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Development of rhythmic melatonin synthesis in cultured pineal glands and pineal cells isolated from chick embryo

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Abstract. The chick pineal gland exhibits circadian rhythms in melatonin synthesis under in vivo and in vitro conditions. A daily rhythm of melatonin production was first detectable in pineal glands isolated from chick embryos at embryonic day 16 and incubated under a LD cycle. All pineal glands isolated from 17-day-old and older embryos were rhythmic while no gland isolated at embryonic day 14 and 15 exhibited a daily rhythm in melatonin synthesis. Melatonin production in static cultures of embryonic pineal cells was rhythmic over 48 h if the cells were kept under a LD cycle. When embryonic pineal cells were incubated in constant darkness the rhythm in melatonin production was damped within 48 h. These results suggest that chick pineal cells from embryonic day 16 onwards are photosensitive but that the endogenous component of the melatonin rhythm is not completely developed at that age. A soluble analogue of cAMP stimulated and norepinephrine inhibited melatonin synthesis in cultured embryonic pineal cells. These findings indicate that the stimulatory and inhibitory pathways controlling melatonin synthesis in the mature pineal gland are effective in pineal cells isolated from chick embryos at least 2 days before hatching.
Key words. Chick; embryo; birds; rhythms; circadian; cyclic AMP; norepinephrine; pineal cells.

The chick pineal gland contains endogenous circadian oscillators and the rhythmic melatonin production persists under in vitro conditions[1,2]. The integrity of the pineal gland is not necessary for melatonin rhythm generation as the rhythm is present in the isolated pineal gland, in dispersed cell cultures and even in individual pineal cells[3–5].

Melatonin production in the chick pineal gland is primarily controlled by a circadian oscillator located in the gland itself. It stimulates melatonin biosynthesis through elevated cyclic AMP production at night[1,6]. During the day norepinephrine activates the pineal 2-alpha adrenoceptors and inhibits adenylate cyclase and subsequently both cAMP and melatonin synthesis[7]. Compounds that raise cAMP (forskolin, increased calcium) as well as analogues of cAMP (8-bromocyclic AMP) immediately increase melatonin synthesis. Agents that lower cAMP levels, such as norepinephrine or light, acutely inhibit melatonin production[8]. In addition to the circadian regulation of melatonin output by cAMP there are at least two neural inputs that regulate melatonin synthesis in the chick pineal gland. The primary neural pathway is a noradrenergic input from the sympathetic nervous system, mediated via alpha-2 adrenergic receptors[9,10]. The mechanism of action of alpha-2-adrenoreceptors is linked to an inhibition of adenylate cyclase. The inhibitory effect of norepinephrine on melatonin production can be overcome by increased cAMP[10]. The second neural input represents vasoactive intestinal polypeptide (VIP)-containing fibers. VIP markedly stimulates melatonin production in chick pineal cells, acting through cAMP stimulation[11]. Nothing is known about the ontogeny of this pathway in birds.

The chick pineal gland has been used successfully, both in vivo and in vitro, for studies on biochemical and molecular mechanisms of rhythmic melatonin production. Incorporation of developmental aspects into this model may further increase its relevance for studies of molecular control of circadian rhythmicity. In birds, enzymes involved in melatonin synthesis are present in the pineal gland of chick embryo[12,13] and melatonin synthesis starts during embryonic life[14–16]. A daily rhythm in melatonin production was observed in 18-day-old chick embryos, both in the pineal gland and the eyes[17].

So far, there has been no information about the development of daily rhythmicity in melatonin production in the chick pineal gland under in vitro conditions. Therefore, the aim of our study was to find out at what age isolated embryonic pineal glands begin to express rhythmic melatonin production, whether this rhythmicity is circadian, and what mechanisms can control rhythmic melatonin synthesis in cultured embryonic pineal cells.

Materials and methods

Eggs of broiler breeder hens were incubated in a forced draught incubator with automatic turning every hour at

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a temperature 37.5 ± 0.5 °C under a light (L):dark (D) cycle of 12:12 (light on from 06.00 h till 18.00 h) or LD 16:8 (light 06.00 h–22.00 h). Light was provided by a white fluorescent tube (Tesla, 16 W) located inside the incubator providing illumination in the range of 400–600 lux.

Experiment 1. Embryos at 14 ,15, 16, 17, 19, and 21 days of development were removed in the middle of the light phase and killed by decapitation. The pineal glands were quickly dissected and placed in 2 ml RPMI 1640 culture medium with L-glutamine (2 mM), HEPES (25 mM), 10% heat-inactivated fetal calf serum, penicillin (10^5 U/l), streptomycin (25 mg/l) and kanamycin (100 mg/l). Glands were washed and transferred to 24-well tissue culture plates containing 1 ml of RPMI 1640 medium per well with other ingredients. Pineals were maintained in medium through day 3 in a tissue culture incubator supplied with a mixture of 95% O_2 and 5% CO_2 under LD cycle 12:12. White fluorescent light with an intensity of 2000 lux was used. Temperature was kept at 37.8 °C and medium was exchanged at least daily. On day 4, the pineals were washed twice with culture medium and subsequently media (0.5 ml) were collected for melatonin assay. Medium (0.5 ml) was changed every 2 or 4 h starting at 10.00 h. Samples during the darktime were collected under dim light (1 lux).

Experiment 2. Embryos were decapitated on day 19 in the middle of the lighttime. Pineal glands were immediately dissected and placed into phosphate-buffered saline (PBS). The glands were incubated in trypsin (0.25% in PBS) two times for 30 min at 37 °C on a shaking platform. After trypsinization, the cells were collected by centrifugation (800 *g*, 10 min, 4 °C) and resuspended in the culture medium RPMI 1640 described for experiment 1. The cell suspension was seeded on a 24-well plate. There were six replicates per group. Cells from 40 glands were used in one experiment. Cells were fed by exchange of medium (1 ml) at least daily. Cells were maintained in the medium through day 4 under 5% CO_2 in air in the tissue culture incubator lit by fluorescent light (2000 lux) and lighting cycles of LD 12:12 and LD 16:8 were used. Cells were exposed to the same LD cycle as the embryos in the incubator. On day 5, pineal cells were washed three times in culture medium, and samples of the media were collected at 2-h intervals over a period of 24 h.

Experiment 3. Embryonic pineal cells, isolated on embryonic day 19 and preincubated for 4 days at LD 16:8 with daily exchange of medium, were used. The control pineal cells were cultured in LD 16:8 and sampled over 48 h at 2-h intervals. To find out whether a LD cycle was necessary for the expression of a daily rhythm of melatonin synthesis, other cells were kept in constant darkness for 48 h following 4 days of preincubation at LD 16:8. Media were collected at the same intervals as in the controls. Samples were subsequently stored at −20 °C until melatonin content was assayed.

Experiment 4. Dibutyryl cyclic AMP (dBcAMP; Sigma) and norepinephrine (NE, bitartate salt; Sigma) were applied to cultured pineal cells isolated from 19-day-old chick embryos and preincubated for 4 days in LD 12:12. Medium was exchanged daily during preincubation. Both drugs were administered at a dose of 10^{-6} M in the middle of the light and dark period at 12.00 h and 24.00 h respectively. Dibutyryl cAMP was dissolved in medium (M) and NE in 0.1 M citric acid in medium (MC). The drugs were applied to the exchanged medium (0.5 ml) in a 10 µl volume. Melatonin production was measured before and 4 h after administration of drugs.

Assay of melatonin. Melatonin concentrations were measured by a direct radioimmunoassay[18]. The validity of hormone measurement was tested by parallelism. There was a close parallelism between serial dilutions of samples collected at night (concentration 1200 pg/ml) and the standard curve. The standard curve was prepared in medium.

^{3}H-labelled melatonin with a specific activity of 3.056 TBq/ mM (NEN Du Pont) was used. The activity of added labelled melatonin was about 10 000 cpm/tube and the binding in B_0 (without unlabelled hormone) was approximately 30%.

Statistical analysis. Data are expressed as mean ± SEM. The rhythm of melatonin synthesis was evaluated by multiple cosinor procedure (sinusoidality test)[19,20]. Effects of drug administrations were evaluated using one-way analysis of variance and the statistical significance between specific means were determined by *t*-test.

Results

No apparent daily rhythm of melatonin synthesis was detectable in pineals dissected from 14- and 15-day-old chick embryos and maintained in vitro at LD 12:12 (fig. 1). High standard deviations of melatonin concentrations were recorded on both days, and there was a moderate increase in melatonin content over the 24-h period in pineals of 14-day-old embryos. Starting from embryonic day 16 a rhythmic profile of melatonin synthesis was measured. Levels were low during the light phase and high during the dark phase of the LD cycle. The appearance of rhythmicity was accompanied by a decrease in the variability of the melatonin values. In some of the pineals isolated on this day concentrations were still low and did not express rhythmic fluctuations (fig. 2). A distinct daily rhythm of melatonin synthesis was found in pineals of 17-day-old and older chick embryos. The amplitude of the rhythm increased during the last days of embryonic development and the maximum amplitude was found in 19-day-old embryos. A

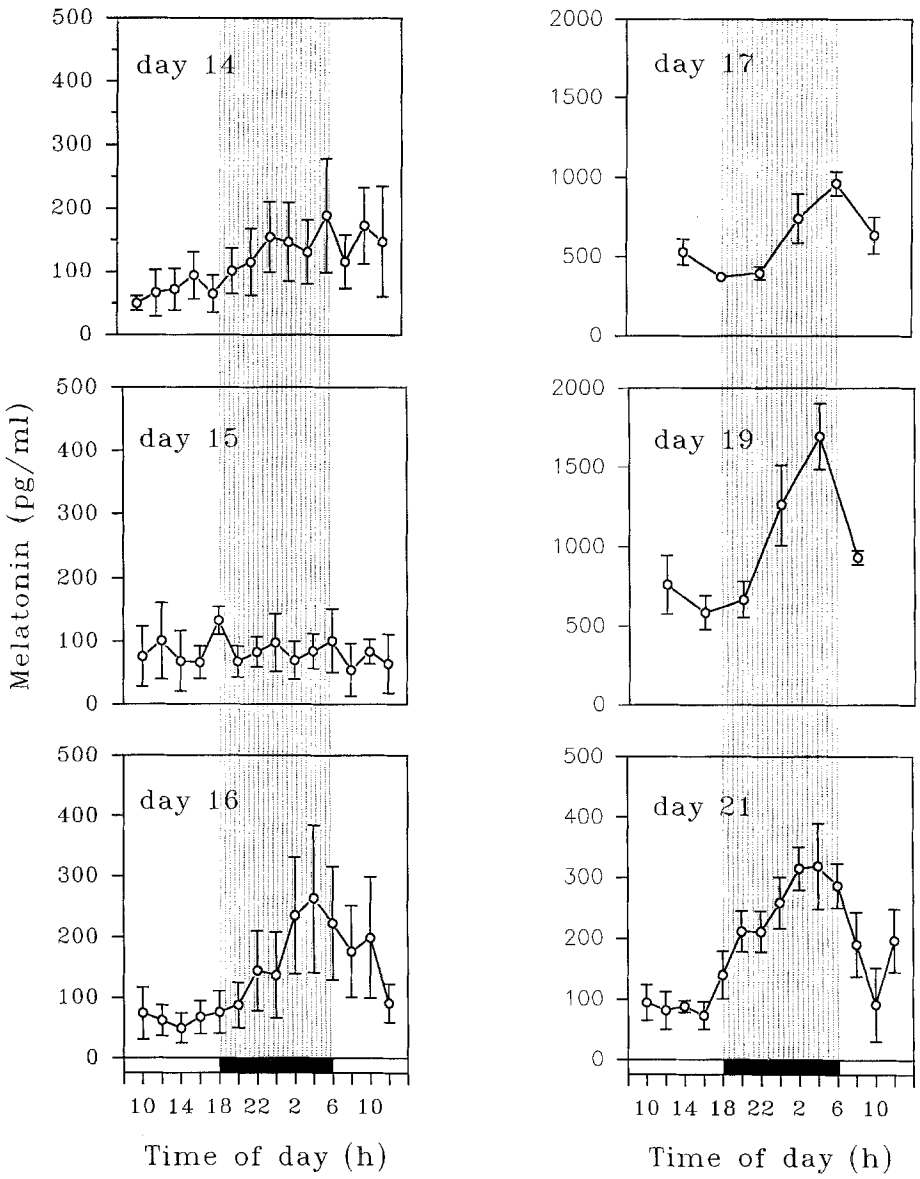

Figure 1. The melatonin rhythm in pineal glands isolated from chick embryos on day 14, 15, 16, 17, 19 and 21. Glands were maintained under in vitro conditions as described in 'Materials and methods'. Media were collected and replaced at 2- and 4-h intervals. Note the different scale for pineal glands isolated from 17- and 19-day-old embryos sampled at 4-h intervals. Data are means ± SEM from three to five wells. Bars below figures indicate the LD cycle.

typical daily rhythm was found also in 21-day-old hatchlings.

Pineal cells isolated from 19-day-old embryos and kept in a steady state culture system under two different LD cycles showed rhythmic melatonin production with high concentrations found during the darktime (fig. 3). The rhythm persisted with the same amplitude for at least two cycles when pineal cells were cultured in LD (fig. 4). However, when the pineal cells were kept for two days in constant darkness the amplitude of the rhythm decreased and the rhythm was damped by the second day in DD (fig. 4).

The analogue of cAMP stimulated melatonin production in cultured embryonic pineal cells. The increase was more pronounced during the day (fig. 5A) than during the night (fig. 5B). Norepinephrine inhibited melatonin synthesis in the pineal cells at night (fig. 5B). There was no effect of NE on melatonin synthesis during the lighttime when basal melatonin concentrations were significantly lower than during the darktime.

Discussion

A daily rhythm of melatonin synthesis was first detectable at embryonic day 16 but not all glands exhibited rhythmic melatonin production at this age. In contrast, all pineal glands isolated from 17-day-old and older chick embryos showed a distinct rhythm in melatonin production. These results corroborate our previous in vivo studies[16]. The developmental appearance of

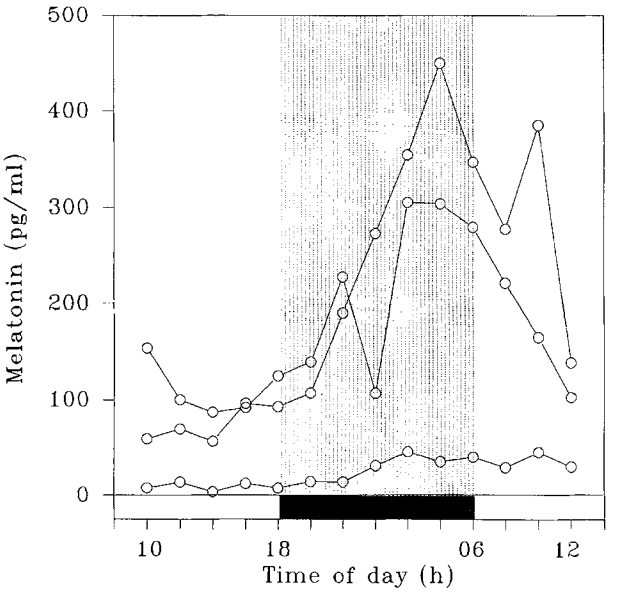

Figure 2. Melatonin production in pineal glands isolated on embryonic day 16. Each curve represents melatonin production of one pineal per well. Glands were maintained as described in 'Materials and methods'. Media were collected at 2-h intervals.

hydroxyindole-*O*-methyltransferase (HIOMT), the second enzyme involved in melatonin production catalyzing the formation of melatonin from *N*-acetylserotonin, has been observed between embryonic day 16 and 18[12]. Recent molecular studies indicate that HIOMT mRNA is present as early as embryonic day 12 in chicken[21]. In agreement with the presence of both enzymes in the pineal gland a distinct rhythm in melatonin production was found in both the pineal gland and the eyes of 18-day-old chick embryos[17]. The presence of melatonin has been noted at embryonic day 10 in the cultured pineal gland[15].

Thus, melatonin production in the chick pineal gland starts relatively early during embryonic development. This contrasts with the situation in mammals, in which melatonin synthesis is a postnatal phenomenon[22,23]. Available data suggest that in chickens melatonin may be synthesized during the second week of embryonic life but that the rhythmic pattern develops later, around embryonic day 16.

For our studies on dispersed pineal cells isolated from pineal glands we used static cultures of cells from 19-day-old chick embryos. The static culture of pineal glands and pineal cells[1,4] has been used for analyzing the molecular mechanisms of rhythmic melatonin production. Under these in vitro conditions there is no differentiation of photoreceptors in embryonic pineals[24] and preincubation of pineal cells or pineal glands is not expected to influence melatonin production. The pattern of the melatonin rhythm in LD was as expected, high concentrations occurring during the darktime and low ones during the lighttime. The amplitude and the general pattern of the rhythm did not change during the

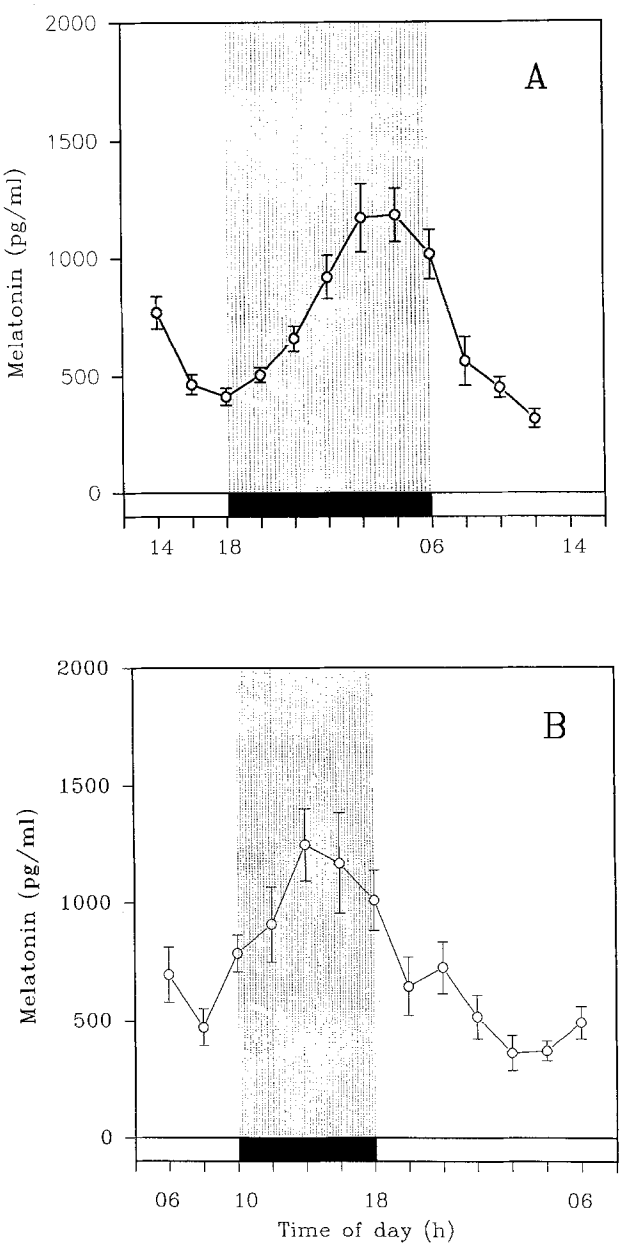

Figure 3. Effect of different lighting cycles on the melatonin rhythm in pineal cells isolated from pineal glands of 19-day-old chick embryos. Cells were maintained in LD 12:12 (*A*) and LD 16:8 (*B*) through day 4, with media replaced every day. Media were collected during day 5 at 2-h intervals. Data are means ± SEM from six wells. Bars below abscissa indicate LD cycles.

two consecutive days during which samples were taken. The strong rhythmicity of melatonin production in pineal cell culture suggests that the cells are photosensitive themselves. They are able to synchronize their melatonin synthesizing activity with the LD cycle. Consequently, the intact pineal organ or close cell-to-cell contact is not essential for the development and maintenance of the rhythm.

The rhythm in melatonin production was damped within 48 h when pineal cells were incubated in constant darkness. A similar damping of the rhythm within 3

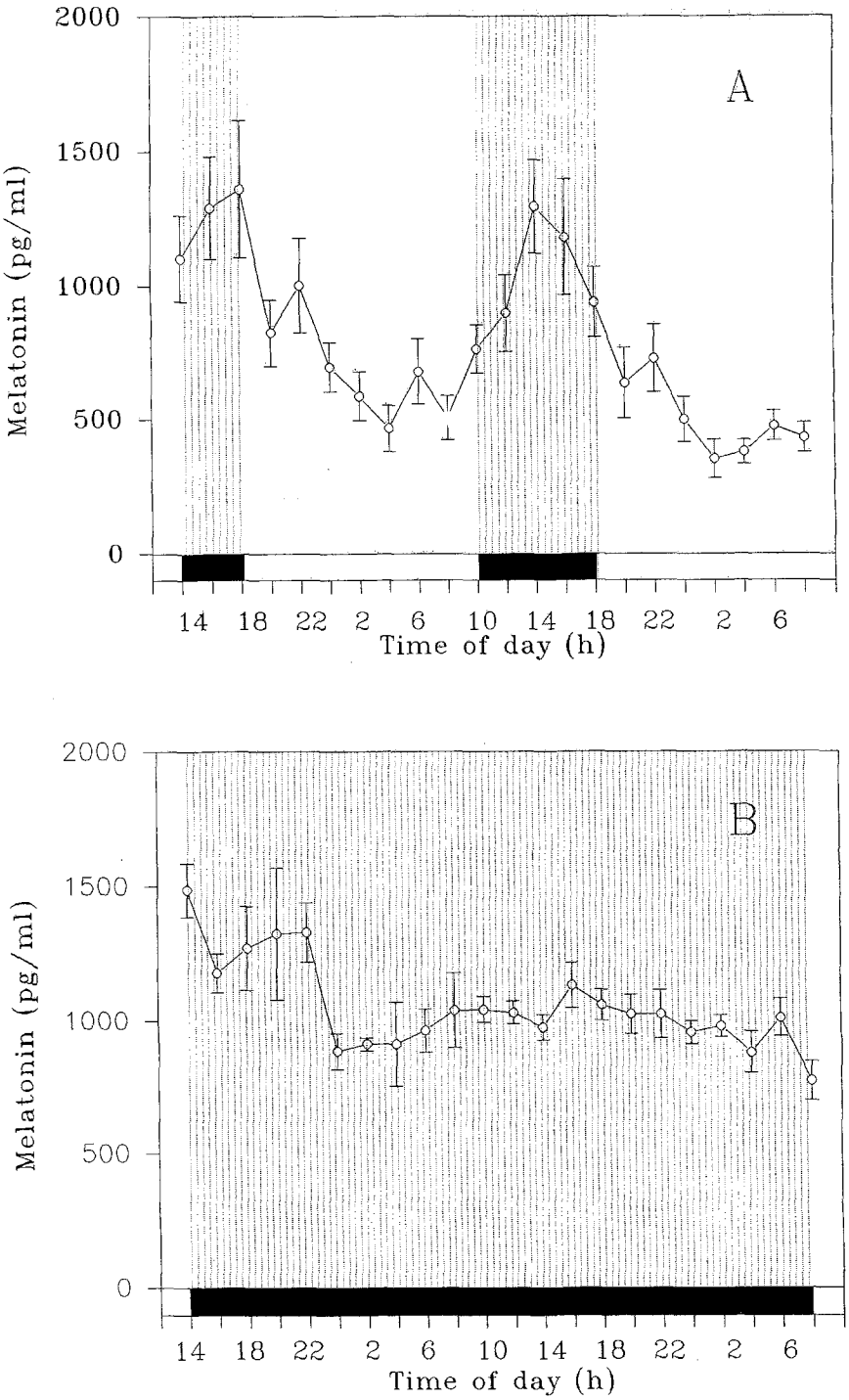

Figure 4. Melatonin production measured for 42 h in pineal cells isolated from 19-day-old chick embryos. Cells were maintained under LD 16:8 until day 5. Media were collected during day 5 and 6 in 2-h intervals under the LD cycle in the control group (*A*) and in constant darkness (*B*). Data are means ± SEM from 5 wells. Bar below figures indicates the LD cycle and darkness.

days has been seen in cultures of pineal cells isolated from 0–2-day-old chicks[8]. The low standard deviation of measured melatonin from different wells suggests that the rhythm is damped and its disappearence is not simply an effect of desynchronization of populations of oscillators. A rhythm in melatonin production in pineal cells isolated from mature chicken pineal glands was shown to persist for 6 days in DD[3].

The developmental appearance of the melatonin rhythm under in vivo conditions coincides with the development of a behavioral response of chick embryos to a light stimulus[17]. The presence of the rhythm in dispersed cell culture suggests that photoreceptive elements are already developed in pineal cells of chick embryos at the end of the embryonic period. It is possible that the photic input from the environment is a key factor

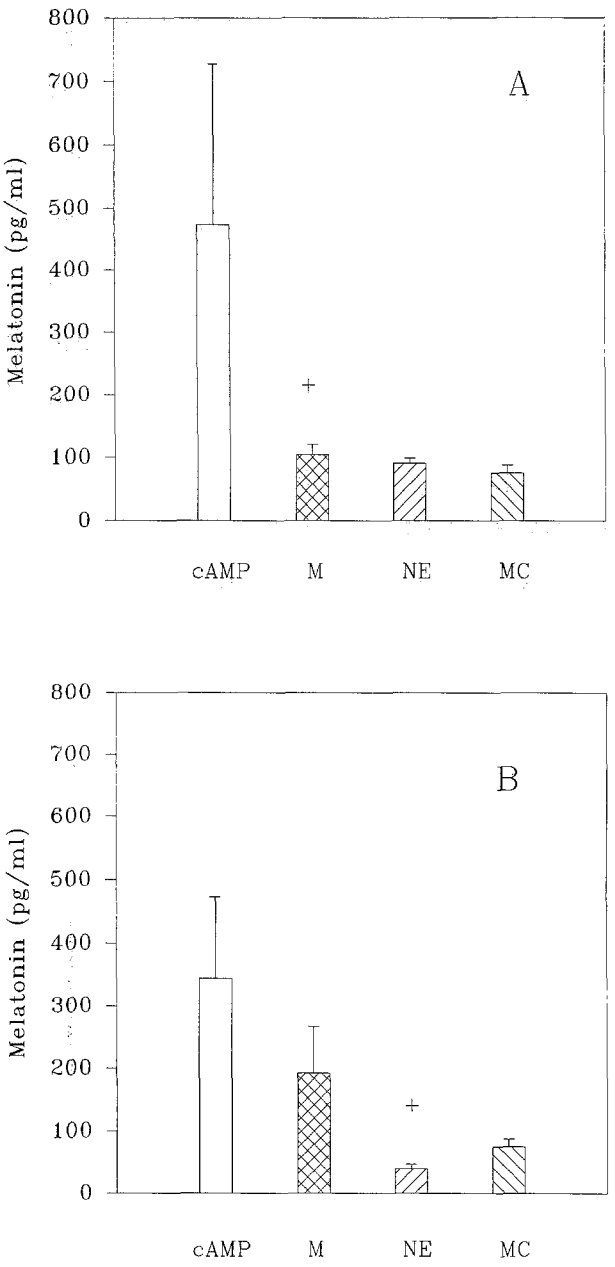

Figure 5. Effects of cAMP and norepinephrine (NE) on the melatonin output in cells isolated from pineal glands of 19-day-old chick embryos. Cells were maintained in LD 12:12 through cycle 5 when the drugs were administered. NE and cAMP were applied for 4 h at a dose of 10^{-6} M in the middle of the light (*A*) and dark (*B*) period (at 12.00 h and 24.00 h). M – growth medium (control for cAMP group), MC – 0.1 M citric acid in medium (control for NE, see 'Material and methods, Experiment 4'). Data are means ± SEM from six wells. $+p < 0.05$.

required for expressing a daily rhythm in melatonin synthesis.

Melatonin synthesis in the mature chicken pineal gland is regulated by cAMP, and factors which increase cAMP concentrations also raise melatonin synthesis (Takahashi et al.[6] for a review). Our results extend this conclusion to embryonic stages showing that a soluble analogue of cAMP stimulates melatonin production in cultured embryonic pineal cells both during lighttime and darktime. The stimulatory effect of cAMP is higher during the daytime when basal melatonin levels are lower than during the darktime when basal concentrations are higher. Concentrations after stimulation did not differ between the darktime and lighttime.

Norepinephrine, which inhibits melatonin production in mature chick pineal glands[6], was able to decrease melatonin production when applied during the darktime. Administration during the lighttime, when melatonin production is naturally low, did not decrease melatonin production. Sympathetic innervation has been found 3 days before hatching in quail embryo[24] and may contribute to rhythm development in the avian embryo. Taken together, our data suggest that the two principal pathways controlling melatonin synthesis are developed in the chick embryo pineal gland by the end of embryonic life. Studies are in progress which analyze both these control pathways in chick embryos of different ages, prior to and after the expression of rhythmic melatonin production.

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