Retinal ganglion cells send visual and circadian information to the brain regarding the environmental light-dark cycles. We investigated the capability of retinal ganglion cells of synthesizing melatonin, a highly reliable circadian marker that regulates retinal physiology, as well as the capacity of these cells to function as autonomous circadian oscillators. Chick retinal ganglion cells presented higher levels of melatonin assessed by radioimmunoassay during both the subjective day in constant darkness and the light phase of a light-dark cycle. Similar changes were observed in mRNA levels and activity of arylalkylamine N-acetyltransferase, a key enzyme in melatonin biosynthesis, with the highest levels of both parameters during the subjective day. These daily variations were preceded by the elevation of cyclic-AMP content, the second messenger involved in the regulation of melatonin biosynthesis. Moreover, cultures of immunopurified retinal ganglion cells at embryonic day 8 synchronized by medium exchange synthesized a遗址3H遗址melatonin-like indole from遗址3H遗址tryptophan. This遗址3H遗址indole was rapidly released to the culture medium and exhibited a daily variation, with levels peaking 8 h after synchronization, which declined a few hours later. Cultures of embryonic retinal ganglion cells also showed self-sustained daily rhythms in arylalkylamine N-acetyltransferase mRNA expression during at least three cycles with a period near 24 h. These rhythms were also observed after the application of glutamate. The results demonstrate that chick retinal ganglion cells may function as autonomous circadian oscillators synthesizing a melatonin-like indole during the day.

In vertebrates, the circadian system that controls many physiological and behavioral rhythms includes the retina, which is responsible for photoreception and for synchronizing endogenous clocks to environmental lighting conditions (1–7). The retina is, itself, rhythmic, generating daily rhythms in melatonin production (8–10) and gene expression (7, 11–13). Melatonin is a key marker of the circadian system activity and may act, regulating retinal physiology (14–18).

The issue of which cell type(s) are responsible for melatonin synthesis within the retina is still open. Several approaches have been used to attempt to answer this question. For example, melatonin levels were assessed after the destruction of the inner retina of Xenopus (9) and chick (19). Since melatonin synthesis persisted after this treatment, it was suggested that it takes place in photoreceptors (PRCs). In addition, it has been shown that the specific activity of arylalkylamine N-acetyltransferase (AA-NAT) increases in retinal cell cultures by treatments that augment the proportion of PRCs (20). Therefore, available evidence supports the idea that circadian clocks located in PRCs drive retinal melatonin rhythms independently of the rest of the retina (9). However, the possibility that another retinal cell type(s) is also able to synthesize melatonin cannot be formally ruled out at present.

PRCs and oscillators located in the pineal gland of nonmammalian vertebrates are both photoreceptive and capable of producing melatonin during the night (9, 21–25). Retinal ganglion cells (RGCs) also express novel photopigments (26, 27) and display a daily rhythm in phospholipid biosynthesis (28). Excitatory amino acids such as glutamate have been shown to mediate the photic entrainment of circadian rhythms through the retinohypothalamic tract (29–32). Glutamate has also been shown to participate in several functions related to retinal physiology, light responsiveness, and survival and growth of RGCs (33, 34).

Here we investigated RGCs as autonomous circadian oscillators examining their capacity to synthesize methoxyindoles. These studies comprised in vivo and ex vivo experiments using RGCs isolated from other retinal cell types and from systemic regulators. In the present report, we demonstrate that 1) chick RGCs synthesize a melatonin-like methoxyindole in a rhythmic manner with levels peaking during the day, and 2) cultures of purified embryonic retinal ganglion cells (eRGCs) biosynthesize a遗址3H遗址melatonin-like methoxyindole from a radioactive precursor with a significant daily variation and show a circadian rhythm in the expression of AA-NAT, a key enzyme in the biosynthesis of this compound.
Melatonin levels and synthesis of a \[^{[H]}\text{melatonin}-\text{like indole in RGCs}\]

| Developmental age | Melatonin levels | Time of labeling | Cells | Medium |
|------------------|-----------------|-----------------|-------|--------|
| day | ng/mg of protein | h | ng/mg of protein |
| E8 | 0.61 ± 0.03 | 8 | 1.77 ± 0.01 |
| E15 | 2.28 ± 0.21 | 12 | 0.70 ± 0.08 |
| P10 | 0.47 ± 0.06 | 4 | 2.70 ± 0.32 |
| 0.03 | 0.47 | 8 | 4.90 ± 0.40 |
| 0.05 | 4.00 | 0.05 | 6.50 ± 0.70 |

**EXPERIMENTAL PROCEDURES**

**Animal Handling**—Cobb Hardig chicks were reared from hatching until day 7 on a 12:12 light-dark (LD) cycle, at ~25 °C with food and water ad libitum. Then a group of animals remained under the LD cycle while the other was released to constant darkness (DD) for 48 h, and on day 10, they were killed at different zeitgeber times (ZTs) that correspond to the phases of the previous LD cycle, at which lights were turned on and off, respectively. Both eyes were dissected out, and the eye cups were rinsed twice in 4 mL of cold 0.25 M sucrose, immediately frozen in liquid N\(_2\), and lyophilized. For animals maintained in DD or killed during the dark phase of the LD cycle, washing, the remaining bound RGCs were harvested in DMEM and B27-DMEM medium (76.4 μM of tryptophan) at time 0 and fed 25 μCi/ml l-\[^{[5]}\text{H}\text{tryptophan}\] (25 Ci/mmol, Amersham Biosciences) with a specific activity of 323 μCi/μmol for 8 h at different phases across a 24-h period. After the end of labeling, the culture medium was separated from the cells and transferred to glass tubes. Then cells were resuspended, and media were mixed with 0.1 M HCl and kept on ice until melatonin extraction was carried out as described under "Melatonin Assay." Before extraction, aliquots from the cells were collected for protein quantification (40). Blanks (medium without cells incubated with \[^{[5]}\text{H}\text{tryptophan}\] or zero time point of incubation) were included to discard the nonenzymatic production of melatonin or any eventual contamination during the incubation. In all cases, blanks were subtracted from the total radioactivity measured, and they represented less than 10–15% of the total labeling. Results of \[^{[H]}\text{melatonin}-\text{like indole}\] are presented as ng/mg protein (Fig. 7 and Table I). To calculate ng of methoxyindole produced, it was considered that 1 mmol of \[^{[5]}\text{H}\text{tryptophan}\] (specific activity 325 μCi/μmol) generates 1 mmol of \[^{[5]}\text{H}\text{melatonin}\] (e.g. 710 dpm/mg protein = 1 × 10\(^{-9}\) mmol = 0.232 ng).

**Melatonin Assay**—Retention preparations were homogenized in 0.1 M HCl and melatonin was extracted with 5 mL of dichloromethane and determined in each sample by radioimmunoassay (RIA) as previously described (41) or by two-dimensional TLC (42). The organic phase was washed twice with 2% NaHCO\(_3\) and distilled water. For the RIA, aliquots of the organic phase dried under vacuum were resuspended in 200 μL of buffer and mixed with a \[^{[H]}\text{melatonin}\] standard melatonin curve. The possible interference of serotonin in the melatonin assay was carried out as described below for in situ hybridization.

**Circadian Rhythms in Retinal Ganglion Cells**

The utilization of RIA for the assessment of melatonin levels in chicken retinas was validated in parallel studies in which serial dilutions of retinal extracts of samples from retinal layers yielded displacement curves that were parallel to standard melatonin curves. The possible interference of serotonin in the

**Experimental Procedures**

**Preparation of PRCs and RGCs**—Highly enriched preparations in PRCs and RGCs were obtained as previously described by Guido et al. (35). Briefly, after lyophilization, each retina was glued to adhesive tape by the RGC layer (white color), and this layer was separated from the rest of the retina by attaching another piece of tape to the pigment epithelium surface, pressing, and removing the second piece of adhesive tape with a cell layer attached. Each time this procedure is repeated, a different retinal layer is obtained attached to the tape. The pigment epithelium (dark gray color) was removed on the first piece of tape, whereas the PRC layer (orange color) was obtained attached to the second and third tapes, and cells from the inner nuclear layer (INL) were removed on the fifth and sixth. Remaining cells from the PRC layer and INL obtained on the fourth and seventh tapes, respectively, were discarded. This procedure was repeated eight times with each retina until a preparation of RGC was obtained on the basal tape.

**Purification and Culture of eRGCs from Chicken Embryos**—eRGCs were purified from embryonic day 8 neural retinas dissected in ice-cold Ca\(^{2+}\)-Mg\(^{2+}\)-free Tyrode’s buffer containing 25 mM glucose according to Brocco and Panzetta (36). Briefly, cells were trypsin-treated and rinsed with soybean trypsin inhibitor and Dulbecco’s modified Eagle’s medium (DMEM). After dissociation, the cell suspension from 30–60 retinas with soybean trypsin inhibitor and Dulbecco’s modified Eagle’s medium (from animal decapitation to freezing of the eye-cups in liquid handling was carried out in dim red light (~3 lux), and the whole procedure (from animal decapsulation to freezing of the eye-cups in liquid N\(_2\), and lyophilization) did not take longer than 1 min. Animal handling was performed according to Ref. 100.

**Synthesis of a \[^{[H]}\text{Melatonin}-\text{like indole in ERGC Cultures After Incubation with l-\[^{[5]}\text{H}\text{tryptophan}\]}**—Immunopurified eRGCs cultured as described above were synchronized by exchange with 2 ml of fresh B27-DMEM medium (76.4 μM of tryptophan) at time 0 and fed 25 μCi/ml l-\[^{[5]}\text{H}\text{tryptophan}\] (25 Ci/mmoll, Amersham Biosciences) with a specific activity of 323 μCi/μmol for 8 h at different phases across a 24-h period. After the end of labeling, the culture medium was separated from the cells and transferred to glass tubes. Then cells were resuspended, and media were mixed with 0.1 M HCl and kept on ice until melatonin extraction was carried out as described under "Melatonin Assay." Before extraction, aliquots from the cells were collected for protein quantification (40). Blanks (medium without cells incubated with \[^{[5]}\text{H}\text{tryptophan}\] or zero time point of incubation) were included to discard the nonenzymatic production of melatonin or any eventual contamination during the incubation. In all cases, blanks were subtracted from the total radioactivity measured, and they represented less than 10–15% of the total labeling. Results of \[^{[H]}\text{melatonin}-\text{like indole}\] are presented as ng/mg protein (Fig. 7 and Table I). To calculate ng of methoxyindole produced, it was considered that 1 mmol of \[^{[5]}\text{H}\text{tryptophan}\] (specific activity 325 μCi/μmol) generates 1 mmol of \[^{[5]}\text{H}\text{melatonin}\] (e.g. 710 dpm/mg protein = 1 × 10\(^{-9}\) mmol = 0.232 ng).

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**Circadian Rhythms in Retinal Ganglion Cells**

The utilization of RIA for the assessment of melatonin levels in chicken retinas was validated in parallel studies in which serial dilutions of retinal extracts of samples from retinal layers yielded displacement curves that were parallel to standard melatonin curves. The possible interference of serotonin in the
Circadian Rhythms in Retinal Ganglion Cells

RIA was ruled out, since this indoleamine was retained in the alkaline aqueous phase during extraction, and the cross-reaction of the melatonin antisem with serotonin was <0.4%. No significant interference was seen with 5-methoxytryptamine, 5-methoxytryptophol, N-acetyltryptamine, N-acetylserotonin, or 5-hydroxytryptophol after assessing three different concentrations (10, 100, and 500 nM) for each compound, but among them, only 5-methoxytryptophol competed with the binding of [3H]melatonin to the antisem at the highest concentration (500 nM) with a displacement lower than 15%. In addition, N-acetylserotonin and N-acetyltryptamine did not significantly displace [3H]melatonin binding to the anti-melatonin antisem used, competing less than 2.5 and 0.2%, respectively. For the TLC, the organic phases containing the [3H]methoxyindoles from the labeling experiments of eRGC cultures were dried under vacuum and resuspended in 50 μl of chloroform/methanol (9:1). [3H]Melatonin and other methoxyindoles were separated by two-dimensional TLC on silica gel 60-precoated plates with fluorescent indicator UV254 (Macherey-Nagel, Germany) and developed in the first direction with a solvent system composed of chloroform/methanol/glacial acetic acid (90:10:1 by volume) and in the second direction with ethyl acetate (42). Standards of tryptophan, N-acetyltryptamine, N-acetylserotonin, methoxytryptophol, methoxyindole acetic, and melatonin were visualized by UV. The radioactivity in the areas corresponding to melatonin and tryptophan was assayed by scraping and counting in a liquid scintillation counter.

**cAMP Level Assessment**—Each retinal cell preparation was homogenized in 1 ml of 0.5 mM 3-isobutyl-1-methylxantine and boiled for 2 min. Homogenates were centrifuged at 5000 × g for 5 min at 4 °C. Cyclic AMP content was measured in the supernatants by RIA after acetylation with acetic anhydride/triethylamine as previously described (41). Briefly, the acetylated products were incubated overnight with 110 Ci/g cyclic AMP (15,000–20,000 dpm, specific activity 140 μCi/mmol) and a rabbit antisem against cyclic AMP (Amersham, Buckinghamshire, England) at 4 °C. After adding 2 ml of ethanol with 2% bovine serum albumin, the antigen-antibody complexes were precipitated by centrifugation at 2000 × g for 30 min, supernatants were separated by aspiration, and radioactivity was determined. Intra- and interassay coefficients of variations for the RIA were <5% and <10%, respectively. The limit of detection of cAMP by RIA was 20 fmol.

**In Situ Hybridization and Northern Hybridization**—Total RNA from the PRC and RGC preparations was extracted following the method of Chomczynski and Sacchi (43) using the TRIzol™ kit for RNA isolation (Invitrogen). Finally, the RNA was resuspended in diethyl pyrocarbonate-treated water and kept frozen at −70 °C. 25 μg of total RNA from each sample was separated by electrophoresis in formaldehyde-agarose gels and then blotted onto charged nylon membranes (Amersham Biosciences). The RNA was fixed onto the membrane by UV irradiation for 2 min. Membranes were prehybridized at 42 °C for 2–4 h in a solution containing 5× SSC, 20 mM NaH2PO4, pH 7.2, 5× Denhardt’s solution, 7% SDS, and 250 μg/ml salmon sperm DNA. Hybridization was carried out at 42 °C for −24–36 h in the hybridization buffer (Sigma) containing 5 ml million cpm/ml of [3H]-radio-labeled AA-NAT antisem. After hybridization, the membranes were washed twice with 2× SSC and 1% SDS at 50 °C for 30 min and twice with 1× SSC and 1% SDS at 50 °C for 30 min. Finally, blots were exposed to the phosphor image or film or after exposure to the phosphor image screen were assessed by optical densitometry using Scion Image Software, and the signal generated was quantified using a standard internal international.

**Probes**—Synthetic oligonucleotides were labeled with [35S]dATP or [32P]dATP by the 3′ deoxyxycytidinyl transferase reaction for in situ or Northern blot hybridization, respectively. Prior to use, labeling probes were centrifuged through a Sephadex G-25 column (Amersham Biosciences). The cDNA oligonucleotides were as follows: AA-NAT 1, 5′-GAT CTC GAA CAC GCT GAC GTC ATC CTC GGT GAC ATT GA-3′; AA-NAT 2, 5′-TTG TCTCCA TCA AGA GAT CCC CTG GAG AGC CCG C-3′ (45); α-tubulin, 5′-GGG CCC GGT AGA TGG ACT ACA TCT GGT ACT TCT TCG C-3′ (45); Thy-1, 5′-GCT TTG GTG ATG CTT CTG GAG AAG AAC GAG ATG ACC A-3′ (45). The sense oligonucleotide for AA-NAT was 5′-TGA GAT CCC CTA CTT AAC GCT GTG CCG C-3′ (45).

**Assay of AA-NAT Activity**—PRC and RGC preparations were homogenized in cold 0.05 M phosphate buffer, pH 6.8. 30 μl of the homogenates were incubated for 20–30 min at 37 °C in the presence of 5 μl of 5.6 mm 5-hydroxytryptamine (Sigma), 10 μl of 25 μmol/liter acetyl [1-14C]-coenzyme A, and 10 μl of 0.05 M buffer phosphate, pH 6.8 (49). The reaction was stopped by the addition of 100 μl of 0.2 M borate buffer, pH 10, and 1 ml of chloroform, agitated for 3 min, and centrifuged. The organic phase was washed with 100 μl of 0.2 M borate buffer, pH 10, 0.5 ml of the organic phase was dried by evaporation, and radioactivity was determined. Controls including blanks and zero time point of incubation were included in all experiments, represented less than 12% of the total labeling, and were subtracted from the total radioactivity measured.

**Statistics**—Statistical analyses involved one- or two-way analysis of variance (ANOVA) with Newman-Keuls post hoc tests when appropriate.

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![Graph](image-url)  
**Fig. 1.** Daily variation in melatonin levels of PRCs (A) and RGCs (B) from chicks maintained in constant darkness for 48 h. Data are mean ± S.E. (n = 7–9 in each group) from three independent experiments. Melatonin levels showed significant diurnal variations in both retinal layers but with clearly different patterns. In PRCs, melatonin levels began to increase at midnight peaking at ZT 22, whereas in RGCs, they were higher during the subjective day, peaking at ZT 5 (p < 0.01 by ANOVA). *, p < 0.05; **, p < 0.01 (marked values were compared with basal levels). The hatched and solid bars above the graph denote when lights were on or off, respectively, in previous days.
RESULTS

Daily Rhythm of Melatonin Synthesis in RGCs in Constant Darkness—We assessed melatonin levels by RIA in purified retinal preparations of PRCs and RGCs from 10-day-old chicks kept for 48 h in DD after 7 days of entrainment to a 12:12-h LD cycle. In PRCs, the typical profile of retinal melatonin content, peaking during the subjective night, was observed (Fig. 1A) (9, 14, 21, 23, 51). As shown in Fig. 1B, RGCs also exhibited detectable levels of melatonin, although the maximum melatonin content in these cells after normalization to total protein content was found to be 10-fold lower than the peak level observed in PRCs. RGC melatonin also showed a strikingly different daily pattern, with peak levels observed during the subjective day, rather than the subjective night (Fig. 1B). In contrast to both PRCs and RGCs, only trace levels of melatonin were detected in cell preparations of the INL, with no significant changes across time (data not shown).

Daily Variation of AA-NAT mRNA and Enzyme Activity—The presence of AA-NAT was studied in RGCs and compared with that in PRCs. The expression of mRNA for AA-NAT and its enzymatic activity were assessed at different time points across a 24-h period in DD and after 48 h in this constant illumination condition. A 1.6-kb transcript corresponding to the mRNA for AA-NAT (45) was observed by Northern blot analysis in both PRCs and RGCs (Fig. 2A) but with clearly different temporal patterns of expression (Fig. 3, A and B). In PRCs, AA-NAT mRNA levels exhibited a significant increase around midnight, peaking at ZTs 19–20 (Fig. 3A), whereas in RGCs, levels of AA-NAT transcripts were elevated during the day over those observed at night (Fig. 3B). Our results show that there is a significant daily variation of AA-NAT expression in RGCs, with mRNA levels at ZT 3 significantly higher than those found at all other time points studied. In addition, Fig. 2B shows the cellular distribution of AA-NAT mRNA in the retina at ZT 3 during the subjective day and at ZT 22 during the subjective night from chickens maintained in DD. AA-NAT transcripts were mainly expressed in the RGC layer at ZT 3 with background levels also observed in the PRC layer at this phase. Furthermore, 75–80% of the cells in the RGC layer showed a positive hybridization signal for AA-NAT mRNA at ZT 3. The nocturnal expression of AA-NAT mRNA in the retina at ZT 22 was stronger and substantially restricted to the PRC layer (Fig. 2B, middle panel) as previously reported for different animal species (45, 52). In accordance with these findings, studies carried out by M. Iuvone, D. Klein and co-workers (45, 53) have previously reported a weak expression of transcripts for differ-
ent enzymes of melatonin synthesis in the chicken RGCs.

In order to establish the grade of purity of each retinal preparation and to discard contamination of RGCs with the PRC preparation and vice versa, the expression of specific markers for each cell population such as Thy-1 and rhodopsin (47, 48) were tested in blots containing total mRNAs from RGCs and PRCs. As shown in Fig. 2C, Thy-1 mRNA, an RGC marker whose protein product has been successfully used for cell immunopurification and culture (36–39, 54), was only detected in the RGC preparations. By contrast, the photopigment rhodopsin, well known to be located only in rod outer segments of PRCs (48, 55, 56), was selectively expressed in the PRC preparations. In addition, Fig. 2D shows the cellular distribution of Thy-1 and rhodopsin mRNAs in the retina according to their hybridization signals. Results further confirm the specificity of the markers used and denote the exclusive labeling of the RGC layer for Thy-1 and of the PRC layer for rhodopsin.

When AA-NAT activity in the PRC and the RGC preparations were compared, a differential pattern of the enzymatic activity was observed for both retinal cell layers (Fig. 3, C and D). In RGCs, the highest values of AA-NAT activity were observed during the early subjective day (Fig. 3D), whereas higher values of this activity during the late subjective night were found in PRCs (Fig. 3C).

Daily Variation of cAMP Levels in Retinal Cells—cAMP has been proposed to serve as the main second messenger involved in the pathway of melatonin biosynthesis in the vertebrate retina (8, 20, 41, 57, 58) and/or in the regulation of the protosomal proteolysis of AA-NAT (59, 60). Fig. 4 shows that levels of cAMP oscillated in both PRCs and RGCs across a 24-h period in DD but, again, with a different pattern for each cell type. In RGCs, cAMP content was significantly elevated during the subjective day, whereas in PRCs it was significantly higher at midnight. In each cell type, daily fluctuations in cAMP content peaked at phases that preceded the increase in the expression of AA-NAT mRNA, AA-NAT activity, and melatonin production.

Melatonin and AA-NAT Activity in RGCs during the LD Cycle—When melatonin levels and AA-NAT activity were measured in RGCs from animals maintained under an LD cycle since hatching, similar findings to those observed after 48 h in DD were found. As shown in Fig. 5, the highest levels of melatonin and AA-NAT activity were seen during the day, with levels decreasing significantly during the night.

Synthesis of a Melatonin-like Indole and AA-NAT mRNA Expression in Cultures of Embryonic RGCs—Since RGCs could be affected by other retinal cell types or by systemic influences to generate the rhythms observed, we examined the synthesis of [3H]melatonin-like indole and AA-NAT mRNA levels in cultures of immunopurified eRGCs over time. Cells were purified from retinas of chicken embryos at embryonic day 8 as described by Brocco and Panzetta (36), and, after being synchronized by changing the culture medium, they were maintained in culture for 24–72 h in constant darkness. Immunological characterization using specific markers (Thy-1, GAP-43, and 200-kDa neurofilament) and the expression of gangliosides of the gangliotetraoxyl series, which characterizes mature retinal neurons, indicated that 85–97% of the cells were mature RGCs (Fig. 6) (36, 37). RGC markers were also expressed in more than 90% of cultured cells after 4 days in vitro (36). This immunocharacterization was clearly comparable with that found at seeding time, pointing out that the cell composition in...
eRGC cultures remained unchanged over time, and they did not dedifferentiate (36). In addition, background hybridization signal levels for the mRNA of the PRC marker, rhodopsin, were seen in these cultures at embryonic day 8 (data not shown).

As shown in Table I, melatonin levels in cell culture were detected by both RIA and metabolic labeling after adding \[^{3}H\]tryptophan to the culture medium. Increasing levels of melatonin were observed in purified eRGCs as a function of the developing stage from embryonic day 8 to postnatal day 10 (Table I, left columns). Moreover, cultures of eRGCs biosynthesized a radioactive melatonin-like indole with the highest levels 8 h after the precursor administration, whereas increasing levels of this compound were released to the medium over time after its synthesis occurred (Table I, right columns). When we examined the biosynthesis of \[^{3}H\]methoxyindoles in eRGC cultures across a 24-h period after synchronization at time 0 by medium exchange, we found a significant daily variation in levels of \[^{3}H\]melatonin-like indole produced by the cells and released to the medium (Fig. 7). Values of labeling peaked 8 h after synchronization and declined by 10–14 h in the cells and by 12–16 h in the medium. The presence of hydroxyindole-O-methyltransferase, the last enzyme in the biosynthesis of melatonin, has not yet been determined in RGCs. It is unknown whether this or another O-methyltransferase could be involved in the conversion of N-acetylserotonin to melatonin; remarkably, the radiolabeled compound synthesized in eRGCs from \[^{3}H\]tryptophan comigrates with the standard of melatonin in the two-dimensional TLC assay.

To further investigate the autonomous capacity of eRGCs to generate self-sustained rhythms, we assessed the levels of AA-
Circadian Rhythms in Retinal Ganglion Cells

Experimental Procedures

At time 0 on day 1, the medium was replaced by fresh B27-DMEM (2 ml), and cells were fed \(^{1}H\)tryptophan (25 \(\mu\)Ci/ml; specific activity 323 \(\mu\)Ci/\(\mu\)mol) for 8 h at different phases across a 24-h period. For the labeling at time 6 h, \(^{1}H\)tryptophan was given 2 h before synchronization and replaced at time 0 when medium was changed. Radiolabeled melatonin from the cells and culture medium was isolated as described under “Experimental Procedures” and separated by a two-dimensional TLC. Data are mean ± S.E. (n = 4/group) from three independent experiments; 250–500 \(\mu\)g of protein were recovered from the cultures. A \(^{1}H\)melatonin-like indole was separated by the two-dimensional TLC that comigrated with the standard of melatonin. Levels of this labeled compound exhibited a significant daily variation (\(p < 0.0001\) for the cells and \(p < 0.0001\) for the culture medium by ANOVA), with levels peaking at 8 h after synchronization, rapidly declining in the cells, and persisting elevated in the culture medium for a few hours later. Post hoc comparisons revealed that levels of this \(^{1}H\)melatonin-like indole in cells at 8 h and in the medium at 6, 8, and 10 h were significantly greater than those at other times examined. Blanks (zero time point of incubation, time 0) were subtracted from the total radioactivity.

Fig. 7. Temporal regulation of the synthesis of a \(^{1}H\)melatonin-like indole in eRGC cultures from chick embryos at the embryonic day 8 (E8). RGCs from chick embryos were immunopurified and cultured in 2 ml of B27-DMEM as described under “Experimental Procedures.”

Effect of Glutamate on AA-NAT mRNA Expression in eRGC Cultures—In order to elucidate potential physiological synchronizing signals acting on the RGCs, cultures were incubated in the presence of glutamate (100 \(\mu\)M) or vehicle (B27-DMEM) without medium replacement and harvested at different hours after and up to a maximum of 60 h. Excitatory neurotransmission has been shown to enhance the survival and growth of developing RGCs (34). In our cultures, the application of glutamate at this concentration did not cause cell death for at least the first 3 days of exposure, during which our studies were carried out. As shown in Fig. 8B, the addition of glutamate to the eRGC cultures significantly promoted the expression of AA-NAT mRNA or synchronized the cell population with a significant daily variation as compared with controls treated with vehicle only and examined at the same time points. Expression of AA-NAT messenger exhibited a circadian fluctuation with the highest levels at \(~7, 32,\) and \(52\) h after glutamate application (Fig. 8B). On the contrary, controls that just received the vehicle without medium exchange did not present a significant major effect of time on AA-NAT mRNA expression across the 60-h range examined.

DISCUSSION

Our observations demonstrate that melatonin levels in RGCs were significantly higher during the subjective day or the light phase of the LD cycle. In addition, our results provide the first conclusive evidence demonstrating that RGCs are capable of synthesizing N-acetylserotonin. In this sense, we found detectable levels of melatonin by RIA in RGCs together with the presence of the key enzyme involved in the pathway of melatonin biosynthesis: AA-NAT. Although melatonin levels and AA-NAT mRNA and activity were substantially higher in the PRCs, as expected, these parameters in RGCs were consistently detectable and significantly different from the blanks. However, at this point, we can not ensure that the complete pathway of melatonin synthesis takes place in these cells; the presence of hydroxyindole-O-methyltransferase (50), the last enzyme in the biosynthesis of melatonin, has not been established yet in RGCs, and it is unknown whether this or another O-methyltransferase could be present in these cells and involved in the conversion of N-acetylserotonin to melatonin. Nevertheless, our findings reveal that the synthesis of a melatonin-like indole in RGCs shows a daily variation that peaks during the day, the opposite phase of peak melatonin production by the pineal gland and retinal photoreceptors. Consistent with these observations is the evidence that the enzyme AA-NAT also exhibits a circadian pattern of mRNA expression and activity in RGCs, with higher levels during the day as well as the content of cAMP.

A daily retinal rhythm may be controlled by a circadian oscillator, by direct responses to environmental lighting levels, or by a combination of these mechanisms. Since our results were observed in animals maintained in constant darkness as well as in those under a stable LD cycle, it seems likely that such daily variation reflects a truly circadian rhythm. It is also remarkable that in the population of RGCs, daytime lighting does not have the typical inhibitory effect described for melatonin synthesis and AA-NAT activity in other retinal cell types or tissues (9, 14, 17, 41, 45, 51, 52, 61). However, although melatonin levels and the amplitude of RGC melatonin rhythms assessed by RIA appeared comparable in both DD and LD, AA-NAT activity seemed to be more affected by the photic input with activity levels smaller in LD compared with DD, probably reflecting a possible differential effect of light on the enzyme.
activity. This unique response of melatonin production apparatus to the presence of light may reflect a distinct regulatory mechanism that has been found exclusively so far in RGCs and needs to be further investigated. This response may be related to the specialized electrical and cellular properties of this cell population. In PRC cultures, K⁺/H₁¹₀₁₀⁻-evoked depolarization stimulates cAMP accumulation and induces AA-NAT activity (62, 63). Moreover, in PRCs of rodents, a circadian clock gates melatonin synthesis by modulating, at least in part, the expression of the type 1 adenylyl cyclase and synthesis of cAMP (58).

On average, a considerable number of RGCs show depolarization in response to the photic input, whereas PRCs are depolarized in the dark. It is in this context that light could be the main signal acting on RGCs. However, we postulate that in the absence of light, a clock-controlled mechanism operates in these cells, which probably involves a cAMP signal that precedes the elevation of melatonin synthesis. Interestingly, levels of cAMP remained elevated in RGCs throughout the subjective day, whereas AA-NAT mRNA and activity peaked early in the day and decreased before dusk. These differences may reflect that AA-NAT can be regulated by other second messengers and/or regulatory transcription factors besides cAMP. The decline of AA-NAT protein could be due to an abrupt inhibition of AA-NAT synthesis or to accelerated enzyme degradation. In this sense, Iuvone et al. (64) have shown that chicken AA-NAT protein in the retina turns over rapidly, reflecting a balance between de novo synthesis and proteasomal proteolysis.

A remarkable feature of the isolated eRGCs is their capacity to synthesize a melatonin-like indole from a labeled tryptophan and to generate self-sustained oscillations when purified and maintained in culture for several days. This observation strongly supports the possibility that a circadian clock located in the RGC layer drives the rhythms described. The early developmental expression of circadian rhythms observed at embryonic day 8 highlights the status of RGCs as autonomous oscillators. At this stage, RGCs purified by immunopanning and after exhaustive washing are postmitotic and mature (see Ref. 56 for a review), whereas PRCs differentiate later and express opsin photopigments only after embryonic day 16 (56). Thus, it seems possible that early in development, RGCs are the primary retinal oscillators. eRGCs in culture were able to synthesize a radioactive melatonin-like indole from [³H]tryptophan that comigrated with the standard of melatonin after separation by a two-dimensional TLC. The newly synthesized labeled indole was rapidly released to the culture medium and exhibited a daily variation with levels peaking 8 h after synchronization, which declined a few hours later. This observation resembles the findings reported in vivo in the mammalian retina.
retina with higher levels of melatonin 8 h after the light was turned off (41). In addition, increasing amounts of a radiolabeled melatonin-like indole were released to the culture medium after its synthesis and underwent a daily fluctuation. Also, rising amounts of melatonin produced in the RGCs were observed from developmental ages (embryonic days 8–15) to postnatal day 10.

Cultures of purified embryonic RGCs exhibit sustained circadian oscillations in gene expression at least for 3 cycles of ~24 h each, that may be synchronized either by changing the culture medium or by the application of glutamate without medium replacement. Based on the former observations, it is noteworthy that a simple medium exchange is able to synchronize RGC cultures for several days in terms of gene expression. This type of synchronization may respond primarily to a mechanical/physical stimulus and probably not to a natural synchronizing signal. Because of this, glutamate was added to the cultures (without medium replacement), left there during the entire experiment, and compared with cultures treated with vehicle only with no medium exchange. Glutamatergic neurotransmission has been involved in a number of functions related both to the mechanisms of photic entrainment of the circadian system and to the retinal physiology (29–33, 65, 66).

Our findings in RGC cultures suggest that in the retina, glutamate may act as a physiological signal capable of synchronizing the cells to display rhythms in gene expression with a period close to 24 h.

Circadian rhythmicity in gene expression has been previously demonstrated in a variety of cell lines and peripheral tissues (67, 68). However, the results presented herein may be considered of special significance based on the identity and potential roles played by the retinal cells examined.

Light is the main synchronizer of the circadian system, and RGCs ultimately send information that adjusts the suprachiasmatic nucleus to environmental changes in illumination conditions. Remarkably, in mammals photic entrainment persists in the absence of photoreceptors, as occurs, for example, in the blind mole rat (69) or in animals with retinal degeneration (70–72). Although there are important species differences in the organization and function of the vertebrate circadian system, the role of the retina in the mechanisms of photic entrainment is very well documented and seems to be evolutionarily conserved (5, 7, 13, 73–75). Light selectively induces the expression of c-fos and other immediate early genes in RGCs of different vertebrate species (12, 76–79). In addition, cells of the inner retina, mainly RGCs, express the clock genes cryptochromes (cry) 1 and 2 (26, 80) and the putative photopigment melanopsin (27), which has been recently shown to contribute significantly to the magnitude of the light responses in circadian photentrainment in mammals (81, 82). In particular, the subset of RGCs that innervates the suprachiasmatic nucleus contains melanopsin (83–85) and is photosensitive (86). These observations strongly suggest that some RGCs may act as the primary photoreceptors for the circadian system. Melatonin has been shown to participate in the control of various aspects of retinal physiology (14–16, 18, 65, 87). Melatonin exerts its action through a family of G-protein-coupled receptors (reviewed in Ref. 88). These receptors have been shown to be present mostly in the inner nuclear layer, in the inner plexiform layer, and in the retinal pigment epithelium within the retina as well as in the optic chiasm, optic tract, retinohypothalamic tract, and other visually related structures (88, 89). Recently, three subtypes of melatonin receptors have been described to be expressed in the chicken retina with differential localization and rhythmicity (90). Melatonin may also alter sensitivity of the retina to photic input on a circadian basis, thereby regulating the information sent to the suprachiasmatic nucleus and other cerebral structures (91). Also, it should be considered that as the contribution of RGC melatonin to the total content of retinal melatonin seems to be small (less than 10%), this methoxyindole could be acting locally to regulate the physiology of the inner retina.

Strikingly, in contrast with the nocturnal appearance of melatonin in PRCs, melatonin peak and AA-NAT activity in the chicken RGCs is observed during the day. Previous reports in the whole chicken retina have shown higher levels of AA-NAT activity at night (14, 21–22), which may strongly reflect PRC melatonin production. It is noteworthy that the fish retina for some species also present higher levels of melatonin and AA-NAT activity during the day or late in the afternoon, displaying opposite rhythms to those observed in the pineal gland (92–97). A detailed review recently published by Falcon et al. (99) has considered the causes for such marked differences between the two organs in fish, which may involve genetic, temporal, and developmental controlled regulation of melatonin synthesis. In addition, AA-NAT mRNA has been shown to be expressed in different sets of retinal cell layers in rats (99). These studies taken together with our results strongly suggest the existence of differential and independent mechanisms for the generation of circadian signals in the vertebrate timing system involving melatonin that may control the physiology of the entire organism in relation to the ambient illumination conditions.

Retinal cells may need differential timing signals to set their own oscillatory mechanisms involved in the clock adjustment. These regulatory signals may include both photic input and melatonin production, which can converge at some time points to adjust the master circadian clock. The findings presented herein demonstrate that RGCs are autonomous circadian oscillators and as such are proposed to constitute a fundamental component of the vertebrate circadian timing system.

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