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

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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 this study 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 antagonists 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 et al, 1988a;Takahashi et al, 1989]. Recently, we 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, 1989b; Zatz et al, 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, we compared the effect of various Ca^{2+} antagonists on the light-induced phase advance and those on the phase delay. Here, we report 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-arginine 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; Crossin, 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 x g. The supernatant was decanted and 10 ml a trypsin solution (0.5 mg/ml, Sigma)

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 x 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 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 [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, and 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 (23.00 h) for phase advance and ZT 10 (17.00 h) for phase delay [Murakami et al, 1995], with ZT12 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 x 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

We 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 1A shows the individual data from 4 wells at the same time in a single experiment and figure 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 (23.00 h) caused both the acute inhibition of melatonin release and large phase advance as shown in figure 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. 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. 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 (17.00 h) caused a phase delay with a magnitude of 4.2 h together with acute inhibition of melatonin release (Fig. 4B). TPS did not reduce the magnitude of this phase delay. The pulses of TPS itself given at ZT 10 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. 4B). The magnitude of acute reduction of melatonin by TPS or Cyc themselves was dependent on time and dose (Fig. 2B, Fig. 3B). The dose over 1 μM of TPS or 30 μM 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, we re-examined this using the smaller phase delay induced by low intensity light pulse. As shown in figure 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. 3B, and Fig. 4C).

The pulses of ryanodine receptor antagonist, dantrolene (Dan) or ruthenium red (Rut) also blocked the light-induced phase advance (Fig. 5B, Fig. 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. 6, Fig. 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. 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 Mullen, 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.

We could not identify the mechanism for light-induced phase delay in this study.

In chick pineal cells, however, there are many distinctive mechanism 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) and high at expected light off (CT16-18). 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.

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Figure Legends

Fig. 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. 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. 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. 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 $\pm$ 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. 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. 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. 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 $\pm$ 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.

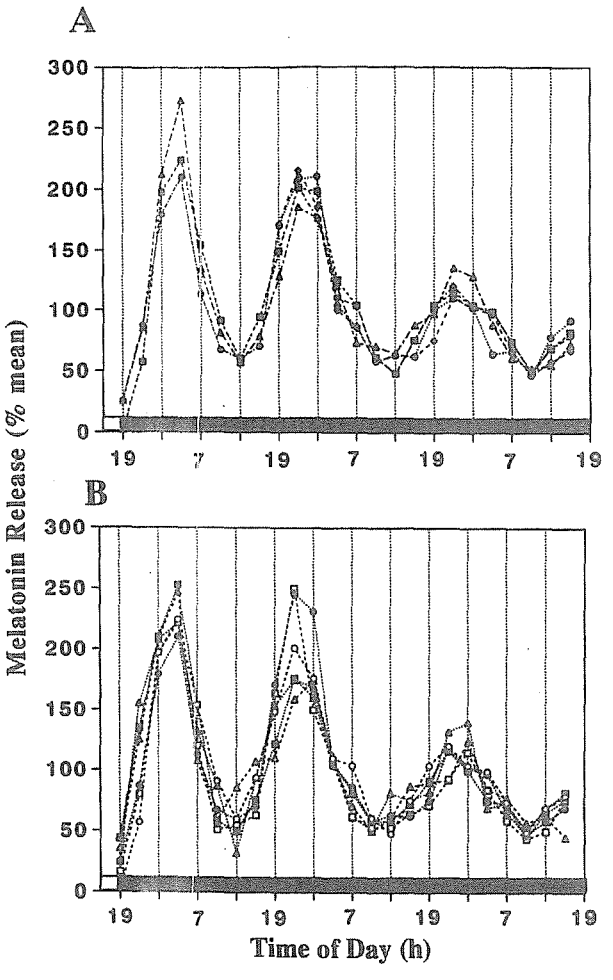
A
B
300
250
200
150
100
50
0
19
7
19
7
19
7
19
Melatonin Release (% mean)
Time of Day (h)

Fig.1

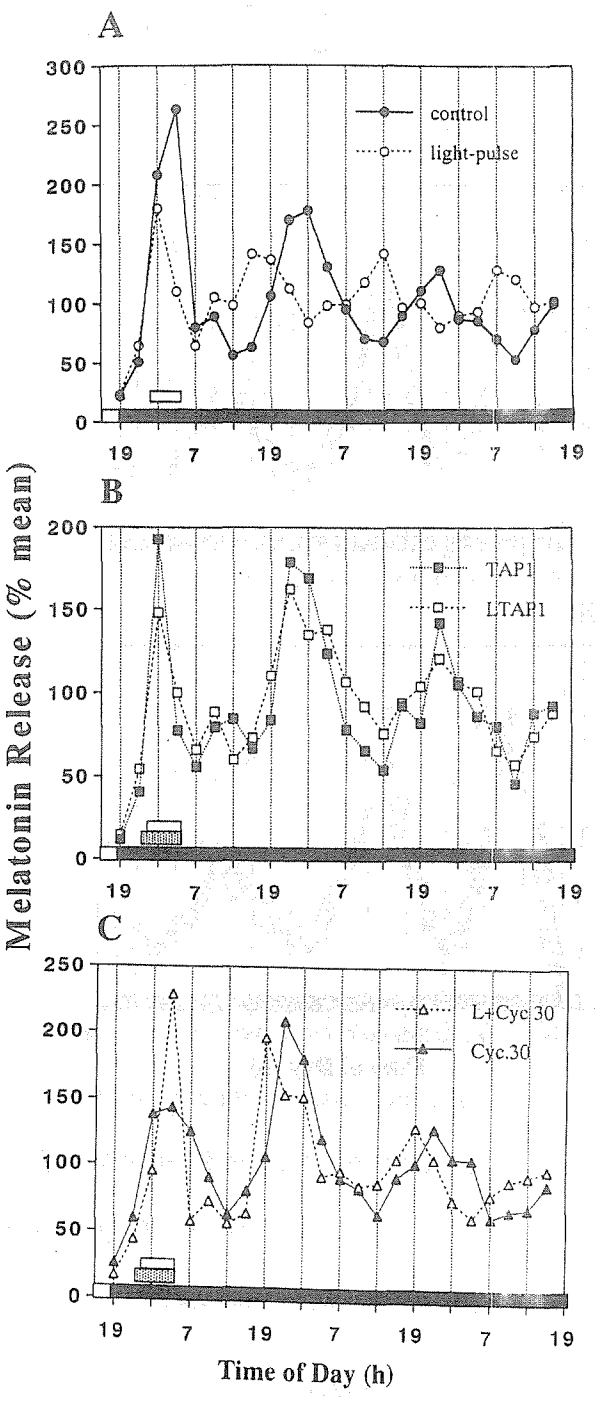
A
300
250
200
150
100
50
0
control
light-pulse
19
7
19
7
19
7
19
B
200
150
100
50
0
TAP1
LTAP1
19
7
19
7
19
7
19
C
250
200
150
100
50
0
L+Cyc.30
Cyc.30
19
7
19
7
19
7
19
Melatonin Release (% mean)
Time of Day (h)

Fig. 2.

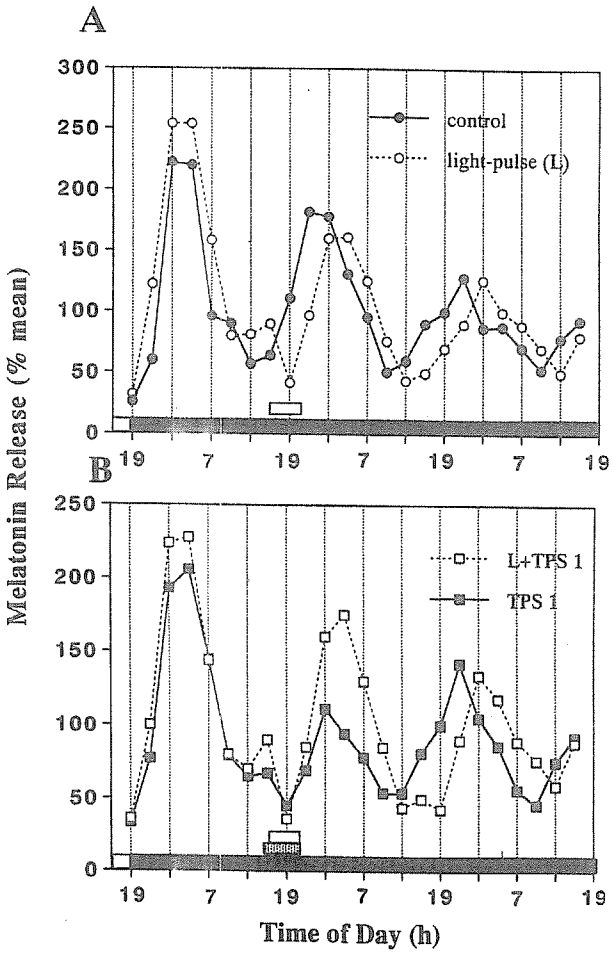
A
B
control
light-pulse (L)
L+TPS 1
TPS 1
Melatonin Release (% mean)
Time of Day (h)
300
250
200
150
100
50
0
19
7

Fig. 3

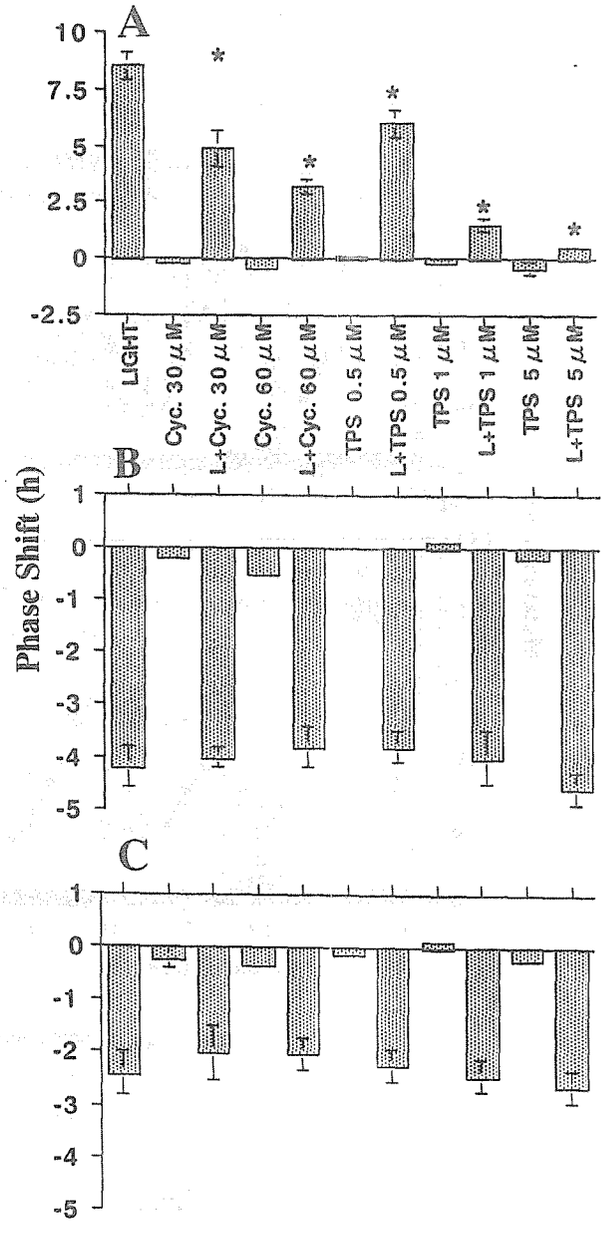
A
B
C
Phase Shift (h)
10
7.5
5
2.5
0
-2.5
1
0
-1
-2
-3
-4
-5
*
LIGHT
Cyc. 30 μM
L+Cyc. 30 μM
Cyc. 60 μM
L+Cyc. 60 μM
TPS 0.5 μM
L+TPS 0.5 μM
TPS 1 μM
L+TPS 1 μM
TPS 5 μM
L+TPS 5 μM

Fig. 4

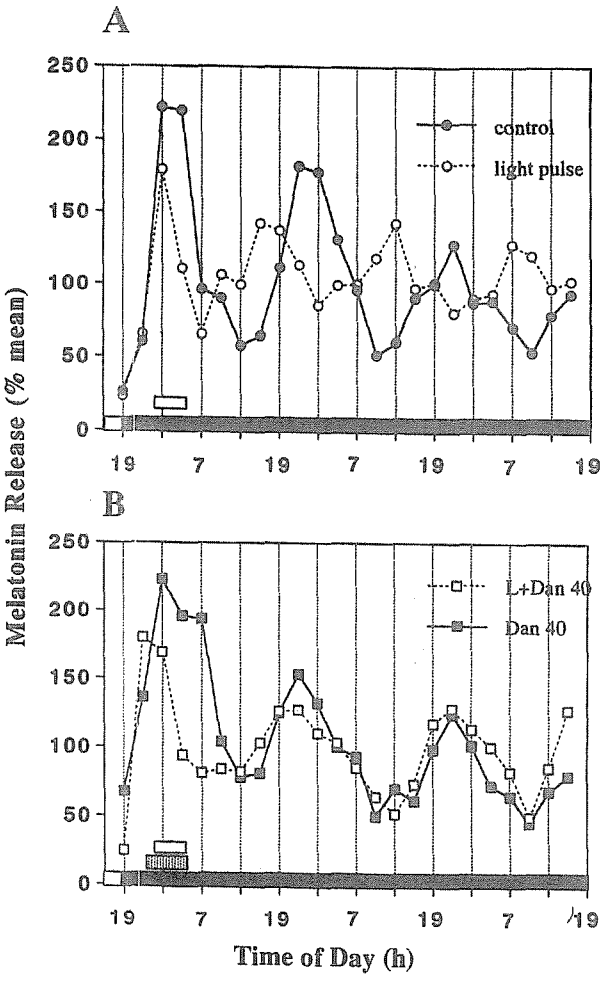
A
250
200
150
100
50
0
control
light pulse
19
7
19
7
19
7
19
B
250
200
150
100
50
0
L+Dan 40
Dan 40
19
7
19
7
19
7
19
Melatonin Release (% mean)
Time of Day (h)

Fig. 5

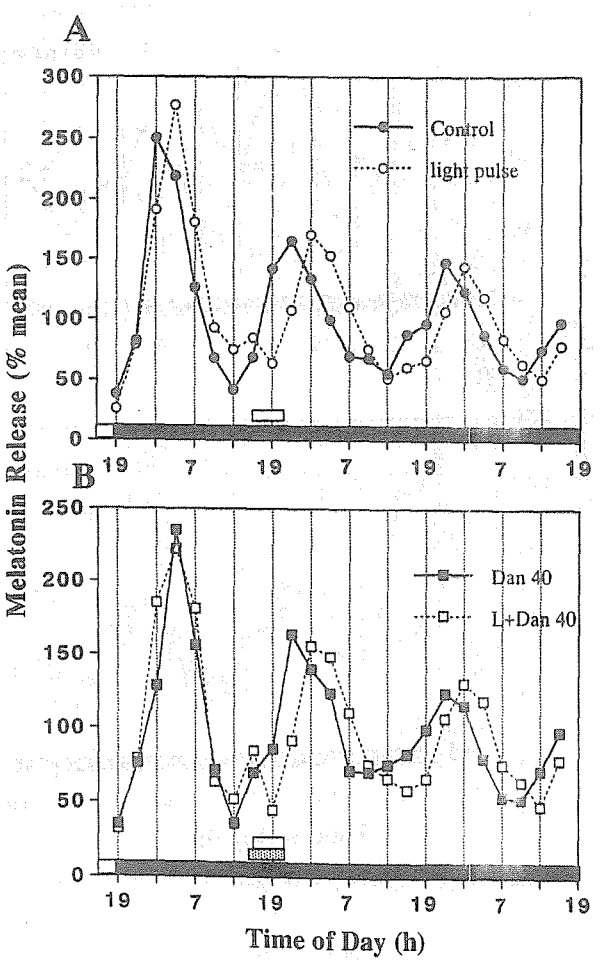
A
B
Melatonin Release (% mean)
Time of Day (h)
Control
light pulse
Dan 40
L+Dan 40
300
250
200
150
100
50
0
19
7

Fig. 6

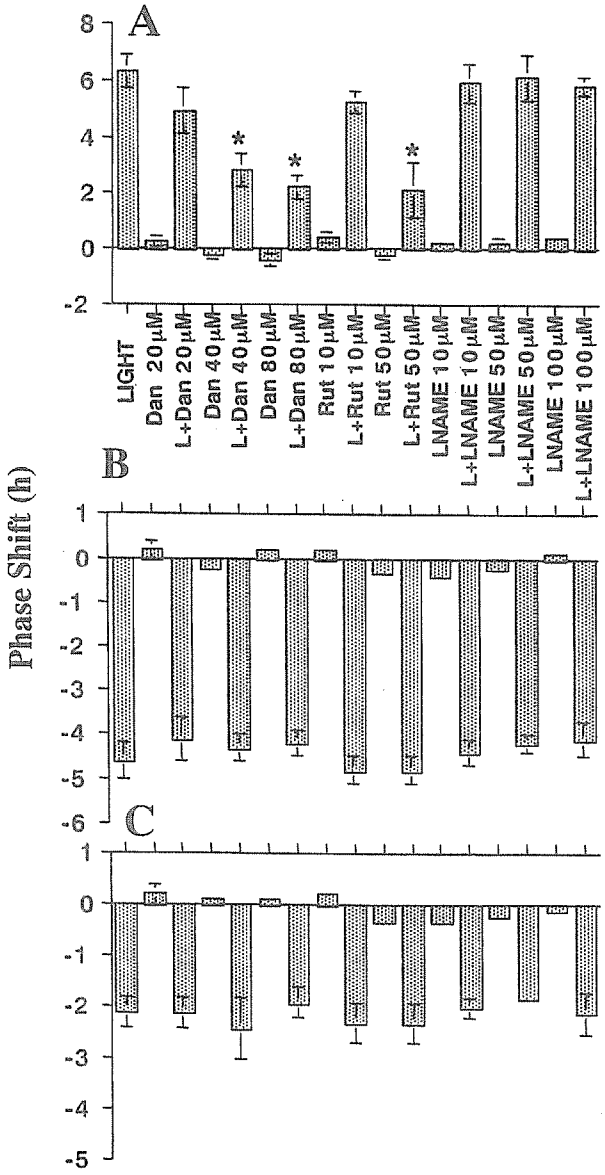
A
B
C
Phase Shift (h)
LIGHT
Dan 20 µM
L+Dan 20 µM
Dan 40 µM
L+Dan 40 µM
Dan 80 µM
L+Dan 80 µM
Rut 10 µM
L+Rut 10 µM
Rut 50 µM
L+Rut 50 µM
LNAME 10 µM
L+LNAME 10 µM
LNAME 50 µM
L+LNAME 50 µM
LNAME 100 µM
L+LNAME 100 µM

Fig. 7