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Differential Regulation of Arylalkylamine *N*-Acetyltransferase Activity in Chicken Retinal Ganglion Cells by Light and Circadian Clock

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Retinal ganglion cells (RGCs) contain circadian clocks driving melatonin synthesis during the day, a subset of these cells acting as nonvisual photoreceptors sending photic information to the brain. In this work, the authors investigated the temporal and light regulation of arylalkylamine *N*-acetyltransferase (AA-NAT) activity, a key enzyme in melatonin synthesis. The authors first examined this activity in RGCs of wild-type chickens and compared it to that in photoreceptor cells (PRs) from animals maintained for 48 h in constant dark (DD), light (LL), or regular 12-h:12-h light-dark (LD) cycle. AA-NAT activity in RGCs displayed circadian rhythmicity, with highest levels during the subjective day in both DD and LL as well as in the light phase of the LD cycle. In contrast, AA-NAT activity in PRs exhibited the typical nocturnal peak in DD and LD, but no detectable oscillation was observed under LL, under which conditions the levels were basal at all times examined. A light pulse of 30–60 min significantly decreased AA-NAT activity in PRs during the subjective night, but had no effect on RGCs during the day or night. Intraocular injection of dopamine (50 nmol/eye) during the night to mimic the effect of light presented significant inhibition of AA-NAT activity in PRs compared to controls but had no effect on RGCs. The results clearly demonstrate that the regulation of the diurnal increase in AA-NAT activity in RGCs of chickens undergoes a different control mechanism from that observed in PRs, in which the endogenous clock, light, and dopamine exhibited differential effects. (Author correspondence: mguido@fcq.unc.edu.ar)

Keywords: Arylalkylamine *N*-acetyltransferase, Circadian rhythms, Dopamine, Light regulation, Melatonin, Photoreceptor cells, Retinal ganglion cells

INTRODUCTION

The circadian system that controls most physiological and behavioral rhythms in vertebrates includes the retina, which is responsible for photoreception and for synchronizing endogenous clocks to the changing ambient illumination conditions (Doyle & Menaker, 2007; Dunlap et al., 2004; reviewed in Guido et al., 2010). The retina is capable of generating a number of daily rhythms in gene expression and diverse biochemical activities, such as melatonin production and lipid biosynthesis (Bernard et al., 1999; Besharse & Iuvone, 1983; Cahill & Besharse, 1993; Garbarino-Pico et al., 2004a; Storch et al., 2007; Tosini & Menaker, 1996). Melatonin in the retina is an important neuromodulator, acting as a key marker of the circadian system that has been shown to be locally implicated in the temporal regulation of retinal physiology (Besharse & Dunis, 1983; Faillace et al., 1996; Hamm & Menaker, 1980; Tosini, 2000; Reiter et al., 2010). In chicken retina, there are different types of melatonin receptors located in the PRs and inner retina with differential rhythmicity in gene expression (Natesan & Cassone, 2002; Reppert et al., 1995). Moreover, three different melatonin receptor subtypes were shown to be expressed in retinal and extraretinal ocular tissues of the chick eye with diurnal rhythms of protein expression in the retina-retinal pigment epithelium (RPE)-choroid (Rada & Wiechmann, 2006).

Photoreceptor cells (PRs) in the retina and oscillators located in the pineal gland of nonmammalian vertebrates are both photoreceptive and capable of producing melatonin with a nocturnal peak (Bernard et al., 1999; Cassone, 1998). However, in the chick, retinal ganglions cells (RGCs) contain circadian oscillators and synthesize melatonin at higher levels during the day (Garbarino-Pico et al., 2004b). These cells also display a daily rhythm in phospholipid biosynthesis under constant

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illumination conditions (Garbarino-Pico et al., 2004a, 2005; Guido et al., 2001).

The regulation of melatonin synthesis and arylalkylamine *N*-acetyltransferase (AA-NAT) activity in PRs and pinealocytes has been extensively studied, and an acute photic suppression of melatonin synthesis in both PRs and pineal organ has been demonstrated (Bernard et al., 1999; Klein, 2007). In this context, the neurotransmitter dopamine synthesized in the inner retina preferentially by amacrine cells appears to mimic, at least partially, the effect of light on melatonin and AA-NAT activity in PRs of different vertebrates acting on D2,4 receptors (Cahill & Besharse, 1993; Iuvone et al., 2005, 2008; Zawilska & Nowak, 1994a, 1994b). However, it is still not known how light and/or dopamine regulates AA-NAT activity in RGCs. To investigate this we carried out studies in highly enriched preparations of RGCs and PRs from retinas of wild-type animals, in some experiments extending our investigation to an avian model of retinal degeneration, the GUCY1* chickens (Semple-Rowland et al., 1998). These chickens carry an autosomal recessive mutation in the photoreceptor-specific *guanylate cyclase 1* (GC1) gene that abolishes phototransduction and progressively affects the survival of cones and rods (Semple-Rowland et al., 1998). They, nevertheless, respond to light, which synchronizes their biological clock, and display sustained circadian rhythms in feeding behavior (Valdez et al., 2009), making them a very useful model for examining RGC activity in the absence of functional cone and rod PRs. We further investigated RGCs as distinct retinal circadian oscillators, examining first the circadian regulation of AA-NAT activity under different illumination conditions or under a pathological situation (GUCY1* chickens) in which there is lack of functional cones and rods. We then examined the effect of brief light pulses or dopamine administration on regulation of AA-NAT activity in RGCs and compared it with responses observed in PRs.

MATERIALS AND METHODS

Animal Handling and Eye Dissection

Cobb Hardig (wild-type) and blind (GUCY1*) chicks ranging from 10 to 15 d of age were reared from hatching until d 7 in a light-dark cycle (LD; 600 lux, cool white fluorescent light) of 12 h each, with food and water ad libitum and room temperature of 25°C as indicated for each assessment. Lights used were standard Sylvania fluorescent lights, F 18 Watts/154-T8, daylight, recyclable, made in Germany with the emission spectrum ranging from 390 to 627 nm.

One group of animals remained under the LD cycle, whereas the other groups were placed in constant darkness (DD) or light (LL; 600 lux, cool white fluorescent light) for 48 h. On d 10, the animals were killed at different zeitgeber times (ZTs) during the subjective day and night under the same illumination conditions in which they had been kept for 48 h. Since chickens have free-running periods very close to 24 h that would not have shifted significantly after 48 h of DD, times of treatments are designated with respect to the previous entraining LD cycle (or zeitgeber). Thus, ZT 0 corresponds to the phase of the previous dark-light transition (subjective dawn), whereas ZT 12 corresponds to the time of the light-dark transition (subjective dusk) when lights are turned off. To distinguish the time points between the LD and the constant illumination conditions tested (DD and LL), we have used circadian time (CT) for DD and LL experiments. At the end of the stated phases, both eyes were dissected out and the eye-cups rinsed twice in 4 mL of cold .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, handling was carried out in dim red light (<3 lux). The entire procedure from animal decapitation to freezing of the eye-cups in liquid N_2 took no longer than 1 min.

Animal handling was performed according to the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care and approved by the local animal care committee (School of Chemistry, National University of Cordoba, Exp. 15-99-39796). All ocular procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the experimental protocol conformed to international ethical standards (Portaluppi et al., 2010).

Light Stimulation

On d 10, after 48 h in DD, a group of animals (n = 10/time point) was exposed to brief light pulses (600 lux, cool white fluorescent light) at circadian time (CT) 3 and CT 17.5 for different periods of stimulation (0, 30, and 60 min). The animals were sacrificed by decapitation and the controls killed in DD. The eyes were subsequently dissected and after elimination of the frontal part, rinsed twice in cold .25 M sucrose. The eye-cups were frozen in liquid N_2 and lyophilized overnight.

Intravitreal Administration of Dopamine (DA)

On d 10, after 48 h in DD, a group of chicks (n = 7/time point) were injected intravitreally with 2.5 mM dopamine (Sigma, St Louis, MO, USA) resuspended in distilled water (50 nmol in 20 μL/eye) at CT 17.5 of their subjective night and sacrificed by decapitation at different times (CTs 17.5, 18, 18.5). In these experiments, control eyes were from animals injected with vehicle. The eyes were dissected and after elimination of the frontal part, rinsed in .25 M cold sucrose. The eye-cups were frozen in liquid N_2 and lyophilized overnight.

Preparation of PRs and RGCs

Highly enriched preparations in PRs and RGCs were obtained as previously described (Garbarino-Pico et al., 2004b; Guido et al., 1999). Briefly, each lyophilized retina was sandwiched between adhesive tape attached to the RGC and retinal pigment epithelium (RPE)

surfaces. After pressing and removing the upper piece of adhesive tape with RPE attached, another piece of tape was applied to the remaining retina and, again, the layer bound to the uppermost tape was removed. This process was repeated successively, such that the RPE (dark gray color) was attached to the first piece of tape and the photoreceptor cell (PR) layer (orange color) to the second and third tapes. Remaining cells from the PR layer and inner nuclear layer (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. The various cell preparations obtained were homogenized in .05 M phosphate buffer, pH 6.8, by sonication at 30 W for 20 s on ice. To establish the degree of purity of each retinal preparation, and to discard contamination of RGCs with the PR preparation and/or other retinal layer cells, such as inner nuclear layer (INL) cells and vice versa, the expression of specific markers for each cell population were tested by Northern or Western blots containing total mRNAs or proteins from RGCs, PRs, or the INL. As previously shown, Thy-1 mRNA and protein, a RGC marker, was only detected in the RGC preparations, whereas the photopigment rhodopsin and its kinase, markers for rod outer segments of PRs, were selectively expressed in the PR preparations (Garbarino-Pico et al., 2004b, 2005). When parvalbumin was assessed as a marker of amacrine cells in the INL, the INL preparations exhibited high levels of this marker, whereas almost no detectable signals of rhodopsin kinase or Thy-1 were observed (Garbarino-Pico et al., 2005). When the immunostaining of each marker was normalized by the signal generated in the blots by α-tubulin, levels of RGC contamination with PRs or INL or vice versa were less than 5–10% (data not shown).

Determination of Total Soluble Proteins

Quantification of total soluble proteins was performed by the Bradford method (Bradford, 1976) using comassie brillant blue as colorant (Bio-Rad, Hercules, CA, USA), and optic density was measured at 595 nm.

Assay of AA-NAT Activity

PR and RGC preparations were homogenized in cold .05 M phosphate buffer, pH 6.8. 50 μg of protein (≈30 μL of the homogenates) were incubated for 30 min at 37°C in the presence of 5 μL of 5.6 mM 5-hydroxytryptamine or tryptamine (Sigma), 10 μL of 25 μmol/L of acetyl (1-^{14}C) coenzyme A (Champney et al., 1984). The reaction was stopped by the addition of 100 μL of .2 M borate buffer, pH 10, and 1 mL of chloroform, and the preparations were then agitated for 3 min and centrifuged. The organic phase was washed with 100 μL of .2 M borate buffer, pH 10; .7 ml of the organic phase was dried by evaporation, and radioactivity was determined in a liquid scintillation counter with the high-safety scintillation cocktail Optiphase "Hisafe" 3 (PerkinElmer, Waltham, MA, USA).

Statistics

Statistical analyses involved one-, two, or three-way analysis of variance (ANOVA) with Newman-Keuls post hoc tests, when appropriate (significance at $p < .05$).

RESULTS

Daily Rhythm of AA-NAT Activity in RGCs Under Different Illumination Conditions

The enzyme activity of AA-NAT was studied in RGCs and compared with that in PRs from wild-type chickens subjected to different illumination conditions. The activity was assessed at different times along a 24-h period in animals that were synchronized for 7 d to a regular 12 L:12 D cycle and then maintained under the same LD cycling condition or released to DD or LL for 48 h up to the different times when the retinas were dissected.

Substantial differences were observed between the profiles of AA-NAT activity in PR and RGC preparations for both retinal cell layers examined (Figure 1). In RGCs, highest values of AA-NAT activity were observed during the early subjective day for all illumination conditions tested (LD, DD, LL), with levels decreasing significantly during the night (Figure 1, right panels). Statistical analysis of AA-NAT activity in RGCs by ANOVA with a time factor showed significant main effect of time in LD ($p < .0005$), DD ($p < .00001$), and LL ($p < .05$), whereas pairwise comparison revealed differences between activity levels during the subjective day (CTs 0–5) and subjective night (CTs 12–20). Remarkably, the enzyme activity rhythm in LD presented lower amplitude than under other illuminations conditions, whereas the rhythm observed in LL exhibited a bimodal profile (see Table 1 to compare the rhythm amplitude among the different illumination conditions examined). AA-NAT activity in PRs on the other hand exhibited higher values during the mid- and late subjective night under LD ($p < .01$ by ANOVA) and DD ($p < .01$ by ANOVA) conditions, but no time effect under LL (N.S.) (Figure 1). Pairwise comparison showed that activity levels in PRs at ZTs/CTs 15–22 differ from those assessed during the subjective day of the LD cycle or DD. Activity levels under LL did not differ from one time to another and were significantly diminished with respect to LD and DD.

Effect of Light Pulses on AA-NAT Activity

In order to further assess the responsiveness to light of retinal oscillators located in RGCs and compare this with PR responses to the photic input, we measured AA-NAT activity in the two retinal populations from animals subjected to DD for 48 h and exposed to light pulses of 30 or 60 min during the subjective day (CT 3) or night (CT 17.5) compared with controls maintained in the dark. As shown in Figure 2, the known inhibitory effect of light on AA-NAT activity in PRs was observed when the stimulus was provided for 30 or 60 min during the subjective night, compared with controls kept in the dark.

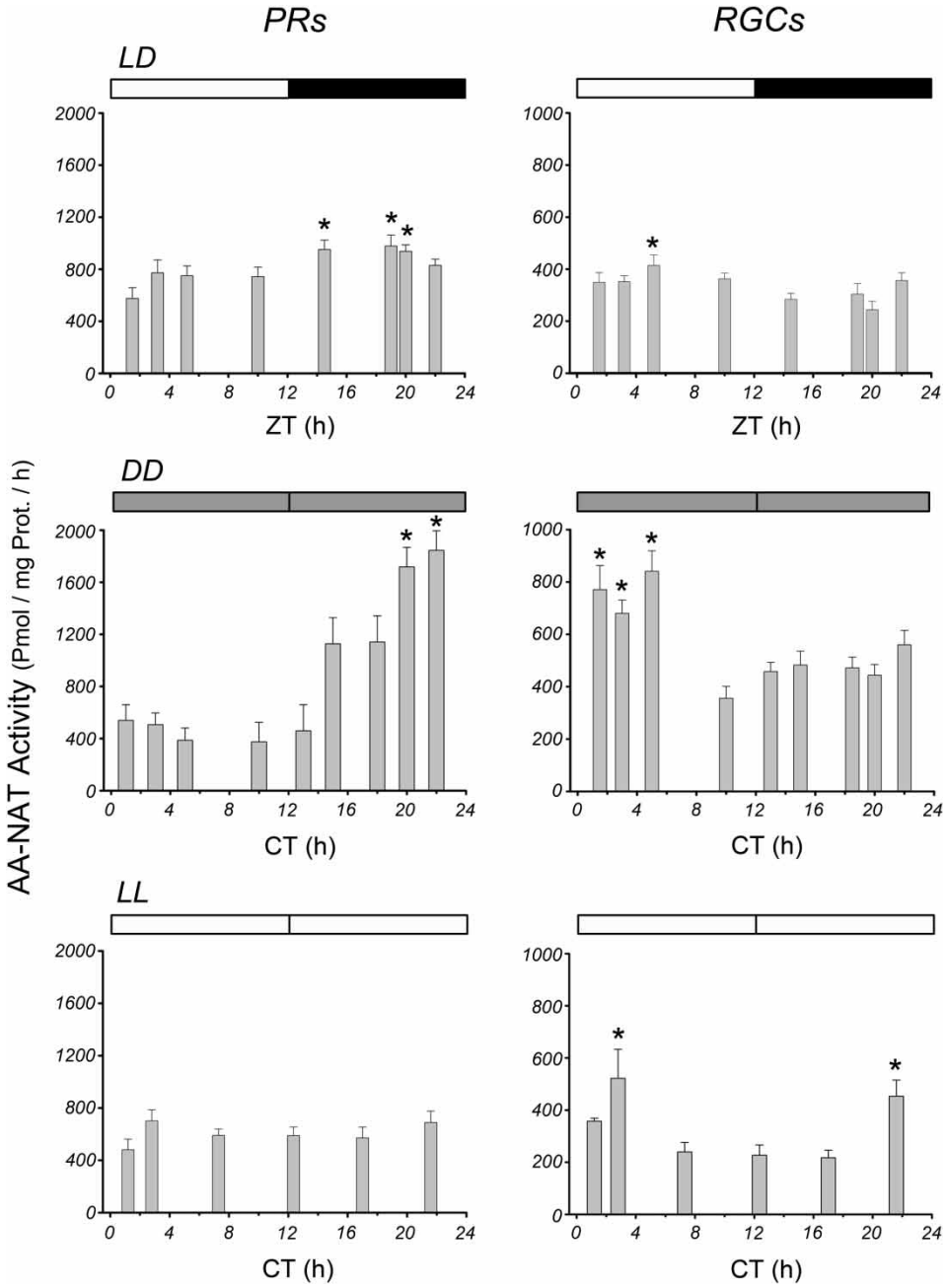

FIGURE 1. Daily variation of AA-NAT activity in PRs and RGCs of chicks maintained in LD, DD, or LL. In PRs, AA-NAT enzyme activity showed highest levels during the night, whereas in RGCs, levels peaked during the day. Significant effect of time was observed for AA-NAT activity in both cell layers in DD and LD, whereas in LL only RGCs showed significant daily rhythmicity. Solid bars above the graphs denote whether lights were on or off during previous days. Data are mean ± SEM (n = 10/group) from three independent experiments. *$p < .05$ as determined by post hoc comparisons; labeled values were compared with lowest levels at each illumination condition. See text for further details

ANOVA revealed significant effect of time ($F = 23.011$, $p \leq .0003$), light ($F = 72.029$, $p \leq .001$), stimulation duration ($F = 2.464$, $p \leq .022$), and interaction ($F = 4.257$, $p \leq .022$). In addition, light was also found to have some effect on levels of PR enzyme activity during the day, exhibiting a 33.5% decrease with respect to dark levels. Light pulses of different durations were observed to have no effect on AA-NAT activity in RGCs compared with dark controls during either the subjective day or night. ANOVA revealed no significant effects of time ($F = .057$, N.S.), light ($F = .755$, N.S.), stimulation duration ($F = 2.682$, N.S.), or interaction ($F = 1.186$, N.S.).

Differential Effect of Dopamine on AA-NAT Activity in RGCs and PRs

Dopamine (DA) is mainly produced by amacrine cells in the INL of the retina and regulates locally a number of functions related to retinal physiology (disc shedding, melatonin synthesis, etc.) (Besharse & Iuvone, 1992).

TABLE 1. Rhythm amplitude and analysis of variance (ANOVA) for the AA-NAT activity of photoreceptor cells (PRs) and retinal ganglion cells (RGCs) of chickens subjected to different illumination conditions (LD, DD, LL)

	Statistical significance	Amplitude (%)	Maximum value$_{(ZT/CT)}$ (pmol·mg prot^{-1}·h^{-1})	Minimum value$_{(ZT/CT)}$ (pmol·mg prot^{-1}·h^{-1})
Photoreceptor cells (n = 10–15/experiment)				
LD	$p < .00001$	16.5	$979_{(ZT\ 19)}$	$576_{(ZT\ 1.5)}$
DD	$p < .01$	51.2	$1845_{(CT\ 22)}$	$375_{(CT\ 10)}$
LL	N.S.	—	—	—
Retinal ganglion cells (n = 10–15/experiment)				
LD	$p < .0005$	19.3	$413_{(ZT\ 5)}$	$244_{(ZT\ 20)}$
DD	$p < .00001$	33.0	$841_{(CT\ 3)}$	$356_{(CT\ 10)}$
LL	$p < .05$	35.6	$701_{(CT\ 3)}$	$480_{(CT\ 1.5)}$

Factor of time (ZT/CT).
Amplitude was estimated as a ratio between the media of results at all ZTs/CTs for each experiment and the average value in the phase showing the highest levels of enzyme activity, which was considered 100%. Maximum and minimum values are also included for each illumination situation to denote the amplitude of each oscillation across a period of 24 h. Statistical analysis was performed by a one-way ANOVA with results from three independent experiments (n = 10–15/group).

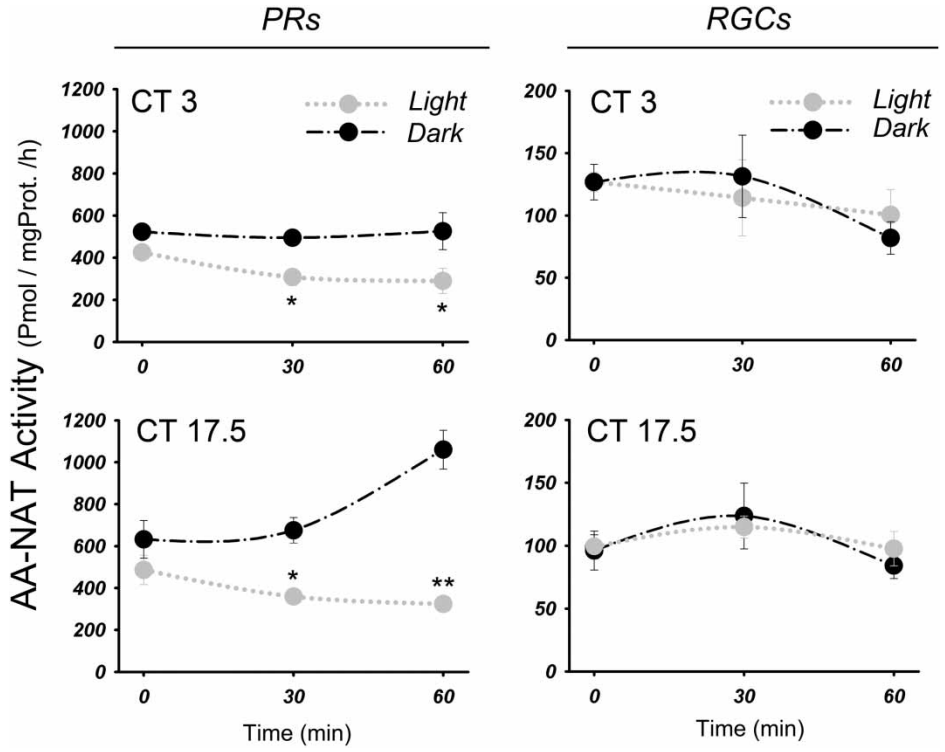

FIGURE 2. AA-NAT activity in PRs and RGCs of chickens exposed to light at different times during their subjective day or night (CT 3 and CT 17.5). Light applied at CT 3 (top left panel) had little effect on PR AA-NAT activity, whereas light applied at CT 17.5 exhibited the typical suppressive effect (lower left panel). In RGCs, light had no significant effect when applied at either CT 3 or 17.5 (right panels). Results are mean ± SEM (n = 10/group) from two independent experiments. *$p < .05$, **$p < .01$ as determined by post hoc comparisons; labeled values from the light condition (*, **) were compared with dark controls. See text for further details.

Since DA has been shown to mimic, at least in part, the effect of light on melatonin synthesis and AA-NAT activity in PRs, causing significant inhibition of enzyme activity and melatonin production (Zawilska, 1994; Zawilska et al., 2003), we tested the activity of AA-NAT during the subjective night in both retinal cell populations after DA administration (50 nmol/eye) (Figure 3). As expected, intraocular injection of DA caused significant decrease in AA-NAT activity of PRs in animals maintained in the dark after only 30 min of DA treatment as compared with vehicle-treated controls (factor of time: $F = .6068$, $p \leq .553$; treatment: $F = 64.3090$, $p \leq .0001$; and interaction: $F = 5.6881$, $p \leq .0095$) by ANOVA). On the contrary, intraocular injection of DA had no effect on AA-NAT in RGCs from animals maintained in the dark at any of the post-injection times tested (factor of time: $F = 1.988$, N.S.; treatment: $F = .078$, N.S., and interaction: $F = .282$, N.S., by ANOVA).

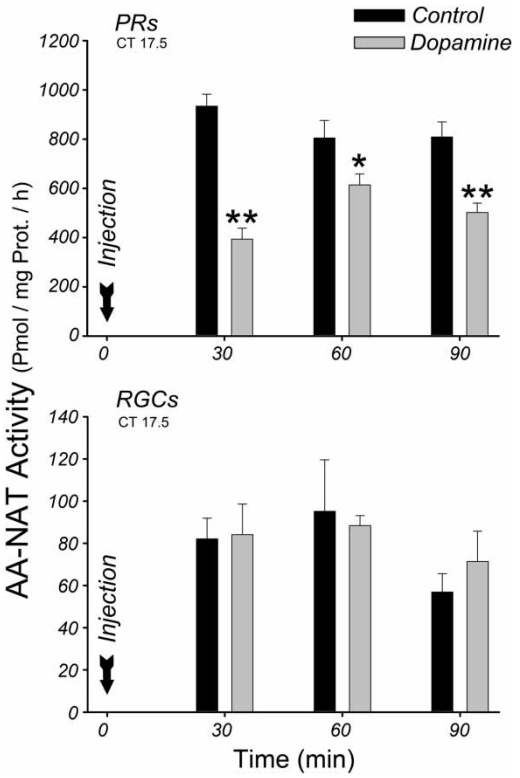

FIGURE 3. DA effect on AA-NAT activity in PRs and RGCs of chickens during the subjective night (CT 17.5). Dark-adapted animals were intraocularly injected or not with DA (50 nmol/eye) at CT 17.5. DA administration in the middle of the night had significant suppressive effect on AA-NAT activity in PRs (top panel), but no significant effect in RGCs (lower panel). Data are mean ± SEM (n = 7/group) from two independent experiments. *$p < .05$, **$p < .01$ as determined by post hoc comparisons; labeled values from the DA-treated samples (*, **) were compared with control samples from vehicle-treated animals. See text for further details.

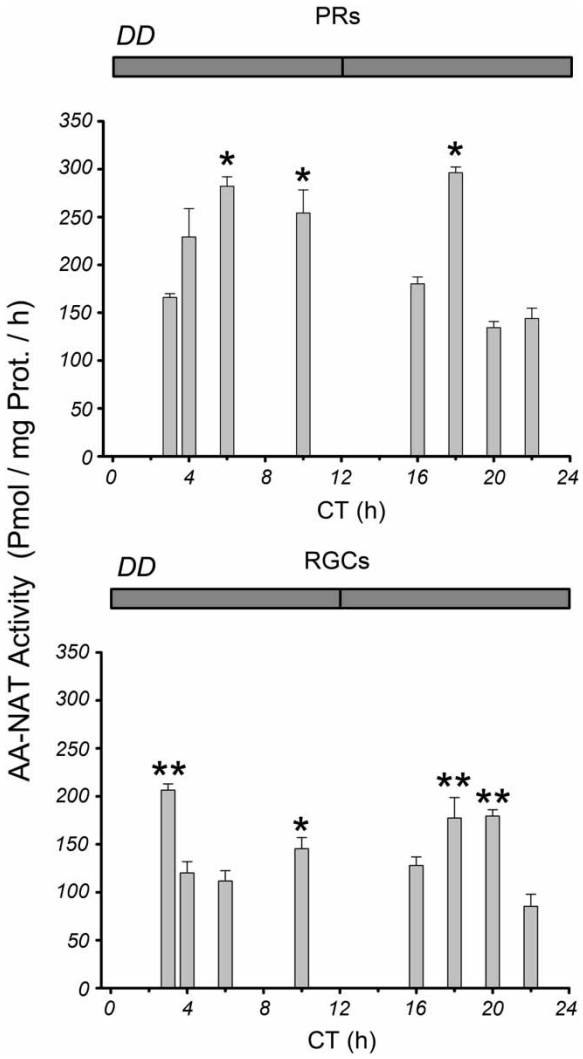

FIGURE 4. AA-NAT activity in PRs and RGCs of GUCY1*chicks maintained in DD. AA-NAT activity in both retinal cell populations was significantly affected as compared with normal controls in DD (Figure 1). PRs exhibited lower levels at all times studied and a diurnal peak around CT 6 during the subjective day in addition to the typical nocturnal elevation of activity at CT 18. In RGCs, AA-NAT activity also differed from that in controls kept in DD (see Figure 1). As in the case of PRs, AA-NAT in RGCs also exhibited a bimodal profile of activity with diurnal peak at CT 3. Levels at other times of the day remained diminished until the middle of the night, with levels rising again at CTs 18–20. Data are mean ± SEM (n = 10/group) from three independent experiments. *$p < .05$, **$p < .01$ as determined by post hoc comparisons; labeled values (*, **) were compared with lowest levels of each illumination condition. See text for further details.

AA-NAT Activity in GUCY1* Birds

In order to investigate retinal clocks in the avian model of blindness—GUCY1* chickens lacking functional PRs—we assessed AA-NAT activity in RGCs and PRs from 10- to 15-d-old blind chickens at different times during the subjective day and night in DD. It should be noted that although at this early age the PRs look morphologically normal, the chickens are in fact already blind, since the phototransduction cascade does not operate in rods and cones due to a specific GC1 mutation (Valdez et al., 2009). Under this pathological condition, AA-NAT activity in both retinal cell populations (Figure 4) was significantly affected as compared with normal controls in DD (Figure 1). As shown in Figure 4, the profile of enzyme activity in PRs exhibited lower levels at all times studied and a diurnal peak around CT 6 during the subjective day, in addition to the typically elevated nocturnal activity which was restricted at CT 18. The statistical analysis revealed significant effect of time ($F = 14.011$, $p \leq .0001$ by ANOVA). There was no difference between the levels assessed at CTs 4, 6, and 10 during the subjective day and at CT 18 during the night, but these levels did differ from the values at other times. In RGCs, the profile of AA-NAT activity across time also differed from that of normal controls kept in DD (see Figure 1). As with PRs, AA-NAT in RGCs exhibited a bimodal activity profile with a diurnal peak at CT 3, low levels of activity at other times of the day, and elevated levels again in the middle of the night (CTs 18–20). Statistical analysis revealed significant effect of time ($F = 19.0519$, $p \leq .0001$ by ANOVA). Levels at CT 3 during the day and at CTs 18–20 did not differ

from one another, but were higher than at the other times assessed.

DISCUSSION

Our results characterize for the first time the differential regulation of AA-NAT activity, the key enzyme in melatonin synthesis, in RGCs. In contrast to PRs and pinealocytes, in RGCs the activity peak occurs during the early day, and no major light or DA effects were observed. Here, we show that this activity is driven by the circadian clock, as observed in the two constant illumination conditions tested (DD and LL). Under all experimental situations examined, the daily rhythm of AA-NAT activity persisted in RGCs, with higher levels during the subjective day in both DD and LL and in the light phase of the LD cycle (Figure 1). Remarkably, in addition to AA-NAT activity, RGC preparations presented detectable levels of the hydroxyindole-*O*-methyltransferase (HIOMT), an enzyme that catalyzes the last step of melatonin biosynthesis, in samples collected at different times during the day or night with no significant variations across time (HIOMT activity [pmol/mg protein/h $\times 10^5$]: $Day_{(CT\ 6)} = 306.2 \pm 223.0$; $Night_{(CT18)} = 676.4 \pm 1004.5$; n = 3–6, N.S.). In this respect, HIOMT mRNA expression and immunoreactivity were previously reported to be found in PRs and cells of the inner retina (Guerlotte et al., 1996), as well as in immunopurified chicken embryonic RGCs (Contin et al., 2006).

Circadian Rhythmicity and Differential Light Effects

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 obtained with animals maintained in constant illumination (DD or LL) and those under a stable LD cycle, the daily variation of AA-NAT activity in RGCs reflects a truly circadian rhythm, generated endogenously. 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 (Bernard et al., 1999; Cahill & Besharse, 1993; Hamm & Menaker, 1980; Hamm et al., 1983). The enzyme activity profiles seen in RGCs differ significantly from the typical nocturnal peak in melatonin synthesis and AA-NAT expression and activity previously reported for PRs in DD by other groups and this laboratory (Bernard et al., 1999; Garbarino-Pico et al., 2004b; Hamm & Menaker, 1980). In addition to the antiphase occurrence of AA-NAT rhythms between RGCs and PRs, AA-NAT activity in PRs under LL was shown to be both reduced with respect to that in DD, and arrhythmic. This could be the result of masking effects of light on the oscillators located in PRs and/or simply the inhibitory effect of light on the enzyme activity, damping out the rhythm in the activity and the typical nocturnal peak (Bernard et al., 1997; Hamm et al., 1983). Although AA-NAT activity in RGCs is regulated by the retinal circadian clock, it appears to be less sensitive to the inhibitory masking effects of light, since the enzyme activity rhythm persisted even in LL. These opposite responses to the imposed ambient illumination conditions may also reflect differential mechanisms of regulation. In fact, we previously reported that melatonin, cyclic adenosine monophosphate (cAMP), and AA-NAT mRNAs also peak during the subjective day in RGCs (Garbarino-Pico et al., 2004b), and cAMP has been proposed to be the signal involved in the regulation of AA-NAT (Iuvone et al., 2005; Klein, 2007).

It is noteworthy that the retina of some fish species also presents higher levels of melatonin and AA-NAT activity during the day or late in the afternoon, displaying opposite rhythms to those observed in the PRs and pineal gland (Bayarri et al., 2003; Benyassi et al., 2000; Falcón & Collin, 1991). Moreover, in some fish species, two AA-NAT isoenzymes have been differentially described, the AA-NAT1 in the retina and the AA-NAT2 in the pineal gland, which may play important and distinct roles in the diurnal profile of melatonin production in these two tissues (Besseau et al., 2007; Coon et al., 1999; Vuilleumier et al., 2007). These species-related observations may implicate a differential genetic, temporal, and developmental controlled regulation of melatonin synthesis (reviewed in Falcon et al., 2003; Guido et al., 2010; Iuvone et al., 2005; Tosini et al., 2008). In this respect, AA-NAT mRNA has been shown to be expressed in different sets of retinal cell layers in the rat (Liu et al., 2004) and chicken (Bernard et al., 1997; Garbarino-Pico et al., 2004b) as well as in primary cultures of RGCs (Garbarino-Pico et al., 2004b) and dispersed retinal cells of chicken embryo (de Lima et al., 2011).

Light is the major synchronizer of the circadian system, and RGCs ultimately send photic information to the brain that entrains the suprachiasmatic nucleus (SCN) to environmental illumination conditions. Under the in vivo conditions tested, the photic input did not appear to have significant effect on AA-NAT activity in RGCs (Figure 2) as compared with the typical acute suppressive effect on melatonin and AA-NAT activity of PRs (and pineal gland). Light selectively induces expression of immediate early gene protein c-Fos in RGCs of different vertebrates (Caputto & Guido, 2000; Sagar & Sharp, 1990; Yoshida et al., 1993) as well as in RGC line RGC-5 cells (Nieto et al., 2011). Inner retinal cells, such as RGCs, express a number of clock genes (Ruan et al., 2006; reviewed in Guido et al., 2010) and the nonvisual photopigment melanopsin (Contin et al., 2006, 2010; Provencio et al., 2000; Valdez et al., 2009; Verra et al., 2011), which has been shown to confer intrinsic photosensitivity to immortalized cell lines (Qiu et al., 2005). This capacity appears early in development, even before classical photoreceptors become mature, as observed in both mammals and chickens (Contin et al., 2006, 2010; Hannibal & Fahrenkrug, 2004; Hao & Rivkees, 1999; Schmidt

et al., 2008; Sekaran et al., 2005; Tarttelin et al., 2003; Verra et al., 2011; and reviewed in Guido et al., 2010; Sernagor, 2005). These observations strongly suggest that some RGCs may act as the primary photoreceptors for the circadian system. In addition, the early developmental expression of circadian rhythms was observed at embryonic d 8 (E8) (Contin et al., 2006; Garbarino-Pico et al., 2004b), when RGCs are postmitotic and mostly mature (Mey & Thanos, 2000). Strikingly, when purified and maintained in culture for several days, isolated embryonic RGCs synchronized to different external cues (medium exchange, glutamate, or the LD cycle) were able to synthesize ^{3}H-melatonin and generate self-sustained oscillations in AA-NAT mRNA levels for at least 3 cycles of ~24 h each (Contin et al., 2006; Garbarino-Pico et al., 2004b). These observations strongly support the idea that an autonomous circadian clock located in the RGC layer drives the rhythms described and that circadian clocks and nonvisual photoreceptors may converge together in the same retinal cell population.

Melatonin and Dopamine in the Retina

Melatonin has been shown to participate in the control of various aspects of retinal physiology (Besharse & Dunis, 1983; Guido et al., 2010; Hamm & Menaker, 1980; Iuvone et al., 2005; Reiter et al., 2010; Tosini, 2000). Moreover, melatonin may also alter sensitivity of the retina to photic input on a circadian basis, thereby regulating the information sent to the SCN and other cerebral structures (Faillace et al., 1995). Also, as the contribution of RGC melatonin to total retinal melatonin content is small, it could be acting locally to regulate the physiology of the inner retinal circuitries during the first hours of light. In contrast to total retinal melatonin, the peak of DA synthesis and release occurs during the day in the retina from different vertebrate species (Besharse & Iuvone, 1992; Djamgoz & Wagner, 1992; Mangel, 2001; Nir et al., 2000). DA is synthesized in the inner retina, particularly in subsets of amacrine cells (Djamgoz & Wagner, 1992). Light positively modulates dopamine release and turnover as well as the activity of the key enzyme in dopamine synthesis, tyrosine hydroxylase (Besharse & Iuvone, 1992; Djamgoz & Wagner, 1992). In some species, daily variations observed in retinal dopamine content have been shown to be driven by a circadian oscillator (Jaliffa et al., 2000; McCormack & Burnside, 1993; Wirz-Justice et al., 1984). DA may mediate, at least in part, the mechanisms of light adaptation and participate in the regulation of a variety of functions related to retinal physiology; among these functions, DA has been involved in inhibition of melatonin synthesis (Iuvone et al., 2005; Jaliffa et al., 2000) through activation of D2,4 receptors, whereas DA acting on D1 receptors can alter AA-NAT activity in chicken pineal gland (Zawilska et al., 2004). Indeed, our results show that DA significantly inhibited AA-NAT activity in PRs (Figure 3); however, this compound was not observed to have any effect on enzyme activity in RGCs. These observations clearly demonstrate that the same neurotransmitter has totally different effects on AA-NAT activity in the retina, depending on the cell population and receptor implicated. Thus, we can speculate that D2,4 receptor activation mediates melatonin synthesis inhibition in PRs, whereas the activation of D1 receptors by the diurnal peak in DA content at the inner retina has no effect on AA-NAT activity in RGCs.

AA-NAT activity did not appear to be significantly affected by photic input in RGCs compared to PRs, likely reflecting differential effects of light and DA on the two retinal populations. This may also indicate the existence of a distinct regulatory mechanism, so far found exclusively in RGCs and which requires further investigation. Moreover, in the absence of light, a clock-driven mechanism operates in these cells, possibly involving a cAMP signal that precedes elevation of melatonin synthesis. In fact, levels of cAMP remained elevated in RGCs throughout the subjective day (Garbarino-Pico et al., 2004b). It is clear that cellular and retinal circuit integrities are required to visualize the rhythm in AA-NAT activity, since retinas of blind animals exhibited altered profiles of enzyme activity in both cell populations studied. Although at a systemic level the circadian timing system appears to be functioning correctly in these animals, since the food-intake rhythms were clearly observed to be synchronized by photic cues (Valdez et al., 2009), it is likely the retinal clock is not functioning adequately due to progressive PR degeneration.

Concluding Remarks

The two types of retinal cell populations examined (RGCs and PRs) may need differential timing signals to set their own oscillatory mechanisms for clock adjustment, either triggered by light and mediated, at least in part, by dopamine or through mechanisms less affected by photic input or continuous light exposure, or that are independent of exogenous DA. Such mechanisms also require the integrity of diverse retinal cell populations as well as of retinal circuits that are likely damaged in pathological conditions (GUCY1* chickens). Taken together, our results indicate that this differential regulation can substantially modulate the generation of circadian signals involving melatonin to regulate the physiology of the entire organism in association with ambient illumination conditions.

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