Reports

Light-triggered rod disc shedding in *Xenopus* retina in vitro. JOHN G. FLANNERY AND STEVEN K. FISHER.

The present study was designed to determine whether light-induced rod outer segment disc shedding occurs when the eye is isolated from the rest of the organism. Whole eye explants of Xenopus laevis *were maintained in vitro and either exposed to 5 min of light or kept in darkness. In both the in vitro eyes and in vivo controls exposed to the same conditions, a significant degree of disc shedding occurred within 1 hr of the light stimulus. The disc shedding response was larger in the explanted eyes than in the intact animals. In explants exposed to [^3H]leucine for 24 hr, a radioactive band formed at the base of rod outer segments. Thus both light-stimulated disc shedding and disc synthesis occur in the eye kept in vitro. The results of this study are compatible with other recently reported results suggesting that rod disc shedding is initiated within the eye.*

Rod photoreceptors in the retina synchronously shed packets of discs in response to the onset of light.[1, 2] Although in albino rats the burst of rod disc shedding occurs according to a circadian rhythm,[2] the results of Basinger et al.[1] indicated that synchronous shedding in the amphibian retina is triggered by the light stimulus itself.* In rats and frogs maintained on a light/dark cycle, the maximum amount of rod disc shedding occurs within 2 hr of light onset.[1, 2] Once shed from the receptor tip, packets of discs are ingested by the retinal pigment epithelium (RPE) and then digested by lytic enzymes of the RPE cells. Ingested disc packets (phagosomes) can be recognized in the light microscope, and thus quantitative data can be derived for different experimental conditions by counting the numbers of phagosomes per unit length of RPE.[1-4]

Following the reports that a burst of rod disc shedding follows the onset of light,[1, 2] the involvement of various neuroendocrine systems in controlling the disc shedding response was examined in several investigations.[1,4-7] The object of this study is to determine whether light-triggered disc shedding occurs in the absence of any connection between the retina and other neuroendocrine systems of the organism. Thus we examined the disc shedding response in cultured whole eye explants from *Xenopus laevis* and compared this to the shedding response for eyes in vivo.

Methods. Adult *X. laevis* (African clawed frog) were kept in a central vivarium colony on a 12 hr light/12 hr dark cycle for several months but were transferred to a controlled environment room (25° C) with the same lighting cycle 5 days prior to use in the experiments. The results reported here are compiled from three identical experiments performed over several weeks time; four frogs were used in each. Four hr into the dark half-cycle when the rod shedding rate should be low,[1, 3] eyes were excised from two animals under a dim sodium vapor safelight (a monochromatic (598 nM) light source that emits at a minimally sensitive portion of the *Xenopus* rod absorbance spectrum), placed in culture medium, and returned to absolute darkness. The intensity of light striking the animals during the dissection was less than 2 lux. Two intact animals exposed to the same lighting and temperature conditions served as the in vivo controls. Following 55 min in darkness, explants from one experimental and one control animal were exposed to 5 min of illumination from a Bausch & Lomb 6.5 V tungsten filament microscope illuminator at an intensity of 600 lux and then returned to darkness. Explanted eyes from the other frog plus the second in vivo control animal remained in darkness. One hour after the light stimulus, eyes were excised from the two intact frogs under the sodium vapor light; then all the eyes were fixed and processed for light and electron microscopy by immersion of the whole eye in 1.5% glutaraldehyde buffered with 0.067M sodium cacodylate (pH 7.2). The tissue was postfixed in 2% osmium tetroxide in Veronal acetate buffer

Table I. Comparison of the average number of phagosomes/mm of RPE in the cultured eye explants (in vitro) and eyes in vivo maintained in constant darkness or exposed to 5 min of light

Condition	*Treatment*	*Avg. phagosomes/ mm of RPE*
In vitro	Dark-maintained	74.03 ± 17.52
	Light-stimulated	117.81 ± 25.95*
In vivo	Dark-maintained	73.53 ± 10.52
	Light-stimulated	99.81 ± 21.02*

*Statistically significant difference between the means, $p < 0.01$, Student's t test.

*Studies in which *Xenopus* tadpoles are kept in constant darkness show that there is also a circadian component to their synchronous shedding of rod discs.[3]

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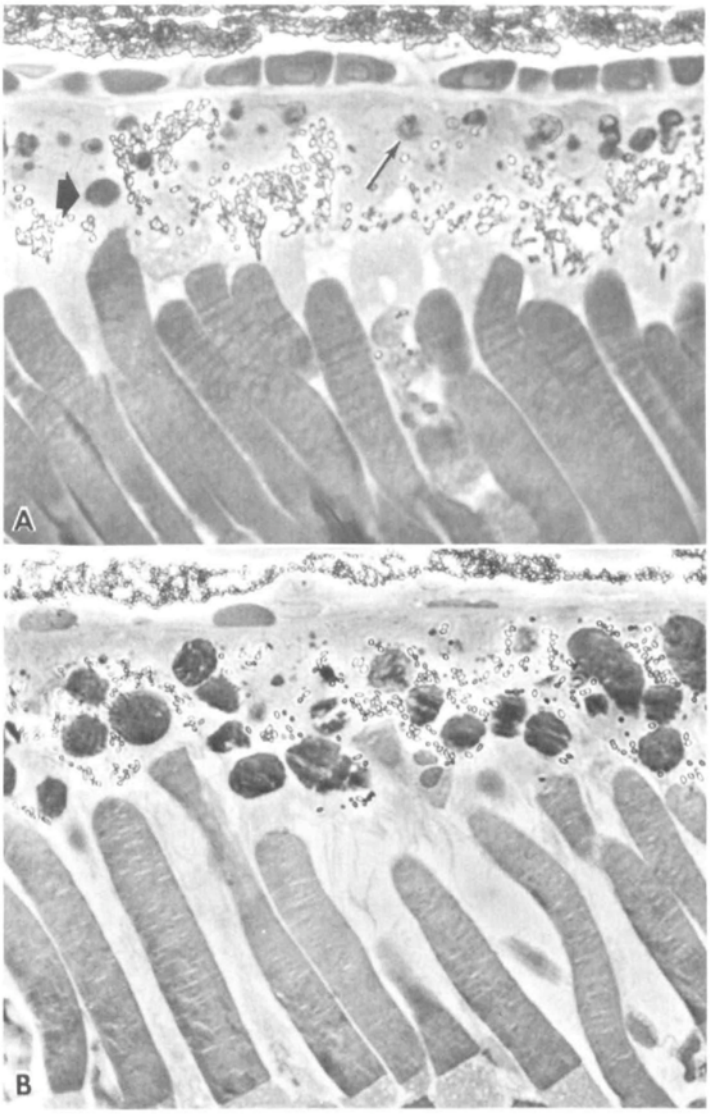

Fig. 1. Light micrographs of *Xenopus* eye explants kept in vitro for 2 hr. **A**, In explants kept in darkness the phagosomes were mainly in the middle to basal zones of the RPE (thin arrow) although some freshly shed phagosomes also occur (thick arrow). **B**, Appearance of the RPE in explants exposed to 5 min of light and fixed 1 hr later. Many large, freshly shed phagosomes appear in the apical to middle zones of the RPE. (×640.)

(pH 7.2) and embedded in Araldite 6005. During the course of this study we found it to be essential to leave the eye intact during incubation in the culture medium in order to prevent detachment of the retina from the RPE.

Results from our earlier attempts to maintain adult *Xenopus* eyes in culture for more than a few hours showed that a simple saline-based culture medium was insufficient. Explanted eyes could, however, be maintained for up to 4 days, with minimal signs of degeneration in either photoreceptor or RPE cells, if kept in sterile Falcon dishes (Falcon Plastics, Oxnard, Calif.) containing a 5 ml mixture of Liebovitz L-15 medium (Flow Laboratories, Rockville, Md.) supplemented with 10% fetal calf serum (GIBCO, Grand Island, N. Y.). The tonicity of the culture medium was adjusted to 200 mOsm by dilution with sterile distilled water. We found it necessary to change the medium when the phenol red indicator incorpo-

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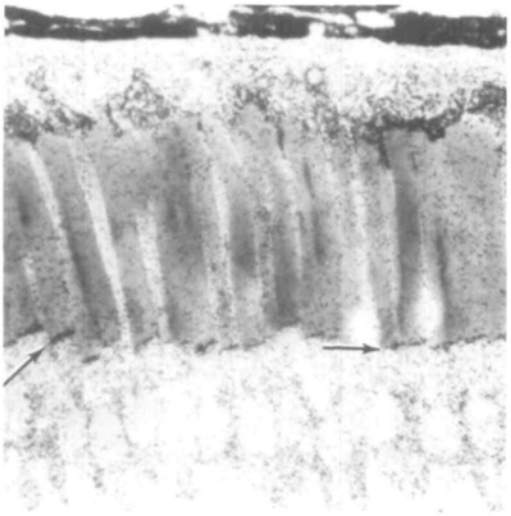

Fig. 2. Autoradiogram from a *Xenopus* eye explant kept for 24 hr in medium containing [^{3}H]leucine and then fixed. A radioactive band (arrows) occurs at the base of each rod outer segment. (×400.)

rated in Liebovitz L-15 indicated that the pH had fallen below 6—otherwise the tissue rapidly degenerated.

To determine whether the photoreceptors in explants were synthesizing new discs and to establish whether the retina had access to amino acids from the culture medium (since the eyes were left whole), we maintained a pair of explants for 24 hr in medium containing 27 μCi/ml 1-[4,5-^{3}H]leucine (Amersham/Searle Corp., Arlington Heights, Ill.). We fixed these explants at the end of the 24 hr period as described above. One-micrometer thick tissue sections on glass slides were coated with Kodak NTB-2 emulsion (diluted 1:1 with distilled water), exposed for 4 weeks at 5° C., and then developed for 3 min in Acufine developer. Autoradiograms were stained with 1% methylene blue in sodium borate.

For each of the four experimental conditions (dark-maintained explants and in vivo eyes, light stimulated explants and in vivo eyes), phagosome counts were made from thirty-three 1 mm lengths of retina containing well-aligned rod outer segments. Sections for phagosome counts were also stained with methylene blue. Thin sections for electron microscopy were placed on formvar-coated single-hole grids and stained with uranyl acetate and lead citrate.

Results. Placing the eyes in culture was not found to alter the general ultrastructural integrity of the photoreceptors or RPE cells. With the criteria of Besharse et al.,[3] we could identify large (4 to 8 μM) and small (1 to 4 μM) phagosomes in each of the eyes examined, although large phagosomes were much less frequent in retinas kept in the dark. Since small phagosomes occur at a relatively constant level in the RPE throughout a 24 hr cycle,[3] we included the entire size range of phagosomes in the total counts. Table 1 shows the average number of phagosomes per millimeter of RPE for each of the four experimental conditions. The nearly identical phagosome counts from the retinas kept in the dark suggested that simply explanting the eye and maintaining it for 2 hr in vitro had little or no immediate effect on disc shedding. In both the explanted and in vivo eyes, however, the number of phagosomes increased dramatically within 1 hr following exposure to a 5 min light stimulus. The difference in the appearance of RPE cells in the explanted eyes kept in the dark and those exposed to light are shown in Fig. 1. RPE cells from both of these conditions contained phagosomes of various sizes, although 1 hr after light stimulation there was an abundance of freshly shed, large phagosomes above the rod tips and within the apical RPE. Phagosomes in the retinas kept in the dark were generally located in the basal portion of the RPE cells. They stained densely with methylene blue and showed ultrastructural characteristics of enzymatic degradation. Thus they may be phagosomes remaining from an earlier shedding event.

Although the autoradiograms from the explants contained a very high level of background labeling in comparison to similar experiments done on intact *Xenopus,* they clearly showed an accumulation of radioactivity as a band at the rod outer segment bases (Fig. 2). The high level of background labeling may be due to the contamination with free [^{3}H]leucine, since explants were kept in labeled medium up to the time of fixation and glutaraldehyde is known to crosslink free amino acids in tissues.[8]

Discussion. The results of this study show the following. (1) Removing the eye and placing it in culture does not, in itself, induce the disc shedding response. (2) A 5 min exposure to light gives rise to an increase in disc shedding from photoreceptors in eyes in vitro and in vivo. (3) Amino acids from the culture medium can enter the excised eyes (by an unknown route). (4) Rod receptors in the explants continue to incorporate amino acids into protein used to assemble new discs.[9]

The first two of these conclusions are compati-

ble with a growing body of evidence suggesting that synchronous disc shedding is initiated within the eye, independent of any central neuroendocrine control. Recent studies by Tamai et al.[4] and LaVail and Ward[10] have shown that pinealectomy, superior cervical ganglionectomy, hypophysectomy, or thyroid-parathyroidectomy does not affect the circadian rhythm of rod disc shedding in albino rats. Currie et al.[6] demonstrated that pinealectomy and frontal organ-pinealectomy also failed to reduce the disc shedding response in *Rana pipiens*. By patching one eye of albino rats and subjecting the animals to constant light (which abolishes the burst of disc shedding), Tierstein et al.[5] show that synchronous disc shedding can occur in one eye independent of the other. The morning burst of disc shedding was abolished in the open eye but not in the patched eye. Since then, Hollyfield and Basinger[7] have shown that in *R. pipiens*, the patching of one eye during the dark part of the cycle is sufficient to suppress the burst of rod shedding in that eye when the animals are exposed to 30 min of light. The shedding response occurs normally in the unpatched eye. Although all these studies, as well as our own, suggest that the control mechanism for initiating disc shedding resides intraocularly, some form of extraocular control may also exist. The original report by LaVail[2] described data showing that reserpine, a drug known to abolish certain circadian rhythms by affecting the pineal gland, blocks the morning burst of rod disc shedding in rats. Our own data suggest that removing the eye from the animal may have the effect of removing some form of extraocular control over the amplitude of the shedding response, inasmuch as the response to a light stimulus in the explants was 24% greater than that induced in vivo.

We found a greater number of phagosomes in our in vivo control animals kept in the dark than has been reported previously for *Xenopus* tadpoles during the middle portion of the dark cycle.[3] It is difficult to determine whether this reflects a difference between *Xenopus* tadpoles and adults, individual variation, or different experimental conditions. An approximation of the magnitude of the morning burst of disc shedding in *Xenopus* tadpoles can be derived from the size-frequency histograms for 0 and 1 hr in Besharse et al.[3] (their Fig. 4), which when compared to the data reported here for light-exposed animals, show a response about 2½ times greater in the tadpoles. A comparison of these results may be complicated by the fact that *Xenopus* tadpoles show an endogenous rhythm of large phagosome production which is apparently synchronized by the onset of light.[3] Our animals received the experimental light stimulus 7 hr before (and were sacrificed 5 hr before) any endogenous peak of shedding would have been expected with our lighting cycle.* Thus it may be that disc shedding can be induced in *Xenopus* by a light stimulus given during the dark period but that the amplitude of the response is reduced because it occurs out of phase with an endogenous rhythm.

The appearance of the radioactive band at the rod outer segment bases in explants labeled for 24 hr is compatible with rod outer segment renewal data in other studies of *Xenopus*.[3, 11] Whether or not disc displacement within the outer segment occurs at the same rate in explanted eyes and intact animals remains to be determined.

The results of our study show that *both* disc shedding and assembly occur in *Xenopus* eyes in vitro. Indeed, it appears that light may act as a more potent stimulus for disc shedding when the eye is removed from some form of extraocular control.

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Key words: photoreceptors, pigment epithelium, disc shedding, tissue culture, autoradiography

*It is important in making this comparison to consider that the animals in the study of Besharse et al.[3] received 60 min of light prior to sacrifice whereas ours received 5 min of light followed by 55 min of darkness.

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Dysgenetic lens (dyl)—a new gene in the mouse.

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A new autosomal recessive gene, dysgenetic lens (dyl), *in the mouse is described. Homozygotes are fully viable and exhibit smaller eye, corneal opacity, adhesion of the iris, cataractous degeneration, and extrusion of the lens nucleus and persistent lens-epithelium attachment. Developmental failure of lens vesicle–ectoderm separation is recognized as the earliest expression of the genetic defect. Possible significance of this mutant in the understanding of Peter's anomaly is indicated.*

Individuals with abnormalities in the eyes appeared spontaneously in the Balb/c line of our mouse colony. Histomorphological observations, developmental studies, and breeding experiments were undertaken to analyze this condition. The findings show that the condition is caused by a new mutation affecting primarily the lens and is inherited as an autosomal recessive trait. In this report, we describe the mutant phenotype, its development, and mode of inheritance. We suggest the gene be designated *dysgenetic lens (dyl).*

Materials and methods. Balb/cLiA mice have been maintained by sib mating under normal conditions of lighting, temperature, and feeding. When individuals with abnormal eyes were first noticed, appropriate matings were set (Table I). All individuals were screened first, at 14 days after birth, when the eyelids have just opened, and again at the time of weaning while under light ether anesthesia, with an ophthalmoscope or a stereo dissecting binocular. Histological observations were made on the normal and the affected eyes after routine procedure. Embryonic stages were obtained by sacrificing pregnant females at known intervals after mating, the day of vaginal plug being counted as day 0. The entire heads were fixed. For postnatal stages, intact eyes were removed from animals at different times after birth.

Table I. Breeding data showing segregation of the dysgenetic lens (dyl) phenotype

Matings	*Number of offspring*	
	Affected	*Unaffected*
Affected × affected	257 (100%)	0
Affected × normal	0	130 (100%)
F_1* × affected	79 (53.4%)	69 (46.6%)
$F_1 \times F_1$	76 (27.3%)	202 (72.7%)

*First generation hybrids.

Results. On superficial examination, most of the affected mice were easily identified by the occurrence of a smaller than normal eye (Fig. 1). This varied between individuals and also sometimes between the two eyes of the same individual. In some cases, the eyes appeared to be of normal proportion, whereas in a few cases the eyelids were completely closed, resembling the condition of anophthalmia, though an eyeball was always present in the orbit.

The affected condition was readily recognized when the mice were observed with an ophthalmoscope or stero dissecting microscope. The pupil was markedly smaller and showed a distorted outline. Pupillary reaction was usually absent. Varying degrees of corneal opacity were encountered and ranged from a few tiny spots in the pupillary area to a completely opaque central cornea. In the older animals, the cornea was often found to be vascularized.

In the sections of the whole eyes (Fig. 2), most pronounced defects were seen in the lens. These were very much reduced in size and irregular in shape. But the striking feature of this anomalous condition was the persistent connection between the lens and the corneal epithelium. The corneal

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