

# Effect of Brefeldin A on Melatonin Secretion of Chick Pineal Cells<sup>1</sup>

Tsuyoshi Hirota,<sup>\*†</sup> Satoshi Kagiwada,<sup>†‡2</sup> Takaoki Kasahara,<sup>\*\*†</sup> Toshiyuki Okano,<sup>\*\*†</sup>  
Masayuki Murata,<sup>†‡3</sup> and Yoshitaka Fukada<sup>\*†3</sup>

<sup>\*</sup>Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033; <sup>†</sup>CREST, Japan Science and Technology Corporation; and <sup>‡</sup>Laboratory of Ultrastructure Research, Department of Molecular Physiology, National Institute for Physiological Sciences, Nishigonaka 38, Myodaiji, Okazaki, Aichi 444-8585

Received September 29, 2000; accepted October 19, 2000

Melatonin is secreted from the pineal gland in a circadian manner. It is well established that the synthesis of melatonin shows a diurnal rhythm reflecting a daily change in serotonin *N*-acetyltransferase (NAT) activity, and the overall secretion of melatonin requires a cellular release process, which is poorly understood. To investigate the possible involvement of Golgi-derived vesicles in the release, we examined the effect of brefeldin A (BFA), a reversible inhibitor of Golgi-mediated secretion, on melatonin secretion of cultured chick pineal cells. We show here that treatment with BFA completely disassembles the Golgi apparatus and reduces melatonin secretion. In more detailed time course experiments, however, the inhibition of melatonin secretion is only observed after the removal of BFA in parallel with the reassembly of the Golgi apparatus. This inhibition of melatonin secretion is not accompanied by accumulation of melatonin in the cells. These observations indicate that chick pineal melatonin is released independently of the Golgi-derived vesicles, and suggest inhibition of melatonin synthesis after the removal of BFA. By measuring the activities and mRNA levels of melatonin-synthesizing enzymes, we found that the removal of BFA specifically inhibits NAT activity at the protein level. On the other hand, BFA causes no detectable phase-shift of the chick pineal oscillator regulating the circadian rhythm of melatonin secretion. The results presented here suggest that the Golgi-mediated vesicular transport is involved in neither the melatonin release nor the time-keeping mechanism of the circadian oscillator, but rather contributes to the regulation of NAT activity.

**Key words:** circadian rhythm, melatonin, pineal gland, secretion, serotonin *N*-acetyltransferase.

The pineal gland secretes (*i.e.*, synthesizes and releases) melatonin in a daily rhythmic manner with a peak at night. The resulting diurnal rhythm in circulating melatonin acts as a hormonal signal of the day/night cycle and/or the photoperiod, and plays a central role in controlling circadian activity/rest cycles and seasonal breeding of various

vertebrate species (1, 2). The rhythm of pineal melatonin synthesis is mainly due to a change in the activity of serotonin *N*-acetyltransferase (NAT; EC 2.3.1.87), which catalyzes the acetylation of serotonin (3, 4). Dispersed chick pineal cells in culture show a persistent circadian rhythm in both NAT activity and melatonin secretion even in constant darkness (5–7), indicating regulation by the circadian oscillator located in individual cells (8). Such intracellular regulation of the pineal melatonin rhythm in non-mammalian vertebrates is in contrast with that controlled by the central pacemaker located in the suprachiasmatic nuclei in mammals (9). In all species, however, it is poorly understood how melatonin is released from the pineal cells, and no direct evidence has been provided that the synthesis and release of cellular melatonin take place simultaneously. There are two possible mechanisms for the process of melatonin release. That is, melatonin synthesized in the pineal cells may be (i) stored in vesicles, which then fuse with the plasma membrane for the release (exocytosis), or (ii) released directly through the plasma membrane either actively *via* a specific transporter or passively by spontaneous diffusion. For example, serotonin, a precursor of melatonin, and other monoamines are taken up into secretory vesicles *via* vesicular monoamine transporters (10, 11), and then released through exocytosis (12).

A fungal metabolite brefeldin A (BFA), an inhibitor of

<sup>1</sup> This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan. T.H. and T.K. are supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

<sup>2</sup> Present address: Department of Biological Sciences, Nara Women's University, Nara 630-8506.

<sup>3</sup> To whom correspondence should be addressed at: Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033. Tel/Fax: +81-3-5802-8871, E-mail: sfukada@mail.ecc.u-tokyo.ac.jp (Y.F.) or Laboratory of Ultrastructure Research, Department of Molecular Physiology, National Institute for Physiological Sciences, Nishigonaka 38, Myodaiji, Okazaki, Aichi 444-8585. Tel: +81-564-55-7815, Fax: +81-564-52-7913, E-mail: mmurata@nips.ac.jp (M.M.)

Abbreviations: BFA, brefeldin A; BODIPY, boron dipyrromethene difluoride; BSA, bovine serum albumin; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIOMT, hydroxyindole-*O*-methyltransferase; MeOH, methanol; NAS, *N*-acetylserotonin; NAT, serotonin *N*-acetyltransferase; PBS, phosphate-buffered saline; SAM, *S*-adenosyl-L-methionine; ZT, zeitgeber time.

vesicle formation from the Golgi apparatus, is a very useful tool for investigating the vesicular transport *via* the Golgi apparatus. In a variety of cells, BFA accentuates the retrograde transport of the Golgi components (proteins and lipids) to the endoplasmic reticulum (ER), and as a consequence, the drug causes disassembly of the Golgi apparatus and hence inhibits protein secretion (13–15). Importantly, the effects of BFA on the Golgi structure and function are reversible. Upon removal of the drug from the culture medium, the intact Golgi apparatus is reconstructed (reassembled) in the perinuclear region of BFA-treated cells and concomitantly the vesicular transport is restored, indicating that BFA induces transient perturbation of the Golgi apparatus without causing irreversible cellular damage.

In this study, we found that treatment of cultured chick pineal cells with BFA reduced melatonin secretion, but the reduction was not observed during the BFA treatment. Instead, the secretion was reduced just after the removal of BFA, and the temporal profile of its restoration was well correlated with the reassembly of the Golgi apparatus. Interestingly, the reduction in melatonin secretion after the removal of BFA was caused by inhibition of NAT activity. We conclude that, in chick pineal cells, (i) melatonin is released with no contribution of the Golgi-derived vesicles, and (ii) the removal of BFA reduces melatonin secretion by inhibiting NAT activity at the protein level.

#### MATERIALS AND METHODS

**Materials**—Defatted BSA, melatonin, *N*-acetylserotonin, and *S*-adenosyl-L-methionine were purchased from Sigma; brefeldin A, tryptamine, and acetyl-CoA from Wako Pure Chemical Industries; and BODIPY FL C<sub>5</sub>-ceramide from Molecular Probes. All other chemicals were of analytical grade and obtained from commercial sources.

**Pineal Cell Culture**—Animals were treated in accordance with the guidelines of The University of Tokyo and National Institute for Physiological Sciences. Newly hatched male chicks were purchased from local suppliers, and their pineal glands were isolated on the next day. The pineal cells were dispersed with the aid of nylon meshes (pore size, 100 μm), and then suspended for plating in Medium 199 (Life Technologies) supplemented with 10 mM HEPES-NaOH (pH 7.4), 100 U/ml penicillin G (Biowhittakar), 100 μg/ml streptomycin (Biowhittakar), 0.25 μg/ml fungizone (Biowhittakar), 3 μg/ml cytosine β-D-arabinofuranoside (Sigma), 10% fetal bovine serum (JRH Biosciences), and 2.2 mg/ml NaHCO<sub>3</sub>. The cells from 4 pineal glands (approximately 2.4 × 10<sup>6</sup> cells) were plated onto a well of a 24-well plate, or those from 8 glands (approximately 4.8 × 10<sup>6</sup> cells) onto a 35-mm dish. The plated cells were cultured at 37°C under 95% air/5% CO<sub>2</sub> in an incubator equipped with a fluorescent lamp (400 lux at dish level) and exposed to a 12-h light/dark cycle (LD cycle), where the 12-h light period acts as “day” and starts at zeitgeber time (ZT) 0, and the 12-h dark period acts as “night” and starts at ZT 12. The days in culture were numbered from the day of plating (day 1). In some experiments, the cells were kept in constant darkness from day 6. The culture media were exchanged at constant intervals (2–12 h, depending on the experiment), and the collected media were subjected to quantitation of the secreted melatonin (see below). All manipulations in the dark period were performed under infrared light (>800

nm) with the aid of dark field goggles (NEC).

**BFA Treatment**—BFA was dissolved in methanol (MeOH) and stored at –20°C as a stock solution (0.1 mg/ml). When applied to cells, BFA was diluted to 0.1 μg/ml with the medium. The dispersed chick pineal cells were incubated with the BFA-containing medium for a certain period (4–12 h), washed twice with fresh medium to remove BFA completely, and then cultured in the normal medium again.

**Staining of the Golgi Apparatus with BODIPY-Ceramide**—The Golgi stacks of living pineal cells were stained with BODIPY FL C<sub>5</sub>-ceramide (BODIPY-ceramide), a fluorescent lipid that specifically labels the Golgi apparatus in living cells (16). BODIPY-ceramide (50 nmol) was dissolved in 200 μl of ethanol and mixed with 10 ml of a serum-free medium containing defatted BSA (0.34 mg/ml), and then the mixture was dialyzed against the serum-free medium. The pineal cells plated on a 35-mm glass-bottom dish were incubated with the BODIPY-ceramide/defatted BSA complex (final 5 μM) for 30 min at 37°C. After being washed twice with fresh medium, the cells were observed under a fluorescence microscope, Axiovert (Carl Zeiss), using a fluorescein channel.

**Quantitation of Melatonin**—Melatonin in the collected medium was quantitated with a HPLC system, NanoSpace SI-1 (Shiseido), as described (17) with some modifications. Briefly, a 10–50 μl aliquot of the collected medium was injected for deproteinization into a MF Cartridge column (4.0 mm × 10 mm; Shiseido) at the flow rate of 500 μl/min, pre-equilibrated with Buffer P1 (100 mM sodium phosphate and 10 mM sodium 1-octanesulfonate, pH 5.0). A delayed eluate containing melatonin was introduced into a Capcell Pak C<sub>18</sub> UG120 5-μm guard column (2.0 mm × 35 mm; Shiseido) at the flow rate of 250 μl/min. The melatonin-containing fraction was eluted with Buffer P2 (100 mM sodium phosphate and 10 mM sodium 1-octanesulfonate, pH 3.0) supplemented with 20% (v/v) acetonitrile, and then separated on a Capcell Pak C<sub>18</sub> UG120 3-μm reverse phase analytical column (1.5 mm × 150 mm; Shiseido) at the flow rate of 100 μl/min. The cartridge column and analytical column were kept at 40°C. The fluorescence intensity of the final eluate was monitored continuously at excitation and emission wavelengths of 264 and 370 nm, respectively.

Melatonin that accumulated in the pineal cells was quantitated according to the method of Chin (18) with some modifications. That is, the pineal cells plated on a 35-mm dish were washed twice with PBS(+) (10 mM phosphate containing 140 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl<sub>2</sub>, and 0.5 mM MgCl<sub>2</sub>, pH 7.4) and then collected in the same solution. They were centrifuged at 20,000 ×g for 1 min at 4°C, and then the cell pellet was stored at –80°C until use. The frozen cells were homogenized with 150 μl of 50 mM perchloric acid containing 0.74 mM cysteine hydrochloride and 0.32 mM disodium EDTA. Then, they were centrifuged at 20,000 ×g for 10 min at 4°C, and 50 μl of the supernatant was analyzed using the HPLC system, as described above.

**Measurement of Enzyme Activities**—NAT activity of the pineal cell homogenate was estimated by measuring the amount of *N*-acetyltryptamine synthesized from exogenously added substrates, tryptamine and acetyl-CoA, according to the method of Fajardo *et al.* (19) with some modifications. Briefly, the pineal cells plated on a 35-mm dish were homogenized with 600 μl of ice-cold 250 mM potassium



phosphate (pH 6.5) containing 1.4 mM acetyl-CoA, and then 120  $\mu$ l of the homogenate was mixed with 40  $\mu$ l of 8 mM tryptamine in 250 mM potassium phosphate (pH 6.5) for incubation at 37°C. After incubation for 10, 20, and 30 min, the reactions were stopped by mixing a 50- $\mu$ l aliquot of each reaction mixture with 10  $\mu$ l of 6 M perchloric acid. Then, the mixture was centrifuged at 20,000  $\times g$  for 10 min at 4°C, and the supernatant was subjected to the HPLC analysis of the *N*-acetyltryptamine content. The HPLC conditions were modified from those for melatonin analysis as follows; 20  $\mu$ l of the supernatant was injected into the Capcell Pak C<sub>18</sub> UG120 5- $\mu$ m guard column (2.0 mm  $\times$  35 mm; Shiseido), and the *N*-acetyltryptamine-containing fraction was separated on the Capcell Pak C<sub>18</sub> UG120 3- $\mu$ m reverse phase analytical column (1.5 mm  $\times$  150 mm; Shiseido) equilibrated with Buffer A (118 mM sodium acetate and 158  $\mu$ M EDTA, pH 5.2) supplemented with 15% (v/v) acetonitrile at the flow rate of 100  $\mu$ l/min. The wavelengths for fluorescence excitation and emission were 285 and 360 nm, respectively.

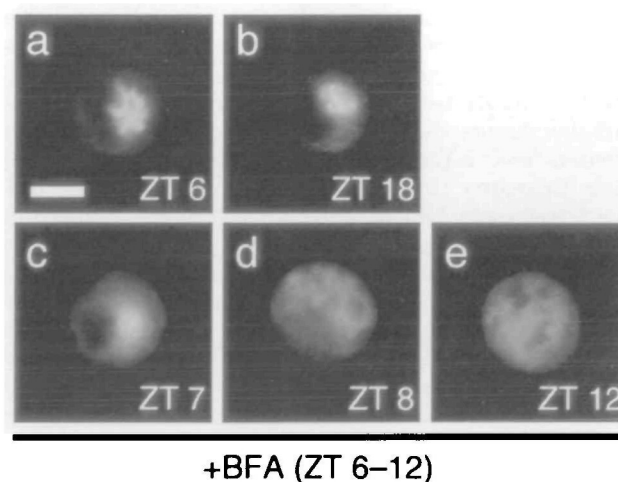
Hydroxyindole-*O*-methyltransferase (HIOMT; EC 2.1.1.4) activity of the pineal cell homogenate was estimated by measuring the amount of melatonin synthesized from exogenously added substrates, *N*-acetylserotonin (NAS) and *S*-adenosyl-L-methionine (SAM), according to the method of Itoh *et al.* (20) with some modifications. That is, the pineal cells plated on a 35-mm dish were homogenized with 300  $\mu$ l of ice-cold 50 mM sodium phosphate (pH 7.9), and then 120  $\mu$ l of the homogenate was mixed with 55  $\mu$ l of 3.2 mM NAS and 0.32 mM SAM in 50 mM sodium phosphate (pH 7.9) for incubation at 37°C. After incubation for 10, 20, and 30 min, the reactions were stopped by mixing a 48- $\mu$ l aliquot of each reaction mixture with 12  $\mu$ l of 6 M perchloric acid. Then, the mixture was centrifuged at 20,000  $\times g$  for 10 min at 4°C, and 20  $\mu$ l of the supernatant was subjected to the HPLC analysis of the melatonin content.

**RT-PCR Analysis**—The pineal cells plated on a 35-mm dish were washed twice with PBS(+), homogenized with 1 ml of TRIzol reagent (Life Technologies), and then stored at -80°C until use. According to the manufacturer's protocol, total RNA fractions were isolated from the homogenate and treated with DNase I (Takara Shuzo). Then, 0.8  $\mu$ g of the total RNA was reverse transcribed with SUPERScript II (Life Technologies) at 42°C for 50 min using anchored (dT)<sub>25</sub> primers. One-hundredth of the reaction mixture (0.2  $\mu$ l) was subjected to PCR in a reaction mixture (25  $\mu$ l) composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each of the dNTPs, 1  $\mu$ M each of the gene specific primers, and 0.625 U AmpliTaq Gold (PE Biosystems). The primer pairs used were, 5'-ATGAGATCCGC-CACTTCCTAAC-3' (forward) plus 5'-AATCCTCGCAT-GAGCAG-3' (reverse) for chicken NAT (GenBank accession number; U46502), 5'-CCAATACCGTCTACTGTGCTG-3' (forward) plus 5'-TCTCCTAACTTGAACCTCCCTG-3' (reverse) for chicken HIOMT (X62309), and 5'-ACCACTGTCCATGCCATCAC-3' (forward) plus 5'-TCCACAACACGGTTGCTGTA-3' (reverse) for chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH; K01458). Each thermal cycle comprised 94°C for 15 s, 55°C for 30 s and 72°C for 30 s, and the number of cycles was determined for each amplification reaction so as to maintain a linear correlation between the amplified products and the amounts of template DNA. The optimal cycle numbers for quantitative

analysis were 24 for NAT, 23 for HIOMT, and 17 for GAPDH under the experimental conditions used. The PCR products were subjected to 6% polyacrylamide gel electrophoresis, stained with SYBR Green I (Molecular Probes), and then detected with an image analyzer FLA-2000 (Fuji Film). The amounts of the products with the predicted sizes were quantitated with MacBAS software (Fuji Film).

## RESULTS

**Disruption of the Golgi Apparatus by BFA**—As described in previous reports (5–7), dispersed chick pineal cells comprised two types of cells after several days in culture; one is small round cells (approximately 10  $\mu$ m in diameter) and the other is fibroblasts, the former of which are melatonin-secreting pinealocytes (21). We examined the effect of BFA on the morphology of the Golgi apparatus in the pinealocytes by using BODIPY-ceramide, a lipid marker of Golgi membranes, under a fluorescence microscope. In the absence of BFA, BODIPY-ceramide brightly stained the perinuclear structure, a typical construction of the Golgi apparatus. The morphology of the Golgi apparatus did not change significantly between in the daytime (ZT 6, Fig. 1a) and in the nighttime (ZT 18, Fig. 1b). Upon exposure of the cells to 0.1  $\mu$ g/ml BFA from ZT 6, the Golgi apparatus began to disassemble within 1 h (Fig. 1c), and was completely dispersed throughout the cytoplasm within 2 h (Fig. 1d). This dispersed state continued for 6 h in the presence of BFA (Fig. 1e). Similar changes were observed with 5  $\mu$ g/ml BFA-treatment (data not shown), and hence the lower dose (0.1  $\mu$ g/ml) was used in subsequent experiments. Compared with chick pineal fibroblasts, whose Golgi apparatus was disrupted by BFA within 30 min (data not shown), the complete disassembly of the Golgi apparatus in the chick pinealocytes was relatively slow. Such a delayed response to BFA has also been reported for a primary culture of rat



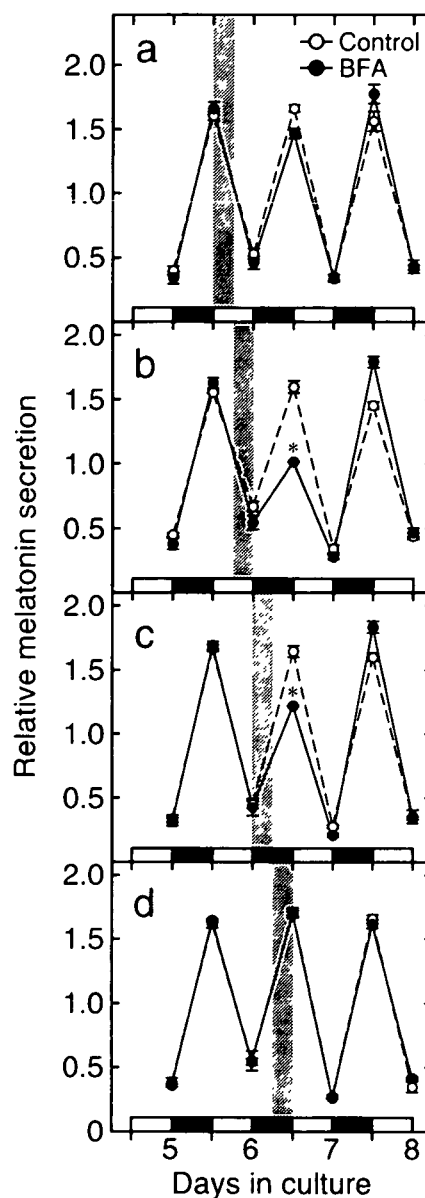
**Fig. 1. Effect of BFA on the morphology of the Golgi apparatus in chick pinealocytes.** Primary cultured chick pineal cells were stained on day 6 with BODIPY-ceramide, which labels the Golgi apparatus. (a, b) The morphology of the Golgi apparatus of a chick pinealocyte in the daytime (ZT 6, a) or in the nighttime (ZT 18, b). (c–e) The time course of the disassembly of the Golgi apparatus upon exposure to BFA. Cells stained with BODIPY-ceramide at ZT 5 were exposed to 0.1  $\mu$ g/ml BFA from ZT 6, and then incubated for 1 h (ZT 7, c), 2 h (ZT 8, d), or 6 h (ZT 12, e). Bar (in panel a), 10  $\mu$ m.

adrenal chromaffin cells (22). Thus, the difference in responsiveness to BFA may depend on the cell type.

**Transient Reduction in Nocturnal Melatonin Secretion Caused by Removal of BFA**—We next examined the effect of BFA on melatonin secretion from pineal cells into the medium. In this article, we use the term “secretion” for a process composed of “synthesis” and “release.” To evaluate the phase-dependent effect of BFA treatment, cultured chick pineal cells were subjected to BFA at four different phases. That is, the cells were treated with BFA or MeOH (control) on day 6 at ZT 0–6/ZT 6–12 (when melatonin secretion is low) or ZT 12–18/ZT 18–24 (when melatonin secretion is high), and then the melatonin secreted into the medium was quantitated (Fig. 2, a–d). The 6-h treatment with BFA in the afternoon (ZT 6–12, Fig. 2b) and early at night (ZT 12–18, Fig. 2c) reduced the subsequent nocturnal melatonin secretion by 36% and 26% of the control value, respectively, and on the next day (day 7), the level of nocturnal melatonin secretion returned to the control value. In contrast, similar BFA treatment in the morning (ZT 0–6, Fig. 2a) or late at night (ZT 18–24, Fig. 2d) had little or no effect on the secretion. These results, particularly the lack of an evident effect of BFA applied late at night (Fig. 2d), suggested that the transient reduction in nocturnal melatonin secretion occurs after the removal of BFA rather than during the treatment.

The possibility described above was examined by measuring melatonin secretion at 2-h intervals during and after 6-h BFA treatment (Fig. 3, a and b). When the cells were treated with BFA at ZT 12–18 on day 6 (Fig. 3a, squares), melatonin secretion was not affected during the treatment but decreased just after the removal of BFA, as compared with the control values (MeOH-treated cells; Fig. 3a, circles). Prolonged exposure to BFA (*i.e.*, without removal of BFA at ZT 18) also reduced melatonin secretion (Fig. 3a, triangles), but the inhibitory effect was delayed and smaller than that in the case of removal of BFA (Fig. 3a, squares). Clearly, the reduction in melatonin secretion caused by 6-h BFA treatment (Fig. 2c) is mainly ascribable to the effect of removal of BFA. A similar or more pronounced effect was observed when the cells were treated with BFA at ZT 6–12 (Fig. 3b). The removal of BFA remarkably suppressed the nocturnal increase in melatonin secretion for 4 h (until ZT 16), followed by a delayed increase thereafter (Fig. 3b, squares). In contrast, the prolonged treatment had no effect on the initial increase in nocturnal melatonin secretion (until ZT 14), although marked inhibition was observed in a later period (Fig. 3b, triangles). With respect to the morphological change of the Golgi apparatus, we observed the disrupted state at 4 h after the removal of BFA at ZT 12 (ZT 16, Fig. 3c), but the dispersed Golgi membranes migrated to the perinuclear region within 6 h (ZT 18, Fig. 3d) and reassembled to show an intact morphology within 8 h (ZT 20, Fig. 3e). It is noteworthy that this time course of reassembly of the Golgi apparatus correlated well with the gradual increase in melatonin secretion (*i.e.*, delayed recovery) after 6-h BFA treatment (Fig. 3b, squares).

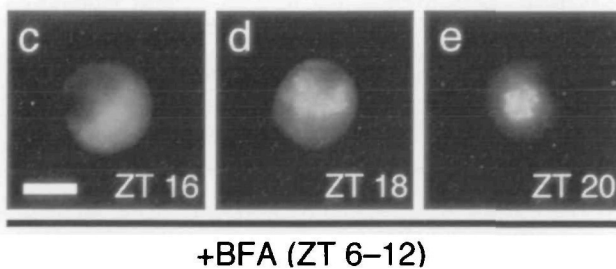
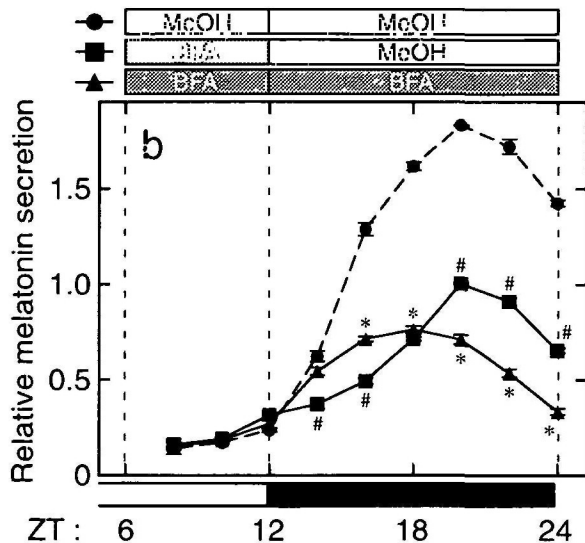
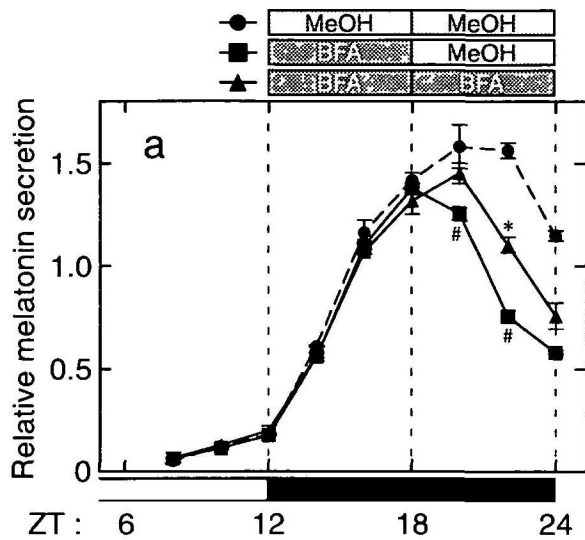
**Inhibition of NAT Activity Caused by Removal of BFA**—To determine which process of melatonin secretion (*i.e.*, synthesis or release) is inhibited after the removal of BFA, the cellular melatonin contents were compared between BFA-treated and control (MeOH-treated) cells. After 6-h



**Fig. 2. Effect of BFA on melatonin secretion.** Chick pineal cells were cultured in LD cycles (white and black bars at the bottom), and the media were exchanged at ZT 0 and at ZT 12. On day 6, the cells were treated with 0.1  $\mu\text{g/ml}$  BFA (in 0.1% MeOH, closed circles) or 0.1% MeOH (control, open circles) for 6 h at ZT 0–6 (a), ZT 6–12 (b), ZT 12–18 (c), or ZT 18–24 (d), the period of which is depicted by a gray bar in each panel. All the melatonin content values for the collected media were expressed relative to the mean value for melatonin secreted on day 5 and plotted at the time when the media were collected. The data are the means  $\pm$  SEM (bars) values of three independent cultures. \* $p < 0.005$ , compared with the control value by Student's *t* test.

treatment with BFA or MeOH (at ZT 6–12 or ZT 12–18), the cells were incubated in the presence of 100  $\mu\text{M}$  eserine, an inhibitor of melatonin deacetylase (23), to prevent the degradation of melatonin. Then, the media and cells were collected separately at ZT 23.5, the end of the dark period, for quantitation of the melatonin content. It was confirmed that, even in the presence of eserine, the amount of melatonin secreted into the medium was markedly reduced after





6-h BFA treatment at the two different phases (Table I, Secreted). Under the conditions used, however, elevated accumulation of cellular melatonin was not observed irrespective of the BFA treatment (Table I, Cellular), suggesting that the removal of BFA inhibits melatonin synthesis rather than its release.

Melatonin is synthesized from serotonin through a two-step enzymatic process sequentially catalyzed by serotonin *N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT), the former of which is a key enzyme

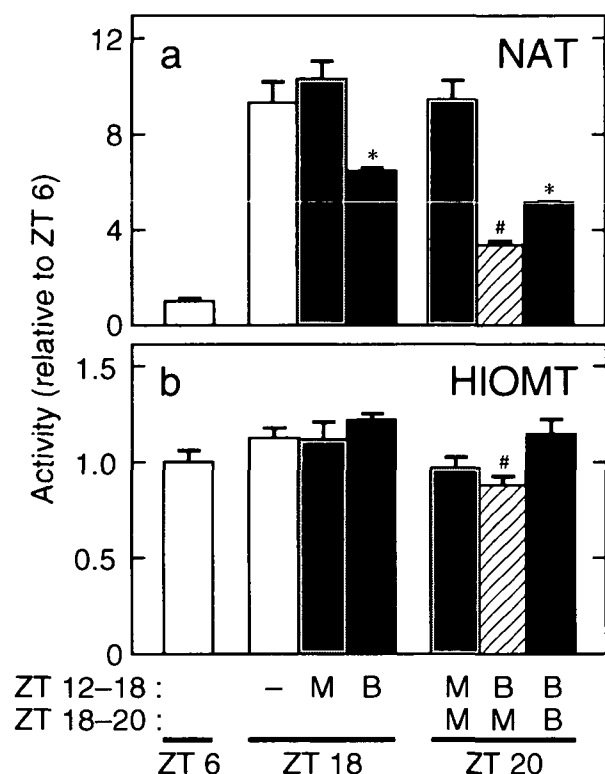
Fig. 3. Effects of removal of BFA on melatonin secretion and on the morphology of the Golgi apparatus. (a, b) Effects of the administration and removal of BFA on melatonin secretion. Chick pineal cells were cultured in LD cycles (white and black bars at the bottom), and the media were exchanged at 2-h intervals on day 6. (a) Cells were treated with 0.1% MeOH at ZT 12–24 (circles), with 0.1  $\mu$ g/ml BFA (in 0.1% MeOH) at ZT 12–24 (triangles), or with BFA at ZT 12–18 and then with MeOH at ZT 18–24 (squares). (b) Cells were treated with MeOH at ZT 6–24 (circles), with BFA at ZT 6–24 (triangles), or with BFA at ZT 6–12 and then with MeOH at ZT 12–24 (squares). All the melatonin content values for the collected media were expressed relative to the mean value for melatonin secreted on day 5 and plotted at the time when the media were collected. The data are the means  $\pm$  SEM (bars) values of three independent cultures. \* $p < 0.01$ , compared with the circle; # $p < 0.01$ , compared with the triangle by Student's *t* test. (c–e) The time course of the reassembly of the Golgi apparatus after the removal of BFA. Cells were treated with BFA for 6 h (ZT 6–12) on day 6, stained with BODIPY-ceramide at ZT 10, and then washed twice with fresh medium to remove BFA at ZT 12. Then the cells were incubated in the normal medium for 4 h (ZT 16, c), 6 h (ZT 18, d), or 8 h (ZT 20, e). Bar (in panel c), 10  $\mu$ m.

TABLE I. Effect of BFA on the cellular content of melatonin. Chick pineal cells were cultured in LD cycles and treated with 0.1  $\mu$ g/ml BFA (in 0.1% MeOH) or 0.1% MeOH (control) at ZT 6–12 or ZT 12–18 on day 4. After the treatment, the cells were incubated in the presence of 100  $\mu$ M eserine, and then the melatonin contents of the cells (Cellular) and the media (Secreted), both of which were collected at ZT 23.5, were determined. The data are the means  $\pm$  SEM values of three independent cultures.

Treatment	Melatonin (ng/dish)	
	Cellular (at ZT 23.5)	Secreted (ZT 12–23.5)
ZT 6–12		
Control	0.071 $\pm$ 0.006	182.6 $\pm$ 1.2
BFA	0.064 $\pm$ 0.011	95.7 $\pm$ 4.3 <sup>a</sup>
ZT 12–18		
Control	0.074 $\pm$ 0.012	190.9 $\pm$ 14.2
BFA	0.062 $\pm$ 0.002	132.5 $\pm$ 2.0 <sup>a</sup>

<sup>a</sup> $p < 0.02$ , compared with the control value by Student's *t* test.

responsible for the nocturnal increase in pineal melatonin (24). To investigate the effect of the removal of BFA on the activities of these enzymes, cultured pineal cells were treated with BFA at ZT 12–18 and collected for analyses just before (ZT 18) and 2 h after (ZT 20) its removal. Consistent with previous reports (4, 25), the NAT activity level in the cell homogenate prepared at ZT 18 in the nighttime was about 10-fold higher than that at ZT 6 in the daytime (Fig. 4a, open bars), whereas the level of HIOMT activity at ZT 18 was similar to that at ZT 6 (Fig. 4b, open bars). Exposure of the cells to BFA from ZT 12 reduced the NAT activity by 38% at ZT 18 or by 45% at ZT 20 of the control (MeOH-treated) value (Fig. 4a, compare the shaded and filled bars for ZT 18 or ZT 20, respectively). Importantly, the removal of BFA further reduced the NAT activity by 35% (compare the hatched bar with the filled bar at ZT 20). In contrast, the administration and removal of BFA had almost no effect on the HIOMT activity (Fig. 4b). These results, together with the finding that melatonin did not accumulate in the cells (Table I), indicate that the reduction in melatonin secretion caused by the removal of BFA (Fig. 3a) is predominantly attributable to the inhibition of NAT activity. BFA seems to have dual effects on NAT activ-

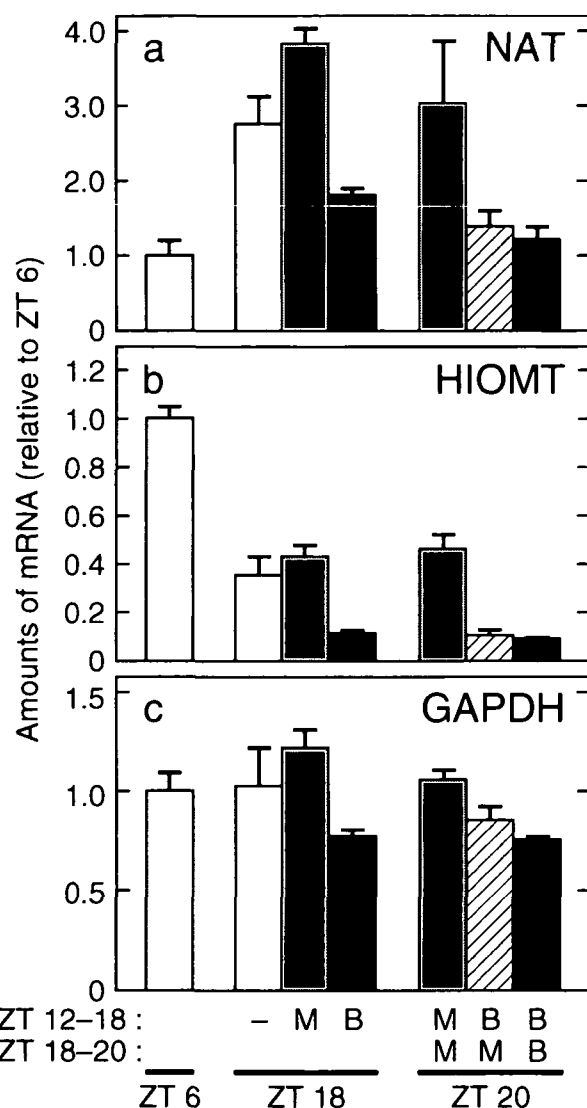


**Fig. 4. Effects of BFA on the activities of NAT and HIOMT.** Chick pineal cells were cultured in LD cycles for 5 days and subsequently cultured in the normal medium (–, open bars), or treated with 0.1% MeOH (M) from ZT 12 (shaded bars), with 0.1  $\mu$ g/ml BFA (in 0.1% MeOH, B) from ZT 12 (filled bars), or with BFA at ZT 12–18 and then with MeOH at ZT 18–20 (hatched bars) on day 6. They were collected to prepare cell homogenates at ZT 6, ZT 18, or ZT 20, as indicated at the bottom, followed by measurement of the activity of NAT (a) or HIOMT (b). All the activity values for the homogenates were expressed relative to the mean value for those at ZT 6. The data are the means  $\pm$  SEM (bars) values of three independent samples. \* $p$  < 0.02, compared with the shaded bar; # $p$  < 0.02, compared with the filled bar by Student's *t* test.

ity, that is, an inhibitory effect during the treatment and an after-effect upon its removal (see "DISCUSSION").

**Effect of BFA on NAT mRNA Level**—Next, by means of quantitative RT-PCR analysis, we investigated the mRNA levels of NAT, HIOMT, and GAPDH in pineal cells, which were cultured under the same conditions as described in the legend to Fig. 4. As reported previously (25, 26), the amount of NAT mRNA at ZT 18 in the nighttime was much higher (about 3-fold) than that at ZT 6 in the daytime (Fig. 5a, open bars), and conversely the amount of HIOMT mRNA at ZT 6 was about 3-fold higher than that at ZT 18 (Fig. 5b, open bars). Under the conditions used, the amount of GAPDH mRNA remained almost unchanged (Fig. 5c, open bars).

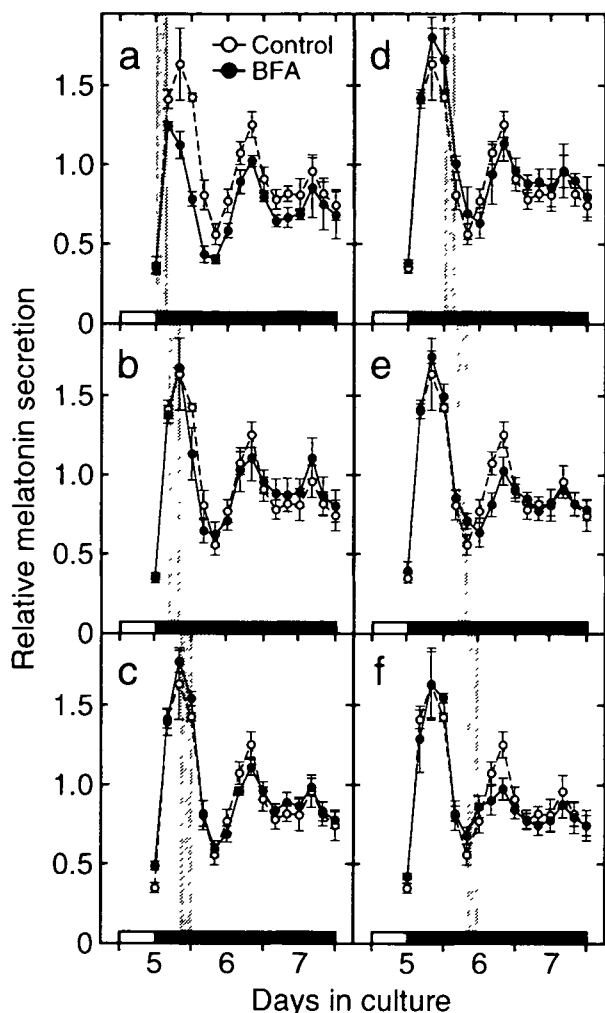
Exposure of the cells to BFA from ZT 12 decreased the amount of NAT mRNA by 53% at ZT 18 or by 60% at ZT 20 of the control (MeOH-treated) value (Fig. 5a, compare the shaded and filled bars for ZT 18 or ZT 20, respectively). The removal of BFA, however, had no further effect on the amount of NAT mRNA (compare the hatched bar with the filled bar at ZT 20). Similarly, the administration of BFA decreased the amounts of HIOMT mRNA (Fig. 5b) and



**Fig. 5. Effects of BFA on the mRNA levels of NAT, HIOMT, and GAPDH.** Chick pineal cells were cultured under the conditions described in the legend to Fig. 4. Total RNA was isolated from cells collected at ZT 6, ZT 18, or ZT 20, and then the relative amounts of mRNA of NAT (a), HIOMT (b), and GAPDH (c) were estimated by quantitative RT-PCR. All the amounts of mRNA were expressed relative to the mean value for those at ZT 6. The data are the means  $\pm$  SD (bars) values of two independent samples.

GAPDH mRNA (Fig. 5c), and its removal caused no further decrease. Thus, the BFA treatment decreased the amounts of all the mRNAs examined here, but its removal had no further effect.

**Effect of BFA on the Pineal Circadian Oscillator**—The BFA-dependent decreases in the amounts of various mRNAs (Fig. 5) raised the possibility that the BFA treatment shifts the phase of the circadian oscillator in chick pineal cells. This is because the molecular machinery of the circadian oscillator is generally considered to contain a negative feedback loop based on the transcription and translation of clock genes (27). We investigated whether or not 4-h treatment with BFA for various time periods within a day shifts the phase of the free-running rhythm of melatonin secretion in constant darkness (Fig. 6, a–f). BFA treatment



**Fig. 6. Effect of BFA on the circadian rhythm of melatonin secretion.** Chick pineal cells were cultured in LD cycles (white and black bars at the bottom) for 5 days and then transferred to constant darkness. The media were exchanged at 4-h intervals. The cells were treated with 0.1  $\mu\text{g/ml}$  BFA (closed circles) for 4 h at ZT 12–16 (a), ZT 16–20 (b), ZT 20–24 (c), ZT 0–4 (d), ZT 4–8 (e), or ZT 8–12 (f), the period of which is depicted by a gray bar in each panel. Control data for cells not treated with BFA are indicated by open circles. All the melatonin content values for the collected media were expressed relative to the mean value for melatonin secreted on day 4 and plotted at the time when the media were collected. The data are the means  $\pm$  SEM (bars) values of three independent cultures.

at ZT 12–16 (Fig. 6a), ZT 4–8 (Fig. 6e), or ZT 8–12 (Fig. 6f) induced a transient reduction in melatonin secretion after removal of BFA, this being consistent with the time-of-day-specific effect of the drug shown in Fig. 2 (*i.e.*, effective from the afternoon to early night). Despite such a clear acute effect of BFA, there was no significant phase-shift of the circadian rhythm of melatonin secretion between BFA-treated and non-treated cells (Fig. 6, a–f). We concluded that the phase of the chick pineal circadian oscillator is unaffected by BFA treatment given at any time of the day.

#### DISCUSSION

##### *Mechanism of Melatonin Release from Chick Pineal*

**Cells**—In the present study, the relation between chick pineal melatonin release and vesicular transport *via* the Golgi apparatus was investigated by using a fungal metabolite BFA, an inhibitor of Golgi-mediated secretion. We demonstrated that BFA disrupted the Golgi apparatus of chick pinealocytes (Fig. 1) and reduced melatonin secretion (Fig. 2). It should be emphasized that, although the Golgi apparatus was completely disrupted in the BFA-treated cells (Fig. 1), melatonin secretion was not affected during the 6-h treatment with BFA (Fig. 3, a and b). Moreover, no significant amount of melatonin was accumulated not only in the BFA-treated cells but also in the control cells (Table I), indicating the absence of cellular machinery for storing melatonin in chick pineal cells. Taken together, these results first demonstrate that, unlike other amine hormones, chick pineal melatonin could be released independently of the Golgi-derived secretory vesicles. Since melatonin is a rather hydrophobic compound, it may possibly diffuse in and pass through the plasma membrane passively. Alternatively, synthesized melatonin might be released actively by a specific transporter through the plasma membrane or by a Golgi-independent exocytotic pathway. These possibilities have not been evaluated properly so far.

**Effect of BFA on Melatonin Synthesis**—Although the Golgi-derived vesicles were not involved directly in the pineal melatonin release, melatonin secretion was reduced by treatment with BFA. An unexpected finding in this study was that the removal of BFA reduced melatonin secretion (Fig. 3, a and b). The lack of accumulation of cellular melatonin after the removal of BFA (Table I) suggested inhibition of melatonin synthesis, and in fact we found a stepwise decrease in NAT activity not only during but also after BFA treatment (Fig. 4a). These two effects are discussed separately below.

First, the inhibition of NAT activity during the BFA treatment (Fig. 4a) can be ascribed to the decrease in its mRNA level (Fig. 5a). Because the turnover of the NAT protein is very fast (within a few minutes, 28), the decrease in its mRNA level may cause acute reduction of its activity due to the reduced supply of newly synthesized proteins. In contrast, HIOMT is a highly stable protein (>24 h, 29). Therefore, its activity did not decrease during the BFA treatment (Fig. 4b), even when its mRNA level was decreased (Fig. 5b). Although NAT is thought to be the rate-limiting enzyme in pineal melatonin biosynthesis (24), we found that the reduction in NAT activity (Fig. 4a) induced no change in melatonin secretion during 6-h treatment with BFA (Fig. 3a). A possible explanation for this is that at midnight when the NAT activity reaches a peak level, the amount of NAT substrate (*i.e.*, serotonin) limits the rate of melatonin biosynthesis. This idea is based on the observation that prolonged BFA treatment starting at ZT 12 (Fig. 3a, triangles) had an inhibitory effect on melatonin secretion only after the peak of NAT activity at midnight. On the other hand, the effect of the prolonged treatment was more pronounced when BFA was administered from ZT 6 (Fig. 3b, triangles). This is possibly because melatonin synthesis is limited by NAT activity from the afternoon, and hence the subsequent increase in melatonin secretion at night should be markedly reduced due to the lowered levels of NAT mRNA and activity. In fact, it was reported that daytime treatment with inhibitors of RNA synthesis blocks the nocturnal increase in melatonin secretion more effec-



tively than nighttime treatment, and these inhibitors have only minor effects on melatonin secretion during the treatment (21).

Second, the after-effect of treatment with BFA on melatonin secretion is distinguishable from the effect of prolonged treatment, in that the former had a stronger and more acute inhibitory effect than the latter (Fig. 3, a and b). This is attributable to additional inhibition of the NAT activity caused by the removal of BFA (Fig. 4a). It should be stressed that the inhibition was not accompanied by a further decrease in the mRNA level (Fig. 5a), indicating regulation of NAT activity at the protein level. Even after the translation, the pineal NAT activity is regulated by several mechanisms, such as protein thiol:disulfide exchange (30, 31), proteasomal proteolysis (32), and phosphorylation by protein kinase A (33). The apparent coincidence in time course between the restoration of melatonin secretion (Fig. 3b) and the reassembly of the Golgi morphology (Fig. 3, c-e) after the removal of BFA suggests a possible linkage between one or some of the mechanisms responsible for the post-translational regulation of NAT activity and the activation of ER-to-Golgi transport.

*No Direct Linkage between the Golgi Apparatus and the Circadian Oscillator*—BFA treatment for 4 h (Fig. 6) or for 12 h (data not shown) caused no detectable phase-shift of the chick pineal oscillator regulating the circadian rhythm of melatonin secretion, while the Golgi apparatus was completely disrupted by BFA treatment (Fig. 1). This indicates that the time-keeping mechanism of the oscillator is independent of the Golgi apparatus. Considering that an inhibitor of protein synthesis shifts the phase of the oscillator (21), we conclude that the Golgi-dependent vesicular transport of synthesized proteins is not involved in the oscillator system. Consistently, all known clock components are cytosolic/nuclear proteins (27).

It is proposed that the clock components constitute a transcription/translation-based negative feedback loop (27). For instance, the transcription of *Drosophila* clock genes *per* and *tim* is inhibited by a combination of PER and TIM proteins, thus generating fluctuations in the amounts of their mRNAs and proteins (34). Recently, mammalian and avian homologues of *per* gene were identified, and their mRNA levels were shown to fluctuate in a circadian manner (35–42). In this study, we found that BFA treatment lowered the mRNA levels of proteins such as NAT, HIOMT, GAPDH (Fig. 5), and pinopsin (43, data not shown) to various degrees, whereas the drug had no effect on the oscillator (Fig. 6). This suggests that the effect of BFA on mRNA levels is restricted to certain genes, or that a transient decrease in the mRNA levels of chick clock genes does not affect the central oscillator mechanism of pineal cells.

In general, the Golgi apparatus plays a very important role in various cellular functions, such as the glycosylation, sorting, and transport of synthesized proteins/lipids, and the secretion of bioactive compounds. In chick pineal cells, however, it is unlikely that the Golgi-dependent vesicular transport plays a role in the melatonin release. The results presented here rather suggest a linkage between the reassembly of the Golgi apparatus and regulation of NAT activity at the protein level. BFA has been widely used as a highly specific blocker of Golgi functions, but it should be noted that BFA may have an effect on mRNA levels through an unidentified mechanism.

We wish to thank Dr. Kamon Sanada and Mr. Yuichiro Hayashi for the technical advice regarding chick pineal cell culture and melatonin quantitation. We also thank Mr. Masao Doi for the technical advice regarding RT-PCR analysis.

## REFERENCES

1. Cassone, V.M. (1990) Effects of melatonin on vertebrate circadian systems. *Trends Neurosci.* **13**, 457–464
2. Tamarkin, L., Baird, C.J., and Almeida, O.F.X. (1985) Melatonin: a coordinating signal for mammalian reproduction? *Science* **227**, 714–720
3. Klein, D.C. and Weller, J.L. (1970) Indole metabolism in the pineal gland: a circadian rhythm in *N*-acetyltransferase. *Science* **169**, 1093–1095
4. Binkley, S., MacBride, S.E., Klein, D.C., and Ralph, C.L. (1973) Pineal enzymes: regulation of avian melatonin synthesis. *Science* **181**, 273–275
5. Deguchi, T. (1979) A circadian oscillator in cultured cells of chicken pineal gland. *Nature* **282**, 94–96
6. Robertson, L.M. and Takahashi, J.S. (1988) Circadian clock in cell culture: I. Oscillation of melatonin release from dissociated chick pineal cells in flow-through microcarrier culture. *J. Neurosci.* **8**, 12–21
7. Zatz, M., Mullen, D.A., and Moskal, J.R. (1988) Photoendocrine transduction in cultured chick pineal cells: effects of light, dark, and potassium on the melatonin rhythm. *Brain Res.* **438**, 199–215
8. Nakahara, K., Murakami, N., Nasu, T., Kuroda, H., and Murakami, T. (1997) Individual pineal cells in chick possess photoreceptive, circadian clock and melatonin-synthesizing capacities *in vitro*. *Brain Res.* **774**, 242–245
9. Klein, D.C. (1985) Photoneural regulation of the mammalian pineal gland. *Ciba Found. Symp.* **117**, 38–56
10. Erickson, J.D., Eiden, L.E., and Hoffman, B.J. (1992) Expression cloning of a reserpine-sensitive vesicular monoamine transporter. *Proc. Natl. Acad. Sci. USA* **89**, 10993–10997
11. Liu, Y., Peter, D., Roghani, A., Schuldiner, S., Privé, G.G., Eisenberg, D., Brecha, N., and Edwards, R.H. (1992) A cDNA that suppresses MPP<sup>+</sup> toxicity encodes a vesicular amine transporter. *Cell* **70**, 539–551
12. Moriyama, Y., Yamamoto, A., Yamada, H., Tashiro, Y., and Futai, M. (1996) Role of endocrine cell microvesicles in intercellular chemical transduction. *Biol. Chem. Hoppe Seyler* **377**, 155–165
13. Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., and Ikehara, Y. (1988) Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J. Biol. Chem.* **263**, 18545–18552
14. Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S., and Klausner, R.D. (1989) Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* **56**, 801–813
15. Chardin, P. and McCormick, F. (1999) Brefeldin A: the advantage of being uncompetitive. *Cell* **97**, 153–155
16. Pagano, R.E., Martin, O.C., Kang, H.C., and Haugland, R.P. (1991) A novel fluorescent ceramide analogue for studying membrane traffic in animal cells: accumulation at the Golgi apparatus results in altered spectral properties of the sphingolipid precursor. *J. Cell Biol.* **113**, 1267–1279
17. Sanada, K., Hayashi, Y., Harada, Y., Okano, T., and Fukada, Y. (2000) Role of circadian activation of mitogen-activated protein kinase in chick pineal clock oscillation. *J. Neurosci.* **20**, 986–991
18. Chin, J.R.L. (1990) Determination of six indolic compounds, including melatonin, in rat pineal using high-performance liquid chromatography with serial fluorimetric-electrochemical detection. *J. Chromatogr.* **528**, 111–121
19. Fajardo, N., Abreu, P., and Alonso, R. (1992) Determination of kinetic properties of serotonin-*N*-acetyltransferase in bovine pineal gland using HPLC with fluorimetric detection. *J. Pineal Res.* **13**, 80–84
20. Itoh, M.T., Hattori, A., and Sumi, Y. (1997) Hydroxyindole-O-



- methyltransferase activity assay using high-performance liquid chromatography with fluorometric detection: determination of melatonin enzymatically formed from *N*-acetylserotonin and *S*-adenosyl-L-methionine. *J. Chromatogr. B* **692**, 217–221
21. Takahashi, J.S., Murakami, N., Nikaido, S.S., Pratt, B.L., and Robertson, L.M. (1989) The avian pineal, a vertebrate model system of the circadian oscillator: cellular regulation of circadian rhythms by light, second messengers, and macromolecular synthesis. *Recent Prog. Horm. Res.* **45**, 279–348
  22. Xu, J. and Tse, F.W. (1999) Brefeldin A increases the quantal size and alters the kinetics of catecholamine release from rat adrenal chromaffin cells. *J. Biol. Chem.* **274**, 19095–19102
  23. Oommen, A. and Balasubramanian, A.S. (1978) Aryl acylamidase of monkey brain and liver: response to inhibitors and relationship to acetylcholinesterase. *Biochem. Pharmacol.* **27**, 891–895
  24. Klein, D.C., Berg, G.R., and Weller, J. (1970) Melatonin synthesis: adenosine 3',5'-monophosphate and norepinephrine stimulate *N*-acetyltransferase. *Science* **168**, 979–980
  25. Bernard, M., Klein, D.C., and Zatz, M. (1997) Chick pineal clock regulates serotonin *N*-acetyltransferase mRNA rhythm in culture. *Proc. Natl. Acad. Sci. USA* **94**, 304–309
  26. Grève, P., Voisin, P., Grechez-Cassiau, A., Bernard, M., Collin, J.P., and Guerlotti, J. (1996) Circadian regulation of hydroxyindole-*O*-methyltransferase mRNA in the chicken pineal gland *in vivo* and *in vitro*. *Biochem. J.* **319**, 761–766
  27. Dunlap, J.C. (1999) Molecular bases for circadian clocks. *Cell* **96**, 271–290
  28. Binkley, S., Klein, D.C., and Weller, J.L. (1976) Pineal serotonin *N*-acetyltransferase activity: protection of stimulated activity by acetyl-CoA and related compounds. *J. Neurochem.* **26**, 51–55
  29. Bernard, M., Guerlotti, J., Cogne, M., Grève, P., Collin, J.P., and Voisin, P. (1993) Transcriptional regulation of hydroxyindole-*O*-methyltransferase in the chicken pineal gland: day/night changes and long-term effects of light and darkness. *Biochem. J.* **290**, 661–664
  30. Namboodiri, M.A.A., Weller, J.L., and Klein, D.C. (1980) Evidence of inactivation of pineal indoleamine *N*-acetyltransferase by protein thiol:disulfide exchange. *J. Biol. Chem.* **255**, 6032–6035
  31. Namboodiri, M.A.A., Favilla, J.T., and Klein, D.C. (1981) Pineal *N*-acetyltransferase is inactivated by disulfide-containing peptides: insulin is the most potent. *Science* **213**, 571–573
  32. Gastel, J.A., Roseboom, P.H., Rinaldi, P.A., Weller, J.L., and Klein, D.C. (1998) Melatonin production: proteasomal proteolysis in serotonin *N*-acetyltransferase regulation. *Science* **279**, 1358–1360
  33. Klein, D.C., Coon, S.L., Roseboom, P.H., Weller, J.L., Bernard, M., Gastel, J.A., Zatz, M., Iuvone, P.M., Rodriguez, I.R., Bégay, V., Falcón, J., Cahill, G.M., Cassone, V.M., and Baler, R. (1997) The melatonin rhythm-generating enzyme: molecular regulation of serotonin *N*-acetyltransferase in the pineal gland. *Recent Prog. Horm. Res.* **52**, 307–357
  34. Young, M.W. (1998) The molecular control of circadian behavioral rhythms and their entrainment in *Drosophila*. *Annu. Rev. Biochem.* **67**, 135–152
  35. Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G., and Lee, C.C. (1997) *RIGUI*, a putative mammalian ortholog of the *Drosophila period* gene. *Cell* **90**, 1003–1011
  36. Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M., and Sakaki, Y. (1997) Circadian oscillation of a mammalian homologue of the *Drosophila period* gene. *Nature* **389**, 512–516
  37. Albrecht, U., Sun, Z.S., Eichele, G., and Lee, C.C. (1997) A differential response of two putative mammalian circadian regulators, *mper1* and *mper2*, to light. *Cell* **91**, 1055–1064
  38. Shearman, L.P., Zylka, M.J., Weaver, D.R., Kolakowski, L.F., Jr., and Reppert, S.M. (1997) Two *period* homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron* **19**, 1261–1269
  39. Takumi, T., Matsubara, C., Shigeyoshi, Y., Taguchi, K., Yagita, K., Maebayashi, Y., Sakakida, Y., Okumura, K., Takashima, N., and Okamura, H. (1998) A new mammalian *period* gene predominantly expressed in the suprachiasmatic nucleus. *Genes Cells* **3**, 167–176
  40. Takumi, T., Taguchi, K., Miyake, S., Sakakida, Y., Takashima, N., Matsubara, C., Maebayashi, Y., Okumura, K., Takekida, S., Yamamoto, S., Yagita, K., Yan, L., Young, M.W., and Okamura, H. (1998) A light-independent oscillatory gene *mPer3* in mouse SCN and OVL. *EMBO J.* **17**, 4753–4759
  41. Zylka, M.J., Shearman, L.P., Weaver, D.R., and Reppert, S.M. (1998) Three *period* homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron* **20**, 1103–1110
  42. Yoshimura, T., Suzuki, Y., Makino, E., Suzuki, T., Kuroiwa, A., Matsuda, Y., Namikawa, T., and Ebihara, S. (2000) Molecular analysis of avian circadian clock genes. *Mol. Brain Res.* **78**, 207–215
  43. Okano, T., Yoshizawa, T., and Fukada, Y. (1994) Pinopsin is a chicken pineal photoreceptive molecule. *Nature* **372**, 94–97