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Research Article

Regulation of rhythmic melatonin production in pineal cells of chick embryo by cyclic AMP

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Abstract. The pineal cells of chick embryos incubated in vitro exhibited a daily rhythm of melatonin synthesis under a 12:12 light:dark (LD) cycle at the embryonic days 16 and 19. In order to elucidate whether cyclic adenosine monophosphate (cAMP) – a component of the melatonin generating system – is already at work in the embryonic period, we measured the effects of forskolin and isobuthylmethylxantine (IBMX) on melatonin production, cAMP efflux and accumulation. Forskolin (after 10, 20, 30, 45, 60 and 90 min of administration) and IBMX (6 h), when applied during the light phase of LD cycle, stimulated melatonin production and cAMP efflux and accumulation during the embryonic period (at days 16 and 19 of development). Our results suggest that the biochemical pathway involving cAMP, which controls melatonin production in the postnatal period, is developed before hatching and already on embryonic day 19 works in a way similar to that in post-hatched chicks. Differences in response to cAMP stimulation between 16- and 19-day-old pinealocytes seem to be mostly quantitative.

Key words. Chick embryo; circadian rhythms; cAMP; melatonin; forskolin; IBMX; pineal cells.

Melatonin is a hormone implicated in regulation of various circadian rhythms and many other physiological processes. In birds, the pineal gland, which seems to be the major source of circulating melatonin, contains a circadian oscillator that controls the rhythm of melatonin synthesis and release [1, 2].

The melatonin rhythm-generating system in birds is driven by endogenous oscillators. Under in vivo conditions there are at least two oscillators, one in the pineal gland itself [3–5]; another is the avian homologue of the suprachiasmatic nucleus (SCN), which is located in the hypothalamus [6]. Oscillators in pineal cells are also able to generate the melatonin rhythm in vitro. Chick pineal cells in culture are directly photosensitive [7], and therefore the light can act to acutely suppress melatonin at night and to entrain the pineal circadian clock. The acute inhibitory effect of light is mediated by cyclic adenosine monophosphate (cAMP), whereas the pathway mediating the entraining effect of light is probably more complex [8, 9]. Agents which stimulate adenylate cyclase, such as forskolin, isobutylmethylxantine (IBMX) and others, increase cAMP levels and subsequently melatonin production [10–12]. A major inhibitory agent, norepinephrine (NE), decreases melatonin synthesis by inhibition of adenylate cyclase and cAMP [4, 13, 14] and acts through the activation of α_2-adrenoreceptors [15–18].

These biochemical pathways of melatonin biosythesis have been studied in post-hatched chicks. However, less is known about the development and expression of these processes during embryonic life. Melatonin is synthesized during the embryonic period in chick pineal glands in vivo [19, 20] and in pineal glands and cells in

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vitro [21, 22], with high levels observed during the dark phase and low levels during the light phase. A similar profile is well known from studies performed during the postnatal period [7, 23, 24]. The inhibitory effect of NE treatment on melatonin production was observed at both night and day [22]. The stimulatory pathway in the regulation of melatonin synthesis through cAMP was investigated in chick embryonic pineal cells by treatment with stable analogues such as dibutyryl cAMP [22] and 8-bromocyclic AMP [21]. In the present work, we studied the effects of additional agents that raise endogenous cAMP levels, such as forskolin (stimulator of adenylate cyclase) and IBMX (inhibitor of phosphodiesterase), on melatonin production, cAMP efflux and accumulation in cultured chick embryonic pineal cells of two different ages. We compared pineal cells isolated from 16- and 19-day-old chick embryos, because at the earlier age the melatonin rhythm started to be detectable in some, and at the later age the rhythm was developed in all, pineal glands kept in vitro [22]. Therefore, the purpose of this study was to elucidate whether there is a difference in the control of melatonin synthesis by cAMP between these two developmental stages of the embryonic period.

Materials and methods

Eggs of broiler hens were incubated in a forced draught incubator with temperature maintained at 37.5 ± 0.3 °C under a light (L):dark (D) cycle of 12:12 (light from 06.00 h until 18.00 h). Light, produced by a white fluorescent tube (Tesla, 16W) located inside the incubator, provided illumination at a range of 200 to 400 lux as measured at the egg surface.

Pineal cell culture. Embryos at 16 and 19 days of development were removed from the incubator during the light phase and decapitated. Pineal glands were immediately dissected, rinsed by phosphate-buffered saline (PBS) and incubated in trypsin (0.25% in PBS) three times for 20 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 with L-glutamine (2 mmol), HEPES (25 mmol), 10% heat-inactivated fetal calf serum, penicillin (10^5 U l^{-1}), streptomycin (25 mg l^{-1}) and kanamycin (100 mg l^{-1}). The cell suspension was seeded on 24-well tissue culture plates. Cells were fed by exchange of medium at least once a day. There were five replicates per group. Experiments used cells from 24 up to 36 glands in 24 wells. Cells were maintained in serum-containing medium through 2 days and in serum-free medium from day 3 onward (serum-free medium is needed because serum, which contains phosphodiesterase activity [25], compromised the measurement of cAMP efflux and accumulation) in a tissue culture incubator. The incubator was supplied with a mixture of 95% O_2 and 5% CO_2, lit by fluorescent light (2000 lux) under the same LD cycle (LD 12:12) to which embryos had been exposed in the incubator. Pineal cell cultures from embryos of both ages were used in the following experiments.

Experiment 1: Rhythms of melatonin production. Daily rhythms in melatonin production were studied in pineal cells isolated from 16- and 19-day-old embryos. After 2 days in culture, cells isolated from 16- and 19-day-old embryos were washed three times in medium RPMI 1640 without serum. Samples of the medium for measurement of melatonin production were collected every 6 h for 24 h. Every medium collection preceded 1 h incubation with fresh medium.

Experiment 2: Effects of forskolin and IBMX on melatonin production, cAMP efflux and accumulation. On day 3 of pineal cell culture, after the application of 10^{-4} mol l^{-1} of forskolin (Sigma) during the daytime beginning 5.5 h after the onset of light, melatonin, cAMP efflux and cAMP accumulation were measured at intervals of 10, 20, 30, 45, 60 and 90 min. On the same day of incubation, cAMP efflux and accumulation and melatonin production were measured after 6 h of treatment with 5×10^{-4} mol l^{-1} IBMX (Sigma) beginning at the time of lights on. After this treatment the medium was removed and the cells were prepared for measurement of cAMP accumulation by immediate treatment with 200 µl of ice-cold 0.1 mol l^{-1} HCl. Cells were scraped from the bottom of the well with a rubber scraper and the extract was frozen at −20 °C until cAMP was measured by radioimmunoassay. An aliquot of the removed medium was saved for measurement of melatonin. The rest of the medium was heated at 90 °C for 3 min for measurement of cAMP efflux. Forskolin and IBMX were dissolved in dimethylsulphoxide (Serva) at a final concentration of 0.01% or less.

Assay of melatonin. Melatonin concentrations were measured by a direct radioimmunoassay [26] that we had previously validated for measurement of melatonin in medium [22]. ^{3}H-labelled melatonin with a specific activity of 1276.5 GBq $mmol^{-1}$ (NEN Du Pont) was used. The activity of added labelled melatonin was about 8000 cpm/tube and the binding in B_0 (without unlabelled hormone) was approximately 30%.

Assay of cAMP. Cyclic AMP was measured by radioimmunoassay based on the principle of competitive binding [27]. ^{125}I-labelled cAMP and tubes coated by a

monoclonal antibody (kit from Immunotech, Marseille, France) were used.

Statistical analysis. Data are expressed as mean ± SEM. Effects of drug administrations were evaluated using the statistical significance between specific means by *t*-test.

Results

Melatonin production during the 24-h period exhibited a rhythmic profile in static culture of pineal cells isolated from 16- and 19-day-old chick embryos (fig. 1A,B). Melatonin levels were higher in the darkness than in the light period on day 16 (midnight vs. midlight values, $P < 0.05$) and day 19 (midnight vs. midlight values, $P < 0.001$) of development. The activity of pineal glands rose rapidly with successive embryonic development, and melatonin production was 44.2 times higher at midnight and 22.4 times higher at midlight in 19-day-old embryonic pinealocytes as compare to pinealocytes isolated from 16-day-old embryos.

After stimulation of adenylate cyclase by forskolin, we measured melatonin production and cAMP efflux and accumulation. In 16-day-old embryos (fig. 2A) melatonin production was increased 10 to 90 min after forskolin treatment ($P < 0.01$ after 30 min; $P < 0.001$ after 45 min; $P < 0.05$ after 60 and 90 min in comparison to initial values). cAMP efflux was significantly elevated after 20 min ($P < 0.01$), 30, 45 and 60 min ($P < 0.001$) of forskolin treatment. After 90 min of forskolin treatment, cAMP efflux was decreased in comparison with 60 min treatment ($P < 0.001$). In 19-day-old embryos cAMP efflux had already increased after 10 min of stimulation with forskolin (fig. 2B). This trend was present 20, 30, 45, 60 and 90 min after the application of forskolin (20 min, $P < 0.01$; 30, 45, 60 and 90 min, $P < 0.001$). cAMP efflux correlated with the increase of melatonin.

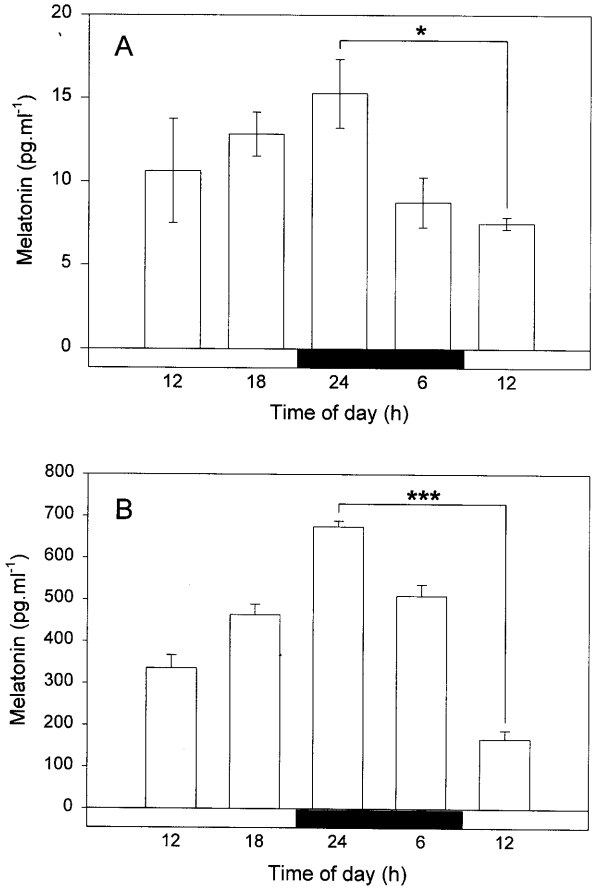

Figure 1. Melatonin production in pineal cells isolated from pineal glands of 16-day-old (*A*) and 19-day-old (*B*) chick embryos in LD cycle 12:12. Samples of medium were collected every 6 h after 1 h incubation with fresh medium. Data are means from five wells ± SEM. Horizontal bars below abscissa indicate LD cycles. $*P < 0.05$. $***P < 0.001$.

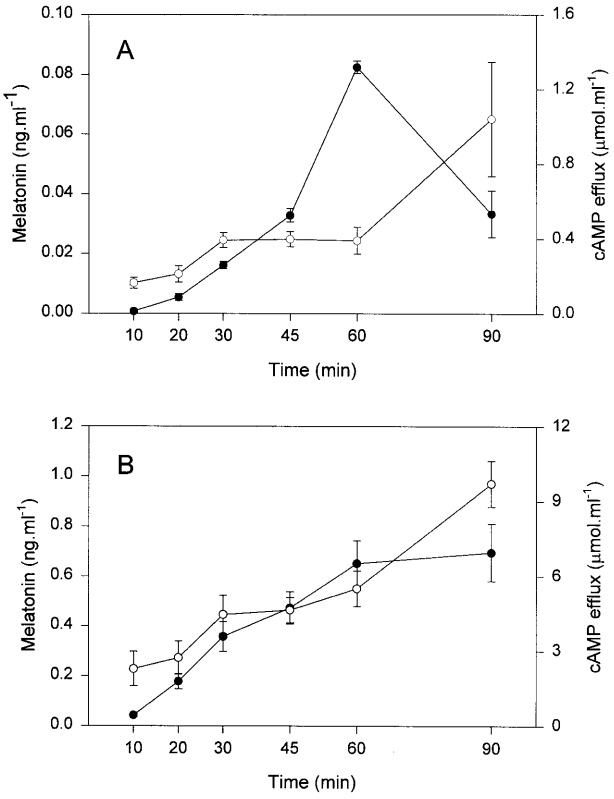

Figure 2. Effect of forskolin (10^{-4} mol l^{-1}) on melatonin output and cAMP efflux in pineal cells isolated from both ages of chick embryos – 16-day-old (*A*) and 19-day-old (*B*). Cells were maintained in LD 12:12 through cycle 3 when the drug was administrated. Forskolin was applied for 10, 20, 30, 45, 60 and 90 min in the middle of the light phase of LD cycle. Melatonin and cAMP efflux were measured by radioimmunoassay from the same collected medium. Data are means from five wells ± SEM. Open circles represent melatonin production, and closed circles represent cAMP efflux.

In 16-day-old embryos cAMP accumulation increased gradually from 10 up to 60 min ($P < 0.001$ in comparison with time 0) of treatment with forskolin (fig. 3A). Ninety minutes of forskolin action brought a decrease in cAMP levels ($P < 0.05$ to time 0; $P < 0.001$ to time 60). In 19-day-old embryos cAMP accumulation peaked 20 min ($P < 0.001$ to time 0) after stimulation with forskolin and then gradually decreased until 90 min ($P < 0.01$ to time 0; $P < 0.05$ to time 60) of treatment (fig. 3B). The total amount of accumulated cAMP in 16-day-old embryonic pinealocytes was severalfold lower than that detected in 19-day-old cells (3.2 times lower after 10 min of treatment; 2.3 times lower after 90 min of treatment).

The relationship between cAMP efflux and accumulation is shown in figure 4. Accumulation of cAMP was calculated from the area under the curves presented in figure 3. In 16-day-old embryos the level of cAMP efflux is not linearly related to the amount of accumulated intracellular cAMP (fig. 4A). However, cAMP efflux is a linear reflection of intracellular levels of cAMP in 19-day-old chick embryonic pineal cells (fig. 4B). The linear regression yielded the following relationship: cAMP efflux = 0.0390 of accumulated cAMP. IBMX evoked an increase of melatonin production and elevated cAMP efflux and accumulation in pinealocytes of both ages (fig. 5A,B). During the light phase the effect of IBMX was more pronounced in 16-day-old embryos (melatonin production was 5-fold higher than in controls, cAMP efflux 10-fold higher and cAMP accumulation 2-fold higher than in controls) than in 19-day-old embryos (melatonin production was 2-fold higher than in controls, cAMP efflux was 5-fold higher and cAMP accumulation only tended to increase as compared with controls).

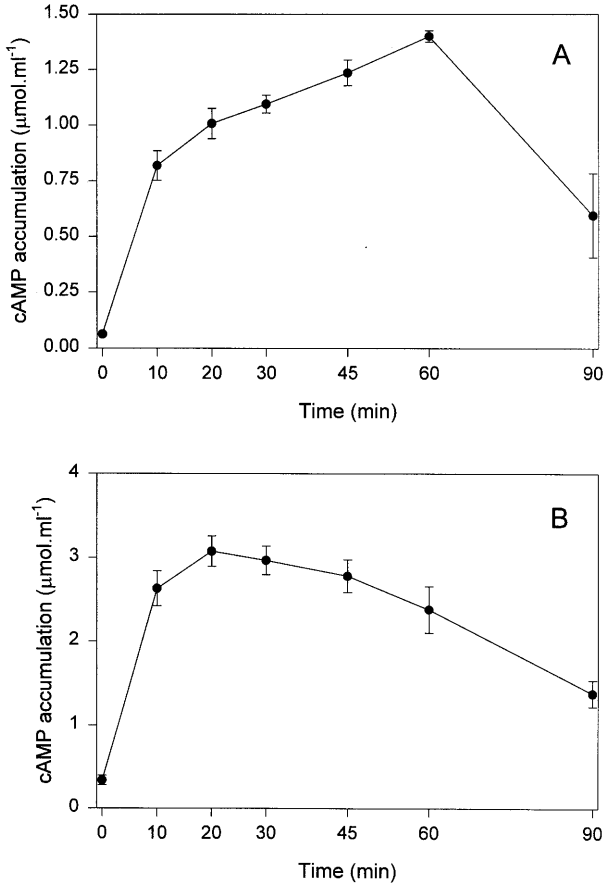

Figure 3. Cyclic AMP accumulation in pineal cells isolated from 16-day-old (*A*) and 19-day-old (*B*) chick embryos measured after treatment with forskolin (dose and time of duration were the same as shown in the text of fig. 2). At the end of each treatment, the medium was removed, and 200 μl of ice-cold 0.1 mol l^{-1} HCl was added immediately to cells. They were scraped, and the extract was frozen at −20°C for measurement by radioimmunoassay. Data are means ± SEM from five wells.

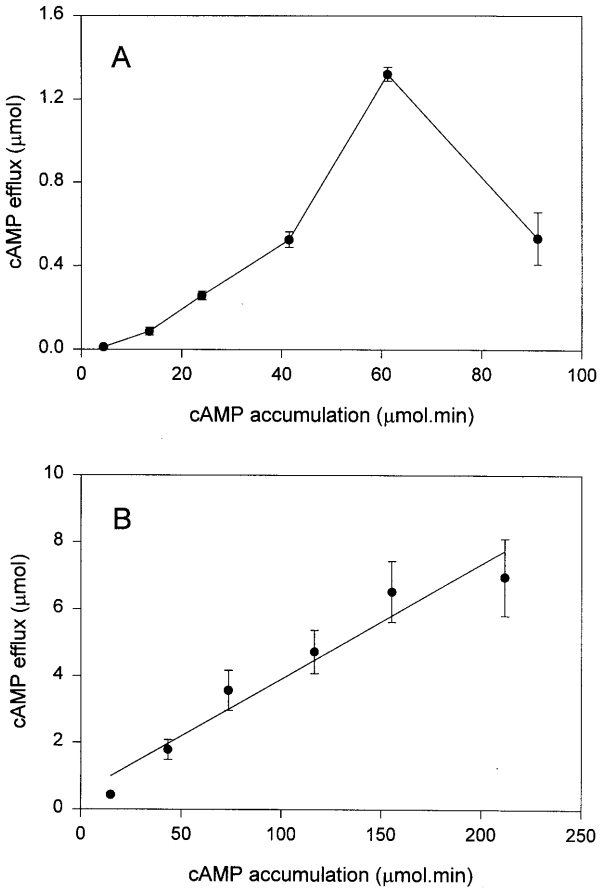

Figure 4. Relationship between cAMP efflux and cAMP accumulation in pinealocytes of 16-day-old (*A*) and 19-day-old (*B*) chick embryos. cAMP efflux is plotted against the total accumulated cAMP for the same duration of forskolin treatment as is shown below figure 2. Total accumulated cAMP was calculated by integrating the area under the curves on figure 3.

Discussion

These results support and extend the conclusions derived from our previous in vitro and in vivo studies [19, 20, 22]. The daily rhythm of melatonin synthesis was present at day 16 of embryonic development, although not all glands exhibited rhythmic melatonin production. However, all pineal glands isolated from 17-day-old or older chick embryos showed a distinct rhythm-melatonin production increased at night and decreased in the daytime [22]. Thus in the present study we investigated the role cAMP plays in control of melatonin production in both 16- and 19-day-old chick embryos. Our results demonstrate that the activity of pinealocytes rapidly increased between days 16 and 19. Melatonin levels were significantly higher in 19- as compared with 16-day-old embryos, confirming the results shown in our previous study [22].

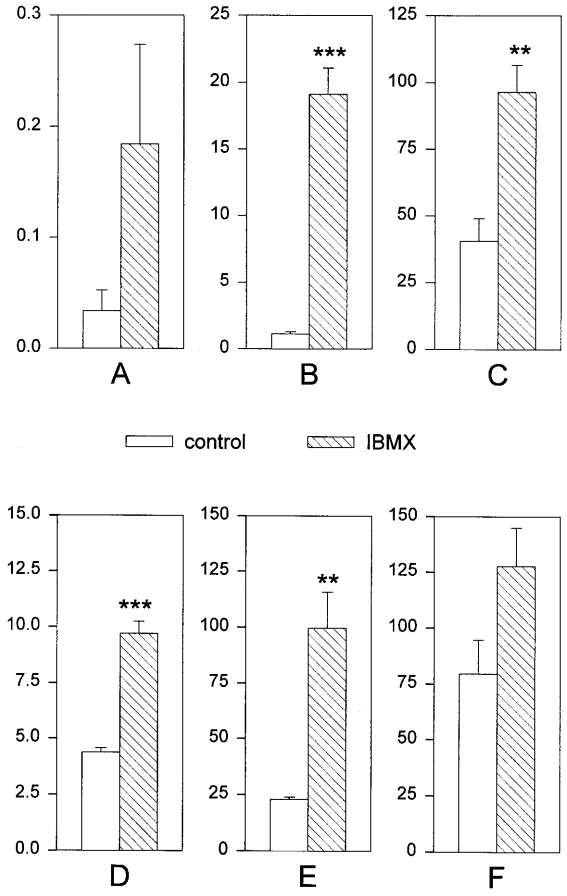

Figure 5. Effect of IBMX (5×10^{-4} mol l^{-1}) on melatonin output, cAMP efflux and accumulation in pineal cells isolated from 16-day-old (*A–C*) and 19-day-old (*D–F*) chick embryos in the light phase of LD cycle 12:12. The drug was applied for 6 h from onset of light to the middle of the light phase. Data are means ± SEM from five wells. (*A*, *D*) melatonin production (ng ml^{-1}); (*B*, *E*) cAMP efflux (nmol l^{-1}); (*C*, *F*) cAMP accumulation (nmol l^{-1}). $^{**}p < 0.01$. *** $p < 0.001$.

Melatonin synthesis in the mature chicken pineal gland is regulated by cAMP, and factors that increase cAMP concentrations also increase melatonin synthesis [2, 4, 7, 11, 24]. Our previous results [22] extended this conclusion to embryonic stages, showing that a soluble analogue of cAMP stimulates melatonin production in cultured embryonic pineal cells both during the light phase and the dark phase. The stimulatory effect of cAMP was higher during the daytime when basal melatonin levels were lower than during the nighttime when basal concentrations were higher.

In the present study we investigated whether changes in melatonin levels during the LD cycle are already clearly connected with cAMP during embryonic development. The increase in cAMP (efflux and accumulation) induced by forskolin was correlated with the increase in melatonin production in 16-day-old embryonic pineal cells, indicating that melatonin synthesis was dependent on stimulation of adenylate cyclase during embryonic development just as in the postnatal period [2]. However, we detected a different response between 16- and 19-day-old embryonic pinealocytes 90 min after forskolin treatment. In younger pinealocytes a significant decrease in cAMP efflux and accumulation was found, while 19-day-old cells reacted similarly to pinealocytes isolated from post-hatched chicks [11].

cAMP efflux is usually a first-order process related to intracellular cAMP accumulation. To determine the relationship between cAMP efflux and cAMP accumulation in embryonic chick pineal cells, we measured the time course of cAMP efflux and accumulation after stimulation of adenylate cyclase by forskolin. In 19-day-old embryonic pinealocytes the amount of efflux of cAMP was linearly related to the amount of accumulated intracellular cAMP. This finding was similar to results from an investigation of pineal cells of post-hatched chicks [11]. However, in 16-day-old pinealocytes cAMP efflux is not a linear reflection of intracellular levels of cAMP.

The inhibition of phosphodiesterase with IBMX increased cAMP efflux and melatonin production in pineal cells isolated from 16- and 19-day-old chick embryos, and this finding is in agreement with results obtained during the postnatal period [11, 12]. The elevated content of cAMP in pinealocytes isolated from both 16- and 19-day-old embryos after treatment with IBMX suggests a diminished degradation of cAMP to 5′-AMP. Results demonstrate that the effect of treatment with IBMX was more pronounced in younger pinealocytes.

Stimulation of melatonin production by forskolin and IBMX is dose-dependent in 1-day-old chicks [11]. Based on these results, we chose the stimulatory dose of forskolin and IBMX for our experiments. Similarly to post-hatched chicks [11], these doses stimulated mela-

tonin production in both 16- and 19-day-old embryonic pinealocytes. The increase in melatonin correlated with the increase in cAMP. In summary, our results suggest a functional biochemical pathway involving cAMP which controls melatonin production in the postnatal period. This biochemical pathway is already developed before hatching, and on embryonic day 19 it works in a way similar to that in post-hatched chicks. Differences in response to cAMP stimulation between 16- and 19-day-old pinealocytes seem to be mostly quantitative.

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