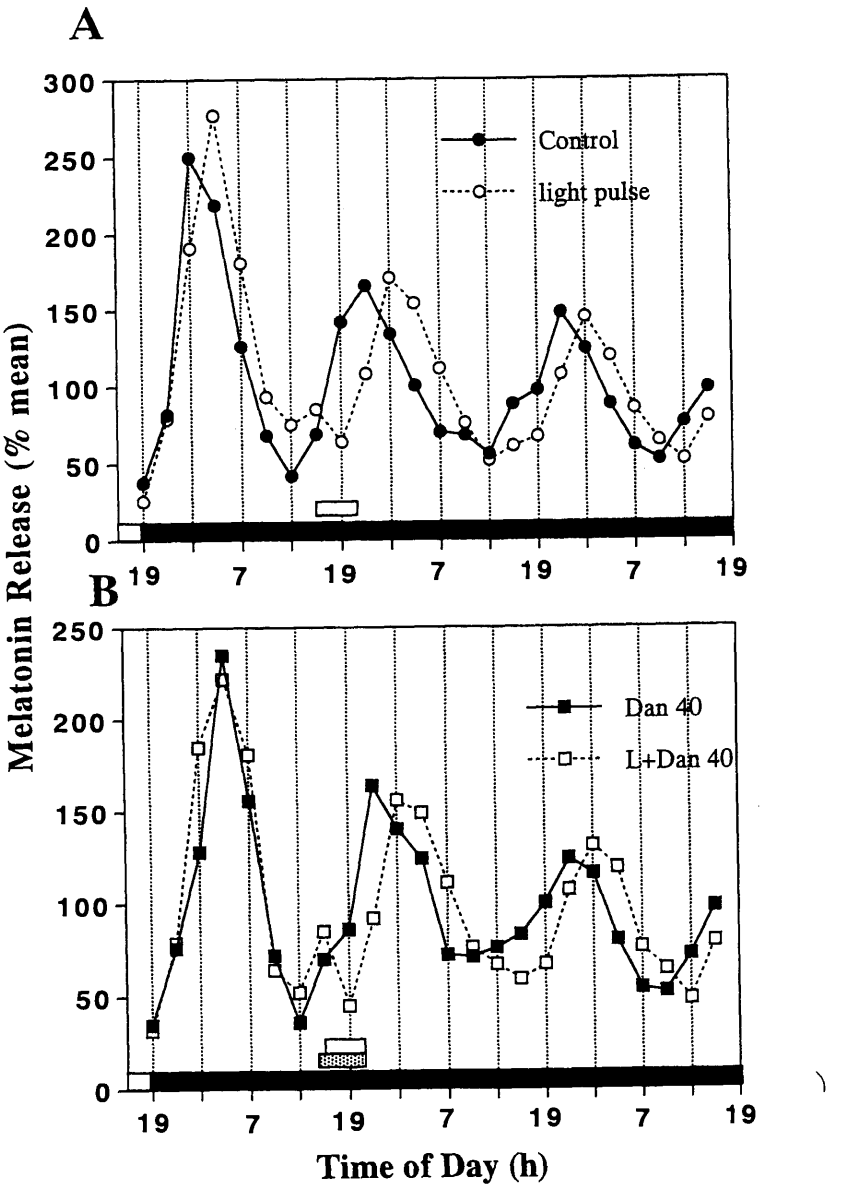


Fig. Ⅳ-6. Effect of Dan on the light-induced phase delay of melatonin rhythm in chick pineal cells. The bar at the bottom of each panel indicates the light-dark cycle. A five-hour light pulse with low illumination was given at ZT 10 indicated by white bar (A). Six-hour pulses of 40 μM Dan was given at ZT 9 indicated by dotted bars. All symbols represent the normalized mean levels of melatonin of four independent experiments.

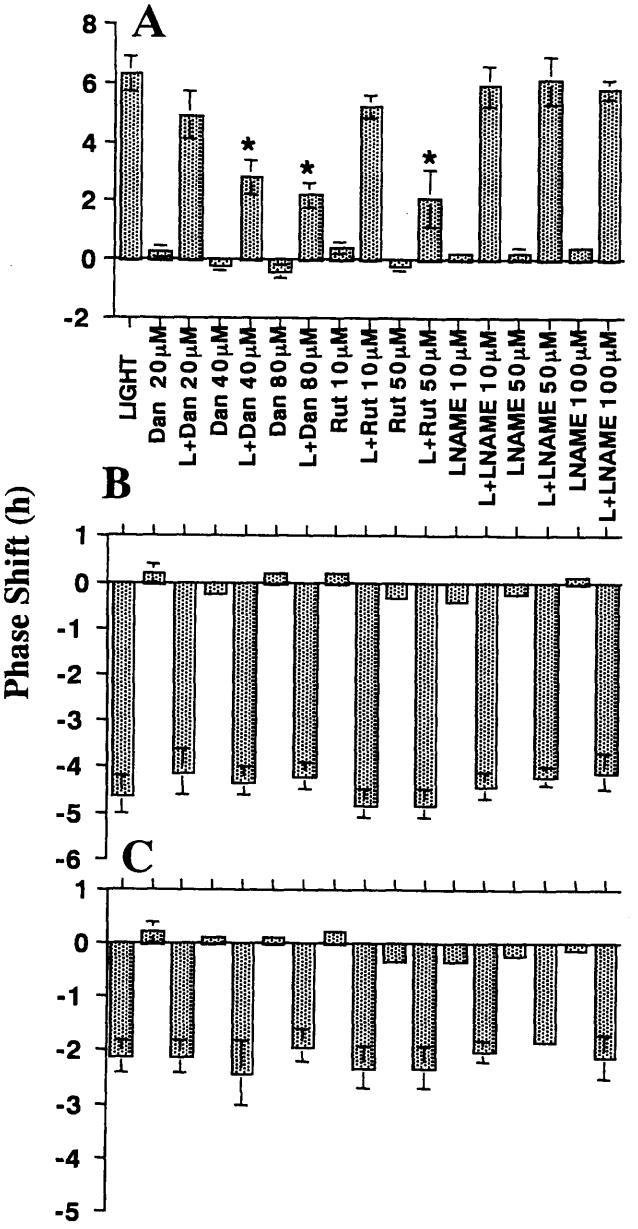


Fig. Ⅳ-7. Effect of various doses of Dan, Rut and LNAM on the light-induced phase advance and delay. Vertical axis represents the magnitude of phase advance (A) and phase delay (B, C). The dotted bar and vertical line represent the mean ± SEM of each 3 to 4 independent experiments. The time of pulses is same as those in Fig. 2, 3. B and C showed the effect of agents on the phase delay caused by high intensity and low intensity light pulse, respectively. The asterisks indicate the significant difference ($P<0.05$) vs light pulse only.

Chapter V

Effect of melatonin on the circadian rhythm, locomotor activity and body temperature in intact house sparrow, Japanese quail and owl.

Abstract:

Through I to IV chapters, I analyzed the cellular mechanism of circadian oscillation of melatonin release. However, it is questionable what role the melatonin rhythm plays. To examine the possibility that melatonin act directly body temperature, locomotor activity or their rhythms, I compared the effect of melatonin on circadian rhythm, body temperature, and locomotion in intact house sparrow, Japanese quails and owl. The daily treatment of melatonin at the fixed time did not entrain the free-running rhythm of locomotor activity in house sparrow and the disrupted rhythm in Japanese quails under constant dim light. However, melatonin inhibited clearly movement during several hours after treatment. The duration of resting after injection of melatonin was dose-dependent. Body temperature was significantly decreased after melatonin treatment with more potent effect during active phase than resting phase. Although this effect of melatonin on body temperature was also dose-dependent, the magnitude of decrease of body temperature by injection of melatonin was larger in house sparrow than in Japanese quail. On the other hand, further large decrease of body temperature by melatonin was observed in nocturnal bird, owl whose pineal gland was degenerated. This decrease of body temperature is larger in active phase than in resting phase. In owl, melatonin did not prevent movement in spite of decrease of body temperature. These results suggest that the effects of melatonin on the circadian rhythm and locomotor activity, and locomotion are different between avian species, and these mechanisms may not be linked each other.

Introduction

Depending upon the avian species, it may be probably established that predominant circadian oscillator located in eye, pineal gland and suprachiasmatic nucleus or multiple combinations [Ebihara and Kawamura, 1981; Gaston and Menaker, 1968; Murakami et al.1994; Norgren, 1990; Simpson and Follett, 1981; Underwood et al.1990]. For examples; transplantation of the pineal gland into an arrhythmic pinealectomized sparrow restores the rhythmicity [Zimmerman and Mwnaker, 1979]. On the other hand, in gallinaceous birds, pinealectomy does not lead to arrhythmicity [Simpson and Follett, 1981]. In Japanese quail, the eyes contain circadian oscillators controlling circadian behavior [Underwood et al.1990]. In columbine birds, neither pinealectomy nor blinding abolishes the circadian rhythm under constant conditions, but pinealectomy and blinding together lead to arrhythmicity [Ebihara et al.1984, Ebihara and Kawamura,1981]. The avian suprachiasmatic nucleus (SCN) also appears to be necessary for expression of circadian behavior in all species examined, but its role, as an oscillator has not been determined [Cassone, 1990; Ebihara and Kawamura, 1981; Norgren, 1990; Simpson and Follett, 1981]. Further, we demonstrated previously that the pineal gland of the owl, a nocturnal bird, degenerated and is not involved in the circadian clock mechanism [Taniguchi et al.1993].

In several species of birds, such as staring and pigeon, daily injection and infusion of melatonin entrains circadian activity rhythm such that the interval of activity proceeds the time of injection [Cassone, 1990; Chabot and Menaker, 1992]. In addition, daily administration of melatonin as a drinking water restored the rhythm from the arrhythmicity of pinealectomized house sparrow [Lu and Cassone, 1993]. The one of the causes of these entraining effects by melatonin may involve the direct reset of master clock by melatonin. The other possible cause may be masking effect

by direct inhibition of locomotor activity through the other brain site than circadian oscillator or muscle by melatonin [Chabot and Menaker, 1992]. Because it has been well known that melatonin affects the thermoregulation in both avian such as chick, house sparrow, and mammalian [Binkley et al.1971; Cagnacci, 1992; Krause and Dubocovich, 1990; Padmavathmma and Joshi, 1994; Rozenboim, 1998; Simpson and Follett, 1981]. Change of body temperature may be also involved in cause of entrainment by daily melatonin injection, since the change of temperature in cell culture system cause the phase-dependent phase shift of circadian rhythm, as well as those by light pulse, in chick pineal cell [Barrett and Takahashi, 1995; Takahashi et al.1989].

In rats, melatonin entrains the circadian rhythm by resetting the circadian clock of suprachiasmatic nucleus [Cassone et al. 1986; Gillette and MacArthur, 1996; . Krause and Dubocovich, 1990; McArthur et al.1991]. In that case, the entraining effects by daily melatonin injections were not different between intact and pinealectomized rats [Marumoto et al. 1996; Redman et al. 1983], suggesting that if entraining effect of melatonin is caused by resetting of circadian clock in suprachiasmatic nucleus, daily injection of melatonin could entrain the circadian rhythm in intact birds as well as pinealectomized ones.

Although I know there are many papers concerning thermoregulation, entraining effect or facilitating effect of synchronization to light [Gwinner, 1994] by melatonin as described above, there is a little report concerning the relationship between them. In this chapter, therefore, I compared the effects of melatonin on the circadian rhythm, body temperature and locomotor activity by daily or single injection of melatonin intact house sparrow, Japanese quail and owl.

Materials and Methods

Twenty-five house sparrows (*Passer domesticus*) were captured in Miyazaki City, Japan, Japanese quail (*Coturnix coturnix*) were reared from hatching from the closed breeding colony in our laboratory. Five adult owls (*Strix uralensis*) were supplied from Miyazaki Phoenix Zoo [Taniguchi et al., 1993]. All birds were kept at the separate room under 12 h light: 12 h dark (LD: lights on 07:00 h) conditions for at least 3 weeks. Illumination was supplied by fluorescent lamps at a light intensity of about 200~250 lux at the cage level. Food and water were available continuously in house sparrow and Japanese quail. In owl, food was given between 16:00 and 19:00 h.

In the first experiment, each bird was measured locomotor activity under LD conditions for one week, following under constant dim light at a light intensity of about 30 lux. The locomotor activity of the animals was measured by rat locomotor activity recording systems (Muromachi Co, Tokyo), which were composed with infrared sensors, interface and computer [Marumoto et al. 1996]. The data was collected at an interval of 15 min and kept in floppy disk. Melatonin (Sigma) was dissolved in 95 % ethanol and then diluted with saline at final concentration 60 mg/ml 1% ethanol-saline. Melatonin (0.001, 0.01, 0.1 or 1mg/kg body wt) or vehicle solution was injected subcutaneously at fixed time every day in 15 house sparrows and 15 Japanese quails, each 5 birds were injected 0.01, 0.1 and 1 mg, respectively. The duration of injection period was sufficient to have passed through the entire free running subjective day and night in intact house sparrow. As the locomotor activity rhythm in Japanese quail became unclear under constant condition, melatonin or vehicle was injected for at least 4 weeks. The day of initiation and end of injections were described in results (figures). Entrainment of free-running activity rhythms by

daily injections of melatonin and vehicle was determined by visual inspection, except in borderline cases that was judged by period gram analysis [Marumoto et al., 1996].

In the second experiment, single injection of melatonin or vehicle was started at 12:00-13:00 or 00:00-01:00 h as subjective day and night respectively, two days after transition from LD to constant dim LL in 12 house sparrows, 18 Japanese quails, and 5 owls. The body temperature was monitored 12 or 15-min intervals. Measuring device consist of small temperature sensor tip (measurable range: from 25 to 50 °C, measuring error: $\pm$0.05 °C) connected with the line (OD: 0.7 mm, length: 45 cm) and the monitor body. Sensor tip was inserted into cloaca and a part of line was fixed in body, then digital signal was transferred to monitor body.

In third experiment, single injection of melatonin or vehicle was performed at 12:00-13:00 during each active phase, in 10 house sparrows, 10 Japanese quails, and at 00:00-01;00 h in five owls. Locomotor activity was recorded before and after injection.

The data was analyzed ANOVA and followed by Fisher's PLSD test *post hoc.*

Results

Daily injection of melatonin did not entrain the free running (Fig. V-1) and disrupted rhythms (Fig, V-2), in 15 intact house sparrow and 15 Japanese quail, respectively, except each one. Only one house sparrow (Fig. V-1.B) injected 0.1 mg/kg melatonin and Japanese quail injected 0.1 mg/kg (Fig. V-2 A) showed the a little correlation between melatonin injection time and initiation of resting phase. The noentrainment might not be due to the insufficient dose of melatonin, since each one entrainment occurred even small dose.

Body temperature was significantly decreased after melatonin injection in all birds. In house sparrow and Japanese quail, effect of melatonin on the body temperature was compared between subjective day and night times under constant dim light. In house sparrow, the body temperature was higher during subjective daytime than the nighttime before melatonin injection, and the magnitude of decrease after injection of melatonin significantly larger in subjective daytime (Fig. V-3, V-4). This magnitude was dose-dependent. Significant decrease was observed from the dose of 0.001mg/kg body weight of melatonin (Fig. V-3). In Japanese quail, the small decrease of body temperature was observed compared with those in house sparrows. But, this decrease was also dose-dependent. In addition, as well as house sparrow, body temperature decrease more potent in subjective daytime (Fig. V-3, V-4).

Single injection of melatonin prevented completely the movement of house sparrow and Japanese quail (Fig. V-5). The duration of resting period after injections was dose-dependent (data not shown). The initiation of resting after melatonin injection was almost coincided with initiation of decrease in body temperature (Fig. V-3, V-5). In the owl kept under LD condition, single injection of melatonin dramatically caused to decrease the body temperature with dose dependent manner (Fig. V-6B, V-7). The recovery of body temperature was very slow. In owl, however, although

the temperature was decreased largely, movement was not inhibited after melatonin injection (Fig. V-6A).

Discussion

It has been reported that daily injection of melatonin into arrhythmic pinealectomized house sparrows at a fixed time can restore their rhythm [Lu and Cassone, 1993]. However, the present findings indicate that daily melatonin injection does not entrain the free running rhythm under dim LL conditions in intact house sparrows, suggesting that daily melatonin does not entrain the circadian clock in intact house sparrows. The entraining effect of melatonin taken in drinking water in pinealectomized house sparrows may have been due to direct inhibition of locomotor activity, rather than an effect on the circadian clock. However, it is still possible that the lack of entrainment of free-running rhythm by daily melatonin injection in intact house sparrows may have been due to maintenance of the endogenous melatonin rhythm from the pineal organ. In rats, the entraining effect was not distinguishable between intact and pinealectomized animals [Marumoto et al., 1996]. In Japanese quails, as the body temperature rhythm is free running even when the locomotor activity become unclear, it is unclear whether or not the free-running rhythm was entrained by melatonin. However, it appears that melatonin does not entrain the disrupted locomotor activity rhythm in the Japanese quail. These findings suggest that the effect of daily melatonin injection differs among avian species and mammals.

On the other hand, injection of melatonin inhibited movement and decreased body temperature in both the house sparrow and the Japanese quail. A thermoregulation by melatonin has been well known in many species including in the house sparrow [Binkley et al., 1971; Cagnacci et al., 1992; John et al.,1978; Pdmavathmma and Joshi, 1991; Rozenboim et al., 1998; Saarela and Reiter, 1993]. It seems likely that the inhibition of movement and the decrease of body temperature are correlated positively, since both of these effects of melatonin showed almost the

same time schedule. Therefore, the endogenous circadian rhythm of melatonin released from the pineal gland appears to control the circadian rhythm of both locomotor activity and body temperature in the house sparrow and Japanese quail, as in the starling or pigeon [Binkley et al.,1971; Chabot and Menaker, 1992; John et al., 1978; Warren and Cassone, 1995]. If this is the case, the differing effect of daily melatonin on the entrainment of circadian rhythm between intact and pinealectomized bird, may due to differences in the location of the master circadian clock. In the house sparrow and Japanese quail, the dominant circadian clock is considered to be located in the pineal gland and/or suprachiasmatic nucleus and eye, respectively [Menaker, 1982; Menaker, 1985; Simpson and B. K. Follett, 1981; Underwood et al., 1990; Zimmerman and Mwnaker,1979]. If melatonin can directly set the circadian oscillator to entrain the circadian rhythm, then the suprachiasmatic nucleus, which contains melatonin receptors, should be the dominant circadian oscillator [Cassone, 1990; Gillette and MacArthur,1996; Krause and Dubocovich, 1990; McArthur et al., 1991]. We expected that the decrease of body temperature induced by daily injection of melatonin would entrain the circadian oscillator, even if the pineal gland or eye was the dominant oscillator, since it has been shown that a pulse of high temperature can cause the same phase-dependent phase shift of the circadian oscillator as a light pulse in chick pineal cells [Barrett and Takahashi, 1995]. This indicates that a low-temperature pulse may cause the same phase shift of free-running rhythm under LL as a dark pulse. However, the present findings indicate that low temperature may not cause a phase shift in the house sparrow and Japanese quail.

In the owl, on the other hand, a single injection of melatonin caused a large decrease in body temperature, but did not prevent movement. We reported previously that the owl pineal gland was degenerate and that blood melatonin levels were very

low during both the day and night [Taniguchi et al. 1993]. Therefore, we speculated that the pineal degeneration was to avoid any melatonin–induced inhibition of movement at night. However, the present findings indicate that this may not be the case, and that the pineal degeneration may instead be used to avoid a decrease of body temperature. Thus in the owl, at least, the body temperature and locomotion responses to melatonin appear to have become separated.

In conclusion, the effects of melatonin on circadian rhythm, locomotor activity and body temperature vary among avian species.

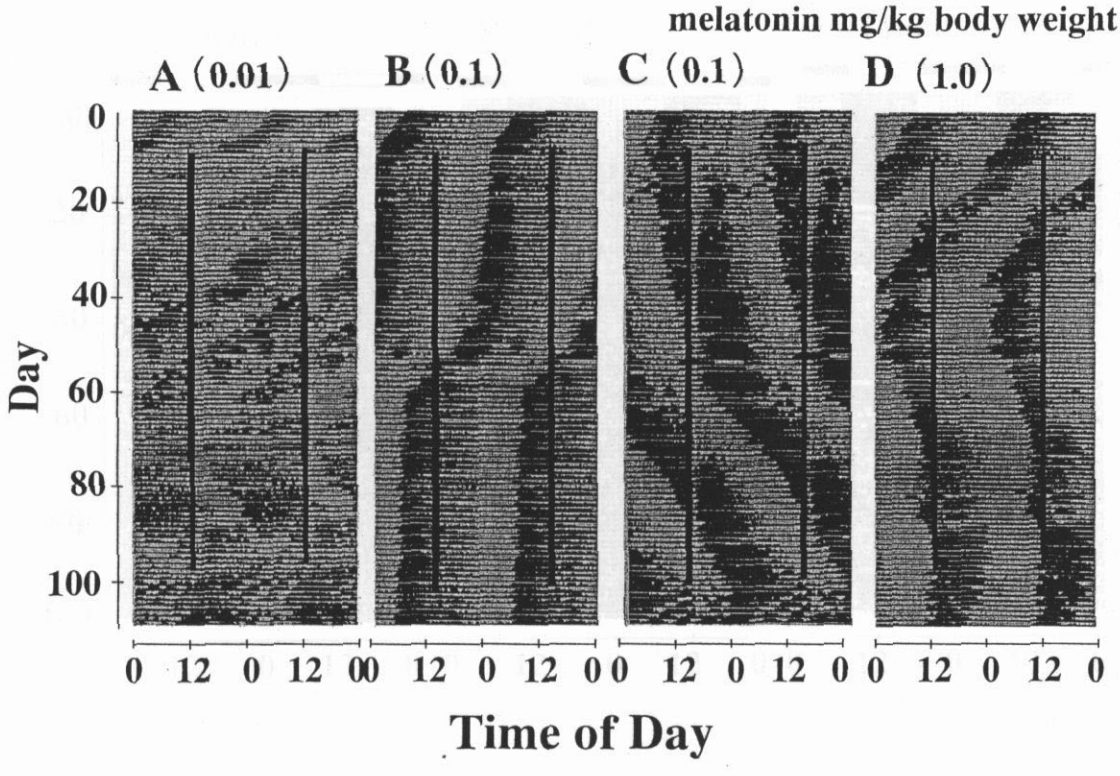


Fig. V-1. Examples of double plotted locomotor activity record before and after daily injection of melatonin or vehicle in intact house sparrow maintained under constant dim light. The melatonin and vehicle only injection times and day was indicated by white and black vertical bar, respectively, in the figures. The dose of melatonin administrated was follows. A: 0.01, B and C: 0.1 and D: 1mg/kg body weight.

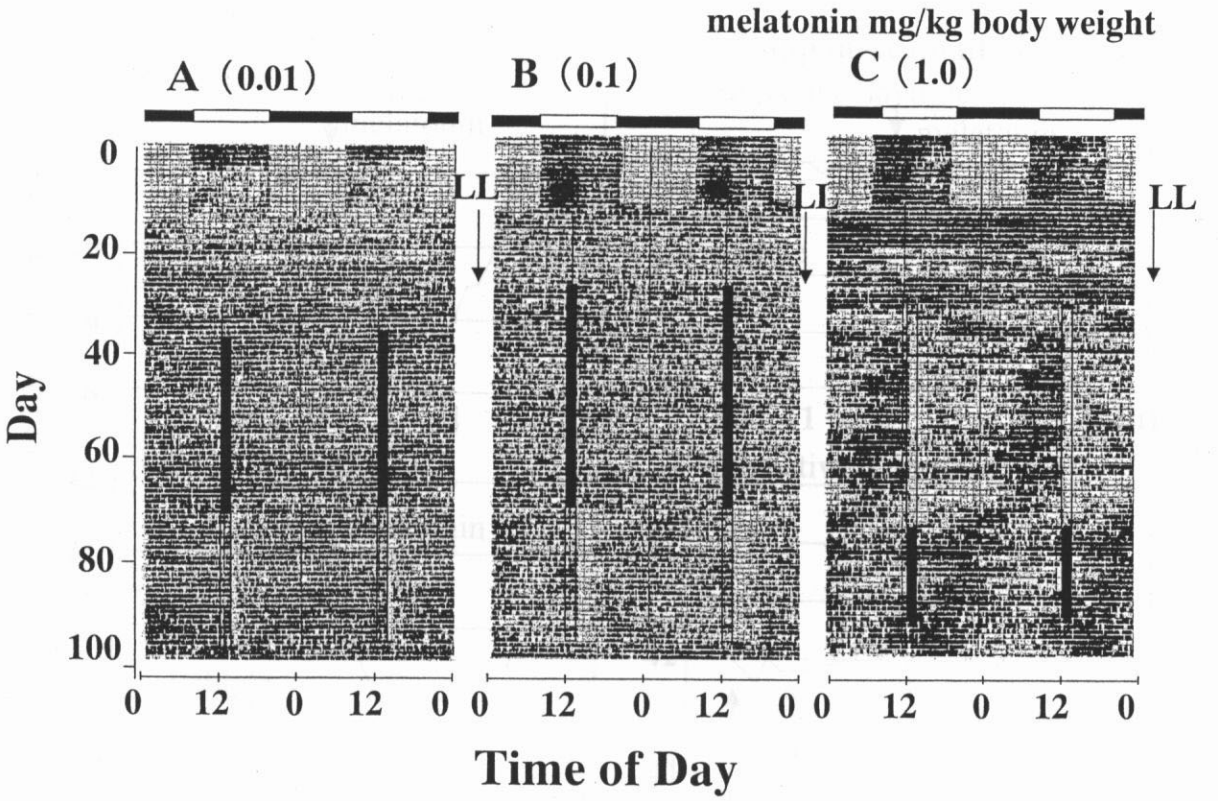


Fig. V-2. Examples of double plotted locomotor activity record before and after daily injection of melatonin or vehicle in intact Japanese quails maintained under constant dim light. The melatonin and vehicle only injection times and day were indicated by white and vertical bar, respectively, in the figures. The dose of melatonin administrated was follows. A: 0.01,B: 0.1 and C: 1mg/kg body weight.

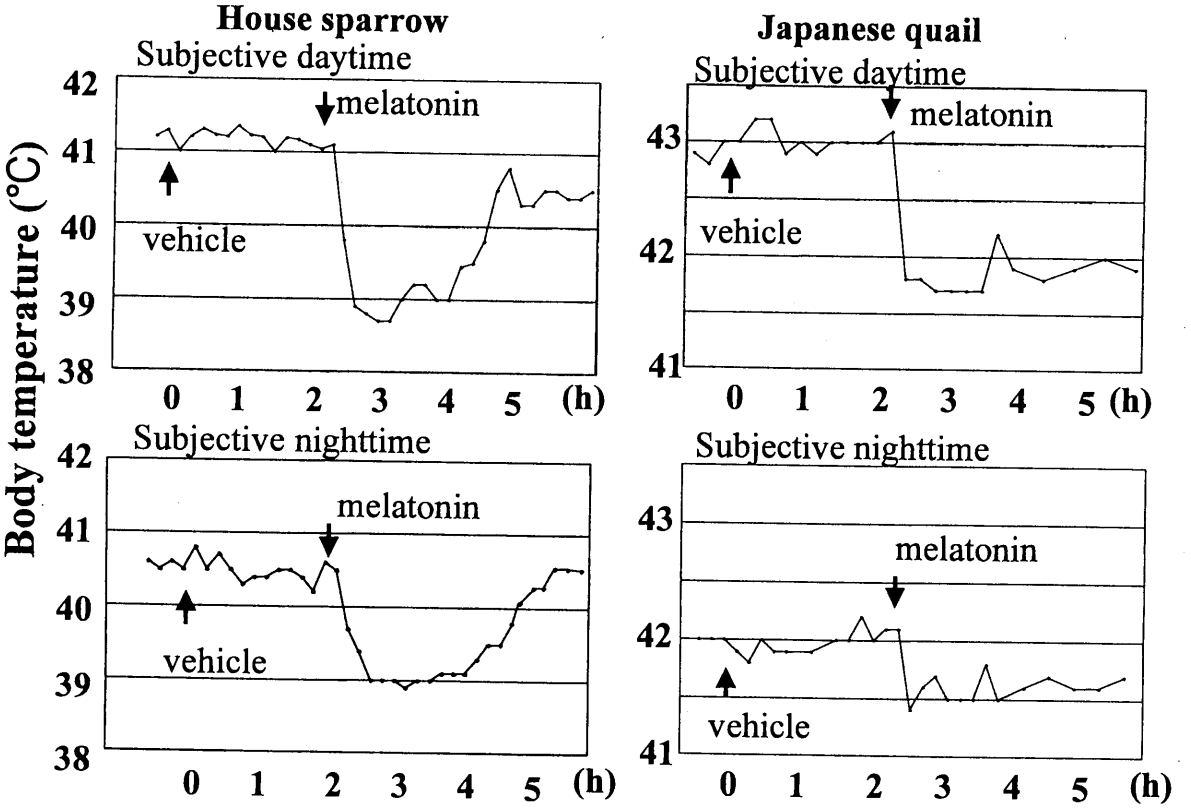


Fig. V-3. Effect of single injection of melatonin on the body temperature in house sparrow and Japanese quail maintained under constant dim light. Injections were started at 12:00~13:00 h and 0:00~01:00 h as a subjective day and night, respectively, two days after transition from LD to dim LL. Body temperature was decreased by melatonin, but not vehicle.

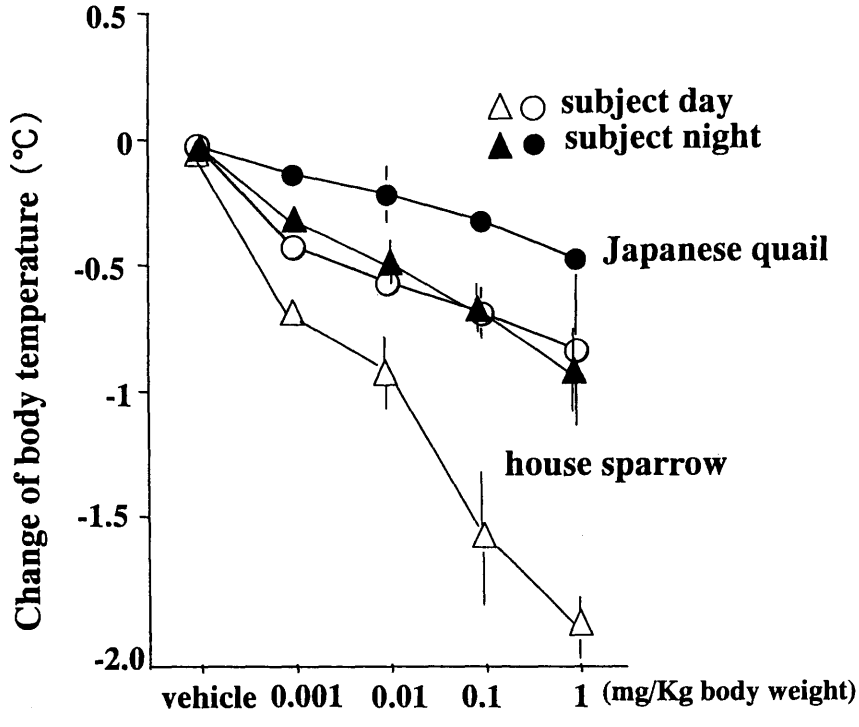


Fig. V-4. Dose-response curve for a single injection of melatonin on the body temperature in house sparrow and Japanese quail maintained under constant dim light. Injections were started at 12:00~13:00 h and 0:00~01:00 h as a subjective day and night, respectively, two days after transition from LD to dim LL. Open and closed triangles represent the decrease of body temperature at the subjective night day and night in house sparrow, respectively. The open and closed circle circles represent the decrease of body temperature at the subjective night day and night in Japanese quail, respectively. Each symbol and vertical bar indicate the mean ±SEM (n=8).

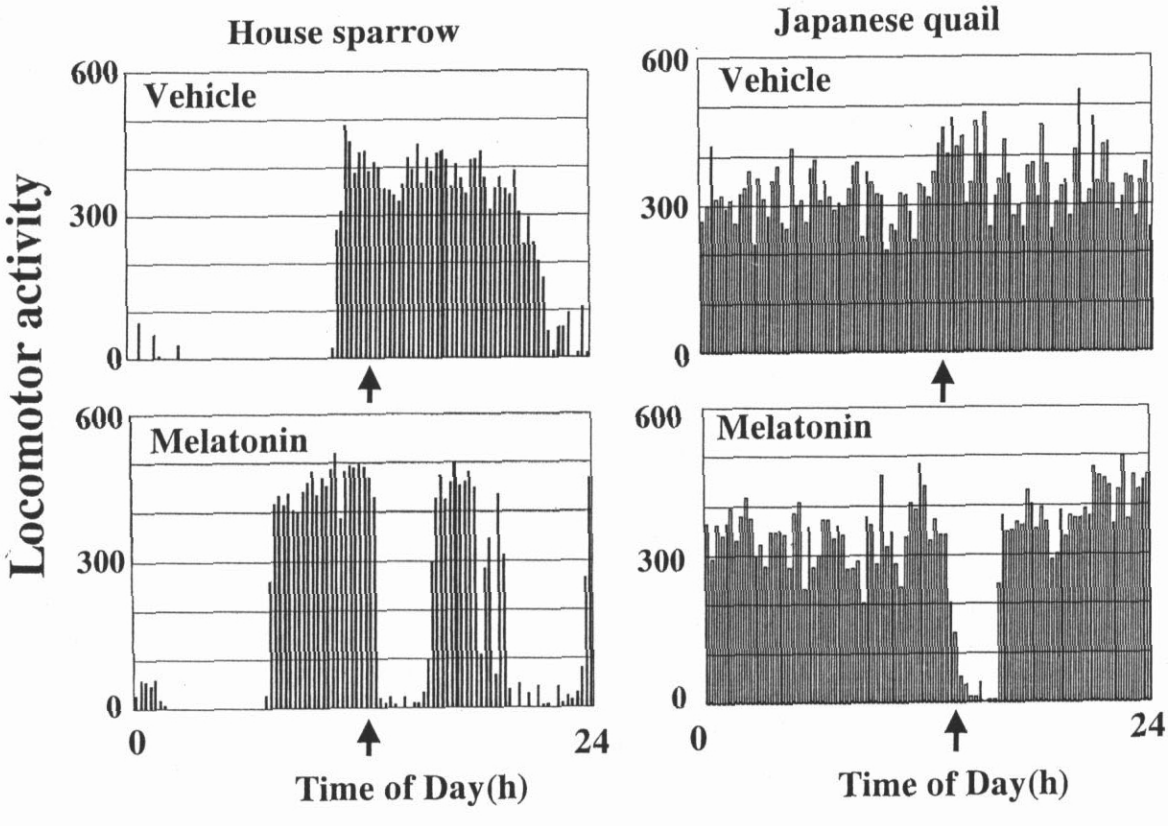


Fig. V-5. Effect of single injection of melatonin on the locomotor activity in house sparrow and Japanese quail maintained under constant dim light. Injections were performed at 12~13:00 h two days after transition from LD to dim LL (indicated by arrow). Locomotor activity was prevented by melatonin, but not vehicle.

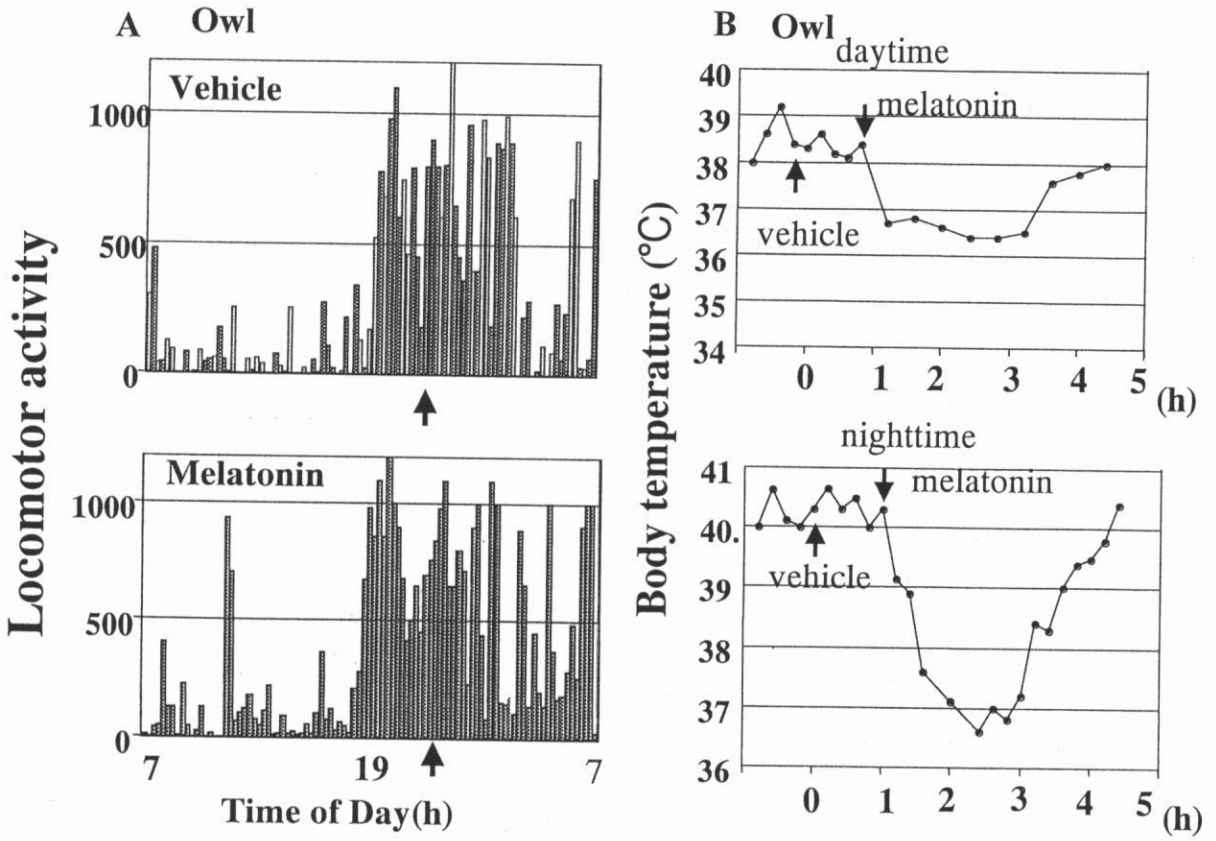
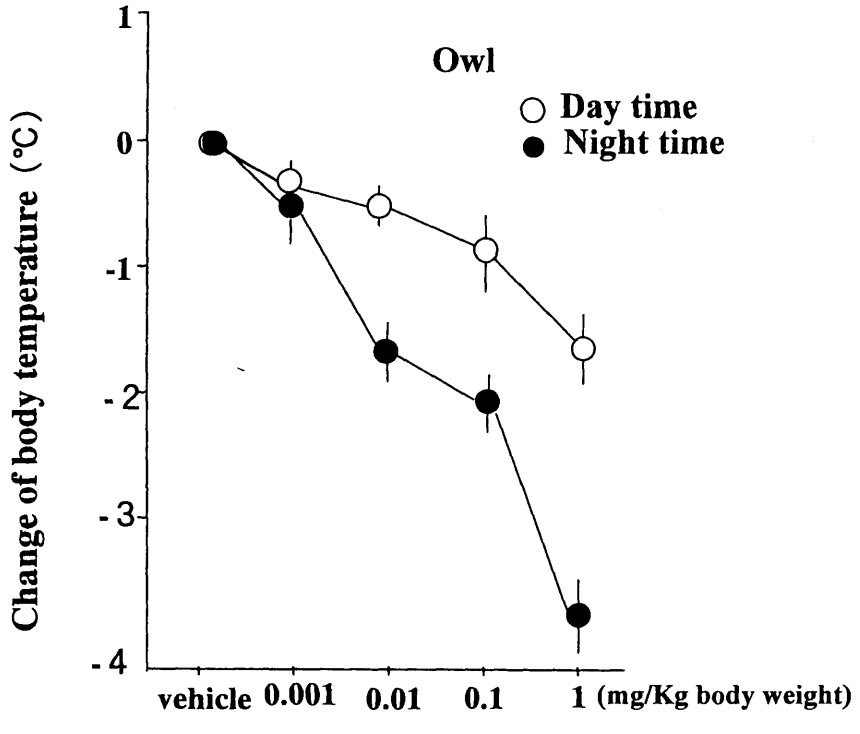


Fig. V-6. Examples of effects of melatonin on the locomotor activity (A) and body temperature (B) in an owl. Concerning about body temperature, injection was started at 12:00~13:00 h and 0:00~01:00 h under light-dark period. Effect of melatonin and vehicle on the locomotor activity was examined during movement judged by recorder under dark period.



Fig, V-7. Dose-response curve for a single injection of melatonin on the body temperature in owl. The open and closed circle circles represent the decrease of body temperature at the night and day time in owl. respectively. Each symbol and vertical bar indicate the mean ±SEM (n=5)

Chapter VI

Effects of microinjection of melatonin into various brain regions of Japanese quail on locomotor activity and body temperature

Abstract

In the previous chapter, I demonstrated that peripheral administration of melatonin resulted in simultaneous decreases in locomotor activity and body temperature in the Japanese quail. To examine the site of these melatonin-induced effects, I performed microinjection of melatonin into various brain regions of the Japanese quail. On the basis of the injection site the effects of melatonin were dissociated into four groups: decrease in only body temperature, decrease in only locomotor activity, decrease in both body temperature and locomotor activity, and neither change. The decrease in only body temperature was observed when melatonin was injected into areas of the diencephalon including the thalamus and hypothalamus, and decrease in only locomotor activity was observed when melatonin was injected close to the nuclei septalis medialis and septalis lateralis. These results suggest that melatonin-induced decreases in body temperature and locomotor activity occur by melatonin acting at discrete sites in the brain.

Introduction

Melatonin secreted from the avian pineal gland is thought to play an important role in circadian systems as a humoral mediator, since the pineal gland is one of the predominant circadian oscillators in avian species [Gaston and Menaker, 1968; Takahashi et al. 1989; Underwood and Goldman, 1987]. In starling, pigeon, and Japanese quail, daily administration of melatonin by injection, infusion, or in drinking water entrains the circadian activity rhythm such that the interval of activity precedes the time of injection [Chabot and Menaker, 1992; Gwinner, 1994; Lu and Cassone, 1993]. These entraining effects may involve direct resetting of the master clock by melatonin. Another possible cause is a masking effect by direct inhibition of locomotor activity by melatonin due to its influence on another brain site rather than by affecting the circadian oscillator or by melatonin actions on peripheral organs such as muscle. If melatonin directly suppresses locomotor activity, the resting phase must occur after the daily melatonin injection as well as the entrainment mentioned above. I demonstrated that a single intramuscular injection of melatonin caused dramatic decreases in body temperature and locomotor activity in a dose-dependent manner in house sparrow and Japanese quail in the previous chapter.

It is well known that melatonin affects thermoregulation in both avian species (such as chick, house sparrow, Japanese quail, and songbird) and mammals [Binkley et al. 1971; Cagnacci et al. 1992; George, 1982; John ea al., 1978; Padmavathamma and Joshi, 1994; Rozenboim et al. 1998; Underwood and Edmonds, 1995]. In avian species, the administration of melatonin always causes a decrease in body temperature, which is not the case in mammals [Binkley et al., 1971; Cagnacci et al., 1992; George, 1982; John et al., 1978; Padmavathamma and Joshi, 1994; Underwood and Edmonds, 1995]. However, melatonin-induced decreases in body temperature and locomotor activity always occurred simultaneously, and hence it is unknown

whether separate mechanisms are involved. Since brown adipose tissue is virtually absent in birds, the maintenance of body temperature is generally believed to be mainly by shivering thermo genesis. This leads to the possibility that either the melatonin-induced decrease in body temperature suppresses locomotor activity, or that the decrease in locomotor activity causes the body temperature to decrease.

The melatonin receptor is widely distributed from central to peripheral organs in avian species [Cozzi et al., 1993; Pang et al., 1995; Panzica et al. ,1994; Zawilska and Nowak, 1996]. As far as I am aware, however, there have been no reports on the targets for melatonin mediating its effects on body temperature or locomotor activity. In the present study, therefore, I examined whether central administration of melatonin also decreases the body temperature and/or locomotor activity in Japanese quail (as does peripheral administration) and, if so, whether these two effects are dependent upon each other or independent. I achieved this by direct microinjection of melatonin into various brain regions of the Japanese quail.

Materials and Methods

Japanese quails (*Coturnix coturnix*) were reared from hatching from the closed breeding colony in our laboratory. Food and water were provided *ad libitum*. All birds were kept under 12-h light/12-h dark (lights on 0700 hours) conditions for at least 2 weeks, and then transferred to constant dim-light conditions. First, to confirm the effect of peripheral injection of melatonin on the body temperature and locomotor activity, 10 male adult quails were injected with saline at ZT (zeitgeber time) 10 h, followed by melatonin (100 μg/kg body weight) at ZT 12 h. In a second experiment, 140 male adult quails underwent a surgical procedure to implant stainless steel cannulae into the head. After the quails had been anesthetized by intramuscular injection of sodium pentobarbital (1 mg/kg body weight), a 26-gauge stainless steel guide cannula was implanted bilaterally into various regions with a brain stereotaxic apparatus. The cannula tip was placed at the following stereotaxic coordinates: at 0.5-mm intervals anterior or posterior from interaural; at 0.5-mm intervals lateral from the midline; and 4, 6, or 8 mm below the dura. The guide cannula was anchored to the skull with machine screws and dental acrylic. For 2 days after surgery the quails were housed under constant dim light (intensity of about 30 lux) and allowed to recover. Four days after the implantation, 1.0 μl of saline was injected at ZT 12 h through a 31-gauge injection cannula connected to a 10-μl Hamilton microsyringe with 15 cm of polyethylene tubing into various brain regions, to confirm the absence of surgery-induced changes in body temperature and locomotor activity. Two days later, 0.5 μg/1.0 μl melatonin solution was injected at ZT 12 h, with the body temperature and locomotor activity being monitored before and after the injections. The locomotor activity of the animals was measured by a rat locomotor activity recording system (Muromachi, Co. Tokyo) comprising infrared sensors, an interface, and a computer [Murakami et al. 1997]. The infrared sensors were placed above the

birdcage, which detected all movements. The data were collected at 15-min intervals and analyzed by CompACT AMS software (Muromachi, Co). The body temperature was monitored at 12- or 15-min intervals using a temperature sensor with a small tip (measurable range: 25–50°C, measuring error: ± 0.05°C) connected with a line (OD: 0.7 mm, length: 45 cm) to the monitor body. The sensor tip was inserted into the cloaca and part of the line was fixed within the body, with the digital signal transferred to the monitor body. I estimated the effect of melatonin on the body temperature and locomotor activity according to the following criteria: If the body temperature decreased by more than 1°C or complete suppression of locomotor activity was observed for at least 30 min after the melatonin injection, I designated this as a melatonin-induced effect. After the end of experiments, the site of injection was confirmed anatomically in the removed brain fixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer.

Results

Locomotor activity rhythms were disrupted under the constant dim-light conditions, and hence saline was injected intramuscularly as a control, followed by melatonin. The melatonin, but not saline, inhibited locomotor activity and decreased body temperature simultaneously as shown in Fig.VI-1. These results are consistent with may previous study shown in Chapter V. Therefore, I examined whether central administration also decreases the body temperature and/or locomotor activity and, if so, I determined whether these two effects are dependent upon each other or not. Twenty-one out of the 140 Japanese quails implanted cannula were excluded from the data because of unevenness of bilateral cannulation, poor recovery after surgery, injection difficulties, and other problems. All the data are summarized in Fig. VI-2, and typical examples are shown in Fig. VI-3. At 4 mm below the dura, mean decreases in body temperature and locomotor activity were observed in response to 26.6% and 6.6%, respectively, of the 45 injections carried out. Most (66.6%) of the melatonin-injected quails did not exhibit any change in body temperature and locomotor activity. In addition, none of the birds showed decreases in both parameters simultaneously. The decreases in body temperature could be induced within a comparatively wide region close to the nuclei septalis medialis and septalis lateralis. At 6 mm below the dura, mean decreases in body temperature and locomotor activity in response to 42.5% and 52.5%, respectively, of the 40 injections carried out. In 34% of these cases, body temperature and locomotor activity decreased simultaneously. At 8 mm below the dura, mean decreases in body temperature and locomotor activity were observed in 70.5% and 49.9%, respectively, of the 34 injections carried out. In 41% of these cases, temperature and locomotor activity decreased simultaneously.

Discussion

In chapter V, when melatonin is injected peripherally into Japanese quail, its depressing effect on locomotor activity and body temperature appear in almost the same time scale. Under the premise that these effects are centrally mediated, this does not exclude separate brain targets for each response, because the peripherally injected hormone should reach different brain targets simultaneously. Indeed, the present study has demonstrated that the two melatonin effects involve separate mechanisms, since there were many sites where melatonin acted only on either locomotion or body temperature. As far as I am aware, this paper is the first to show the central effect of melatonin in avian species.

Contrary to our expectation, the region for melatonin-induced body-temperature decreases extended over a wide area to include the hypothalamic thermoregulatory region. Although the possibility of diffusion of the injected melatonin solution is not excluded, this result suggests that melatonin acts on the hypothalamus through neurotransmitters released over wide regions, since melatonin receptors are widely distributed throughout the central nervous system [Cozzi et al., 1993]. On the other hand, each of the multiple sites for specific suppression of locomotion activity by melatonin were circumscribed but scattered. Especially, the hyperstriatum ventrale and neostriatum intermedium are sites for the suppression of only locomotor activity by melatonin.

Under regular light/dark conditions, the body temperature and locomotor activity generally decreased during the dark phase when the plasma melatonin increased, and these decreases may be due to direct action of melatonin on the central nervous system since the central administration of melatonin to various brain regions decreased body temperature and/or locomotor activity. However, melatonin is not the only possible cause for decreases in body temperature and locomotion, since there

were several exceptions where the circadian rhythms of locomotor activity and body temperature were dissociated. As shown in previous chapter, in Japanese quail, although the body temperature rhythms were maintained under constant dim light, locomotor activity rhythms were disrupted without suppression of activities. In nocturnal birds, such as owl, melatonin has been shown to decrease body temperature but not locomotor activity. Thus in the owl, at least, the body temperature and locomotion responses to melatonin appear to be separated, indicating that administration of melatonin does not always result in decreases in both body temperature and locomotor activity in avian species. I cannot explain these discrepancies from the results of the present study. On the other hand, it has been demonstrated that melatonin affect the blood vessels, and that expression of melatonin receptors in arteries might be involved in thermoregulation [Viswanathan et al., 1990]. Therefore, further studies are required to elucidate the mechanism of melatonin in the regulation of thermogenesis and behavior in avian species.

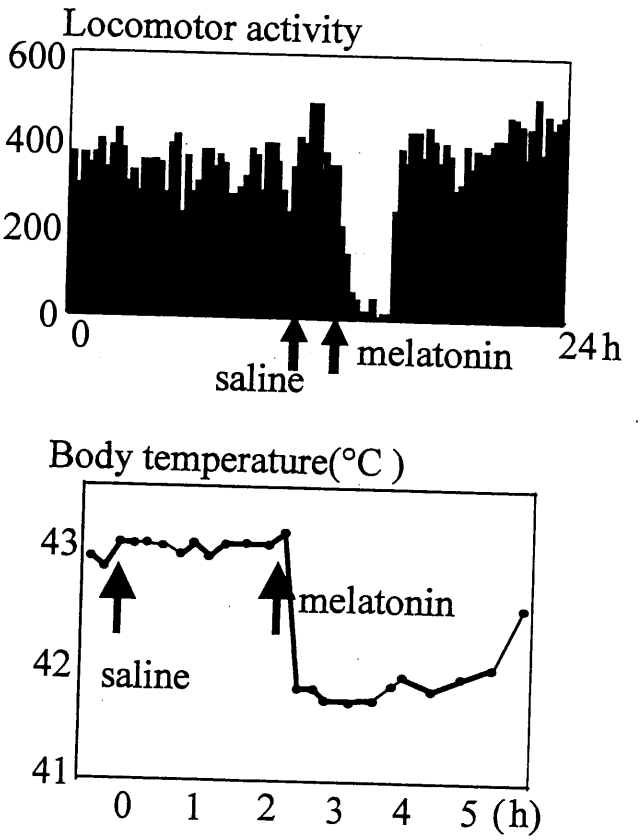


Fig. VI-1. Effect of a single intramuscular injection of melatonin or saline on the locomotor activity and body temperature in Japanese quail maintained under constant dim-light conditions. Saline and melatonin injections were started at 1000 and 1200 hours 2 days after the transition from light/dark (LD) to dim light/light (LL), respectively. Melatonin, but not saline, simultaneously decreased locomotor activity and body temperature.

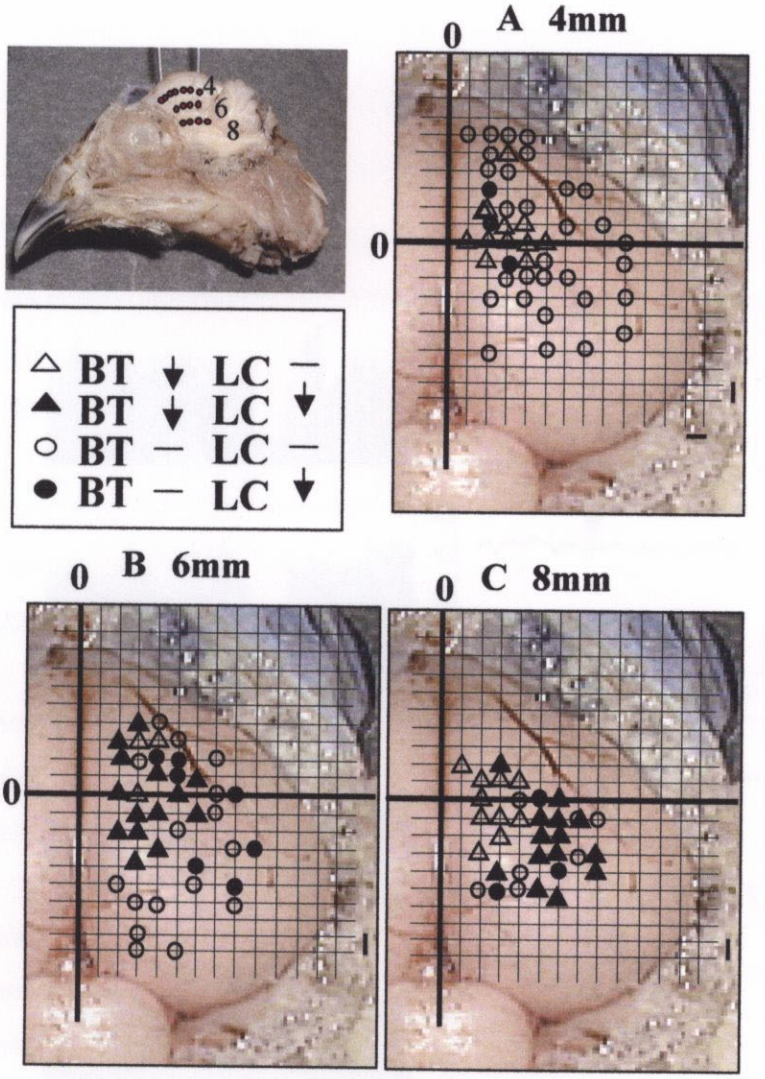


Fig. VI-2. Effect of microinjection of melatonin into various brain regions on the locomotor activity and/or body temperature in Japanese quail. Panels A, B, and C are Japanese quail brains observed from the top, and the vertical and horizontal lines are 0.5-mm intervals from the midline and ear bar, respectively. The depth of the cannula tip (depth of injection) was 4 mm (A), 6 mm (B), and 8 mm (C). Each symbol represents both the location of injection at each depth and the resulting effect of melatonin on the body temperature and/or locomotor activity: open triangles, decrease in only body temperature (BT); closed triangles, decreases in BT and locomotor activity (LC); open circle, decrease in neither BT nor LC; closed circle, decrease in only LC. Upper-left panel represents the sagittal section of Japanese quail brains showing the three kind of depth (4,6,8 mm) of cannula tip.

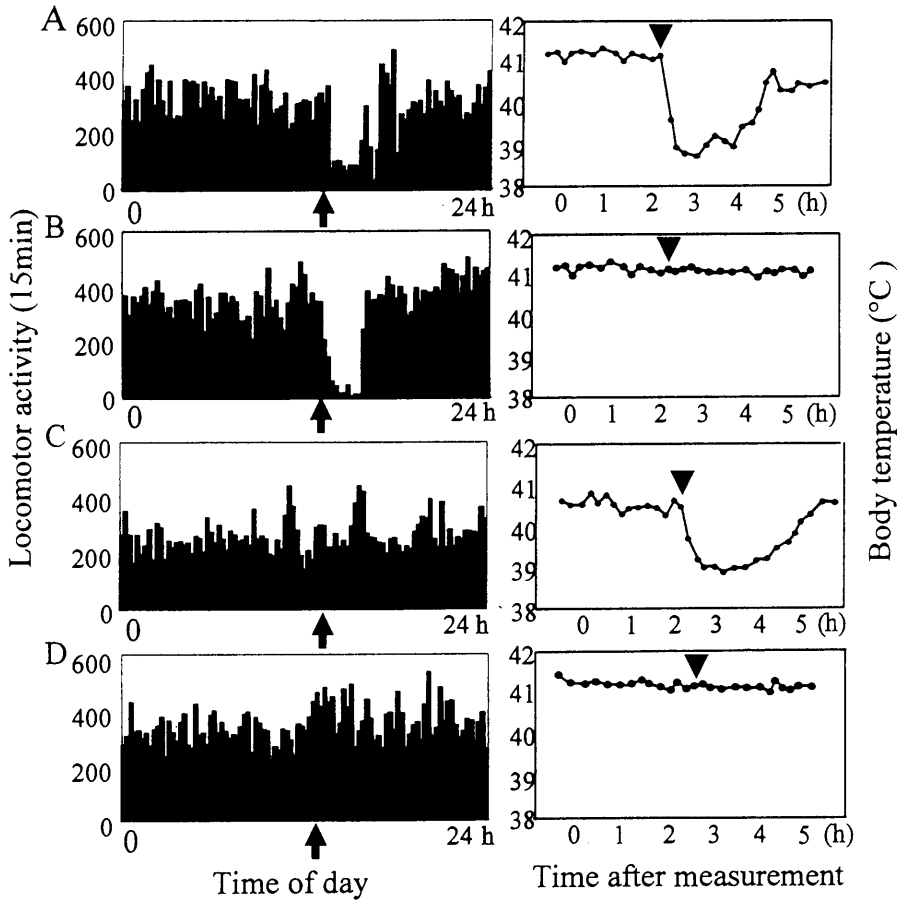


Fig. VI-3. Typical examples showing the effect of microinjection of melatonin into various brain regions on the BT and/or LC. Panels show examples of the decrease in both BT and LC (A), only LC (B), only BT (C), and neither BT nor LC (D). The arrows indicate the time of injection.

General discussion

The suprachiasmatic nucleus (SCN) plays an important role in the generation of various physiological rhythms, and appears to be the predominant circadian oscillator in mammals [Turek, 1985]. On the other hand, many studies have demonstrated that the pineal gland is a more important oscillator than the SCN in avian species. Among these, two outstanding studies provide compelling evidence that the pineal gland is the main avian circadian oscillator. The first showed that pinealectomy abolished the circadian rhythm in locomotor activity in house-sparrows, and that reimplantation of pineal gland tissue into pinealectomized house-sparrows restored this rhythm [Zimmerman and Menaker, 1979]. The second was the discovery by Deguchi (1979b) that chick pineal cells, *in vitro*, exhibited circadian oscillation in the activity of N-acetyltransferase (NAT), a rate-limiting enzyme in melatonin synthesis. This involved an increase during the dark period and a decrease during the light period, and these rhythms were maintained under constant conditions. The latter discovery had a considerable impact on research in the field of biological rhythms because it indicated that chick pineal cells possess a photoreceptor, a circadian clock and melatonin synthetic capacities, and that they develop light-dark entrained circadian oscillation of NAT activity by linking these components together. However, it also raised the fundamental question of whether the photoreceptor, circadian clock and mechanism for melatonin synthesis reside within separate pineal cells, or whether a single pineal cell can in itself generate the circadian rhythm in melatonin secretion. To address this question, I cultured single pineal cells in a Terasaki microplate and measured melatonin secretion every 12 hours under light-dark cycle, dark-light cycle and constant dark conditions. The individual cells secreted more melatonin during the dark period than during the light period under both light-dark cycle and dark-light cycle conditions, and rhythmic secretion persisted during constant darkness. These

results suggest that, in the chick, each individual pineal cell possesses photoreceptive, circadian clock and melatonin-synthesizing capacities, and that generation of the circadian rhythm in melatonin secretion does not require interaction with any cells other than pinealocytes. However, I was unable to determine whether or not the phase and the period of the circadian oscillator in each of the individual cells from a single pineal gland were the same. To resolve this problem, short-interval sampling is required, and this will need the development of a ten-fold more sensitive assay system.

In the first chapter, I showed that melatonin release from individual pineal cells increased during the dark periods and decreased during the light periods of light-dark cycles, and that this rhythmic secretion was maintained under constant dark conditions. This observation resulted in the question of how the circadian oscillator drives the melatonin increase under constant conditions. The pathway most likely to be involved in cellular signal transudation from the circadian clock to the melatonin synthetic system appears to be the cAMP system, because it is well known that cAMP agonists stimulate melatonin release in chick pineal cells synthesis [Zatz and Mullen1988a; Zatz et al., 1988; Takahahshi et al, 1989]. In the second chapter, therefore, I examined the possibility that cyclic AMP and cAMP-dependent protein kinase A may be involved in the subjective nocturnal increase in melatonin release by chick pineal cells cultured under constant darkness. The subjective nocturnal increase in melatonin release was suppressed dose-dependently by H8 (a general protein kinase inhibitor) and H89 (a specific protein kinase A inhibitor), but not by calphostin C (a specific protein kinase C inhibitor) in static cell cultures. In a cell perfusion experiment, 9-hour pulses of H8 and H89 starting at zeitogaber time (ZT) 9 h (CT11.2 h) suppressed the subjective nocturnal increase in melatonin release in a dose-dependent manner without causing a phase shift. An intracellular Ca^{2+} chelator,

BAPTA-AM, and two extracellular C^{2+} chelators, BAPTA and EGTA, all suppressed the subjective nocturnal increases in melatonin release and cAMP levels dose-dependently. These direct pieces of evidence strongly support the hypothesis that cAMP and cAMP-dependent protein kinase A may be involved in the subjective nocturnal increase in melatonin release by chick pineal cells, and that intracellular Ca^{2+} plays an important role in pineal adenylate cyclase activation. Recently, it has been shown that adenylate cyclase type I and VIII are activated by Ca^{2+}-calmodulin [Cali. et al., 1994; Wong et al.,1990]. I am now working on identifying which type of adenylate cyclase is present in the chick pineal gland and on cloning the enzyme.

Although many findings concerning the regulation of melatonin release in the rat pineal gland have accumulated, there is little information on the regulation of melatonin release from the avian pineal gland. Recently, it was reported that pituitary adenylate cyclase-activating polypeptide (PACAP) plays an important role in both the release of melatonin from pineal cells and the glutamatergic regulation of the suprachiasmatic circadian clock in rats [Chik and Ho,1995; Simonneaux et al.1990, Simonneaux et al.1993; Hannibal et al.1997; Hannibal et al, 1998; Harrington et al.1999]. In the third chapter, therefore, I investigated whether PACAP is involved in melatonin release and the circadian oscillation system in chick pineal cells, and if so, whether its effects are mediated by the PACAP-specific receptor (PACAP-r1). Within the dose-range 10^{-10} to 10^{-6} M, PACAP increased melatonin release dose-dependently during the 12-h light period on day 3 of culture, and the degree of stimulation was greater than that produced by VIP. VIP receptor antagonists produced only slight inhibition of PACAP-stimulated melatonin release, while simultaneous addition of VIP and PACAP produced almost additive melatonin release. Under constant dark conditions, 6-h pulses of PACAP started at ZT 15, 21, 3 and 9 h in separate groups of pineal cells did not cause any phase shifts in the

melatonin rhythms of the cells. In addition, PACAP did not affect a light-induced phase advance (ZT 15 h) or delay (ZT 9 h) in the melatonin rhythms. The expression of mRNA for PACAP-r1 (including its splicing variant with a hop cassette) was observed in chick pineal cells. These results suggest that PACAP participates in melatonin release, but not in the circadian oscillator system, via the specific receptor PACAP-r1 in chick pineal cells.

As shown in the first chapter, chick pineal cells have photoreceptive, circadian clock and melatonin synthetic capacities, and exhibit circadian oscillation of melatonin release entrained by light-dark cycles *in vitro*. The entrainment of the circadian clock to light is based on light-induced phase shifts in the circadian clock. That is, application of light pulses to pineal cells causes phase-dependent phase shifts in the circadian melatonin rhythms [Robertson and Takahashi, 1988a; Zatz and Mullen, 1988a; Takahashi et al, 1989]. However, it is unclear how light acts on the circadian clock via the photoreceptor, and which signal transduction pathway is involved in light-induced phase-shifting of the circadian clock [Zatz and Mullen, 1988b; Nikaido and Takahashi, 1989; Takahashi et al, 1989]. To address these questions, in the forth chapter, I analyzed the mechanism underlying light-induced phase-shifting of the circadian rhythm in chick pineal cells. Phase-shifting of the circadian clock occurs immediately after the application of a light pulse. Therefore, I focused on the possibility of the involvement of intracellular Ca^{2+} in light-induced phase-shifting. Accordingly, thapsigargin and cyclopiazonic acid, which deplete the intracellular calcium stores, blocked a light-induced phase advance in a dose-dependent manner. Pulses of a ryanodine receptor antagonist (dantrolene sodium or ruthenium red) also blocked the light-induced phase advance. Most agents did not cause a significant phase shift in themselves. On the other hand, all of the agents used failed to block a light-induced phase delay, even if the magnitude of the

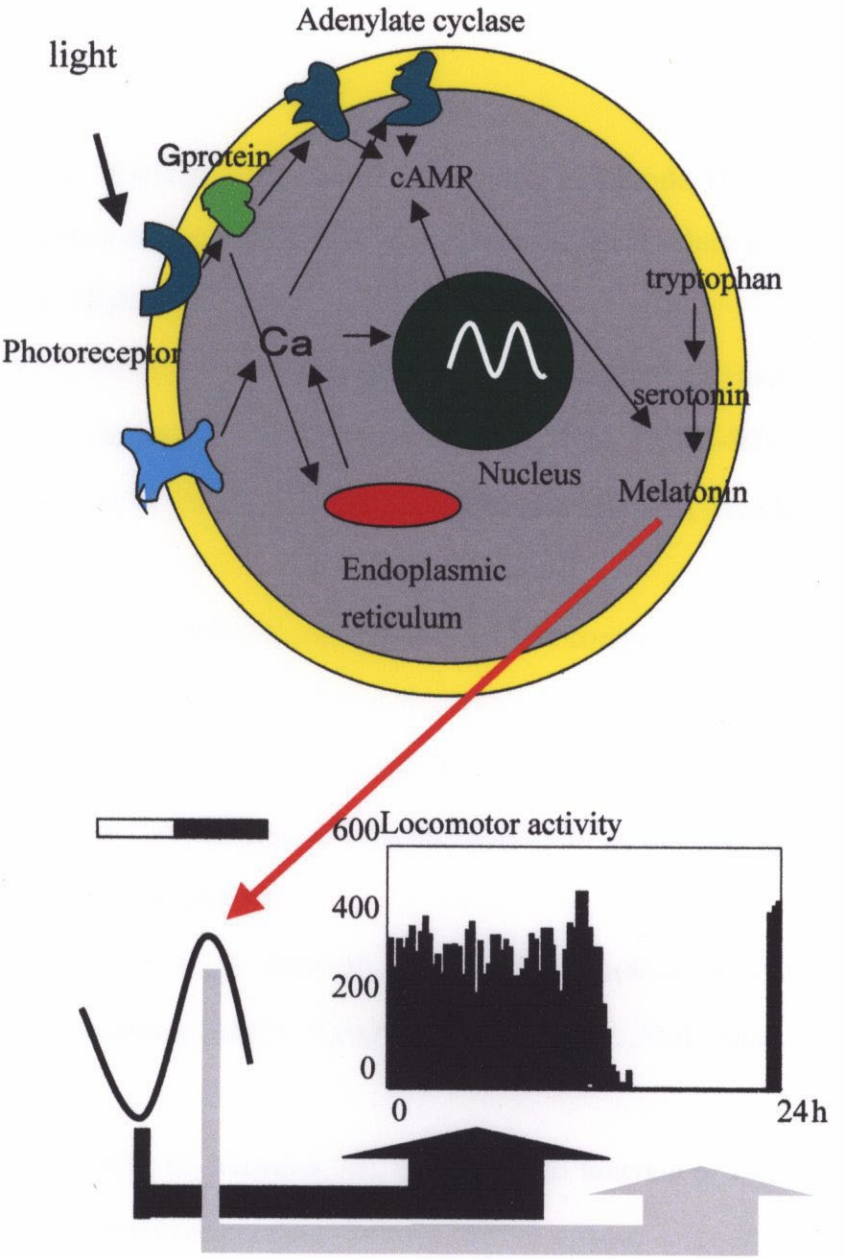
delay was decreased by using low-intensity light. Antagonists of nitric oxide synthase blocked neither the light-induced phase advance nor the light-induced phase delay. These results indicate that intracellular Ca^{2+} plays an important role in light-induced phase advances, but not in phase-delays. The mechanisms by which light-induced phase advances and delays arise in chick pineal cells may therefore be different.

How does the circadian clock regulate so many physiological rhythms, such as the sleep-wake pattern rhythm, the body temperature rhythm and so on? As the pineal gland has no neural output, melatonin may be the mediator of such regulation. To ascertain this possibility, I investigated the effects of a single injection or of repeated daily injections of melatonin on the circadian rhythms in behavior, locomotor activity and body temperature, as described in the fifth chapter. In particular, I compared the effects of melatonin in diurnal birds (house-sparrows and Japanese quails) and nocturnal birds (owls). Daily treatment with melatonin at a fixed time did not entrain the free-running rhythm in locomotor activity in house-sparrows or the disrupted rhythm in Japanese quails kept under constant dim light. However, melatonin clearly inhibited movement for several hours after treatment. The duration of resting after the melatonin injection was dose-dependent. Body temperature was also significantly decreased after melatonin treatment; this effect was more obvious during the active phase than during the resting phase. Although the effect of melatonin on body temperature was also dose-dependent, the magnitude of the melatonin-induced decrease in body temperature was larger in house-sparrows than in Japanese quails. A further large decrease in body temperature was produced by melatonin in an owl with a degenerated pineal gland. This decrease in body temperature was more marked during the active phase than during the resting phase. In the owl, melatonin did not prevent movement despite the decrease in body

temperature. These results suggest that melatonin regulates at least locomotor activity and body temperature. Probably, the night-time increase in melatonin levels acts to decrease locomotor activity and body temperature in diurnal birds. On the other hand, melatonin does not decrease locomotor activity in nocturnal birds. These results raised the question of where melatonin acts to affect locomotor activity and body temperature. Since there are no reports concerning the site of action of melatonin in the central nervous system, in the last chapter, I investigated the site of action by administering microinjections of melatonin into various brain regions of the Japanese quail. The effects of melatonin could be categorized into four groups, depending on the injection site: a decrease in body temperature only, a decrease in locomotor activity only, a decrease in both body temperature and locomotor activity, and no change in either. A decrease in body temperature alone was observed when melatonin was injected into areas of the diencephalon, including the thalamus and the hypothalamus, while a decrease in locomotor activity alone was observed when melatonin was injected close to the nuclei septalis medialis and septalis lateralis. These results suggest that melatonin-induced decreases in body temperature and locomotor activity are caused by melatonin acting at discrete sites in the brain.

In its various chapters, this thesis presents several studies on the characteristics of the circadian oscillation system in the avian pineal gland, as well as on the mechanisms involved in the regulation of melatonin release, light-entrainment, the physiological role of the melatonin rhythm and the site of action of melatonin. I believe that this thesis presents many important suggestions concerning avian biological rhythms. As a summary, on the next page I present a schematic representation of the regulation of the circadian melatonin rhythm, and of the regulation of the circadian locomotor activity rhythm by the melatonin rhythm in avian species. I conclude that the avian pineal gland functions as a biological clock,

which regulates the circadian rhythms of many physiological functions via rhythmic secretion of melatonin.



Schematic of the link system between photoreceptive, oscillator and melatonin synthesis in chick pineal cells

Light is perceived by photoreceptor, and then entrains the circadian oscillator via cellular Ca^{2+}. The rhythm of oscillator drives the melatonin rhythm through Ca^{2+}-cAMP signal transudation pathway. The melatonin increased by oscillator suppresses the locomotor activity and decrease body temperature.

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Acknowledgments (謝辞)

稿を終るに臨み、終始ご指導、ご鞭撻を賜りました宮崎大学農学部獣医学科家畜生理学講座の村上　昇助教授に深甚なる謝意を表します。論文作成および研究のご指導、ご援助を賜りました、東京大学大学院農学生命科学研究科応用動物科学専攻細胞生化学研究室の塩田邦郎教授に心から感謝申し上げます。また、実験の遂行に際し、ご協力頂きました研究室の関係各位に厚く御礼申し上げます。

最後に、これまでいつも励まし、見守って下さった母に心から感謝申し上げます。