Endogenous Circadian Retinomotor Movements in the Neon Tetra (*Paracheirodon innesi*)

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Retinomotor movements of retinal cones and pigment epithelium melanosomes were studied in the neon tetra, *Paracheirodon innesi*. The cone myoids clearly contracted during the daytime, but the migration of the total population of pigment granules was less easy to see. However, when rod-shaped melanosomes were measured separately from granular-shaped melanosomes it became evident that the rod-shaped melanosomes, but not the granular melanosomes, did migrate in response to circadian changes in light intensity. Retinomotor movements of both the cones and the rod-shaped melanosomes persisted for at least 2 days in continuous darkness. Microspectrophotometric measurements of spectral transmission of small groups of melanosomes showed that absorption was greatest at shorter wavelengths, but that there was also a distinct absorbance maximum at about 480 nm. Invest Ophthalmol Vis Sci 24:1203-1210, 1983

It has been known for more than a century that the migration of dark (melanic) pigment and the elongation of the cone myoids in the retina of many vertebrates responds to light.1,2 Yet even now there is little understanding of the mechanisms of such retinomotor movements, the extent to which they respond to the direct action of light, or show circadian rhythms that persist even in constant darkness.

Walls3 has reviewed the occurrence of retinomotor movements in vertebrate eyes. Both retinal pigment epithelium melanosomes and cones migrate extensively in the teleosts and anurans, there is some movement of both in the reptiles and it is fairly rapid and extensive in the birds. In the mammals no retinomotor movements have been reported.

In this study of the persistence of retinomotor movements in constant darkness by cones and rods and by the melanosomes in the retinal pigment epithelium it became clear that there are two distinct shapes of melanosome in the neon tetra eye, and that one of these types migrate and the other does not. The nonmigrating population of melanosomes outnumber and effectively mask the movements of the migrating population, and this might possibly lead investigators to assume that some animals have no retinal pigment migration when, in fact, they do.

The neon tetra (*Paracheirodon innesi*) was used in these investigations because it is cheap, small, and easy to maintain. It is the subject of an investigation into the sensitivity of the iridophore to the direct action of light,4 and a study of the disruptive effect of constant darkness on the cone outer segments, that will be reported later.

**Materials and Methods**

The neon tetras (*Paracheirodon innesi*) used in these experiments were purchased from local aquarists who had in turn imported them from fish breeders in Southeast Asia. In the laboratory the fish were kept in a 12-hr light-12-hr dark regime at a constant temperature of 25°C. Fishes were maintained under these conditions for at least 28 days before they were used.

Twelve hours before sampling began individual fishes were taken from the experimental tank and placed in 250-ml beakers, one-half filled with water. A single fish was placed in each beaker which was covered with fine netting. Those beakers containing fishes that were to be sampled in the dark were wrapped in aluminium foil. Each beaker was then floated in the experimental tank. This procedure meant that a single fish could be removed for sampling with minimal disturbance to the other fish in the tank. In total there were 12 experimental batches processed and the experiments extended over 2 years. Each experiment point represents the results from a single fish, but overall one sample was taken at least within each 60-min period and usually much more frequently. As was practical sampling times were arranged so that the results from one batch were confirmed directly by the results of at least one subsequent batch.
Refraction Index Measurements

Fish were decapitated and the eyes removed in freshwater teleost ringer. A piece of retina was dissected out and teased apart on a microscope slide in one of the following sucrose solutions: 75%, 67.5%, 60%, 52.5%, and 45% w/v, which have refraction index values of 1.48, 1.46, 1.44, 1.425, and 1.41, respectively. Each preparation was covered by a coverslip and viewed under oil immersion using an Olympus H1OO/1.30 oil immersion objective with the diaphragm open.

Microspectrophotometry

Retinal smears were prepared in the same way as above, but were mounted in glycerine (refraction index 1.47). Measurements were made in the Department of Zoology of Queen Mary College, University of London by Dr J. K. Bowmaker. The microspectrophotometer has been described by Knowles and Dartnall, and has been recently improved. An individual melanosome is too small to be measured singly so a clump of melanosomes was measured. Therefore, it is difficult to estimate the optical density of an individual melanosome.

Electron Microscopy

The techniques used were conventional. Whole eyes were fixed in 5% glutaraldehyde in 0.1 M phosphate-phosphate buffer pH 7.4. Eyes from animals that had been killed during a period of illumination were fixed in the light; eyes from animals that had been in darkness were removed in dim light, but were fixed in darkness. After at least 2 hrs fixation the retina was gently dissected from the eye cup, washed in 0.1 M phosphate-phosphate buffer, pH 7.4, and postfixed in 1% osmium tetroxide for 1 hr. The retinas were dehydrated in a series of alcohols and propylene oxide and embedded in Araldite. Radial sections were cut on a Porter Blum ultramicrotome, stained in uranyl acetate followed by 0.2% lead citrate, and viewed on a Philips TEM 300.

Measurements of Retinomotor Movements

Electron micrograph prints were used for these measurements. A rectangular area of retina between the level of Bruch's membrane on the scleral side and the outer limiting membrane on the vitreal side was divided transversely into 10 equal sections, each section being approximately 63 µm × 13 µm. The number of rod-shaped and granular melanosomes was counted within each section, each rectangular area contained at least 500 granular and 100 rod-shaped melanosomes. The percentage of each type within the total sampled area of retina was calculated. The position of the base of at least 10 rod and 10 cone outer segments in the transverse section of retina was also recorded and plotted accordingly.

Results

When a piece of retina is teased apart in physiologic saline and viewed under a high power light microscope, both granular and rod-shaped melanosomes are visible. The granular melanosomes are spherical and have an average diameter of 0.3 µm, SD 0.034. The rod-shaped melanosomes also have a diameter of 0.3 µm, SD 0.01, but have an average length of 2.68 µm, SD 0.63. Under the electron microscope the granules appear to be composed of two concentric spheres, with most of the electron dense pigment deposited between the spheres. Many of the granules have a hollow core (Fig. 1). The rod-shaped melanosomes appear not to have a hollow core, but in longitudinal section appear as if they are slightly segmented transversely (Fig. 2).

When mounted in physiologic saline or water both types of melanosomes appear dark. However, they are highly refractive and act as minute lenses making their true color difficult to discern. Mounted in a higher refractive index (RI) medium such as glycerol or sucrose solutions, the lens effect is largely cancelled and both types take on a reddish-brown color. When viewed by superior illumination against a dark background the needles appear blue-green in color but are not nearly bright enough to suggest that the color by transmitted light is really a structural color. Mounted in a series of sucrose solutions of graded strength it was found that the solutions between 60% w/v, (RI 1.44), and 75% w/v, (RI 1.48) were most successful at cancelling the lens effect of the melanosomes, and, therefore, most closely approximate to their RI. The most effective concentration was, in fact, 67.5% w/v, which has an RI of 1.46.

The spectral transmission of a clump of rod-shaped melanosomes is shown in Figure 3. There is a general increase in spectral absorbance with decreasing wavelength, and there is a marked maximum in spectral absorption at about 480 nm.

Melanosome Migration

Superficial observation of the EM and LM material showed very little difference in the position of the melanosomes between day and night (Fig. 4). The granules outnumber the rod-shaped melanosomes by about 5 to 1, and it is evident that when the two types are considered separately, the rod-shaped melanosomes migrate and the granules do so only slightly or not at all. The very slight indication of migration...
Fig. 1. Granule-shaped melanosomes in the retinal pigment epithelium. These melanosomes frequently have an empty core in these preparations. In some examples two concentric membranes are visible and the dense staining material is deposited between them (bar equals 300 nm).
Fig. 2. Rod-shaped melanosomes lying alongside a cone. Note that these melanosomes appear to be transversely segmented (bar equals 500 nm).

of the granules in the control period (Fig. 5, upper diagram) should be treated with caution. A needle cut transversely would resemble a granule, though a granule could never resemble a needle, however it was cut. The granules remain concentrated in the sclerad part of the retina, whereas in the daytime the rod-shaped melanosomes move vitread to sheath the cone outer segments and ellipsoids. The movement is reversed at night. In continuous darkness a circadian rhythm persists for at least 48 hrs, which was the duration of the experiment (Fig. 5, lower diagram). In this "free running" mode the circadian migration of all the rod-shaped granules away from the sclerad part of the retina was less complete in constant darkness than in the normal light-dark regime.

Photoreceptor Elongation

The elongation and contraction of the cone and rod myoids by day and night, and when free running in constant darkness are shown in Figure 6. A strong circadian rhythm persists for at least 48 hrs in constant darkness with little apparent change in the timing of the movements. During the initial 48 hrs of the experiment, the bases of the cone outer segments were located within a band representing 10% of the width of the retinal pigment epithelium. By the final 12-hr period the positions of the cones had become less regular and they were grouped within a band 20% of the retinal pigment epithelium width.

The elongation of the cone myoids does not begin until about 1 hr after the light goes off at "dusk," and contraction is completed about 1 hr before the light comes on again at "dawn." The rod-shaped melanosomes, however, begin their migration almost at the same time as the light comes on and goes off. There is about 1 hr before dawn, and a similar period after dusk when the cones are in their daytime position, but are not shrouded with rod-shaped melanosomes.

Discussion

In 1907 Raehlmann reported that both the granular and rod-shaped melanosomes occurred in the retina of a 7- to 8-month-old human fetus. Later electron microscope studies show elliptical human melanosomes. In the adult human eye there are also numerous lipofuscin granules, that resemble melanin granules, but which are somewhat less osmiophilic and are autofluorescent. Murray and Durbin and Epiggi showed granular and rod-shaped melanosomes in the frog. Two types of melanosomes also occur in fishes with the rods lying vitread to the granules. Walls mentions that two types of melanosome are to be found in the lower vertebrates and, presumably referring to his own work, states that the rodlet melanosomes migrate, whereas the granules do not. Klyne and Ali had noted that rod-shaped melanosomes occupied a position around the cones in light-adapted brook trout, but attributed this to a transformation of granules into rodlets, rather than a migration of the rodlets but not of the granules. Since we find that in the neon tetra the number of rodlets relative to granules is the same by day and night, we think that Walls' migration explanation is the more likely.

The study of retinomotor movements has a venerable history; the phenomenon was discovered independently in 1877 by Boll and Kühne, and in 1915 Arey was able to devote a substantial review to the subject. The phylogenetic distribution of the phenomenon has been reviewed by Walls and Wagner.

There is no doubt that the cone myoids elongate at night and contract in the daytime, and that in species of amphibians and fishes melanosomes migrate from a position close to the sclera at night to a more vitread position in the daytime. These movements are initiated by the presence of light, and in at least some fishes the circadian rhythm persists (free runs).
in constant dark. Free running retinomotor movements of cones have been reported in the catfish, *Ameirus*,22,23 in the goldfish, *Carassius auratus*24 and *Cichlosoma*.25 Ali26 looked for the phenomenon in *Salmo salar* but could only show free running for the first 24 hrs of constant darkness.

Although free running of cone elongation has been demonstrated with reasonable certainty, free running of retinal melanosome migration is not so well established. Douglas27 has shown a persistent circadian rhythm in total darkness in rainbow trout, *Salmo gairdneri*, that shows marked but transient retinom-

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**Fig. 4.** The migration of both rod-shaped and granular-shaped melanosomes in the neon tetra retina. For each experimental animal the retina was divided into 10 equal sections between the outer limiting membrane (OLM) and Bruchs membrane (BM). The relative number of melanosomes in each section are expressed in the form of a kite diagram each kite representing a single fish. The first 24-hr period represents a normal 12-hr light, 12-hr dark period. The subsequent 48-hr period was total darkness. The arrows represent the exact time that the lights were programmed to switch on or off. There is little more than a hint of melanosome migration when data from both rod-shaped and granule-shaped melanosomes are expressed together, because there are numerically five times more granule-shaped melanosomes than rod-shaped melanosomes.

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**Fig. 5.** Migration of granule-shaped melanosomes (upper diagram) and rod-shaped melanosomes (lower diagram) in the retina. Data expressed as described for Figure 4. The rod-shaped melanosomes show marked migration across the retinal pigment epithelium, whereas the granule-shaped melanosomes do not. Although endogenous pigment migration clearly exists in constant dark, it is less complete than in the normal 12-hr light, 12-hr dark regime.
otor movements at times that correspond to dawn and dusk. However, Ali26 noted free running in *Salmo salar* for 1 day only in total darkness, and Olla and Marchioni28 reported no free running of pigment migration in *Pomatomus*. Our own initial observation was that pigment migration was slight or absent in the neon tetra (Fig. 4), and it was not until we differentiated between the granular and rod-shaped melanosomes that it became evident that the rod-shaped melanosomes migrated and showed free running in continuous darkness.

Although it is the rodlets rather than the granules that migrate in the retina of the neon tetra, granular melanosomes are known to migrate in dermal melanocytes in the lower vertebrates, and so do the granular melanosomes in the occlusable tapeta of some fishes.29 It is, therefore, unlikely that the mechanism of retinal pigment migration requires the melanosomes to be rod shaped. It seems more likely that the rod shape is in some way important in the optical arrangements in the retina. It is interesting that a report in the older literature4 shows rod-shaped melanosomes congregated in that part of the pigment epithelium where the receptor outer segments are located. In daylight the cones are retracted and sheathed with rod-shaped melanosomes. This is thought to have two functions. First to reduce the intensity of light that reaches the rods, and secondly to prevent light that is guided up one cone from leaking across to neighboring cones.30,31 The refractive index and the spectral adsorption of the rod-shaped melanosomes are optical properties that are relevant to these two functions. In this investigation we have found that the RI of the rod-shaped melanosomes is between 1.44 and 1.48, and our best estimate is 1.46. By way of comparison Land32 gives typical RI values of 1.33 for cytoplasm, 1.56 for dry protein such as keratin or chitin, and 1.83 for guanine. A refractive index of about 2.0 is quoted for dry melanin. Our value for the refractive index of 1.46 for the melanosomes means that the melanin (if that indeed is what it is) is mixed with substances of lower refractive index such as water. In any event it is clear that the melanosomes have a substantially greater refractive index than the cytoplasm that surrounds them.

The relatively high refractive index of the melanosomes creates problems in the measurement of their spectral transmission, and it is necessary to reduce the difference in the refractive index of the melanosome and the material that surrounds it. Our measurements, which were done in this way, show a maximum in spectral absorbance at about 480 nm and a further increase at shorter wavelengths. Previous microspectrophotometric measurements by Pakkenberg33 and Jensen34 for melanin-containing tissues from various sources, including the eye, show a maximum at about 430 nm and no further increase in absorption at wavelengths shorter than this. Nickerson35 who extracted melanin from red chicken breast feathers with 0.1 N HCl found a maximum in the absorption curve at 535 nm. On the other hand measurements of the spectral absorption of whole pigment epithelium36,37 show a gradual increase in absorption towards shorter wavelengths with no maximum between 400 and 700 nm. Perhaps the rod-like shape of the migrating melanosomes, and their spectral absorbance which selectively absorbs short-wavelength light helps channel the image forming light along the visual pigment containing outer segment of the cone and prevents stray light, and scattered light from leaking between receptor cells. It should, however, be mentioned that retinal melanins may have functions other than a screening pigment and the absorption of scattered and reflected light within the eye. La Vail38 has reviewed evidence that melanin acts as a free radical sink in the pigment epithelium,
and for this role it is important for the melanosomes to be located near the photoreceptor outer segments.

Key words: melanosomes, retinomotor movements, retinal cones, dark adaptation, circadian rhythms, neon tetra, spectral absorption, pigment epithelium

References