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Histological Changes in the Retina Provoked by Lithium Treatment in a Nocturnal Rodent *(Lagostomus maximus maximus)*

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Key Words
Retina · Lithium · Histology · *Lagostomus*

Abstract
Daily morphological variations have been previously described in the viscacha *(Lagostomus maximus maximus)* retina. The aim of this work was to determine the effects of lithium administration on the histology of retinas from this nocturnal rodent since lithium is a drug that has been shown to affect different parameters of circadian rhythms. Adult male viscachas were divided into 2 groups, injected daily with lithium chloride or vehicle for 35 days, and sacrificed at 08:00, 16:00, and 24:00 h for light and electron microscopy studies. The following morphometric parameters were analyzed: the thickness of the photoreceptor layer, the rod outer and inner segments, and the outer nuclear layer. The control group displayed a true daily cycle of photoreceptor renewal similar to that previously reported by us for (untreated) viscachas in their normal habitat. In all lithium-treated groups, we did not observe histological changes in the thickness measurement of the retinal layers. In these groups, the retinas presented ultrastructural characteristics similar to those observed in control animals sacrificed at 24:00 h. In conclusion, chronic lithium administration abolished the daily histological rhythm in the viscacha retina, probably via inhibition of the phagocytosis process in pigment epithelial cells.

Abbreviations used in this paper

8C	controls at 08:00 h
16C	controls at 16:00 h
24C	controls at 24:00 h
8Li	lithium-treated animals at 08:00 h
16Li	lithium-treated animals at 16:00 h
24Li	lithium-treated animals at 24:00 h
C	choroid
INL	inner nuclear layer
IS	rod inner segments
M	melanin pigment granules
N	nucleus of the pigment epithelial cell
ONL	outer nuclear layer
OS	rod outer segments
P	photoreceptors
PE	pigment epithelium

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Introduction

Numerous human and animal studies have demonstrated the effects of different drugs on circadian rhythms. Lithium lengthens the circadian period in different species and therefore causes a phase delay, an effect that may underlie, at least in part, its mood-stabilizing activity [Klemfuss, 1992; Kronfeld-Schor and Einat, 2012]. Lithium salts have been used in the treatment of bipolar disorder for more than 60 years because they have been shown to have stabilizing effects on circadian rhythms [Bendetti et al., 2001; Goodwin, 2003; Lin et al., 2006]. Lithium adverse effects are observed at similar serum levels in humans and animals. The organs sensitive to the general toxic effect of this ion are comparable in humans and laboratory animals, and their absorption is not significantly different across species [Doshi et al., 1983; Thürauf and Kaschka, 1991]. In relation to the ion effect on the retina, it has been reported that lithium can prevent photoreceptor cells from undergoing apoptosis in a mouse model of retinal degeneration [Yang and Chen, 2008].

Our experimental model, the viscacha *(Lagostomus maximus maximus),* is a rodent with nocturnal habits that inhabits the semiarid zones of central Argentina, lives in large colonies in extensive burrow systems, and usually emerges at dusk or at night to feed. They are seasonal breeders and present a reproductive cycle synchronized by the environmental photoperiod through the pineal gland and its main hormone, melatonin. This cycle is characterized by a gonadal regression period during the short winter days in South America [Dominguez et al., 1987; Fuentes et al., 1991, 2003; Muñoz et al., 2001; Aguilera Merlo et al., 2005; Filippa et al., 2005]. The viscacha has been described to have an avascular, rod-rich retina with a specialized region spanning most of the equator of the eye, suggesting that this equatorial region might be a highly sensitive light detector related to foraging behaviors during the crepuscular or nocturnal hours. This band lies approximately 3.5 mm above the optic nerve, containing the longest photoreceptors and pigment epithelial cells [Lascano et al., 1999]. The retina of the viscacha exhibits a close temporal relation between melatonin levels, 2[^{125}I]-iodomelatonin binding, and daily morphological changes, as previously demonstrated in our works [Calderón et al., 2001, 2002].

In nocturnal or crepuscular animals with rod-dominated retinas, the rods are likely to be of predominant importance for the circadian system as mediators of dawn and dusk signals [Remé et al., 1991]. Independently of the temporal niche, rod photoreceptor disk shedding occurs at dawn across nocturnal and diurnal species [Tabor et al., 1982; Fisher et al., 1983]. Both disc shedding and autophagy follow a circadian rhythm in mammals [LaVail, 1976; Remé et al., 1985].

Previous morphological and biochemical studies have shown the effects of lithium on the brain and retina of this rodent [García Aseff et al., 1998; Calderón et al., 2001; Fuentes et al., 2008].

In addition, it has been shown that the viscacha is more sensitive than the rat to the general toxic effect of lithium [Perez Romera et al., 2000]. This makes the viscacha an interesting model to study the effects of lithium on retina histology. Thus, the aim of this work was to determine the effects of lithium administration on the daily histological rhythms of the viscacha retina.

Materials and Methods

Experimental Animals

Adult male viscachas *(L. maximus maximus)* weighing between 5 and 7 kg were used in this study. They were captured in their habitat near San Luis, Argentina (latitude: 33° 20' S, altitude: 760 m), during spring (October to November). The animals were kept in isolated boxes at 20 ± 2°C under a photoperiod of 14 h light: 10 h dark (lights on from 08:00 to 20:00 h), with free access to food and water. After acclimation for at least 2 weeks, the animals were divided in 2 groups and given daily intraperitoneal injections of lithium chloride (Sigma; 1 mmol/kg of body weight, lithium-treated group) or sterile distiller water (control group) at 08:00–09:00 h for 35 days. Finally, the control (n = 12) and lithium-treated (n = 12) viscachas were intraperitoneally anesthetized with a ketamine (ketamine hydrochloride; Holliday-Scott S.A.):xylazine (xylazine hydrochloride; Richmond Laboratories) solution (10:1 w/v, 0.3 ml/kg of body weight) and sacrificed by intracardiac injection of Euthanyle (0.25 ml/kg of body weight, pentobarbital sodium, diphenylhydantoin sodium; Brouwer S.A.) at local clock times 08:00, 16:00, and 24:00 h (n = 4 per group). At each time point, blood samples were obtained by cardiac puncture, and the serum was separated by centrifugation. The lithium concentration was determined via atomic absorption spectrometry according to the method described by Scott [1982], and the technique was similar to that reported in previous works [Garcia Asseff et al., 1998; Perez Romera et al., 2000].

The experimental design was approved by the local ethics committee and was in agreement with the National Institute of Health (NIH, USA) guidelines for the use of experimental animals. Moreover, the Biodiversity Control Area of the San Luis Ministry of the Environment (Argentina) approved a study protocol for conducting scientific research within the territory of this province (resolution No. 03 PRN-2011).

Tissue Preparation

The eyes to be used for microscopy studies were rapidly removed, and the eye bulbs were divided on the equatorial plane into

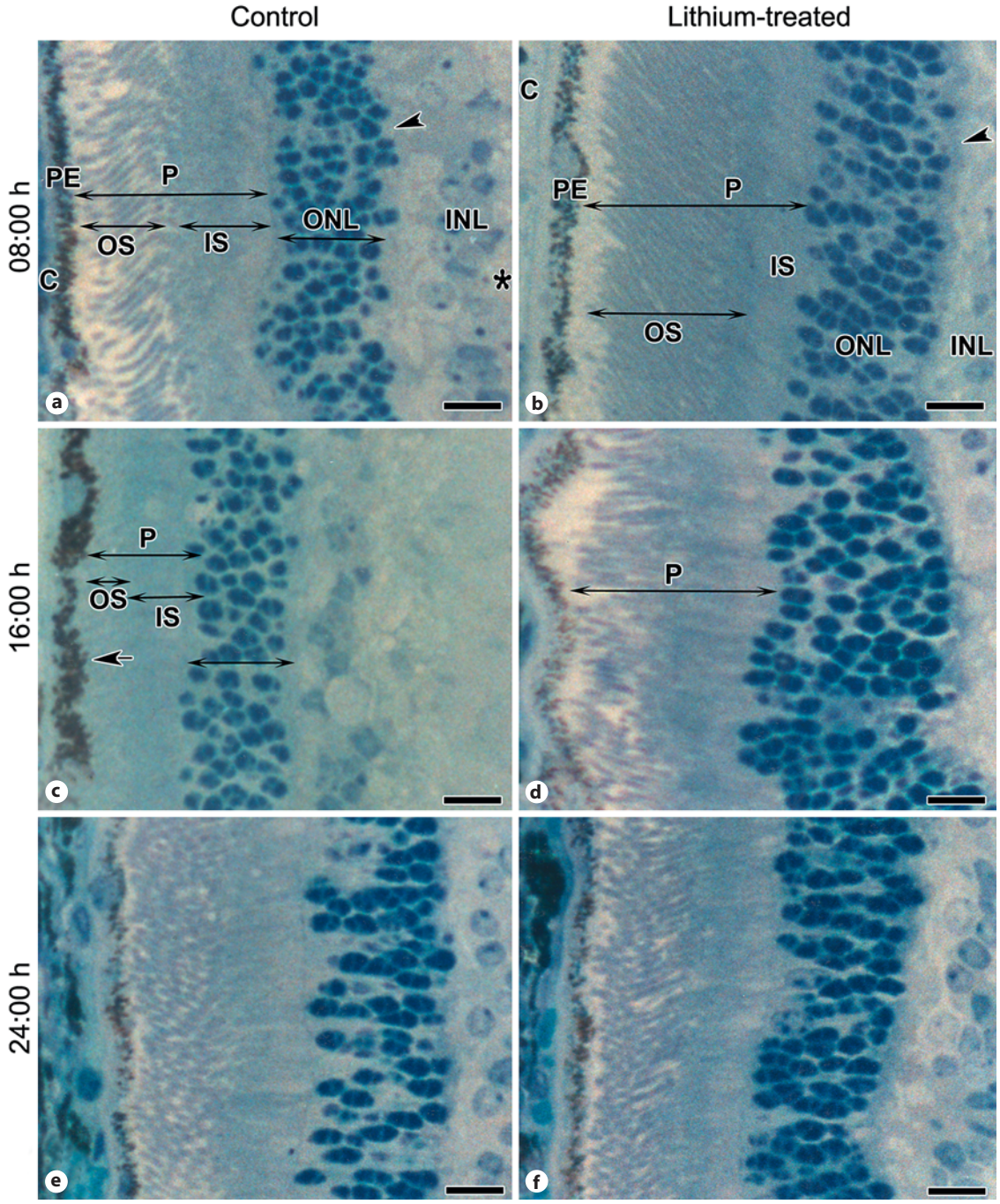

Fig. 1. Light micrographs of the retinas of control (**a**, **c**, **e**) and lithium-treated (**b**, **d**, **f**) viscachas. Sections (1 μm thick) were stained with toluidine blue. **a**, **b** Retinas of viscachas sacrificed at 08:00 h. The arrowheads mark the outer plexiform layer. The asterisk represents the inner plexiform layer. **c**, **d** Micrographs of the retinas of viscachas sacrificed at 16:00 h. **c** Melanin pigment granules of pigment epithelial cells (arrow) are present. Note that the melanin pigment granules are dispersed at 16:00 h, whereas at 08:00 and 24:00 h the granules are aggregated. **e**, **f** Retina of a viscacha sacrificed at 24:00 h. **a**, **c**, **e** In the control group, rhythmic changes were detected mainly at the level of the photoreceptor layer and pigment epithelium. These images reveal an increase in the thickness of the photoreceptor layer at 24:00 h compared to retinas obtained at 08:00 and 16:00 h. This increase was accompanied by a greater thickness in the rod outer segments. The morphologic retina characteristics of the 3 lithium-treated groups (**b**, **d**, **f**) are similar to those of the control group at 24:00 h. PE = Pigment epithelium; P = photoreceptors; OS = rod outer segments; IS = rod inner segments; ONL = outer nuclear layer; INL = inner nuclear layer; C = choroid. **a–f** Scale bars = 25 μm.

anterior and posterior parts. Retinal samples were taken from the specialized band, which lies approximately 3.5 mm above the optic nerve according to reports by Lascano et al. [1999]. The tissues were fixed for electron microscopy with Karnovsky fluid [Karnovsky, 1965] for 6 h. They were postfixed in 2% osmium tetroxide for 1 h at 4°C, washed in phosphate buffer (pH 7.4), and dehydrated in acetone. The tissue blocks were embedded in plastic Spurr's resin.

Sections of retina of 1 μm were cut precisely along the horizontal meridian (for each time point) with a Porter-Blum ultramicrotome (Ivan Sorvall, Norwalk, Conn., USA) and stained with toluidine blue. Ultrathin sections stained with lead citrate and uranyl acetate [Milloning, 1961] were examined using a Siemens Elmiskop I electron microscope (Siemens Co., Berlin, Germany).

Morphometric Analysis

A computer-assisted image analysis system was used to measure the thickness of the complete retina, the photoreceptor layer, the rod outer and inner segments, and the outer nuclear layer. The system consisted of an Olympus BX-40 binocular microscope (magnification ×400) interfaced with a host computer, image processing, and a recording system. The images were captured by a Sony SSC-DC5OA camera and processed with Image-Pro Plus 5.0 software under the control of a Pentium IV computer. The software allowed the following processes: image acquisition, automatic analogous adjustment, thresholding, background subtraction, distance calibration, lineal measurement, and diskette data logging. The images were displayed on a color monitor, and the parameters were measured using the image analysis system. The morphometric study was carried out as follows: 6 sections of each eye were analyzed (n = 4 animals for each time point; 2 retinas per animal). Measurements were taken at 200-μm intervals all along the 1-μm-thick sections stained with toluidine blue, and a minimum of 15 readings were made on each section. Finally, 720 measurements per time point and per group were performed. Measurements were not made when there was evidence of distortion of the sections. The measurements were expressed as percentages of the total retinal thickness in order to allow for regional or individual differences.

Statistical Analysis

Results are expressed as means ± SEM for all data sets. Differences between the two groups (control vs. lithium-treated animals) were evaluated using Student's t test. Comparisons of the percentages measured in the different layers of each group were evaluated using a one-way analysis of variance (ANOVA), followed by a Tukey-Kramer multiple comparisons test. $p < 0.05$ was considered statistically significant.

Results

The lithium chloride concentration in serum at the end of the experiment was 0.620 ± 0.068 mmol/l in lithium-treated viscachas (mean value from the 3 studied time points). The levels of lithium in the control groups were almost undetectable (Student's t test, $p < 0.005$).

The histological study of sections stained with toluidine blue revealed that the retinas of the control and treated animals exhibited all of the histological layers at the 3 time points studied. In the retinal pigment epithelium of control viscachas, a dispersion of melanin pigment granules was observed at 16:00 h and an aggregation of them was seen at 08:00 and 24:00 h. The morphometric study of the thickness of the control retina layers allowed us to observe that the thicknesses of the photoreceptor layer and the photoreceptor outer segment were maximal at 24:00 h and minimal at 16:00 h. However, the thicknesses of the rod inner segments and the outer nuclear layer did not present significant variations. In lithium-treated viscachas, the retinal histology and the analyzed morphological parameters did not show significant differences at any point. The histological characteristics of the lithium-treated retinas were similar to those of the control retinas of viscachas sacrificed at 24:00 h. In addition, significant differences between the control and lithium-treated groups in terms of the thickness of the photoreceptor layer and the outer segment, but not the inner segment and outer nuclear layer, were observed at 08:00 and 16:00 h (fig. 1, 2).

Ultrastructural changes at the level of the pigment epithelium and photoreceptor outer segments in the control viscacha retinas were observed. Abundant dense bodies similar to lysosomes, with variable shapes in the apical cytoplasm of pigment epithelial cells, were found at 08:00 and 24:00 h whereas they were scarce at 16:00 h. In the retinas of animals sacrificed at 08:00 and 16:00 h, a tight structural relationship among the microvilli of the epithelial cells and the rod outer segments was observed. In lithium-treated viscachas, regardless of the sacrifice time, abundant dense bodies like lysosomes, minimal contact between the microvilli of the epithelial cells and the rod outer segments, and undigested membrane remnants detached from the ends of the rods were detected. This situation was observed at the 3 studied time points and was similar to that in the control retinas at 24:00 h (fig. 3).

Discussion

The study of lithium treatment has become an important aspect of therapy for bipolar affective disorder (manic depression) and is useful in other recurrent affective and nonaffective illnesses. Human clinical evaluations indicate that the range of clinical effective concentrations in serum is narrow, i.e. 0.8–1.2 mmol/l for acute manic depression and 0.6–1.0 mmol/l for maintenance therapy

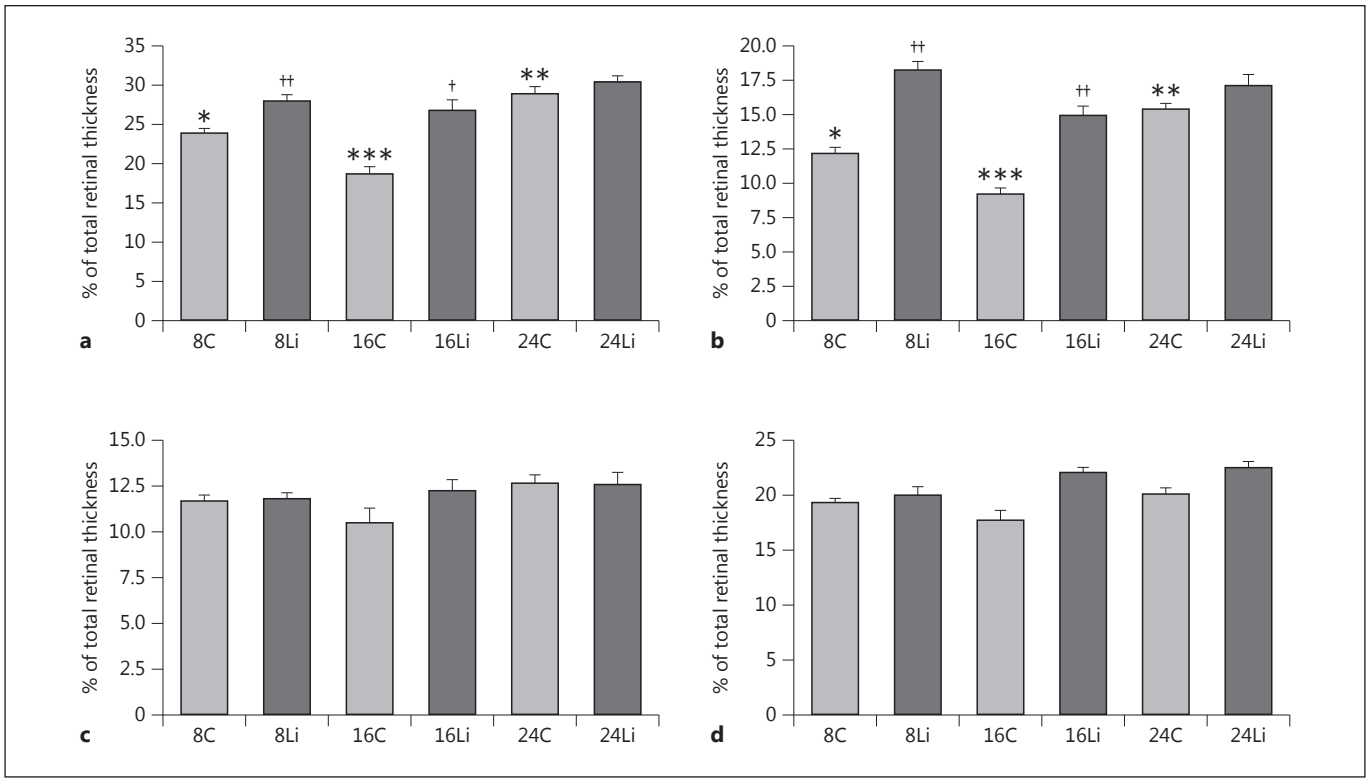

Fig. 2. Morphometric study of the daily variations in retinal layer thickness. Values are expressed as means ± SEM. **a** Photoreceptor layer. **b** Outer segments. **c** Inner segments. **d** Outer nuclear layer. Control group: ANOVA-Tukey's test: * $p < 0.05$, 08:00 vs. 16:00 h; ** $p < 0.01$, 24:00 vs. 08:00 h; *** $p < 0.001$, 16:00 vs. 24:00 h. In lithium-treated viscachas, the retinal layer thickness remained stable at 3 points; ANOVA, not significant. There were significant differences between lithium-treated and control animals in the photoreceptor layer (lithium-treated vs. control animals, Student's t test: at 08:00 h, †† $p < 0.001$, and at 16:00 h, † $p < 0.01$). In the outer segments (lithium-treated vs. control animals, Student's t test: at 08:00 h and at 16:00 h, †† $p < 0.001$). 8C = Controls at 08:00 h; 8Li = lithium-treated animals at 08:00 h; 16C = controls at 16:00 h; 16Li = lithium-treated animals at 16:00 h; 24C = controls at 24:00 h; 24Li = lithium-treated animals at 24:00 h.

[Scott, 1982]. In our work, the lithium serum levels achieved the human range after a 35-day injection treatment (0.620 ± 0.068 mmol/l).

It has been reported that the melanin pigment granules in retinal pigment epithelium are aggregated during the night, when melatonin levels are high [Thumann et al., 2013]. Previous research carried out in our laboratory has demonstrated daily morphological variations in the retinas of viscachas captured in their habitat. A notable dispersion of melanin pigment granules in the retinal pigment epithelium was observed at 16:00 h, whereas at 08:00 and 24:00 h aggregated melanin pigment granules were found [Calderón et al., 2002]. In lithium-treated viscachas, the retinal levels of melatonin did not show significant differences at any point during the light-dark cycle, indicating that the ion abolished the indole rhythm [Calderon et al., 2001]. The present work demonstrates that lithium treatment modified the distribution and aggregation of granules in relation to the control retina, in which variations during the light-dark cycle and aggregation at 08:00 and 24:00 h were observed, suggesting that this ion might alter the dispersion or aggregation of melanin pigment granules.

The circadian rhythms of photoreceptor outer segment renewal and autophagy in the inner segments of the photomembranes have been described in several mammals [Remé, 1999]. The digestive enzyme activity has been related to light, and thus the decrease in multivesicular dense bodies, like secondary lysosomes, from the pigment epithelium is proportional to the light intensity [Chen et al., 2013]. Furthermore, lithium alters the ability of the retina to detect light by modulating the functional environment of the rod photoreceptors in the retina in humans [Seggie, 1988]. Other researchers have

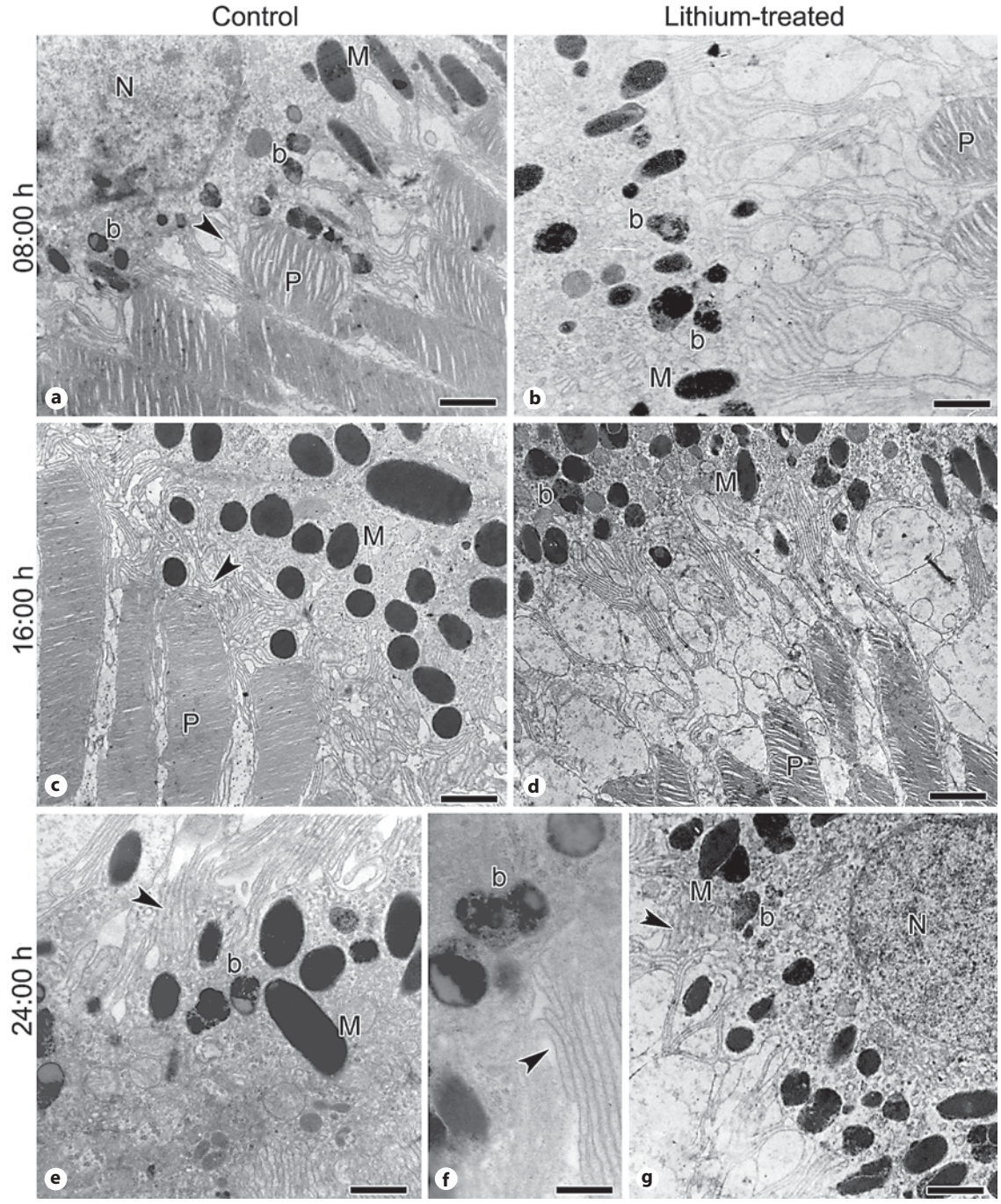

Fig. 3. Electron micrographs of the retinas of control (**a**, **c**, **e**, **f**) and lithium-treated (**b**, **d**, **g**) viscachas. **a**, **b** Micrograph of the retinas of viscachas sacrificed at 08:00 h. There are dense bodies of variable sizes and shapes in the pigment epithelial cells. Scale bars = 4 μm. **c**, **d** Electron micrograph of the retinas of viscachas sacrificed at 16:00 h. **c** A tight structural relationship among the microvilli of the epithelial cell and rod outer segment is observed. Scale bar = 2 μm. **d** Some dense bodies are present. Scale bar = 4 μm. **e–g** Electron micrograph of the retinas of viscacha sacrificed at 24:00 h. There are dense bodies of variable sizes and shapes in the pigment epithelial cells. The contact among the outer segments and the pigment epithelial cells is at a minimum. In the images of lithium-treated animals, lithium abolishment of the daily rhythms of photoreceptor renewal is observed. Scale bar = 2 μm (**e**), 1 μm (**f**), and 4 μm (**g**). Arrowheads indicate microvilli (**a**, **c**, **e–g**). N = Nucleus of the pigment epithelial cell; P = photoreceptor; M = melanin pigment granules; b = dense body.

observed that lithium attenuates the eye rhythms and mainly the outer segment disk shedding in the rat and delays the neuronal activity rhythms in the *Aplysia* isolated eye, suggesting that this ion might produce similar effects on the brain and retinal pacemaker [Woolum and Strumwasser, 1983; Remé et al., 1990]. In the control viscacha retinas of the present study, we observed ultrastructural changes that demonstrate a daily rhythm of retinal photoreceptor renewal. These results are similar to those previously reported by us for viscachas in their normal habitat [Calderón et al., 2002]. On the contrary, the daily variations in the thickness of the photoreceptor layer, and specifically the outer segments, and ultrastructural variations disappeared completely as a result of the action of lithium, unlike the results reported by Remé et al. [1990]. In addition, these authors suggested that lithium may act via platelet-activating factor responses in the rat retina [Remé et al., 1992].

The mechanisms responsible for lithium's chronopharmacological actions are not yet fully clear. Specific alterations at the ionic, second messenger system, and neuromodulator levels have been involved in the clinical and circadian rhythm actions of the ion [Klemfuss, 1992; Jope and Williams, 1994]. Studies have suggested that the second messenger involved in outer segment phagocytosis may be inositol trisphosphate. In addition, as lithium chloride is used to inhibit the hydrolysis of inositol trisphosphate, lithium might reduce outer segment phagocytosis in this way [Heth and Marescalchi, 1994; Heth et al., 1995; Hall et al., 1996]. Our results suggest that the ion inhibits rod photoreceptor disk shedding because the length of the outer segments remains constant along the light-dark cycle. Thus, lithium inhibits daily photoreceptor renewal, probably by inhibiting the process of phagocytosis in pigment epithelial cells. Finally, the histological changes observed in the viscacha retina as an effect of lithium might confirm the property that is attributed to this ion of modification of various parameters of daily or circadian rhythms.

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