

## **Disc Shedding and Autophagy in the Cone-dominant Ground Squirrel Retina**

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The temporal pattern of cone outer-segment disc shedding was examined in the retina of the California ground squirrel, *Spermophilus beecheyi*, under two different lighting conditions. Squirrels were entrained to either 10–180 lx (room lighting) or 1400 lx. Cone shedding during the dark period was biphasic in both conditions, with increases occurring at 2–3 hr and 5–6 hr after light offset. Entrainment to 1400 lx resulted in an increase in shedding at 2 hr after light offset and a slight advance in the timing of both peaks.

Dense granules were often found in photoreceptors, retinal neurons, Müller cells, microglia and vascular cells. These granules, which were found primarily during the dark period and early light period, were lipofuscin-like in their lipophilia, size and autofluorescence. Many of the granules were probably autophagic in origin, but some within Müller cells may have originated via endocytosis of extracellular material which was exocytosed by photoreceptors.

*Key words:* disc shedding; autophagy; cone; lipofuscin; retinal pigment epithelium; ground squirrel.

### **1. Introduction**

The light-sensitive organelle of the vertebrate photoreceptor, the outer segment, undergoes a diurnal turnover of its membranous discs (recent review by Besharse, 1982). A peak in rod disc-shedding activity, as determined by counts of phagocytosed outer-segment disc packets within the retinal pigment epithelium (RPE phagosome), occurs within a few hours after light onset in a number of mammalian and non-mammalian species (Basinger, Hoffman and Matthes, 1976; LaVail, 1976a,b; Besharse, Hollyfield and Rayborn, 1977; Young, 1978; O'Day and Young, 1978; Tabor, Fisher and Anderson, 1980; Herman and Steinberg, 1982; Huang, Spira and Wyse, 1982).

In contrast, cone disc shedding in non-mammalian species predominates 1–4 hr after light offset (Young, 1977, 1978; O'Day and Young, 1978; Bernstein, Breeding and Fisher, 1984). In mammals, the peak of cone disc shedding, or at least significant levels of cone shedding, occurs later in the dark period (tree squirrels: Tabor et al., 1980), during the light period (tree shrews: Immel and Fisher, 1985; cats: Fisher, Pfeffer and Anderson, 1983) or perhaps at both times (rhesus monkeys: Anderson, Fisher, Erickson and Tabor, 1980). Thus, the temporal pattern of mammalian cone disc shedding is not as uniform as it is for rods for, as yet, unknown reasons. It is possible that the different patterns of cone disc shedding reflect underlying differences in cone physiology or in experimental parameters. Since the mammalian studies utilized lower light levels during entrainment than did the non-mammalian studies, differences

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in entraining light intensity potentially could explain the reported differences in cone disc-shedding patterns.

Ground squirrels are a family of rodents with cone-dominant retinas, and as such have proved to be valuable in a variety of studies of cone physiology. In this study, we first examined the temporal pattern of disc shedding throughout the light-dark cycle in a group of California ground squirrels entrained to room light (10–180 lx). In order to determine whether the entraining light intensity could influence this pattern, we then performed a similar study using squirrels entrained to 1400 lx. Preliminary findings from the first study have been reported (Fisher, Long and Anderson, 1983).

## 2. Materials and Methods

Fifty-eight adult and four yearling California ground squirrels, *Spermophilus beecheyi*, of both sexes were trapped locally and housed individually. Squirrels were maintained under a 12 hr light: 12 hr dark regimen for a minimum of 4 weeks (2 months average) prior to each experiment. Light onset is designated as 0000 hr, light offset as 1200 hr. Room illumination (300–380 lx measured in front of the cages) was provided by overhead fluorescent bulbs; cage illuminance (measured a few centimetres above the cage bottom) varied from 10–30 lx in bottom tier cages to 180 lx in the upper-tier cages located directly below ceiling fixtures. In experiments using higher light intensity four 36 W fluorescent bulbs and diffusing panels were placed above two neighboring pairs of the cages; onset and offset of these lamps were synchronized with room lights. Illuminance in these cages (measured at the base of the running wheels) was 1400 lx. Forty squirrels were entrained to cyclic light of 10–180 lx, 22 squirrels to 1400 lx. Entrainment to 1400 lx did not result in retinal damage (squirrels have highly pigmented eyes). We occasionally identified squirrels with disrupted outer segments and apparently dystrophic RPE cells—five such squirrels were excluded from this study.

Each squirrel was anesthetized with an intraperitoneal dose of pentobarbital and then intracardially perfused with 200–300 ml of 1% paraformaldehyde, 1% glutaraldehyde in 0.086 M phosphate buffer (room temperature, pH 7.1, 0.05% CaCl<sub>2</sub>). In some perfusions, 0.1 mM phenylmethylsulfonyl fluoride was present in the fixative and it appeared to improve the quality of the fixations. The squirrels perfused during the dark period and at light onset were handled with the aid of dim red light, and their eyes were shielded from room light during the perfusion. The shield was removed immediately before perfusion from those animals perfused at light onset. Just prior to perfusion, the eyes of the squirrel perfused at 1200 hr were shielded.

Eyes were removed after perfusion and hemisected. The eyecups were placed in cold fixative, where they remained for 5–18 hr before being rinsed in phosphate buffer, divided into quadrants, and then post-fixed for 1.5–2 hr with buffered 2% OsO<sub>4</sub> (4°C). Following osmication, quadrants were rinsed in distilled water, dehydrated in a graded ethanol-water series, transferred through propylene oxide, and embedded in Araldite 6005.

For fluorescence microscopy, right superior quadrants of some eyes were fixed in the aldehyde fixative but were not post-fixed in OsO<sub>4</sub>. The tissue specimens were then dehydrated as described above, and embedded in Spurr's medium. Unstained sections (1–2 μm) were illuminated with transmitted light from an HBO 200 W/4 super-pressure mercury lamp, filtered with BG 38 and BG 12 exciter filters. Sections were viewed and photographed through a 530-nm barrier filter.

For phagosome counts, 1-μm sections were taken from the posterior pole—within 1–2 mm inferior to the central streak of high photoreceptor density (Long and Fisher, 1983). Sections were stained with saturated *p*-phenylenediamine (ppda) followed by a mixture of Toluidine Blue and Azure II in sodium borate. All light-microscopic counts and measurements were performed at a magnification of 1250 (phase 100 objective). Phagosomes were counted in a minimum of 10 mm of RPE adjacent to well-aligned photoreceptors. One section per slide was used for phagosome counts (three to seven slides per timepoint); each section counted was separated from its nearest neighbor by at least 3 μm in order to prevent duplication of

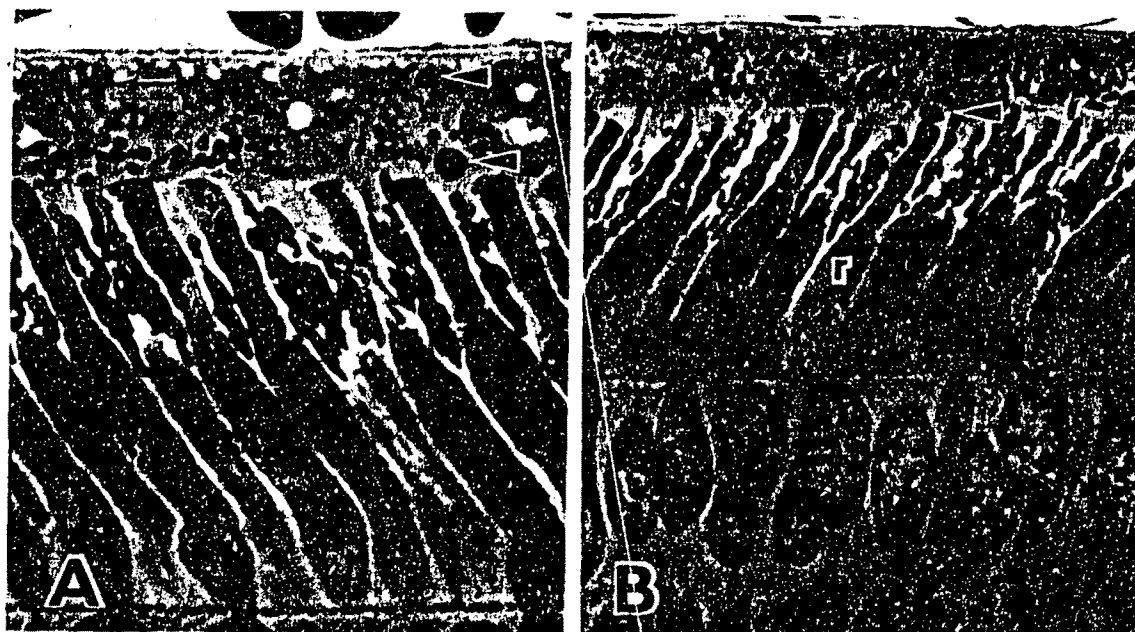


FIG. 1. Light micrographs of photoreceptors and RPE of the California ground squirrel. A, A recently shed phagosome in the apical RPE and an older phagosome in the basal RPE (arrowheads) can be identified by the similarity between their staining characteristics and those of outer segments. Melanin granules (arrow) were often found in the RPE cell bodies.  $\times 1890$ . B, A recently shed phagosome (arrowhead) is present at the distal tip of a rod (r) outer segment. Ground squirrel rods have somewhat longer and narrower outer segments than cones and their nuclei are within the vitreal ONL tier.  $\times 945$ .

counts. Using phase microscopy, phagosomes were identified as dark-staining, non-iridescent RPE inclusions greater than  $0.75 \mu\text{m}$  in diameter.

Small, ppda-positive granules in the RPE and sensory retina were counted in sections used for phagosome counts. For each timepoint, 10  $73\text{-}\mu\text{m}$  lengths of retina were sampled and the number of granules was counted. Small granules ( $<0.5 \mu\text{m}$ ) in the photoreceptor myoid region and in the nuclear layers were difficult to quantify, so they were qualitatively rated. Granules near the outer limiting membrane (OLM), apparently in Müller apical processes, were counted separately from myoid and outer nuclear layer (ONL) granules.

Thin sections for electron microscopy were placed on mesh grids, and stained with 1% uranyl acetate followed by lead citrate.

### 3. Results

Phagosomes, which stain sufficiently more darkly than outer segments to permit their identification and quantification, were found throughout the RPE (Fig. 1A). Similarly dark-staining inclusions that measured less than  $0.75 \mu\text{m}$  long were considered residual bodies and were not quantified. Except in one squirrel (discussed below), rod phagosomes (Fig. 1B) were rarely observed and were included with the RPE phagosome counts. Melanin granules within RPE cell bodies were quite abundant in some squirrels killed during the dark period (Fig. 1A), but were sparse in the squirrels killed during the light period.

*Entrainment to 10–180 lx*

In squirrels entrained to room light, a low level of disc shedding was present from 1 hr after light onset to 1 hr after light offset (0.3–1.6 phagosomes per mm RPE) (Fig. 2). Phagosome numbers increased at light onset, 2–3 hr after light offset and 5–8 hr after light offset, while somewhat elevated levels of disc shedding occurred a few hours before light onset and shortly after light onset. At light onset, one squirrel had numerous phagosomes (13.9 per mm RPE, primarily cone phagosomes), while the other had considerably fewer phagosomes (3.7 per mm), a level similar to the four other squirrels killed within 1 hr of light onset. No definite peak in rod shedding could be discerned.

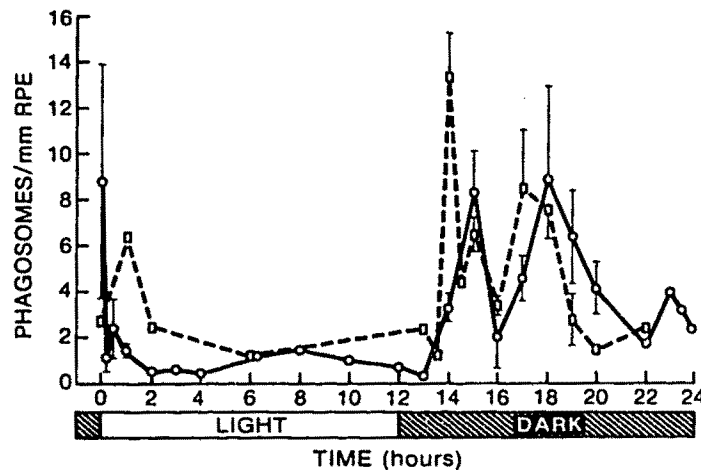


FIG. 2. The temporal patterns of disc shedding in the two lighting conditions: 10–180 lx (circles, solid line); 1400 lx (squares, dashed line). Each point without an error bar represents the mean number of phagosomes per millimeter of RPE in a single squirrel; each point with an error bar represents the mean [ $\pm 1$  s.e.(M.)] of two to four squirrels.

The first increase in cone disc shedding during the dark period was found at 1400 hr, at which time over 70% of phagosomes were in the apical RPE, i.e. recently shed. At 1500 hr the number of phagosomes varied considerably—3–13 per mm RPE in the four squirrels examined. The two squirrels housed in upper-tier cages (160–180 lx) had a mean of 11.4 phagosomes per mm RPE, whereas two squirrels housed in lower-tier cages (10–30 lx) had a mean of 5.3 phagosomes per mm RPE. A second phase of cone disc shedding was evident during the mid-dark period. The number of phagosomes was low at 1600 hr and increased at 1700 hr, at which time phagosomes again were located primarily in the apical RPE. Shedding 'peaked' at 1800 hr, although one squirrel had few phagosomes (1.2 per mm RPE). The only notable difference between this and the other squirrels examined in the mid-dark period was that it was kept in captivity for the longest period of time (13.5 months). At 1900 hr the level of disc shedding was still elevated and the majority of phagosomes were in the mid and basal RPE. By 2000 hr the phagosome number returned to a level which was only slightly greater than that in the light period (1.8–4 phagosomes per mm RPE).

*Entrainment to 1400 lx*

In squirrels entrained to 1400 lx the phagosome number was low at light onset, equivalent to that found 2 hr earlier (Fig. 2). The small peak of disc shedding at 0100 hr was probably due to rod shedding, since the majority of apical phagosomes were identified as rod phagosomes and many short rod outer segments were also present. Phagosome numbers were low at 0200 hr and at 0600 hr. A low level of shedding was also found at 1300 hr and 1330 hr.

An increase in phagosome number occurred at 1400 hr and was significantly greater ( $P < 0.05$ , Student's *t* test) than at the 10–180 lx entrainment intensity. Phagosome number was lower at 1430 hr and 1500 hr than at 1400 hr. Phagosomes in the two squirrels killed at 1500 hr were similar in number, but differed somewhat in their location within the RPE. In one squirrel, only 8 of 58 phagosomes were in the apical RPE while, in the other, 35 of 72 were in the apical RPE, suggesting that shedding in these animals was well under way. Overall, there was 50% more shedding at 1400 hr–1500 hr in 1400 lx than in 10–180 lx, but the only statistically significant difference was found between squirrels entrained to 10–30 lx vs. 1400 lx. At 1600 hr disc shedding was low in both squirrels examined.

As in the squirrels entrained to room light, an increased level of shedding was evident during the middle of the dark period (1700 hr–1800 hr). At 1900 hr and 2000 hr phagosome number decreased, and was lower than that found in the group entrained to room light. We did not test for a statistical difference between the two entrainment conditions at these times due to the small number of animals used.

*Autophagic granules*

In many squirrels, particularly those killed during the dark period, small granules were present in photoreceptors and the inner retina (Figs 3–6). These inclusions were similar to RPE lipofuscin granules in their size (0.2–1.5  $\mu\text{m}$ ), lipophilia (both were ppda-positive), and autofluorescence (Fig. 4A). They were located primarily in the myoid region of photoreceptors and were frequently found near the OLM, apparently within the apical portions of Müller cells (Figs 3 and 4A). These granules were membrane-bound and often associated with lysosomes (Fig. 4B) and cisternae of endoplasmic reticulum (Fig. 4C). Based on the above characteristics these granules can be classified as condensed, late-stage autophagic vacuoles. Their presence in the apical portion of Müller cells was verified in thin sections (Fig. 4C and D). Dense granules were also observed in microglia of the outer plexiform layer (OPL) and inner plexiform layer (IPL) (Fig. 6) and rarely in pericytes and capillary endothelial cells of the inner retina. Microglia of the OPL were infrequently observed (0.5–1.5 per mm). Preliminary counts indicated that 30–70% of microglia contained granules, but no definite diurnal variation was found.

Membranous whorls and vesicular debris were also present within photoreceptors (Fig. 4B) and were occasionally found between photoreceptors and Müller cells (Fig. 5A, B). In a few squirrels killed during the dark period in both lighting conditions, similar debris was present in the extracellular space between photoreceptors (Fig. 5C–E).

The number of autophagic granules within retinal cells varied throughout the light–dark (LD) cycle. In both groups of squirrels the number of granules within the neural retina was generally low from 1 hr after light onset to 1 hr after light offset, while numerous granules were present in many animals during the dark period after

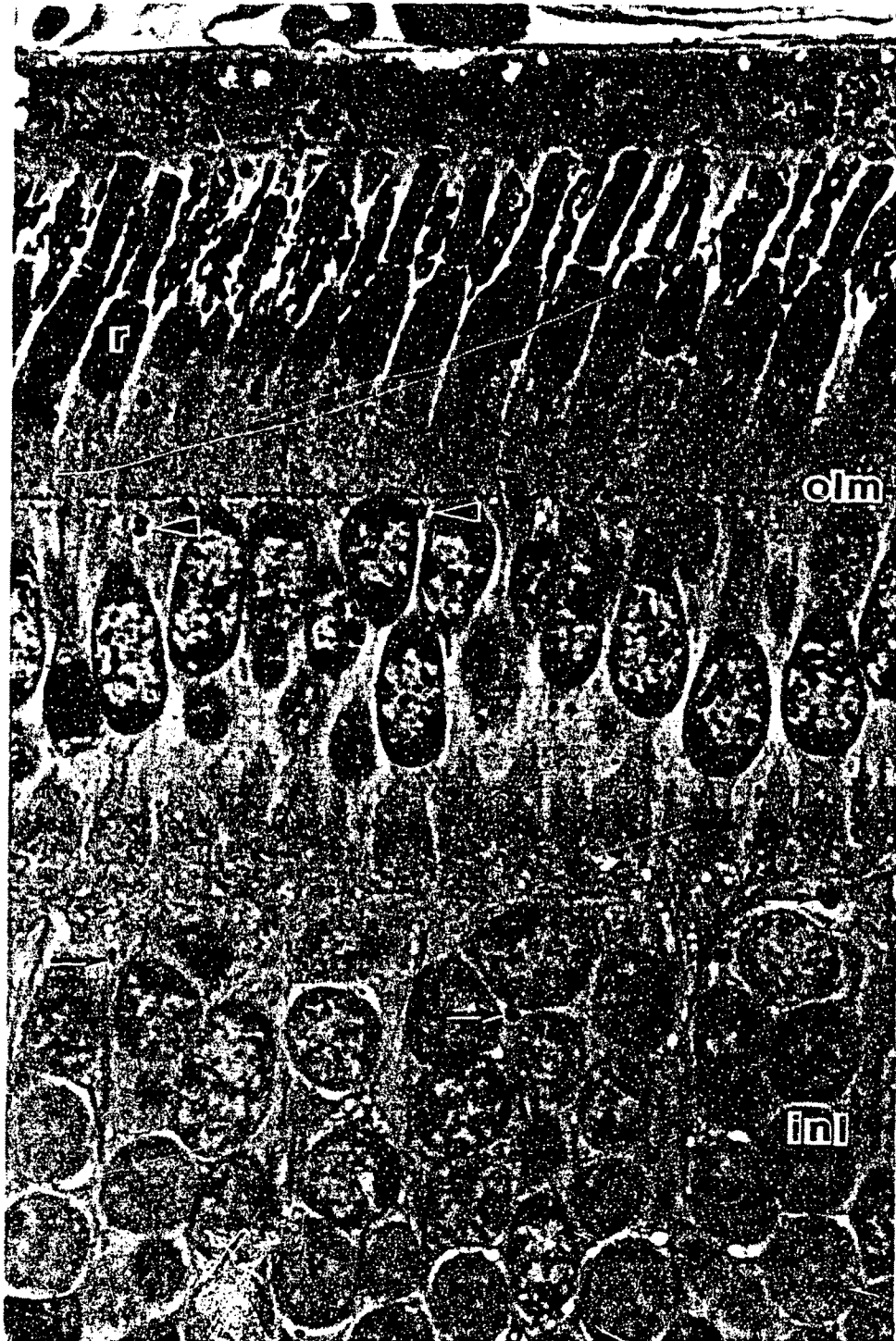


FIG. 3. Light micrograph of the outer portion of a ground squirrel retina (1500 hr, 160–180 lx). Note the numerous granules in the cone myoids, while none are visible in a rod (r). Granules (arrowheads) are present near the outer limiting membrane (olm), apparently in the apical portion of Müller cells (electron-lucent areas adjacent to the olm). Granules (arrows) are also present in the inner nuclear layer (ini) between bipolar nuclei and adjacent to horizontal cells.  $\times 1575$ .

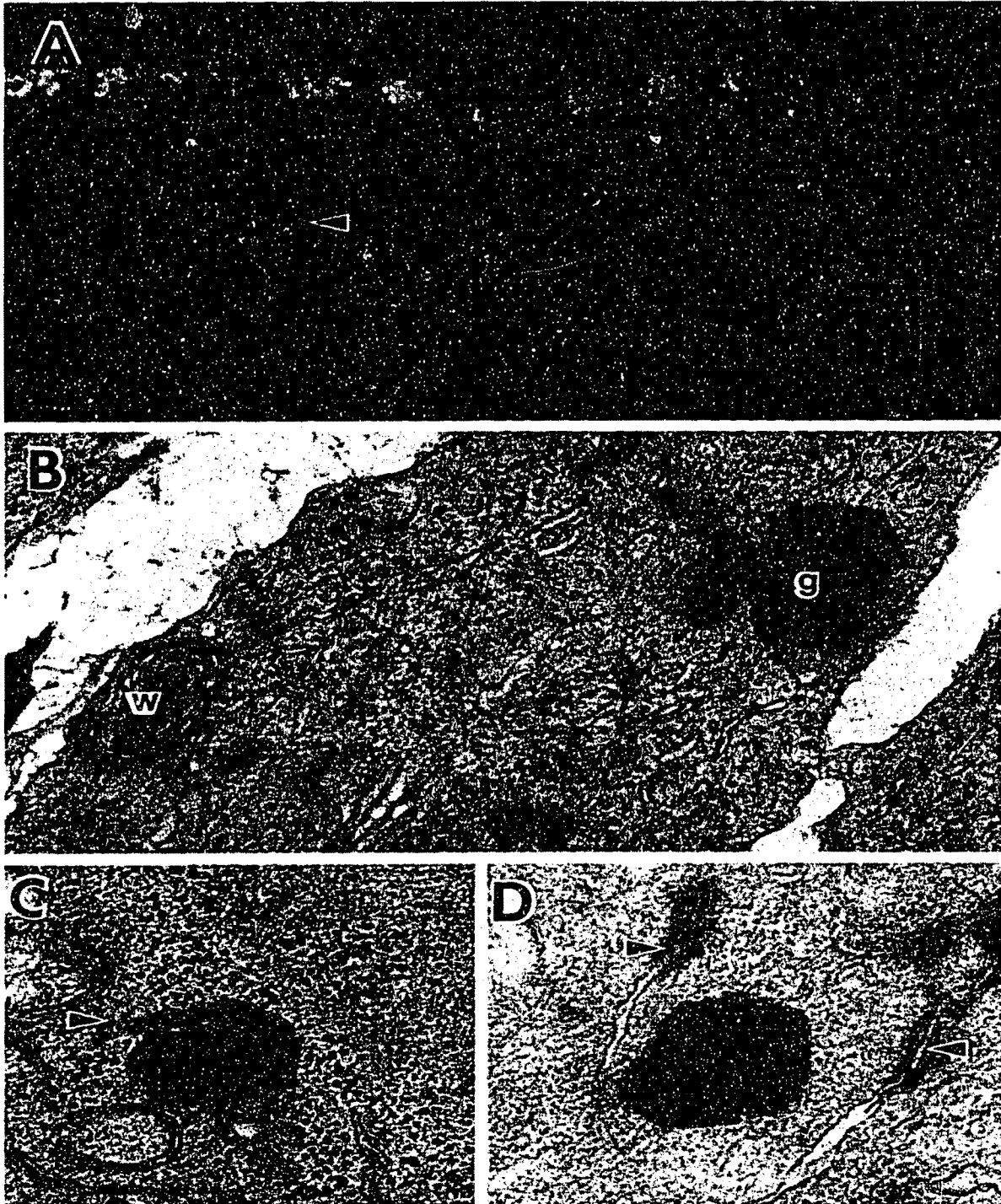


FIG. 4. A, Fluorescent micrograph of the outer retina (1800 hr, 10–180 lx). The RPE contains numerous autofluorescent lipofuscin granules. Lipofuscin-like granules are also present within photoreceptor inner segments and at the level of the olm (arrowhead).  $\times 600$ . B, Electron micrograph of membranous whorl (w) and dense granule (g) with associated lysosome within cone myoid.  $\times 19500$ . C, Dense granule with associated endoplasmic reticulum (arrowhead) is within the apical cytoplasm of Müller cell.  $\times 48000$ . Note the numerous glycogen granules indicative of Müller cells. D, Dense granule with lamellar structure is located at the level of the olm (arrowheads) within Müller cell apical cytoplasm. This inclusion was within cytoplasm which was confluent with Müller apical processes.  $\times 48000$ .



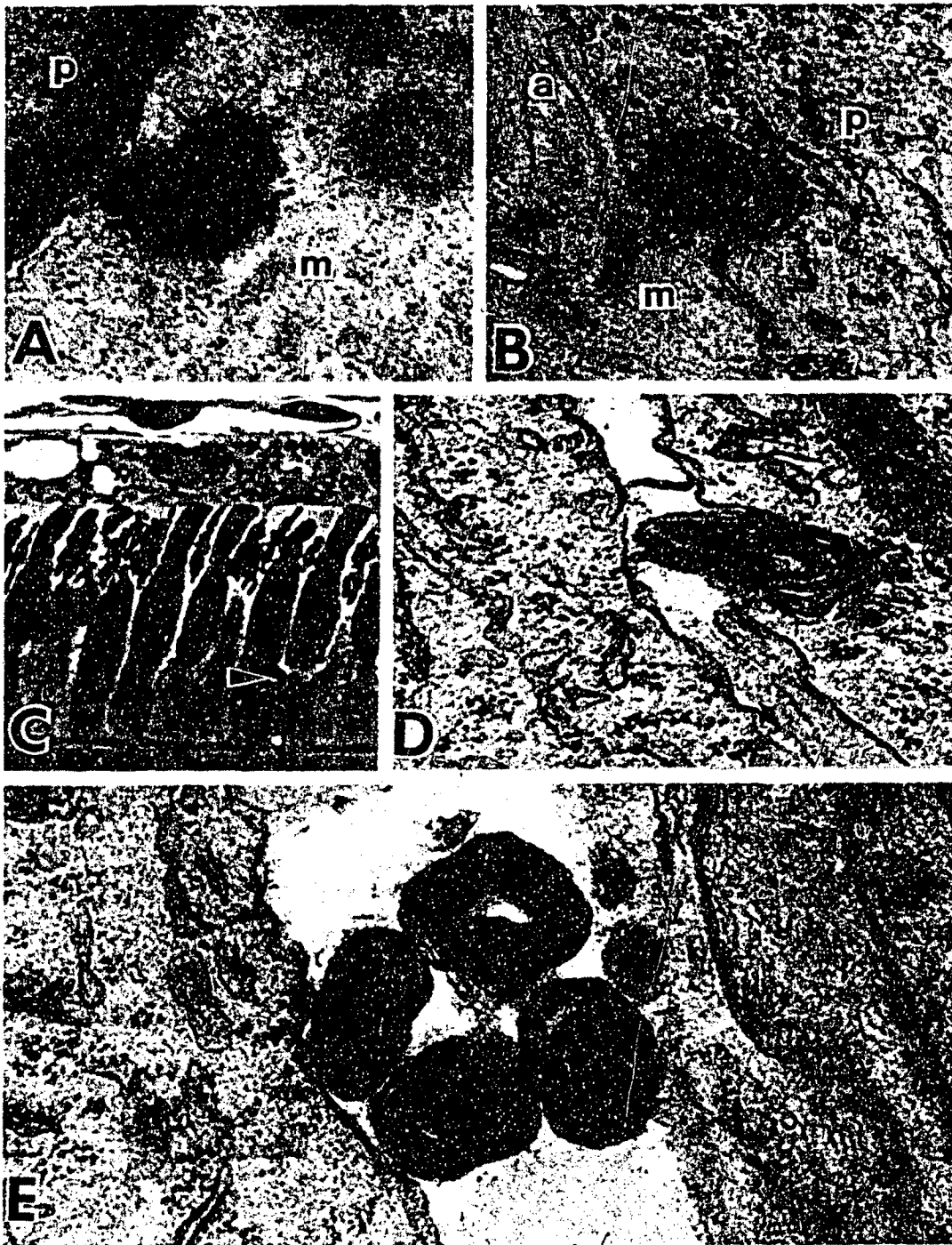


FIG. 5. Membranous intracellular and extracellular debris. A, A dense membranous whorl is present within the electron-lucent cytoplasm of a Müller cell (m), adjacent to a photoreceptor nucleus (p).  $\times 30000$ . B, A membranous whorl is situated between a photoreceptor myoid (p) and a Müller cell (m) near the apical microvilli (a).  $\times 20000$ . C, Light micrograph of extracellular debris between cone myoids (arrowhead).  $\times 945$ . D, E, Extracellular membranous whorls, such as are seen in this electron micrograph, were found between photoreceptor inner segments of some dark-adapted squirrels.  $\times 48000$ .



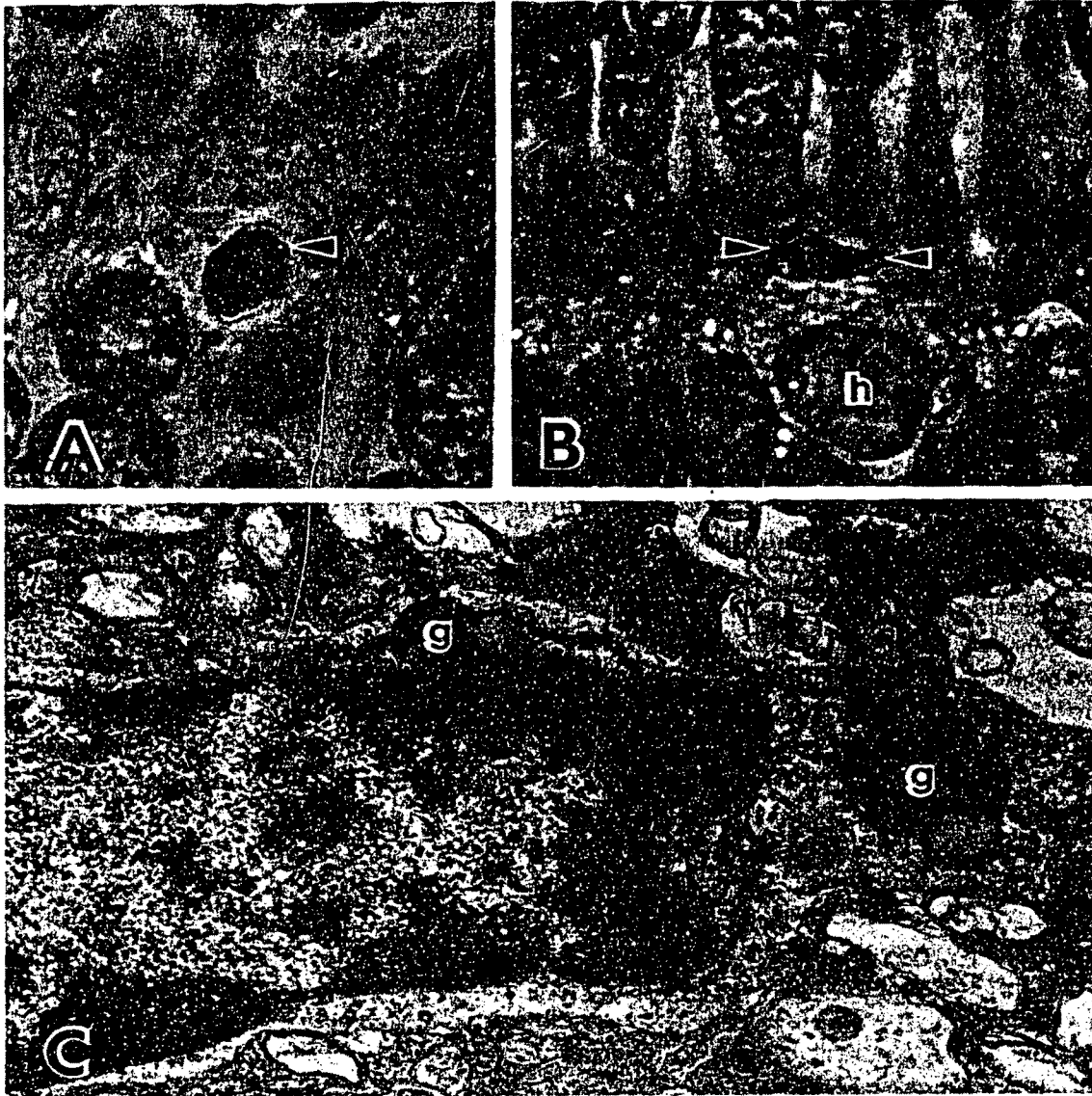


FIG. 6. Small dense granules were found in what are tentatively identified as microglia of the opl (A, B) and ipl (C). A, A microglial cell can be distinguished from cells of the onl and inl by its small size and heterochromatic pattern. A small granule (arrowhead) adjacent to a microglial nucleus can be seen in this light micrograph.  $\times 1890$ . B, A microglial cell with two perinuclear granules (arrowheads) is within the opl, just sclerad to a horizontal cell (h).  $\times 1890$ . C, By electron microscopy the granules (g) were found to be membrane-bound and often vacuolated, as in this ipl microglial cell which is adjacent to the inl.  $\times 13000$ .

1300 hr and shortly after light onset. Overall, the number of photoreceptor granules was four times greater in the dark period, but variability was great. Using the relative amounts of RPE granules (lipofuscin) as an index of age, older animals had up to 100-fold more granules within their photoreceptors than younger animals in 9 of 10 timepoints at which the comparison could be made. No consistent differences in granule number and location were found between the sexes, or between squirrels killed in different seasons.

#### 4. Discussion

Although a diurnal cycle of disc shedding has been identified in several vertebrate retinas, a similar cycle for autophagic events has been characterized only in rat rod photoreceptors (Remé and Sulser, 1977) and lizard RPE cells (Young, 1977). The greater number of granules found in some squirrels during the dark period and early light period suggests that there may be a diurnal rhythm of autophagy in the neural retinal cells of this species as well. Remé and Knop (1980) and Remé, Aeberhard and Schoch (1985) have reported that increasing the light intensity increases rod photoreceptor autophagy both *in vitro* and *in vivo*, and cone autophagy *in vitro*. Although our quantitative data (not shown) are limited and complicated by the apparent influence of age, they support this conclusion for cones *in vivo* as well.

Since photoreceptors have not been observed to normally accumulate the residual bodies formed via autophagy, these inclusions must be either fully digested within the photoreceptors or excreted. Exocytosis of membranous debris by photoreceptor inner segments has been suggested by Remé (1981), who found electron-dense, membranous structures in the extracellular space between rod inner and outer segments of rats which were kept in total darkness for over 2 weeks. Our observations support this suggestion and extend it to dark-adapted squirrels housed in cyclic light. Debris exocytosed into the extracellular space (Figs 5B-E) may then be phagocytosed and degraded by Müller cells (Figs 4C, D, 5A). However, we cannot rule out a phagocytic role for the photoreceptors, since other studies have shown that these cells actively endocytose horseradish peroxidase and components of the extracellular matrix from the subretinal space (Besharse and Forestner, 1981; Hollyfield, Varner, Rayborn, Liou, and Bridges, 1985). Further studies are needed to determine if the membranous whorls are indeed debris exocytosed by the photoreceptors.

Müller cells are known to remove debris derived from degenerating photoreceptors (Caley, Johnson and Liebelt, 1972; Blanks, Adinolfi and Lolley, 1974). Our data suggest they may do so in the normal eye as well. The transference of debris or lipofuscin from neurons to glia or vascular cells has been suggested in many studies on the vertebrate CNS (Issidorides and Shanklin, 1961; Friede, 1962; Manocha and Sharma, 1977; Spoerri, 1978), so it would not be surprising if Müller cells also act as phagocytes or as extraneuronal sites for lipofuscin deposition.

On the basis of structural criteria, the small cells containing lipofuscin granules that lie near or within the OPL are most likely microglia (Vrabec, 1970; Boycott and Hopkins, 1981). Since microglia within the rat IPL have also been reported to contain lipofuscin (Kohn, Inomata and Taniguchi, 1982), these cells may also endocytose extracellular debris within the retina.

While not commonly reported to occur within cells of the neural retina except in certain pathological and experimental conditions, lipofuscin accumulation is the most prominent age-associated change observed in post-mitotic cells (Dolman and Macleod, 1981; Feeney, 1978; Wing, Blanchard and Weiter, 1978). Our observations show that structures having the characteristics of lipofuscin were present within photoreceptors, Müller cells, vascular cells, and microglia in squirrel retinas and may be part of the normal aging process of the eye. The presence of granules within photoreceptors is apparently dependent upon both age and the LD cycle.

A biphasic pattern of cone disc shedding was found in both experimental conditions, but our results are in general agreement with those reports showing maximal cone shedding during the dark period. The early dark period increase in cone shedding is

similar in timing to the peak in cone phagosomes reported by Young (1977, 1978) and O'Day and Young (1978) in the chick, lizard and goldfish retinas. The second period of increased phagosome counts corresponds in time to the peak of cone phagosomes reported in the gray squirrel retina (Tabor et al., 1980). One interpretation of the biphasic pattern would be that there are 'dark-triggered' and circadian components to cyclic cone shedding in the ground squirrel and that the two do not necessarily occur simultaneously. In lizards two such peaks can be sorted out by altering the lighting cycle (Bernstein et al., 1984). Another possibility is that there are two populations of cones which differ in the timing of their shedding response, e.g. blue- and green-sensitive cones (Anderson and Jacobs, 1972; Jacobs and Tootell, 1981). A possible precedent for biphasic cone shedding may be found in Young's (1978) study of chick photoreceptor disc shedding. Young's data show one peak in recently shed cone phagosomes (his Stage I phagosomes), but apparently two peaks in partially degraded phagosomes (Stages II and III).

In the first experiment squirrels were entrained to relatively low levels of illumination—levels far below that which these strongly diurnal animals normally experience in their natural habitat. In order to determine whether lighting intensity affects the amount or pattern of cone shedding in this species, we entrained the second group of animals to a significantly higher illumination level (1400 lx). The results from this second experiment indicate that the increased illumination level led to a slight advance in the timing of peak shedding and an increase in the number of phagosomes shed during the period just after light offset. A comparison of two studies using goldfish entrained to relatively low (32 lx, Balkema and Bunt-Milam, 1982) and high (700 lx, O'Day and Young, 1978) light intensities also suggests a similar advance in the peak of cone shedding at the higher intensity. The effect of increasing the intensity of the entraining light may have been caused by the greater amount of light the squirrels received or by the greater amplitude of the light to dark transition. The present study cannot differentiate between these two possibilities.

It is difficult to assess rod disc shedding in this species since rods make up only 5% of the photoreceptor population in the central retina (Long and Fisher, 1983). The apparent peak in rod phagosomes occurred shortly after light onset, at least in the 1400 lx experiment, as reported for most species studied to date. Cone phagosomes were also found at light onset and in the early light period, but it seems unlikely that cone shedding in this species is associated with light onset as it is in some other mammals (Anderson et al., 1980; Fisher et al., 1983; Immel and Fisher, 1985).

There is considerable interspecific variability in the reported times of maximal cone shedding. Light intensity is one of many possible factors which could influence disc shedding and account for such differences. Entrainment to 1400 lx resulted in a shift in ground squirrel cone disc shedding toward dark onset, but the magnitude of the change was small relative to the different entrainment conditions. While some of the effects of increased lighting intensity may have been masked because our condition of 'room lighting' encompassed a fairly wide range of light intensities, our results suggest that the illumination level during entrainment accounts for only a minor portion of interspecies variability.

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