Developmental sources of conservation and variation in the evolution of the primate eye

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Conserved developmental programs, such as the order of neurogenesis in the mammalian eye, suggest the presence of useful features for evolutionary stability and variability. The owl monkey, *Aotus azarae*, has developed a fully nocturnal retina in recent evolution. Description and quantification of cell cycle kinetics show that embryonic cytogenesis is extended in *Aotus* compared with the diurnal New World monkey *Cebus apella*. Combined with the conserved mammalian pattern of retinal cell specification, this single change in retinal progenitor cell proliferation can produce the multiple alterations of the nocturnal retina, including coordinated reduction in cone and ganglion cell numbers, increase in rod and rod bipolar numbers, and potentially loss of the fovea.

proliferation | nocturnal | diurnal | retinal development | $p27^{Kip1}$

The evolution of the eye has focused research interest ever since Darwin identified the eye with its "inimitable contrivances" as a vexing problem for evolutionary theory (1859). Gradual evolution seemed implausible because "intermediate" forms of the eye seemed unlikely to be adaptive and selectable (1). Since Darwin's original challenge, however, a surprisingly large number of cases of independent evolution of image-forming eyes have been documented (2, 3). Furthermore, various living species with completely functional forms of eye organization are now known, which could be viewed as "intermediate" between a simple photoreceptive patch and the complex image-forming eye seen in cephalopods and most vertebrates (2, 3). Although the fact of repeated evolution of image-forming eyes, as well as the capacity for functional intermediates, is thus firmly established, the mechanism of the evolutionary process is still speculative.

An essential tenet of Darwin's view of the process of evolution is that it must be gradual, because random but adaptive variations in organisms are accumulated over evolutionary time. The empirical basis of the claim that variations are random remains to be explored (4). The variation offered for selection must be nonrandom in several important senses. For example, at the genetic level, recent states of the genome must be more accessible than remote states (5). Development itself evolves to reflect common and repeated challenges. Current studies of "evo–devo," the relationship between developmental programs and patterns of evolution, have demonstrated highly conserved patterns of gene expression and developmental sequencing in the organization of the body plan, the brain, and the eye, quite unlike the cumulative diversification of developmental programs across major taxa that selection on random adaptations would suggest (6–8). Moreover, such conserved patterns often appear to have advantageous features that permit variability while maintaining viability.

In the case of the evolution of the eye, we now know enough about essential features of the patterns of retinogenesis and oculogenesis in "model" systems (principally, the eyes of the mouse, rat, and rhesus macaque) and enough about the anatomical and functional variations of vertebrate eyes to begin to bring the domains of development and functional evolution together. The ordering and identity of the cellular and molecular processes that coordinate retinogenesis appear highly conserved in those animals that have been studied (8, 9). An organizational feature that suggests scalability and potential for coordinated adjustment to new visual niches is the conserved order of photoreceptor and neuron production in the retina. Elements related to nocturnal and diurnal vision are grouped together in developmental time. Specifically, multipotent retinal progenitor cells produce each cell type in an evolutionarily conserved order beginning with ganglion cells, cones, and horizontal cells followed by amacrine cells, Müller glia, bipolar cells, and finally rod photoreceptors (10). If retinal progenitor cell proliferation were advanced or slowed with respect to the changing extracellular specification environment over time, predictable changes in the ratio of the different retinal cell types based on their birth order, and thus diurnal or nocturnal niche, could occur (11). The contributors to the "extracellular specification environment" are multiple, and include the dosage of cell types within the developing retina (12) as well as adjacent tissues, such as the retinal pigment epithelium (13) and the lens ectoderm (14).

At the level of morphology and function, the multiple properties of nocturnal versus diurnal eyes have been much studied across taxa. Diurnal eyes are specialized for acuity, and cone photoreceptors predominate, containing multiple opsins. Convergence from photoreceptors to retinal bipolar cells and ganglion cells is kept limited, and specializations for high acuity, such as the packed photoreceptors of an area centralis or fovea, with oculomotor control allowing the use of a specialized region, are typically seen. Nocturnal eyes maximize sensitivity, containing large numbers of rods specialized for high quantum capture, with high-acuity specializations absent, and often morphological features advantageous for light capture, such as large size, frontal position, and a reflective tapetum allowing multiple chances for light capture. Virtually all mammalian eyes may function in both contexts, however, and evolutionary movement between these niches has occurred repeatedly. The long list of contrasting features between nocturnal and diurnal eyes, from protein expression, to cell types and numbers, to retinal topography and connectivity, and to overall eye morphology, suggests the comparison of the development of these eyes may provide a window into the mechanisms of evolutionary coordination of features of complex organs.

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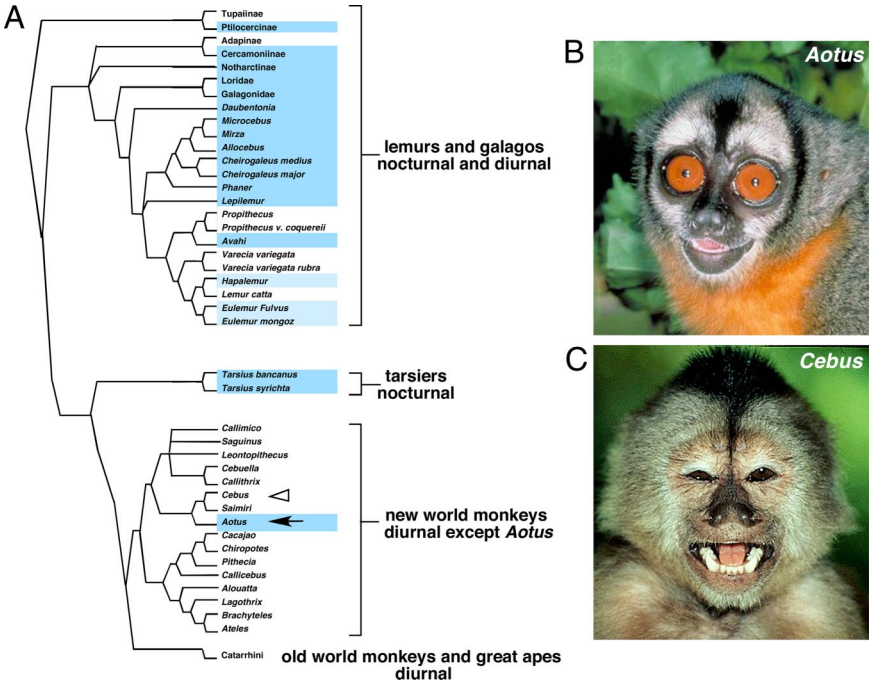

Fig. 1. Nocturnal and diurnal New World monkeys. (*A*) Phylogeny of the primates, in the order of distance from great apes, showing presence of nocturnal (blue) and diurnal species. "Stem anthropoids" begin at *Tarsius bancanus*, which are nocturnal. All past and present Platyrrhine and Catarrhine monkeys (from *Callimico* down) are thought to be diurnal, with the exception of the recently nocturnal *Aotus* (arrow). The nocturnal owl monkey, *Aotus azarae* (*B*), and the diurnal capuchin monkey, *Cebus apella* (*C*), were the two New World monkeys examined in this study.

Here, we investigate specifically the very recent evolution of nocturnal vision in the New World owl monkey, *Aotus azarae*, thought to have evolved from its diurnal ancestors ≈15 million years ago, and which possesses the full suite of morphological features of the nocturnal eye with the exception of the tapetum (15). We examine both the complement of cell classes in the retina of the owl monkey and the early alterations of cell proliferation compared with a diurnal cousin, *Cebus apella*, the capuchin monkey, to determine whether alteration in duration of cell proliferation alone in the context of a preserved pattern of neuronal specification could account for the multiple differences of the two eyes.

Results

Morphological and Cellular Characteristics of Nocturnal Vision in *Aotus* Compared with *Cebus*. In vertebrates, it is believed that the first retinas were diurnal, with low-light vision evolving independently in many lineages. The original mammals were nocturnal. All present primates arose from a nocturnal ancestor ≈55 million years ago (16–18). All present-day monkeys and apes (Anthropoidea) are diurnal, with the single exception of the owl monkey (*Aotus*) (Fig. 1 *A* and *B*), thought to have diverged from its diurnal ancestors recently, perhaps 15 million years ago in South America (15, 19). When compared with diurnal monkeys such as *Cebus* (Fig. 1 *A* and *C*), the *Aotus* retinal surface area is 50% larger than would be expected for a diurnal monkey of comparable brain and body size, permitting greater photon capture (Table S1 and Fig. 2*A*) (20). The cell classes of the retina, including both photoreceptors and neurons relevant to nocturnal versus diurnal vision, are altered: rod numbers are greatly increased, whereas cone and retinal ganglion cell numbers are reduced (Table S1 and Fig. 2 *B* and *C*) (21). Both the numbers and distribution of bipolar cells, as seen in whole mounts and by Chx10 and PKCα (22) immunostaining (this study), are altered in correspondence with the altered rod photoreceptor distribution (Fig. 2 *D–I*). In summary, total eye size, retinal area, both rod and cone numbers, retinal ganglion cell numbers, and rod bipolar cell numbers are altered in *Aotus* compared with *Cebus*.

Analysis of Retinal Progenitor Cell Proliferation in *Aotus* and *Cebus*. Based on the proportion of retinal cell types and the evolutionarily conserved birth order across vertebrate species, we hypothesized that the single change of moving the envelope of exit from the precursor pool toward the later stages of retinal cell specification (Fig. 3*A*) could lead to relatively fewer early-born cell types, such as ganglion cells and cones, and greater numbers of later-born cell types, such as bipolar cells and rods in the *Aotus* retina (Fig. 3 *B* and *C*). If terminal retinal neurogenesis is delayed until the probability of specification of rods is higher, the production of a rod-free area in the central retina would be opposed, which has been hypothesized as the initial and critical step in production of the fovea (20). This prediction integrates all of the alterations seen in *Aotus* compared with its diurnal ancestors: a single change in the timing of exit from the retinal progenitor pool during retinogenesis could lead to the conversion of a diurnal retina into a nocturnal retina.

Although we cannot effectively detect a delayed onset of terminal mitosis in *Aotus* in view of the high amount of ongoing cytogenesis, we can test for delayed offset (Fig. 3*A*). To directly test whether cytogenesis was disproportionately extended in the developing *Aotus* retina as predicted (Fig. 3 *A–C*), we compared the proliferation of retinal progenitor cells across development in fetal eyes from *Aotus* and *Cebus*. We have previously optimized a retinal explant culture system for mouse, rat, and human fetal retinas that recapitulates the cell proliferation, cell fate specification, and differentiation of the retina (23–28). The advantage of this system is that it allows us to perform detailed analysis of cell cycle kinetics and to determine the proportion of proliferating retinal progenitor cells throughout retinal development (29). For these studies, we performed a [^{3}H]thymidine continuous labeling experiment to determine the proportion of proliferating retinal progenitor cells and to derive estimates of the relative cell cycle length, as described in ref. 29. Bromodeoxyuridine (BrdU)/[^{3}H]thymidine colocalization studies were used to confirm the identification of proliferating retinal progenitor cells in *Cebus* and *Aotus* retinal explants (Fig. 3*D*). As retinal progenitor cells progress through the cell cycle and enter S phase, they incorporate the [^{3}H]thymidine. Therefore, the proportion of [^{3}H]thymidine-labeled cells increases until all of the retinal progenitor cells have entered S-phase (Fig. 3 *E–G*). The amount of time required to label all of the retinal progenitor cells is an approximation of the length of G_2–M–G_1 (29). Moreover, the proportion of cells at that time is equivalent to the proportion of proliferating retinal progenitor cells. Therefore, our [^{3}H]thymidine continuous labeling experiment provides data on the proportion of

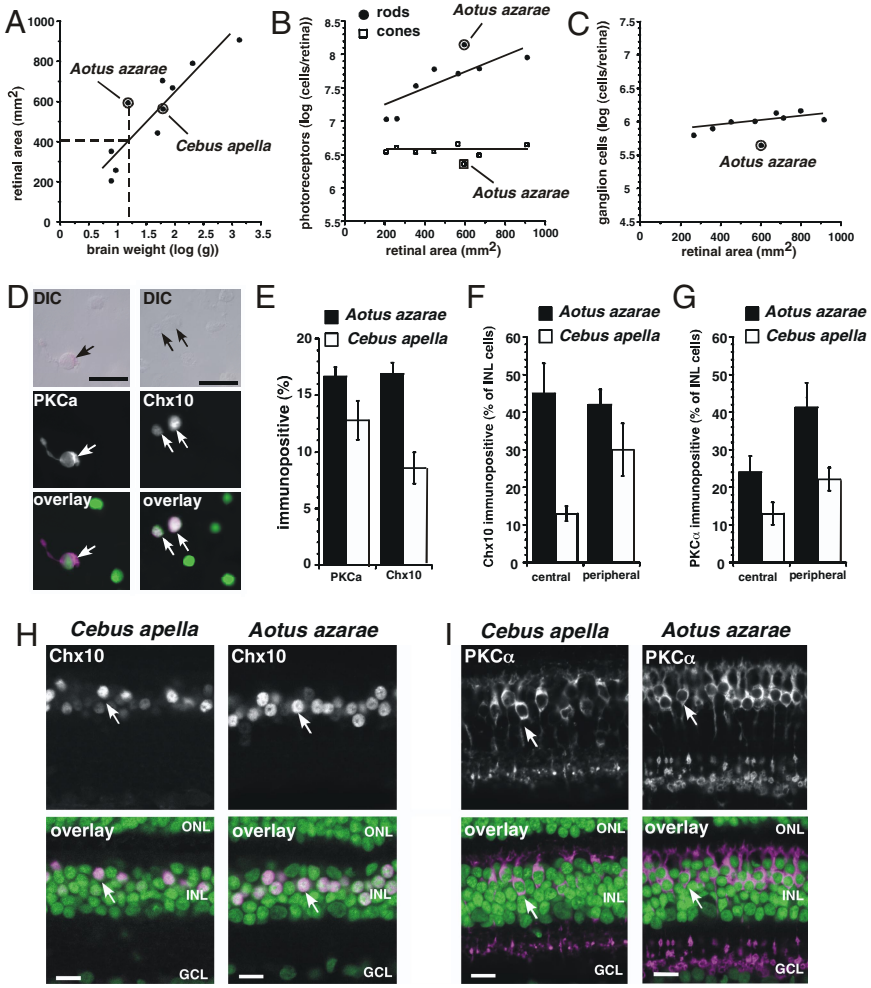

Fig. 2. Nocturnal adaptations of *Aotus azarae* retinas. (*A–C*) Total retinal area (*A*), numbers of cones and rods photoreceptors per retinal area (*B*), and numbers of retinal ganglion cells per retinal area (*C*) as measured from adult primate retinas of different species, including *Aotus azarae* and *Cebus apella*. (*D*) Representative pictures of immunopositive dissociated retinal cells stained with indicated antibodies. Stained cells are shown in purple and nuclei are shown in green. (*E*) The proportion of Chx10- and PKCα-stained cells was determined from three adult *Aotus azarae* (filled bars) or *Cebus Apella* (open bars). (*F* and *G*) Quantification of the proportion of bipolar cells as determined from Chx10 (*F*) or PKCα (*G*) immunostainings in adult retinal sections of both *Aotus* (filled bars) and *Cebus* (open bars). The number of Chx10- or PKCα-stained cells was quantified in central and peripheral areas of the retina. Error bars indicate SEM. (*H* and *I*) Representative pictures of adult retinal sections stained for either Chx10 (*H*) or PKCα (*I*) are shown for both species. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (Scale bar, 10 μm.)

retinal progenitor cells and their cell cycle kinetics throughout development in *Cebus* and *Aotus*.

Because the prediction entails that retina cytogenesis be extended in *Aotus* with respect to its maturational state, both the postconceptional age of embryos must be determined, as well as the equation of their maturational state independent of absolute age. Timed pregnancies were not possible in this research context, so each embryo's age was determined post hoc. Absolute brain size increases as a highly predictable function of age, particularly in closely related species (30, 31), and it was the primary means used to register embryo postconceptional ages between *Aotus* and *Cebus* (Fig. S1*A*) and to register both these primates with the best-studied primate, *Macaca mulatta* (*SI Materials and Methods*). Additional gross maturational features (crown–rump length, head and eye dimensions, finger separation, and eye opening) and brain maturation were used to confirm embryo staging (Fig. S1 *B–F* and Table S2). These features could then be used to derive a species score to employ the "translating time" maturational scale, which employs extensive data on neuroembryological events on 11 species including humans to register each species to a common event scale (9, 32). For example, *Cebus* has a larger brain (62 g) than *Aotus* (16 g) at maturity and takes ≈75 days to reach the generation of the outer layers of the cortex, whereas *Aotus* requires 40 days. The *Aotus* maturational period must therefore be transformed to match the *Cebus* period for best comparison of the relative sequence of maturational events in the two species.

The proportion of proliferating retinal progenitor cells in *Aotus* was higher than in *Cebus* at a similar gestational stage (Fig. 3 *E* and *F*). Moreover, when samples were analyzed from independent animals at similar gestational stages (Fig. 3, compare *F* with *G*) in independent experiments, they provided very similar results highlighting the reproducibility of the explant culture assay used for this study. By extending these studies to retinas that span the gestational stages of *Aotus* and *Cebus*, we found that the period of cytogenesis was protracted in *Aotus* (Fig. 3*H*). Nonparametric analysis of the probability of two groups (each $n = 8$) drawn from the same population to be separated without overlap on the two plotted dimensions is <0.001.

To provide independent confirmation of the protracted period of retinal progenitor cell proliferation in *Aotus* retinas, we performed a series of experiments based on previous studies of the mechanism of cell cycle regulation in the developing retina. It has been shown that the length of the cell cycle increases during retinal development and that this is most pronounced at the later stages of retinogenesis (29). Therefore, one might predict that the lengthening of the cell cycle may occur at an earlier maturational age in *Cebus* than in *Aotus* if cytogenesis is protracted in *Aotus*. We estimated the length of G_2–M–G_1 at each stage of development from the [^{3}H]thymidine continuous labeling data (Fig. 4*A*) as described in ref. 29. These data show that the cell cycle length is increased in the *Cebus* retina at an earlier maturational age than in the *Aotus* retina.

Expression of Cell Cycle Gene in *Aotus* and *Cebus* Retinas. Next, we characterized the expression of several key cell cycle regulators that control retinal progenitor cell proliferation in the mammalian retina. Cyclin D1 is the major D-type cyclin expressed in the developing retina (33, 34). The *Cebus* and *Aotus* genomes have not

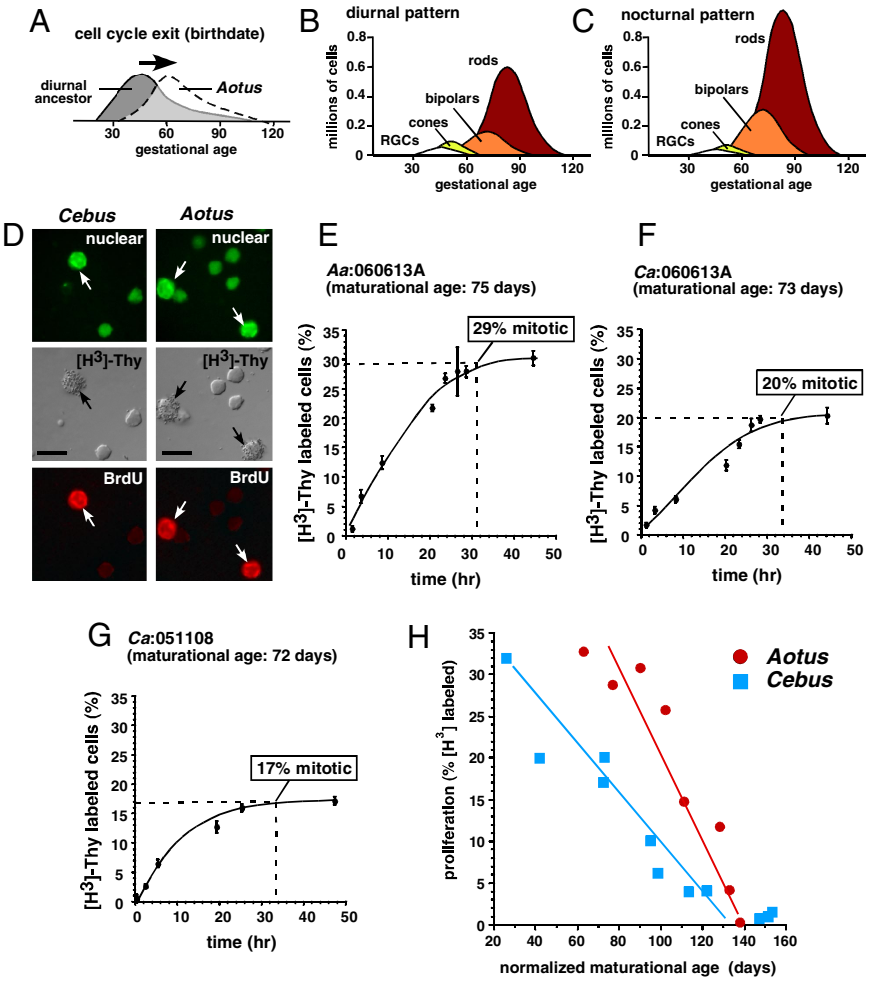

Fig. 3. Retinal progenitor cell proliferation in the developing *Aotus* and *Cebus* retinas. (*A–C*) Predictive illustrations showing how the extension of retinal progenitor cells proliferation during development may lead to changes in retinal cell type distribution as observed in diurnal (*B*) or nocturnal (*C*) primate retina. (*D*) Representative pictures of dissociated retinal progenitor cells previously labeled with markers of DNA synthesis. Retinal explants were cultured in the presence of [^{3}H]thymidine or BrdU (10 μM), dissociated, immunostained for BrdU (red), and overlaid with autoradiographic emulsion. (*E–G*) The kinetics of retinal progenitor cells proliferation was measured by determining the proportion of [^{3}H]thymidine-positive cells in both *Aotus azarae* (*E*) and *Cebus apella* retinas (*F* and *G*). Error bars represent the standard deviation. (*H*) Quantification of the proportion [^{3}H]thymidine/proliferating cells from various developmental stages from both species indicates that the period of cytogenesis is extended in developing retinas of nocturnal species (*Aotus*) compared with diurnal ones (*Cebus*). A total of 250 cells was scored for each sample at each time point in duplicate.

been sequenced, so we developed PCR primers for the cyclin D1 gene for each species based on sequence conservation for other mammalian species. These PCR amplicons were then sequenced and used to design TaqMan real-time RT-PCR probes and primers for cyclin D1 (see *SI Materials and Methods*). Samples were normalized to β-actin expression by using the same approach. There was an extension in the expression of cyclin D1 in *Aotus* compared with *Cebus* when plotted on the same maturational age scale (Fig. 4*B*). Cell cycle exit in the developing retina is regulated in part by the precise balance of the cyclin-D/cyclin-dependent kinase levels and the levels of cyclin kinase inhibitors (11). The major Cip/Kip family cyclin kinase inhibitor expressed in the retina is p27^{Kip1} (23, 35). Real-time RT-PCR analysis of the expression of p27^{Kip1} mRNA showed no significant difference between species (Fig. 4*C*). However, the level of p27^{Kip1} protein is regulated primarily by protein turnover rather than transcriptional control. Therefore, we characterized the expression of p27^{Kip1} protein in dissociated retinas at each stage of development in *Aotus* and *Cebus* (Fig. 4 *D* and *E*). As predicted, the percentage of p27^{Kip1}-immunopositive cells increased at an earlier maturational stage in *Cebus* compared with *Aotus*.

Regulation of Eye Size in *Aotus*. In addition to the relative increase in *Aotus* retinal size and the shift in the composition of the retina to accommodate nocturnal vision, the size of the eye is also disproportionately large (Fig. 5*A*). The coordination of eye size with retinal size could be achieved in many ways; we describe two mechanisms here that have some empirical support. First, there are early genetic mechanisms implicated in regulating the growth of the retina with respect to the growth of the other ocular structures. If these become uncoupled, the retina will not be properly proportioned for the eye size and vision will be disrupted or absent. One example of a gene that coordinates retinal growth with eye growth is the N-myc protooncogene (36). It is possible that the increase in *Aotus* eye size is due to alteration of this genetic mechanism during early development (Fig. 5*B*).

Second, in primates, and a number of other vertebrate species, the activity of the neurons of the retina in early visual experience regulates the size of the eye to bring it into proper focus, a process called emmetropization. If the eye is in focus, with the length of the eye matched to the power of the optics (lens and cornea), and thus receives a high-contrast signal, the activity of the neural retina directly signals that the eye is the correct size, and growth is checked. If the eye is closed, or the image is blurred, the eye continues to grow (reviewed in ref. 37). Both the loss of an opsin and being active nocturnally might reduce the activity of the neural retinal and thus cause the eye of *Aotus* to grow disproportionately. This experience-related process would occur after birth (Fig. 5*C*).

To distinguish between these two possibilities and to determine whether the increase in the relative size of the *Aotus* eye begins during retinogenesis before activity-dependent processes become important, we measured eye size in *Aotus* and *Cebus* across maturational stages. These data indicate that the increase in the relative size of the *Aotus* eye begins well before birth, ruling out that an experience-driven mechanism could be solely responsible for its larger eye, although some role of emmetropization in control of final eye size must certainly occur (Fig. 5 *D* and *E*).

Discussion

We have collected evidence that multiple features of eye morphology, and at least four separate cell classes in the retina vary

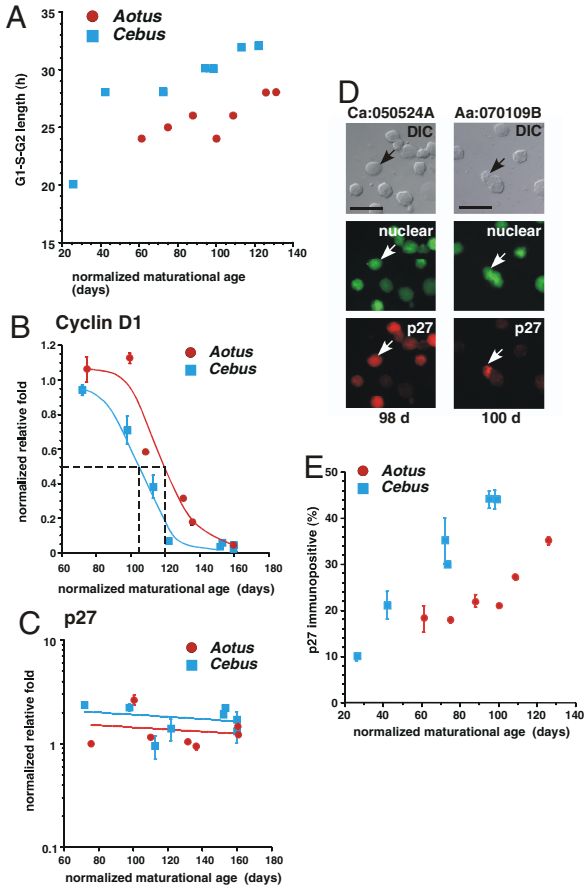

Fig. 4. Expression of cell cycle regulators in developing *Aotus* and *Cebus* retinas. (*A*) Length of G_1, S, and G_2 phases of the cell cycle as measured from various stages of both *Aotus* (red) and *Cebus* (blue) developing retinas. (*B* and *C*) Real-time RT-PCR analysis showing normalized levels of cyclin D1 (*B*), and $p27^{Kip1}$ (*C*) mRNA expression from various developmental stages of *Aotus* (red) and *Cebus* (blue) retinas. All samples were normalized to *GAPDH* expression. (*D*) Representative pictures of dissociated retinal cells immunostained for $p27^{Kip1}$. (*E*) Quantification of the proportion of $p27^{Kip1}$-immunopositive cells from various stages of both *Aotus* (red) and *Cebus* (blue) developing retinas. The expression of cell cycle regulator mRNA and protein consistently matched the changes in cell proliferation during the development of *Aotus* retina compared with *Cebus*.

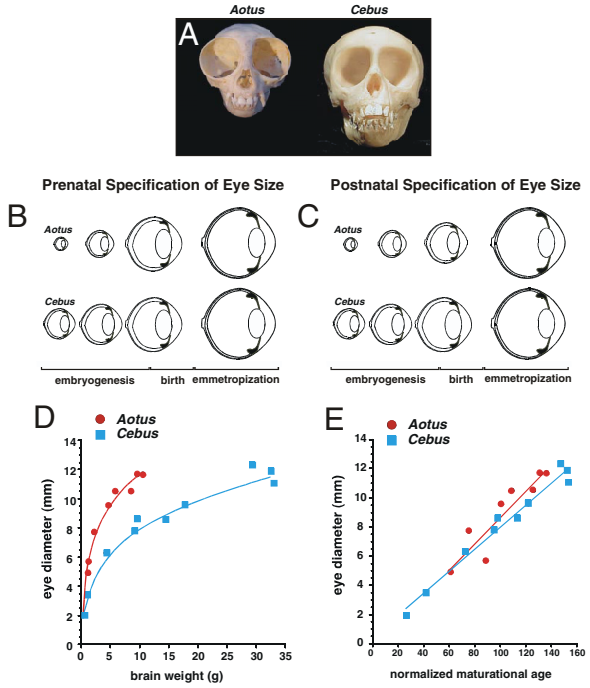

Fig. 5. Developmental regulation of eye growth in diurnal vs. nocturnal primates. (*A*) Representative pictures of *Aotus azarae* and *Cebus apella* skulls. (*B* and *C*) Predictive models illustrating the specification of eye size during the development of *Aotus* and *Cebus* prenatally during retinogenesis or postnatally by emmetropization. (*D*) Quantification of eye diameter in relation to brain weight (in grams) in *Aotus* (red) and *Cebus* (blue). (*E*) Quantification of eye diameter in relation to maturational age in *Aotus* (red) and *Cebus* (blue).

in number as predicted in nocturnal *Aotus* compared with diurnal *Cebus* and proposed that shifting of the envelope of exit from the precursor pool later in *Aotus* with respect to the schedule of retina cell fate specification could account for all of the changes seen. Consistent with this prediction, we have shown that multiple measures of cell proliferation are extended in *Aotus*, both in absolute days of development, and when their schedules are normalized to the basic mammalian plan (9). This observation is the first step in demonstration of the plausibility of the hypothesis, and it is important to note that we have not explicitly disconfirmed all other possibilities for alteration of development, but rather increased the probability that the one we propose is the case.

The principal alternate hypothesis is that the pattern of cell fate specification is altered in a cell class-by-cell class manner in *Aotus*, such as to produce somewhat fewer retinal ganglion cells and cones, more rod bipolars, and many more rods. Although our experiment cannot rule out this possibility, the demonstration that retinal cell fate specification results from a precise balance of intrinsic and extrinsic cues (10, 12) makes this an unlikely and rather cumbersome possibility. One possibility, which should be explicitly entertained, is that special mechanisms for overproduction of rods in *Aotus* may have also been engaged. Although many studies have demonstrated that the majority of retinal progenitor cells are multipotent, capable of producing rods as well as other retinal cell classes, some retinal progenitor cell clones have been shown to contain only rods (38, 39). However, increased numbers of rods are only one of the changes in *Aotus*, so a singular change in the rod precursor pool could not account for all of the changes observed.

All of the diurnal monkeys and apes, as well as *Homo sapiens*, have an eye specialized for high-acuity daytime vision. High numbers of cones are packed into the central retina in a foveal organization unique to primates, including all New World monkeys but *Aotus* (16, 40). In addition, all Old World monkeys and great apes have evolved a third cone photopigment to complement the long- and short-wavelength photopigments found in the majority of mammals, which permits enhanced color vision (41). Thus, the owl monkey (*Aotus*) (Fig. 1*C*), in the relatively short period of its evolution (19), appears to have evolved the full suite of features that optimize night vision, including reduced numbers of opsins, changed photoreceptor complements, changed intraretinal connectivity, loss of the fovea, and increased eye size; only the relective tapetum is absent (16). We propose that the number of developmental changes required to produce this set of nocturnal features could be as low as three. The first two are loss of one opsin and an overall increase in eye and retina size. The third change is the movement of the envelope of retinogenesis with respect to overall maturation. This change reduces cell numbers associated with diurnal vision, increases cell numbers associated with nocturnal vision, consequently alters convergence and connectivity appropriately, and perhaps removes the fovea (20). Essentially, we are proposing a heterochronic change in the nocturnal retina, shifting the envelope of precursor exit with respect to the envelope of the timetable of retinal cell specification, as a simplifying mechanism to produce the complex list of cell population and distribution changes

in the nocturnal retina. Heterochrony in stem and organ populations is of course a classic proposal for a mechanism of evolutionary variation and change (42), and multiple hypotheses for heterochronic change have been made, at every level of analysis. Recently, heterochronic alterations have been demonstrated in the control of organization of body plan. For example, in snakes, the "segmentation clock" runs faster with respect to overall developmental rate than in other vertebrates, producing a much larger number of smaller segments (43).

The sources of an advantageous, evolvable sequence of neurogenesis that can smoothly produce the complement and ratio of cells required for nocturnal or diurnal vision are not hard to discern. The owl monkey (and any extant primate) is the descendant of a line of vertebrates that have traversed the diurnal–nocturnal niche division several times. Evolution may have thus filtered the sequence and organization of retinal development, selecting an order of cell specification that permitted separation diurnal and nocturnal classes in time of specification and permitted their reciprocal regulation. The peripheral retina in any diurnal primate is essentially a nocturnal retina, and *Aotus* could also be viewed as delaying its terminal cytogenesis to produce an all-peripheral retina.

In addition, the sequence of cell specification would appear to have useful features for normal variation and scaling of the retina. Functionally related cell groups are produced close together in time, coordinating their numbers. In addition, across the brain, when neurogenesis is protracted, late-generated cell groups become disproportionately large (44), and this nonlinear feature appears to be used to advantage in the retina as well. To maintain sensitivity in larger eyes, retinal progenitor cells that give rise to rods proliferate at a larger exponent than retinal progenitor cells that give rise to cones, and their late position in retinogenesis may permit "automatic" scaling when retinogenesis is extended to produce a larger nocturnal eye. For the retina, as with the vertebrate body plan (7), not only adult form and function but also the developmental programs that produce them are equally the products of evolution.

Materials and Methods

Determination of Maturational Age for *Cebus* and *Aotus*. A detailed description of the calculations used to determine maturational age is provided in *SI Materials and Methods*.

Explant Cultures. Procedures for maintaining mouse and primate retinal explants cultures are described in ref. 26.

Immunostaining, BrdU, and [^{3}H]Thymidine Labeling. Immunostaining procedures were done as reported in ref. 26. Previously dissociated cells or retinal sections were stained by using the following antibodies and dilutions: BrdU, 1:3 (GE Life Sciences; RPN20); p27, 1:500 (BD Biosciences); chx10, 1:2,000 (ExalphaBio; X1180P); PKCα, 1:10,000 (Upstate; 05-154). For detection, we used Cy3-tyramide (PerkinElmer; NEL704A). Nuclear staining was performed by using Sytox Green at 1:20,000 (Invitrogen; S7020). Labeled cells were visualized by using a Zeiss Axioplan 2 microscope, and images were captured with a Nikon TE2000E2 inverted confocal microscope.

S-phase retinal progenitor cells were labeled in explant culture medium containing 10 μM BrdU (Roche Molecular Biochemicals) or [^{3}H]thymidine (5 μCi/ml; 89 Ci/mmol) for the indicated times. Autoradiography detection was carried out as described in ref. 45.

Real-Time RT-PCR. RNA extraction and cDNA synthesis were performed as described in ref. 45. cDNA produced from retinas of each species was amplified by using primers for conserved regions of mammalian cyclin D1 (5′-cgccctcggtgtcctacttcaaatgt-3′; 5′-tgttctcctccgcctctggcattttg-3′) and β-actin (5′-attgctcctcctgagcgcaagtac-3′; 5′-cacctccctgtgtggacttggga-3′) genes. These PCR amplicons were then sequenced and used to design real-time RT-PCR primers and probes using Primer Express software (ABI). The TaqMan primers and probes for each sequence were as follows: cyclin D1 (forward, 5′-ctgcatgttcgtggcctctaa-3′; probe, 5′-atgaaggagaccatccccctgacgg-3′; reverse, 5′-tgtcggtgtagatgcacagctt-3′); β-actin (forward, 5′-actggaagcgtgaaggtgaca-3′; probe, 5′-cagtcggttggagcgagcatccc-3′; reverse, 5′-tcggccacattgtagaactttg-3′).

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