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Differential regulation of feeding rhythms through a multiple-photoreceptor system in an avian model of blindness

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ABSTRACT **All organisms have evolved photodetection systems to synchronize their physiology and behavior with the external light-dark (LD) cycles. In nonmammalian vertebrates, the retina, the pineal organ, and the deep brain can be photoreceptive. Inner retinal photoreceptors transmit photic information to the brain and regulate diverse nonvisual tasks. We previously reported that even after preventing extraretinal photoreception, blind GUCY1* chickens lacking functional visual photoreceptors could perceive light that modulates physiology and behavior. Here we investigated the contribution of different photoreceptive system components (retinal/pineal and deep brain photoreceptors) to the photic entrainment of feeding rhythms. Wild-type (WT) and GUCY1* birds with head occlusion to avoid extraocular light detection synchronized their feeding rhythms to a LD cycle with light >12 lux, whereas at lower intensities blind birds free-ran with a period of >24 h. When released to constant light, both WT and blind chickens became arrhythmic; however, after head occlusion, GUCY1* birds free-ran with a 24.5-h period. In enucleated birds, brain illumination synchronized feeding rhythms, but in pinealectomized birds only responses to high-intensity light (≥800 lux) were observed, revealing functional deep brain photoreceptors. In chickens, a multiple photoreceptive system, including retinal and extraretinal photoreceptors, differentially contributes to the synchronization of circadian feeding behavior.—Valdez, D. J., Nieto, P. S., Díaz, N. M., Garbarino-Pico, E., Guido, M. E. Differential regulation of feeding rhythms through a multiple-photoreceptor system in an avian model of blindness. *FASEB J.* 27, 000–000 (2013). www.fasebj.org**

Key Words: phototransduction · inner retina · pineal gland

Organisms are exposed to alternating cycles of day and night imposed by the Earth's rotation and have adapted to this light-dark (LD) environment by evolving a number of photodetection systems capable of regulating diverse non-image-forming (NIF) functions [pupillary light responses (PLRs), entrainment of activity rhythms, suppression of pineal melatonin synthesis, seasonal physiology, and masking] characteristic of a more primitive form of vision (1–3). In nature, light is the strongest, but not the only, signal that synchronizes vertebrate physiology and behavior to environmental cycles.

Living beings have developed time-controlled mechanisms involving circadian clocks, which measure time and use this temporal information to regulate physiology and anticipate environmental cycles. The vertebrate circadian system comprises the retina, the pineal gland, the suprachiasmatic nucleus (SCN), and a number of peripheral oscillators distributed in different organs and tissues throughout the body (4). In birds, the pineal gland and retina are key players, but their relative importance varies among species (5, 6). Furthermore, both the retina and pineal gland are able to sense the environmental illumination changes that regulate particular NIF functions (4). From the retina, light information is transmitted to specific brain areas through projections to the SCN and other regions (7, 8) to modulate gene expression, physiology, and behavior. It has recently been shown that mammals and birds having retinal degeneration and lacking functional rod and cone photoreceptor cells (PRCs) still respond to light stimulation that regulates diverse NIF tasks through

Abbreviations: ANOVA, analysis of variance; DD, dark-dark (constant darkness); Enx, enucleation; GC1, guanylate cyclase; ipRGC, intrinsically photosensitive retinal ganglion cell; LD, light-dark; LCA, Leber's congenital amaurosis; LL, light-light (constant light); NIF, non-image-forming; PLR, pupillary light response; Pnx, pinealectomy; PR, photoreceptor; PRC, photoreceptor cell; RGC, retinal ganglion cell; SCN, suprachiasmatic nucleus; WT, wild-type

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noncone, nonrod retinal photoreceptors (PRs); moreover, in mammals, photic responses are lost after enucleation (Enx; refs. 9–12; reviewed in refs. 2, 13). Non-image light responses have been attributed mainly to the presence of a novel nonvisual photopigment, melanopsin (Opn4; 14–18), which was initially discovered in *Xenopus* (19) and later found in the brain, iris, and retinal cells of most vertebrates examined (15, 18–25). Opn4 has been identified as the photopigment conferring intrinsic photosensitivity to a subset of retinal ganglion cells (RGCs; refs. 26–29). In mammals, Opn4 is only expressed in a small subset of RGCs (18, 23–25) that project to the hypothalamic SCN, the intergeniculate leaflet, the pretectal region, and other areas (23) directly involved in NIF tasks (13, 30).

Birds and lower vertebrates possess extraretinal PRs located in the pineal organ and in the hypothalamus, deep in the brain, that operate together with the eyes to mediate light effects on physiology and behavior (13). Extraretinal PRs are responsible for regulating light responses associated with seasonal modulation of reproduction (31); in fact, the work of Menaker and colleagues (32–35) demonstrated the existence of functional extraretinal PRs involved in the photoperiodic control of gonadal growth in birds even after pineal occlusion or pinealectomy. Recent works have shown that deep brain PRs located in the hypothalamus may act through noncanonical photopigments such as Opn5, VA-opsin, and Opn4 as reported in chicken, quail, and turkey (13, 36–39). Encephalic PRs have been also implicated in photic entrainment of daily locomotor activity rhythms in blinded sparrows (32), and pineal PRs have been reported to be involved in the synchronization of circadian locomotor activity in blinded chickens exposed to high light intensities (40).

Very little is known about the individual contribution of each retinal and extraretinal PR on the photic entrainment of daily rhythms in wild-type (WT) and blind chickens and their interactions. To investigate these, we used an avian model of retinal degeneration, the GUCY1* chicken (41, 42) carrying an autosomal recessive mutation in the PR-specific guanylate cyclase 1 (GC1) gene (42, 43). A deletion in the homologous human gene causes Leber's congenital amaurosis (LCA) with a pathology similar to that observed in chickens (44, 45), in which this retinopathy causes blindness at hatching by affecting the classic phototransduction cascade, dramatically affecting cone and rod survival as the pathologic condition progresses (46, 47). However, we recently reported that the inner retina of these animals contains PRs that perceive light to regulate PLRs and the daily synchronization of feeding rhythms (12).

In the present work, we investigated the contribution and interactions of PRs in the multiple photoresponsive system comprising visual and nonvisual retinal, pineal, and deep brain components that may cooperate to entrain the circadian feeding rhythms in WT and GUCY1* chickens.

MATERIALS AND METHODS

Animal handling

Cobb Hardig (WT) and blind (GUCY1*) chicks were reared from hatching until d 10−15 in a 12:12-h LD cycle (600 lux, cool white fluorescent light) with food and water *ad libitum* and a room temperature of 25°C as indicated for each assessment.

Animal handling, Enx, and brain surgeries were performed in agreement with the standards stated in the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals and approved by the local animal care committee (School of Chemistry, National University of Córdoba, Córdoba, Argentina; Exp. 15–99-39796) and according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Feeding activity recording

At 15−20 d of age, WT and GUCY1* chicks were housed individually in cages equipped with feeders modified for continuous recording of feeding activity. Feeding activity data were collected in 20-min bins by computer with a data collector hardware and software package (M. Carbajal, Universidad Nacional de Córdoba, Córdoba, Argentina) and analyzed in double-plotted actograms with El Temps software (A. Nunez Noguera, Universitat Autonoma de Barcelona, Barcelona, Spain). The period under constant conditions was calculated with El Temps.

Occlusion of extraretinal PRs

For light occlusion of the pineal gland (photosensitive in birds) and other deep brain PRs, feathers from the head were removed, and the skull was completely covered with a black leather cap glued to the skin with surgical cyanoacrylate (12).

In a control experiment performed to assess light penetration into the brain, heads from normal or enucleated animals, with or without a black leather cap for head occlusion, were sectioned along the median sagittal or median transverse planes. The sectioned heads were placed on an opaque device with a small hole to fit only the head, adapted for preventing the passage of light and connected to the light detector of a light meter. The light source to provide cool white fluorescent light of different intensities was set to the top of the head, which was then illuminated with light of 250 or 1000 lux. When the light meter was covered with the black leather used for the head occlusion and exposed to light of different intensities, no signal was detected (not shown). Results shown in **Table 1** indicate that the light intensity reaching an enucleated animal through the eye orbits represents $<0.5\%$ of the external light used: ~0.5 and 2.4 lux were detected deep in the encephalon for 250 and 1000 lux of external illumination, respectively.

Pinealectomy (Pnx) surgery

Pnx surgery was performed as described previously(48). In brief, birds were anesthetized with 2.5 ml/kg of equithesin (426 mg chloral hydrate, 96 mg pentobarbital, 212 mg $MgSO_4$, 3.5 ml propyleneglycol, and 1 ml ethanol; final volume 10 ml) and immobilized to prevent movement during surgery. A single midsagittal incision was made in the scalp behind the comb, and the skull was exposed and dried. The pineal gland was exposed by craniotomy and removed ($n=4$).

TABLE 1. *Light penetration into the chicken brain*

	External illumination			
	250 lux		1000 lux	
Condition	Normal	Enucleated	Normal	Enucleated
Sagittal section	0.34 ± 0.05	0.46 ± 0.14	1.15 ± 0.19	2.4 ± 0.46
Transverse section	0.045 ± 0.015		0.07 ± 0.01	
Transverse section with leather cap	0.035 ± 0.005		0.065 ± 0.005	

Heads from normal or enucleated animals, with or without a black leather cap for head occlusion, were sectioned along the median sagittal or median transverse planes. The sectioned heads were placed on an opaque device with a small hole to fit only the head, adapted for preventing the passage of light and connected to the light detector of a light meter. The light source to provide cool white fluorescent light of different intensities was set to the top of the head, which was then illuminated with light of 250 or 1000 lux. Data are means ± SE from 1–3 animals assessed in duplicate. When the light meter was covered with the black leather used for the head occlusion and exposed to light of different intensities, no signal was detected at all (not shown). See text for further details.

The dura and the skullcap were replaced, the incision was closed, and the wound was treated with a topical antibiotic. All birds were allowed to recover for ≥1 wk before further studies. The effectiveness of the surgery was confirmed by visual inspection of all pineal glands removed and postmortem histological analysis of all brains.

Enx

For experiments involving Enx, 20-d-old WT and GUCY1* chickens were anesthetized with 2.5 ml/kg of equithesin, and eyes were surgically removed. Animals were then allowed to recover from the anesthesia, fed *ad libitum*, and synchronized to a 12:12-h LD cycle with white light of 600 lux for 10 d.

Experiment 1

To determine the detection threshold of the inner retina, the animals were subjected to LD cycles of decreasing intensity of cool white light. WT (n=4) and GUCY1* (n=4) chickens with occluded extraretinal PRs were synchronized during the first 10 d to LD cycles of 400 lux and subsequently subjected to 4 h of phase advances and delays accompanied by a reduction in light intensity (400 to 3 lux). The light intensity was determined using a datalogging light meter (Extech Instruments Corp., Nashua, NH, USA) with a range from 0 to 20,000 lux.

Experiment 2

To investigate the effect of different light conditions on the feeding behavior of sighted (n=4) and GUCY1* (n=6) chickens, the animals with or without occluded extraretinal PRs were synchronized to LD cool white light cycles of 600 lux during the first 8 d and then kept in continuous light (100 lux) for 8 d. The chickens were subsequently kept in LD cool blue fluorescent light cycles (~600 lux), and 4 h of phase delays and advances were applied. Blue light generated by a cool blue fluorescent light with an emission wavelength between 350 and 490 nm, peaking around 420 nm, was used for photic entrainment of daily rhythms.

Experiment 3

To assess the functioning of the endogenous clock and the interaction between retina and extraretinal PRs, WT and GUCY1* birds were synchronized to LD cool white light cycles (600 lux) for 7−10 d. A group of animals (WT, n=4; GUCY1*, n=4) was subsequently subjected to constant darkness [dark-dark (DD) cycle] for several days. Animal handling was performed under dim red light (<3 lux). Another group of chickens was kept in constant light [light-light (LL) cycle; 100 lux] for several days. Animal handling was performed at the same time of day.

Experiment 4

To determine the influence of extraretinal PRs (encephalic PRs) on circadian control of feeding rhythms, animals (WT, n=4; GUCY1*, n=8) were subjected to Enx and then kept in an LD white light cycle (600 lux) for 10 d with their heads exposed to achieve correct synchronization. At d 11, after having their pineal glands covered, the animals were subjected to 4 h of phase delays and advances with increasing light intensities (600 to 800 to 1000 lux).

Experiment 5

To further demonstrate the influence of extraretinal PRs (pineal gland and encephalic PRs) on circadian control of feeding rhythms, animals (WT, n=4) were subjected first to Pnx surgery as described above, allowed to recover for 1 wk, and subsequently kept in a 12-h LD cycle with white light (600 lux) for 16 d with their eyes exposed to achieve correct synchronization. After the photic synchronization was achieved, the Pnx animals were subjected to Enx and then kept in a LD cycle with white light (600 lux) for another 12 d. At d 28, animals were subjected to 4 h of phase advances and delays with increasing light intensities (800 to 1000 lux).

Statistics

Statistical analyses involved 1- or 2-way analysis of variance (ANOVA) with t tests or Newman-Keuls *post hoc* tests when appropriate (significance at P<0.05).

RESULTS

Although inner retinal PRs have the capacity to synchronize feeding rhythms under moderate illumina-

tion conditions, the sensitivity and efficiency of the system is enhanced with the participation of the cone and the rod.

To characterize the ability of inner retinal PRs to detect white light, we determined the threshold of intensity for the entrainment of daily food intake rhythms. For this, we recorded the activity of food intake in WT and blind birds with their heads occluded (to avoid extraretinal photoreception) and exposed to white LD cycles of different intensities ranging from 400 to 3 lux (cool white fluorescent light). As shown in **Fig. 1*A***, sighted animals efficiently synchronized their feeding rhythms up to 3 lux with virtually no transient cycles after the 4-h shift (advances or delays) imposed in the LD cycle. In contrast, blind birds synchronized their daily feeding rhythms only up to light stimuli of ~12 lux, but responses to decreasing light intensities showed marked transient states mainly for the 4-h advances of the new LD cycle imposed (Fig. 1*B*); this was clearly visualized in changes from 400 to 200 lux and from 100 to 50 lux. These responses were significantly slower than those in WT (Fig. 1*A*) or GUCY1* birds exposed to brighter light (600 lux) as reported in a previous study (12), in which the adaptation to the newly imposed light schedule was almost immediate. The transient states observed in blind chickens seem to reflect the time window that the circadian system requires for its complete entrainment to the newly imposed LD schedule and might indicate that cone/rod PRs are required for immediate behavioral responses to light (masking effect), at light intensities ≤600 lux. A further difference observed in GUCY1* birds at the lowest intensities tested (12 to 3 lux) was that in the transition from 12 to 6 lux, synchronization of birds subjected to 4-h delays was more efficient than when birds were exposed to a 4-h advance of the LD cycle. Moreover, at 3 lux, blind birds were unable to detect synchronizing changes in light intensity and displayed free-running behavior with a period longer than 24 h.

The period length was assessed for both WT and GUCY1* birds subjected to 4-h phase shifts under LD cycles of decreasing light intensities. Remarkably, at the different light intensities tested (from 3 to 400 lux), WT birds always exhibited a 1440-min period, perfectly matching the 12:12-h LD cycle imposed (Fig. 1*C*). In contrast, blind birds presented only a 24-h period in LD cycles up to 25 lux, with the period lengthening as the intensity decreased. ANOVA revealed a major effect of the light intensity ($P<0.00001$), of genotype (WT *vs.*

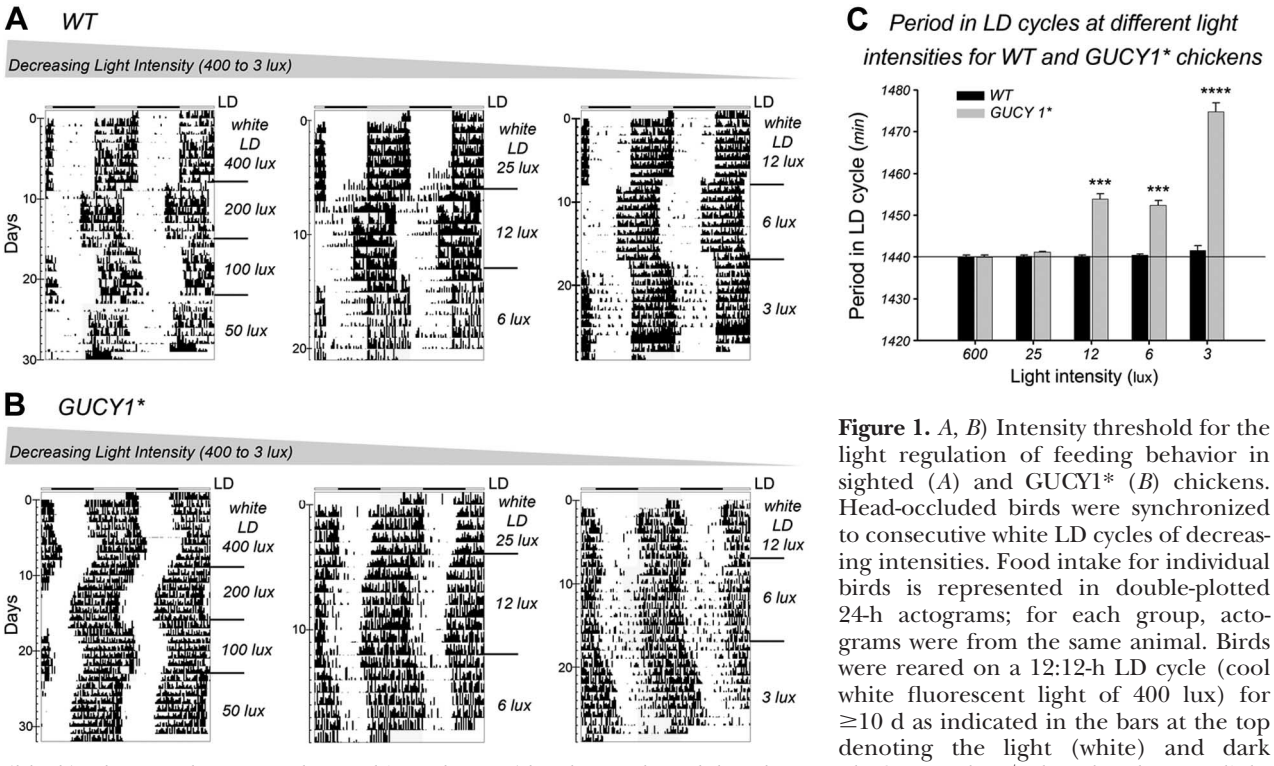


Figure 1. *A*, *B*) Intensity threshold for the light regulation of feeding behavior in sighted (*A*) and GUCY1* (*B*) chickens. Head-occluded birds were synchronized to consecutive white LD cycles of decreasing intensities. Food intake for individual birds is represented in double-plotted 24-h actograms; for each group, actograms were from the same animal. Birds were reared on a 12:12-h LD cycle (cool white fluorescent light of 400 lux) for ≥10 d as indicated in the bars at the top denoting the light (white) and dark (black) phases. They were then subjected to a 4-h advanced or delayed LD cycle for another 7 d each. The new light regimen is denoted by gray squares indicating the onset of each new 12:12-h LD cycle under decreasing light intensities from 400 to 3 lux. *C*) Histograms indicate the period length (min) assessed for both WT (black bars) and GUCY1* birds (gray bars) under regular LD cycles and subjected to 4-h phase shifts under LD cycles of decreasing light intensities. Statistical analysis by ANOVA revealed a major effect of light intensity ($F=118.41$, $P<0.00001$), of genotype (WT *vs.* GUCY1*, $F=396.83$, $P<0.000001$), and of the interaction between the 2 factors ($F=100.70$, $P<0.000001$). Post hoc comparisons showed that the period did not vary at the different intensities tested in WT animals. For GUCY1* birds, the period at 3-lux LD cycles differed from all other lighting conditions and genotypes ($P<0.00001$), whereas the periods at 6- and 12-lux LD cycles did not vary from one another but did differ from other illumination conditions assessed in GUCY1* and WT birds ($P<0.0001$). See text for further details. ***$P < 0.0001$, ****$P < 0.00001$.

GUCY1*, $P<0.000001$), and of interaction ($P<0.000001$). *Post hoc* comparisons showed that the period did not vary at the different intensities tested in WT animals. In the case of GUCY1* birds, however, the period at 3-lux LD cycles differed from that for all other lighting conditions and genotypes, whereas although the periods at 6- and 12-lux LD cycles did not differ from one another, they did differ from all other lighting conditions assessed in GUCY1* and WT birds (Fig. 1*C*). Results for GUCY1* chickens exposed to LD cycles with light from 600 to 25 lux did not differ from those for the WT controls. These observations clearly indicate that the intensity threshold for the photic synchronization of feeding rhythms in blind birds is 12 lux. The detection of light at very low intensities up to ≥3 lux in intact animals further indicates that cone and rod PRCs are responsible for the precise and fine entrainment to light of WT birds.

With the aim of evaluating the efficiency of the head occlusion performed to avoid extraretinal photoreception and to estimate light penetration into the deep brain, we measured the light passing through heads once they were dissected along the medial sagittal or medial transverse planes under the different conditions in which feeding behavioral rhythms were analyzed (Table 1; see details in Materials and Methods). Less than 0.15% of light was able to pass through the head dissected along the sagittal plane, and 0.02% was able to pass along the transverse plane at the different intensities tested. Light penetration was even lower with the leather cap but still sufficient to reach deep brain structures. Nevertheless, we can infer that when the intensity level of the external light is low (3−100 lux), presumably no detectable light would be able to reach the pineal gland or deep brain PRs.

To compare the transient periods observed after 4-h phase shifts under the same light intensity in WT and GUCY1* birds, a series of experiments was performed in LD cycles at 400 lux as shown in Supplemental Fig. S1. Under these illumination conditions, WT chickens immediately adjusted their feeding activity rhythms to either 4-h advances or delays, whereas GUCY1* birds did so for a 4-h delay in the new imposed LD cycle but took between 1 and 3 d to entrain to the 4-h advances of the new LD cycle. These observations further support the idea that different PRs regulate the circadian system in a different manner in response to phase-shift advances or delays. For the phase advances, cone and rod PRCs seem to be essential for immediate responses because GUCY1* birds exhibited 1- to 3-d transient periods; however, for delays, the responses are immediate in both genotypes, indicating that inner retinal PRs are sufficient to rapidly entrain the clock.

Photic modulation of feeding behavior is mediated by diverse retinal and extraretinal PRs

In a series of experiments illustrated in **Fig. 2**, WT (Fig. 2*A*, *B*) or GUCY1* chickens (Fig. 2*C*, *D*) with their heads exposed or totally occluded were entrained to diverse featured LD cycles (cool white or blue fluorescent light of 600 lux) or released to LL of a lower intensity (100 lux). All WT animals entrained to the new light regimens (4-h advances/delays), regardless of whether or not their heads were occluded (Fig. 2*A*, *B*). GUCY1* chickens with occluded heads also entrained to the new light regimen (Fig. 2*D*). However, the feeding behavior of WT animals became arrhythmic in LL for both conditions (exposed and occluded heads; Fig. 2*A*, *B*). GUCY1* birds with occluded heads, on the other hand, efficiently entrained to the 600-lux LD cycle but free-ran and did not become arrhythmic under LL. It is worth noting that, unlike under the other conditions examined (Fig. 2*A*, *C*), under the blue LD cycle both genotypes with occluded heads (Fig. 2*B*, *D*) showed sporadic activity during the dark phase. The

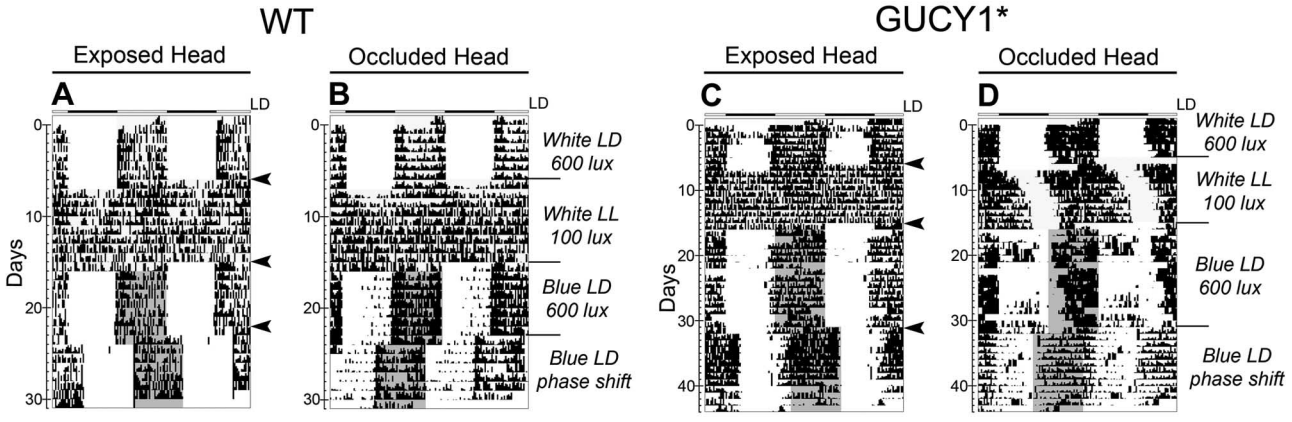


Figure 2. Effect of different light conditions on the feeding behavior of sighted (WT; *A*, *B*) and GUCY1* chickens (*C*, *D*) after occlusion of extraretinal PRs. Double-plotted actograms of feeding activity for WT and GUCY1*chickens with (*B*, *D*) or without (*A*, *C*) head occlusion and exposed to different light conditions (white and blue LD cycles and LL). Birds were synchronized to a 12:12-h LD cycle (light: cool white fluorescent light of 600 lux) for 8 d, as indicated in the bars at the top denoting the light (white) and dark (black) phases. They were then released to LL (cool white fluorescent light of 100 lux) for ~8 d for free running and finally exposed to 12:12-h LD cycles with blue light (cool fluorescent light of ~600 lux) with 4-h delay (*A*, *C*) and 4-h advance (*B*, *D*) as indicated in the right panel. See text for further details.

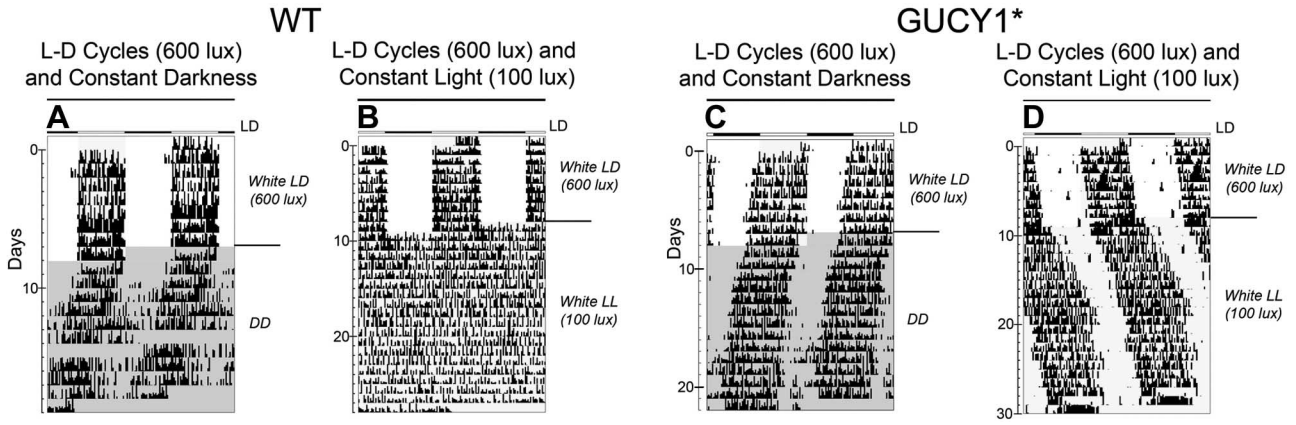


Figure 3. Daily rhythms of feeding in sighted (WT) and GUCY1* chickens under constant illumination conditions (DD and LL) and extraretinal occlusion. *A, C*) Head-occluded WT (*A*) and GUCY1* (*C*) birds were synchronized to a 12:12-h LD cycle (light: cool white fluorescent light of 600 lux) for 7−10 d, as indicated in the bars at the top denoting the light (white) and dark (black) phases, after which a group of birds was released to DD during ~10−12 d for free running. *B, D*) Another group of WT (*B*) and GUCY1* (*D*) head-occluded chickens was released to LL (cool white fluorescent light of 100 lux) during ~20 d for free running.

actograms shown in Fig. 2 suggest that nonclassic PRs located in the inner retina are sufficient to drive the photic regulation of the daily feeding rhythms under different LD cycles; however, other PRs may also be involved under particular illumination conditions. The results clearly show that classic (rods and cones) and extraretinal PRs, presumably pineal PRs, are involved in the loss of rhythmic behavior in LL.

To further investigate the behavioral responses to LL, we assessed the food intake rhythms of WT and GUCY1* birds with their heads occluded for longer exposure times to 100 lux in LL (cool white fluorescent light) or maintained in DD for the same number of days. **Figure 3** confirms the LL observations shown in Fig. 2*B, D*: WT birds with their heads occluded became arrhythmic, whereas GUCY1* chickens free-ran with an endogenous period longer than 24 h (24.50±0.07; Fig. 3*B, D*). In contrast, in DD both WT and GUCY1* birds free-ran with a period of 22.94 ± 0.11 and 23.48 ± 0.10 h, respectively (Fig. 3*A, C*). The statistical analysis revealed a significant genotype effect in DD (WT DD *vs.* GUCY1* DD: $P \leq 0.016$) and a light effect in both groups (WT DD *vs.* GUCY1* LL, head occluded: $P \leq 0.0001$) and (GUCY1* DD *vs.* GUCY1* LL, head occluded: $P \leq 0.0001$) for a group of 4 animals in each condition.

To determine the ability of inner retinal PRs to entrain the circadian system to LD cycles, we analyzed all records for the GUCY1* chicks tested with their heads occluded. In most cases (n=37 of 45, 82% of total) GUCY1* chickens entrain very efficiently to the regular 12:12-h LD cycle of 600 lux with an exact period (τ) of 1440 min and only 8 animals (18% of total) showed poor entrainment to a regular LD cycle, as shown in the actograms of Fig. 3. At this point, we cannot completely rule out the possibility that the pineal and deep brain PRs also contribute to this response; however, as shown in Table 1, light penetration into the brain in chickens with their heads occluded was significantly reduced, and the leather cap proved to be efficient in the experiments under LL conditions (Fig. 3).

Photic regulation of food intake activity

Effect of Enx

To investigate the putative participation of encephalic PRs in the nonvisual photoperception of GUCY1* chicks, we carried out experiments on feeding activity in enucleated animals, both in sighted controls and blind chicks. As shown in **Fig. 4**, enucleated animals with their extraocular PRs exposed to a LD cycle with white light of 600 lux were able to entrain their activity

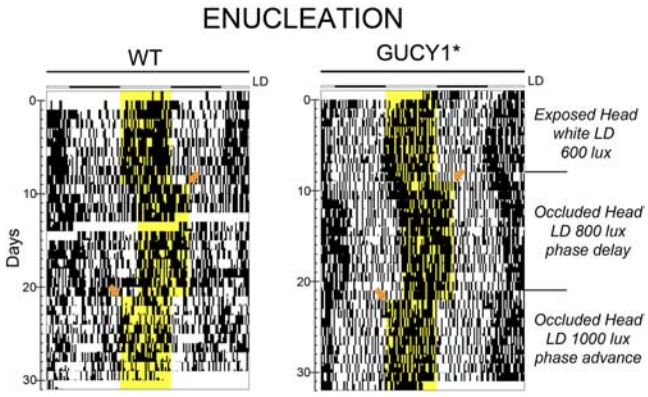


Figure 4. Participation of extraretinal PRs (encephalic PRs) in circadian control of feeding rhythms in sighted, enucleated GUCY1* chickens. The enucleated birds without head occlusion were synchronized to a 12:12-h LD cycle (light: cool white fluorescent light of 600 lux) for 10 d, as indicated in the bars at the top denoting the light (white) and dark (black) phases. The heads were then occluded, and the birds were subjected to 4 h of phase delays and advances with increasing light intensities (600 to 800 to 1000 lux). The main recorded activity rhythms are highlighted in yellow; the orange arrows indicate changes in the illumination condition. See text for further details.

rhythms to the imposed LD cycle. However, after both Enx and head occlusion, GUCY1* chickens were unable to entrain their feeding rhythms in response to a 4-h shifted LD cycle with blue or white light of moderate intensity (≤600 lux). When enucleated and head-occluded WT and GUCY1* chickens were exposed to a 4-h shifted LD cycle with white light of increasing intensities (800−1000 lux, cool white fluorescent light), the 2 genotypes entrained their feeding rhythms in response to 800 and 1000 lux of light, respectively. These observations strongly suggest that encephalic PRs participate in the photic entrainment of daily food intake rhythms in blind and WT chickens exposed to high light intensity levels.

Effect of Pnx and Enx

To further examine the participation of extraocular PRs in the nonvisual photoperception of chickens and particularly of encephalic PRs, we performed experiments on feeding activity in WT animals subjected first to Pnx surgery and then to Enx. As shown in **Fig. 5**, pinealectomized birds with their eyes and heads exposed to light were able to entrain their activity rhythms to the imposed LD cycle. When these pinealectomized birds were enucleated, under exposure of their nonpineal, extraocular PRs to a white LD cycle of 600 lux, their activity rhythms remained entrained to the previous LD cycle. Moreover, when they were exposed to a 4-h shifted LD cycle with white light of increasing intensities (800−1000 lux, cool white fluorescent light), they entrained their feeding rhythms in response to the higher light intensities. It is noteworthy that the offset of daily activity remained adjusted along the days under the new LD regimens. These are the first observations in support of the hypothesis that encephalic PRs participate in the photic entrainment of daily food intake rhythms in enucleated chickens exposed to high light intensity levels.

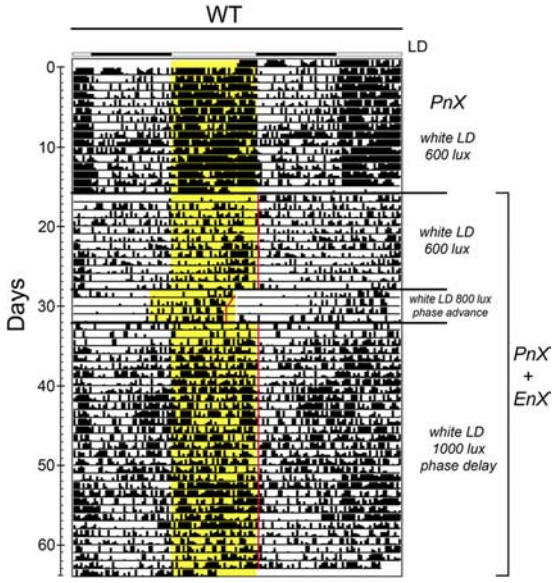


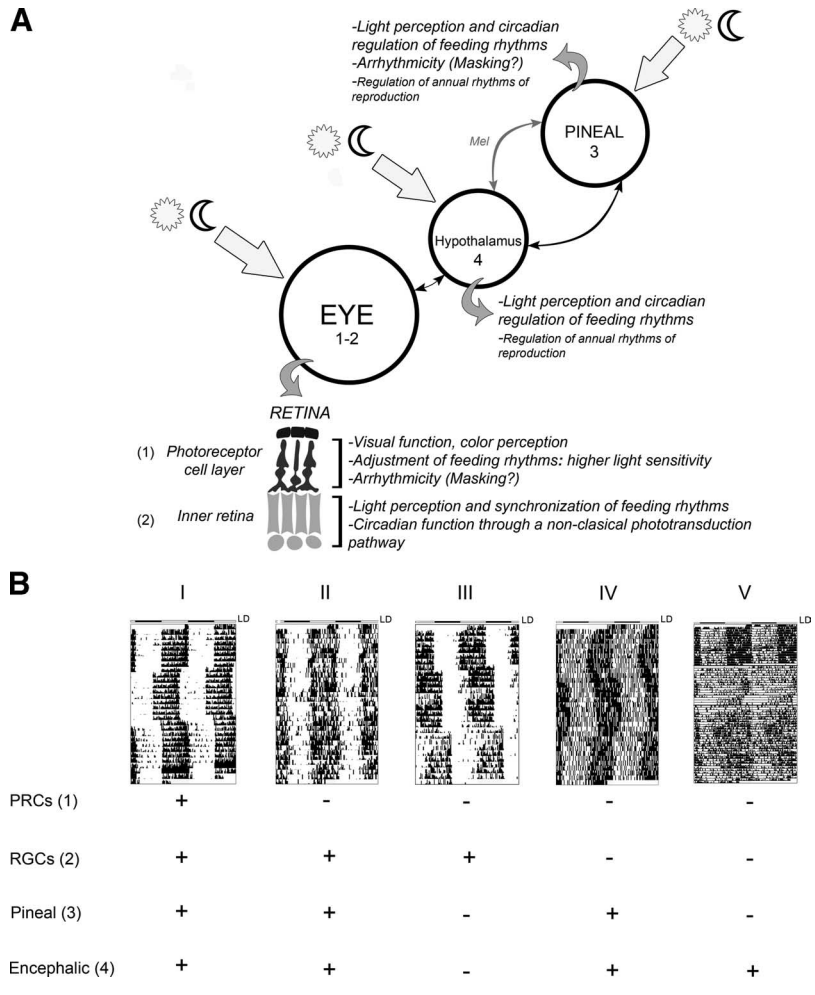
Figure 5. Participation of encephalic PRs in circadian control of feeding rhythms in WT chickens subjected to Pnx and Enx. Chickens (WT, n=4) were subjected to Pnx, allowed to recover for a week and then kept in a 12:12-h LD cycle with white light (600 lux) for 16 d (indicated in the bars at the top denoting the light [white] and dark [black] phases) with their eyes exposed to achieve a correct synchronization. After the photic entrainment, pinealectomized animals were subjected to Enx and then kept in a LD cycle with white light (600 lux) for a further 12 d. At d 28, birds were subjected to 4 h of advance and delay phase shifts with increasing light intensities (800−1000 lux). The main recorded activity rhythms are highlighted in yellow. The red vertical lines indicate the offset of feeding rhythms. See text for further details.

DISCUSSION

In the present work, we demonstrate for the first time the contribution of a multiple PR system to the precise and differential regulation of the daily photic entrainment of food intake rhythms in chickens. These PRs respond to different intensity levels of white and blue light; such responses involve classic rods and cones, inner retinal cells presumed to be the intrinsically photosensitive RGCs (ipRGCs), the pineal organ, and encephalic PRs probably located in the hypothalamus (**Scheme 1**). Unlike in adult mammals, in which the only photoreceptive structure is the retina, photoreception in nonmammalian vertebrates is characterized by multiple light detectors (6, 13). In this respect, we previously reported that blind chickens with their heads occluded to avoid extraretinal photoperception are able to detect light that regulates diverse NIF tasks, revealing the presence of functional inner retinal PRs (12) such as ipRGCs (49, 50).

In this work we have characterized the mutual interaction of different PRs in chickens by using WT birds and a model of blindness in the form of GUCY1* birds with retinal degeneration (42, 43, 46), which first allowed us to dissect the function of inner retinal PRs. In addition, we performed complete occlusion of chicken heads to avoid extraocular photodetection, Enx to abrogate retinal function, Pnx to eliminate pineal gland activity, or Enx and Pnx to analyze the functioning of deep brain PRs with no other active photoreceptive structure. Notably, all PRs were capable of synchronizing circadian rhythms at high-intensity LD cycles.

Another interesting finding of this study was that GC1 is not essential for the functioning of the photocascade in PRs located in the inner retina, pineal gland, or deep brain. Animals carrying a null mutation in the gene for GC1, a key enzyme for the classic phototransduction cascade, responded to light under the different experimental situations investigated (head occlusion, Enx, and Pnx together with Enx); this finding strongly suggests that the biochemical photocascade operating in the nonvisual inner retinal or extraocular PRs does not require the synthesis of cGMP by GC1 as occurs in isolated ipRGCs (49, 50). In the chicken and mammalian retina, another guanylate cyclase, GC2 (also known



Scheme 1. Light perception and interaction between different components of avian circadian system in the regulation of feeding behavior. *A*) The retina, in addition to its major role in vision, acts as a sensor of environmental illumination changes through nonvisual circuitry involved in the photic regulation of different NIF tasks such as behavior (locomotor activity rhythms and feeding). In this article, we demonstrate that blind chickens displayed light responses even in the absence of functional classic photoreceptor cells (cones and rods, in violet) and occlusion of extraocular PRs located in the pineal gland and the encephalon. Light acts directly on nonvisual PRs localized in the inner retina (light blue cells). This nonvisual circuitry involves ipRGCs projecting to different brain areas. The SCN is the site for the master circadian clock regulating the daily coordination of behavior, which receives the nocturnal signal from the pineal gland (photosensitive in birds) through the neurohormone melatonin. Classic PRCs (rods and cones) and the pineal gland appear to be responsible for masking effects and/or loss of rhythmicity in feeding behavior under constant conditions. Light responses were still observed after enucleation and pinealectomy in chickens exposed to high light intensities, indicating that encephalic PRs may play a role in the photic regulation of circadian feeding behavior. *B*) Participation of a multiple photoreceptor system in the regulation of feeding behavior by light in chickens. Photic entrainment of daily rhythms in food intake of WT or GUCY1* chickens synchronized to LD cycles and exposed to different illumination conditions (I−V) as shown in Figs. 1 to 5 to demonstrate the presence (+) or absence (−) of different functional photoreceptive systems including retinal PRs (PRCs and RGCs) and/or extraocular PRs. The main recorded activity rhythms synchronized by the L phase from the different illumination conditions are highlighted in gray.

as GC-F), is also expressed; however, levels of cGMP in GUCY1* chicken retinas are 6-fold lower than those in WT animals, even though animals are blind and PRCs still degenerate (42). Remarkably, the unique guanylate cyclase reported in pinealocytes is GC1 (ref. 51; reviewed in ref. 52); however, the pineal gland in GUCY1* chickens still responds to light (ref. 53 and this study), strongly suggesting that the photocascade operating in this tissue does not involve cGMP.

GUCY1* and WT birds also express 2 melanopsin genes (*Opn4m* and *Opnx*) in their retinas (12, 54). Remarkably, Opn4m has been shown to confer intrinsic photosensitivity to ipRGCs (29, 55, 56), whereas the pineal gland of WT birds expresses 2 different photopigment mRNAs (pinopsin and Opn4x; ref. 57). In this respect, Zatz and Mullen (58, 59) and Zatz *et al.* (60) have proposed that 2 mechanisms of photoendocrine transduction occur in cultured chicken pineal cells, one of which appears to be cGMP-independent. Moreover, Matsushita *et al.* (61) demonstrated colocalization between pinopsin and 2 α subunits of the G-protein ($G_{t1}\alpha$ and $G_{q/11}\alpha$) in cultures of chicken pinealocytes, supporting the idea of a second functionally independent cGMP phototransduction pathway. However, the biochemical nature of the phototransduction cascade in these cells, involving Opn4, is not yet known. The pineal glands of GUCY1* birds showed significantly elevated levels of pinopsin mRNA (53, 62), and a cGMP-independent cascade could be active in these cells. This finding, together with our current results and biochemical and behavioral evidence, leads us to infer that the pineal gland of GUCY1* chickens remains functional.

In 1964, Benoit (31) reported the existence of photosensitive hypothalamic structures in blind ducks; these structures were later related to seasonal reproduction cycles in birds. The expression of a number of different photopigments such as VA-opsin, Opn5, and Opn4 and a rhodopsin-like protein has now been reported in avian hypothalamic nuclei, but little is known about the nature of the biochemical cascade triggered on their photoactivation and its potential involvement in circadian functions. In the pigeon brain, it seems plausible that one of these photopigments activates the cGMP pathway (63); however, in our animal model, the cGMP route by GC1 is severely

impaired, strongly suggesting that an alternative phototransduction pathway could be present in these structures. In the circadian context, it is important to mention that different proposed photosensitive hypothalamic structures in the chicken are interconnected with the suprachiasmatic nuclei (64).

Our results (Figs. 1−5) reveal the complexity of the photoreception system regulating food intake activity in chickens and disclose the presence of ≥4 different PR groups that clearly impinge on avian photoperception and feeding entrainment under different illumination situations. 1) The nonclassic PRs located in the inner retina, such as the ipRGCs, seem to be sufficient for adjusting the feeding behavior to LD cycles of different intensities and qualities (white and blue light) in blind animals with extraretinal occlusion. Nevertheless, inner retinal PRs seem to be less sensitive than classic PRs and, as a consequence, these nonvisual PRs can be less efficient in adjusting the feeding rhythm (Fig. 1*B*). 2) The classic retinal PRs (cones and rods) and the pineal organ appear to strongly participate in and/or interfere with the photic entrainment of behavior, because sighted WT birds and blind chickens with their heads exposed to light became arrhythmic in LL, whereas blind birds with their heads occluded did not (Figs. 2 and 3). 3) Enucleated birds (WT or blind) with their heads exposed to light remained synchronized to the imposed LD cycles, although synchronization was not as precise as in nonenucleated blind birds, indicating the crucial role played by the eyes in the chicken multiphotoreceptor system. 4) Encephalic PRs may also contribute to photic entrainment of behavior because enucleated animals (WT or blind birds) with occluded heads (Fig. 4) responded only to white light of high intensities (≥800 lux). Moreover, WT animals lacking retinal and pineal gland PRs as a consequence of the Pnx surgery and Enx still responded to white light of increasing intensities (≥800 lux) under 4-h shifted LD cycles (Fig. 5).

The fact that GUCY1* chickens with head occlusion showed free-running feeding behavior in LL implies that PRCs or extraretinal PRs mediate masking or disruption of the oscillators that govern the food-intake rhythm. Alternatively, the synergic action of different PRs may be required for this LL effect. In addition, light responses in enucleated birds mediated by the exposed pineal organ, described as morphologically normal (62), and encephalic PRs reveal a weaker contribution of these 2 PR systems to the photic entrainment of behavior because they produce higher basal activity at all times. Moreover, in the case of encephalic PRs, they may require a higher light intensity to exhibit an appreciable response (36–39); in our experimental design, after 1000 lux of white light illumination, the <3 lux reaching deep into the brain appeared to be sufficient to entrain brain oscillators. These responses could be correlated with the presence and expression of a variety of novel photopigments such as Opn4, Opn5, and VA-opsin, all highly expressed in reproduction-related hypothalamic nuclei (36–39).

Pioneering work by Menaker (32) and Menaker and Keatts (33) has shown that the perching activity of enucleated sparrows can be entrained to LD cycles with light intensities even <1 lux when their pineal glands are exposed to light stimulation or when they are subjected to LD cycles after Pnx (32, 65). In addition, these pinealectomized sparrows kept in LL (500 lux) become arrhythmic like those kept in DD and normal birds exposed to LL. In chickens, data from Nyce and Binkley (40) showed that enucleated animals have a daily rhythm of locomotor activity, which entrains to a 24-h LD cycle of a higher light intensity (870 lux); in these experiments, we cannot discard the contribution of PRs both of the pineal gland and deep brain. In our experiments, after Enx, animals having their eye orbits uncovered but head occluded as in previous experiments do not respond to blue or white light of 600 lux and free-run with a period similar to that observed in DD (12); however, animals are entrained to a new LD cycle imposed when light intensity is increased from 800 to 1000 lux. Taken together, our observations demonstrate that either blue or white light of 600 lux is not detected in enucleated animals (12), but light of higher intensities is able to reach the deep brain for synchronizing activity rhythms in the absence of a photodetective pineal gland (head occlusion or pinealectomy). In addition, significant differences in light and intrinsic responsiveness can be observed among different avian species: the perching activity in house sparrows is normally rhythmic in DD, with a period (τ) of >24 h, or in constant dim light (≤1 lux; 66), the period shortening with increases in LL and lengthening with decreases in illumination. These photic responses were mediated by both eyes and the extraretinal brain PRs as observed after bilateral orbital Enx and/or head occlusion. Normal sparrows become arrhythmic under LL of higher intensities (≥10 lux; 67); however, circadian rhythms emerge in blinded birds that free-run under LL of 50−200,000 lux or in intact animals maintained under constant dim light with their heads covered with opaque material (35, 67). In contrast and beyond the evolutionary differences among these avian species (68), both wild-type and GUGY1* chickens display circadian rhythms in feeding activity in DD with a period (τ) <24 h and become arrhythmic under LL, whereas blind birds, with no functional cones and rods, exhibit circadian rhythmicity in LL after head occlusion. Remarkably, in both avian species (house sparrows and chickens), eyes and extraretinal PRs clearly contribute and interact with one another in generating this arrhythmicity.

In addition, different free-running periods were found in WT and GUCY1* birds among different illumination conditions (WT in DD=22.94 h<GUCY1* in DD=23.48 h <GUCY1* with head occlusion (LL)= 24.50 h and GUCY1* in 3-lux LD cycle=24.58 h). These findings clearly show that in DD, when all PRs are present as in the case of WT animals, the free-running

period is shorter than that in GUCY1* chickens having all functional PRs except rods and cones. Moreover, the free-running period was even longer in GUCY1* birds having only the inner retinal PRs exposed to LL of 100 lux with occluded extraretinal brain PRs or when kept in LD cycles of 3 lux (Fig. 1*C*). Taken together, these observations clearly indicate that the GC1 mutation somehow lengthens the period in DD compared with the period in WT birds and that in blind birds the inner retinal PRs alone are sufficient to lengthen the period under specific lighting conditions (LL or dim LD cycles).

We and researchers in other laboratories have described the presence of retinal and extraretinal PR cells in chicken and other avian species, most of them expressing the photopigment Opn4 and being responsible for nonvisual photoreception such as the ipRGCs in the inner retina (49, 53), pinealocytes (57–62), and encephalic PRs located in the reproduction-related hypothalamic nuclei (36–39).

Overall, the photoreception system that synchronizes the daily rhythms of food intake in chickens exhibits a high level of complexity, with each component (retinal and extraretinal PRs) contributing to a significantly different degree (Scheme 1). Our studies revealed that inner retinal PRs are sufficient for the daily entrainment of feeding and that classic retinal PRs (cone and rod) and those located in the pineal gland play a major role by adjusting the phase and masking the clock under LL in which animals become arrhythmic. Moreover, encephalic PRs operate under higher light intensities, presumably resulting in a basal contribution to entrainment of the circadian system under physiological conditions. FJ

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