Retinal development and function in a ‘blind’ mole

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Animals adapted to dark ecotopes may experience selective pressure for retinal reduction. No previous studies have explicitly addressed the molecular basis of retinal development in any fossorial mammal. We studied retinal development and function in the Iberian mole Talpa occidentalis, which was presumed to be blind because of its permanently closed eyes. Prenatal retina development was relatively normal, with specification of all cell types and evidence of dorsolateral regionalization. Severe developmental defects occurred after birth, subsequent to lens abnormalities. ‘Blind’ Iberian moles had rods, cones and rod nuclear ultrastructure typical of diurnal mammals. DiI staining revealed only contralateral projections through the optic chiasm. Y-maze experiments demonstrated that moles retain a photoreception response. Over-representation of melanopsin-positive retinal ganglion cells that mediate photoperiodicity was observed. Hence, molecular pathways of eye development in Iberian moles retain the adaptive function of rod/cone primary vision and photoperiodicity, with no evidence that moles are likely to completely lose their eyes on an evolutionary time scale.

Keywords: Iberian mole; fossorial; evolution; retina development; melanopsin; gliosis

1. INTRODUCTION

About 300 mammalian species have adopted a subterranean lifestyle (Burd et al. 1990; Peichl 2005). Most fossorial mammals retain eyes, although the extent of anatomical regression varies, resulting in a multitude of defective eye phenotypes (Nevo 1979; Burda et al. 1990). Very little is known about the molecular and developmental mechanisms underlying the regression of the visual system in mammals, and recent studies have been performed mainly postnatally in rodents such as the blind mole-rat and the African mole-rats (Némec et al. 2007).

Mammals have a duplex retina containing two types of photoreceptors: cones for colour vision and rods for low-light vision. A small proportion of retinal ganglion cells (RGCs) in the retina contain the visual pigment melanopsin and are involved in photic responses such as entrainment of the circadian cycle and control of pupil size (Nickle & Robinson 2007). Melanopsin is expressed in approximately 1 to 2.5 per cent of the RGC population in rodents and man (Hattar et al. 2002).

True moles (Talpidae) are insectivores of the order Eulipotyphla that show striking adaptations (Gorman & Stone 1990). Their eyes exhibit anatomical regression, although the main ocular structural elements are present (Quilliam 1966; Sato 1977; Carmona et al. 2008; Glösmann et al. 2008). While the domains of the central nervous system required for vision are diminished (Lund & Lund 1966), the structures involved in controlling the biological rhythms, the suprachiasmatic nucleus and the retinohypothalamic projections, are superficially normally developed (Kudo et al. 1991). The adult European mole (Talpa europaea Linnaeus, 1758) has small eyes, about 1 mm in diameter, with eyelids that can open and close. A nucleated and disorganized lens is present; the retina displays a typical laminated organization, with about 2000 RGCs (Quilliam 1966). Talpa europaea successfully performs light/dark discrimination tasks under experimental conditions (Lund & Lund 1965, 1966; Johannesson-Gross 1988), suggesting that photopic vision is of relevance in the ecology of this insectivore. Closely related talpids—the Mediterranean mole (Talpa casca Savi, 1882), the Roman mole (Talpa romana Thomas, 1902), the Balkan mole (Talpa stankovici Martino & Martino, 1931) and the Iberian mole (Talpa occidentalis Cabrera, 1907)—have been considered totally blind because their eyes remain enclosed under the skin throughout life (Dubost 1968; Nevo 1979; Kryštufek 1994; Carmona et al. 2008).

No approaches have explicitly addressed the developmental basis of morphological change in the retina of any fossorial mammal, in spite of the potential to inform the link between developmental biology, ecology and evolution of the eye in a mammalian system. In this study, we characterize the pattern of differentiation of the main retinal cell types in T. occidentalis. We further
study the behavioural response of *T. occidentalis* to light stimuli. We conclude that there remains selective pressure for light detection, but, in contrast to nocturnal mammals, there is no evidence that *T. occidentalis* has undergone any adaptations for optimizing low-light vision. Moles' vision is probably restricted to the detection of sunlight and maintenance of circadian rhythms.

2. RESULTS

(a) Morphological characteristics of the Iberian mole retina

The terminology of retinal lamination is described in figure 1. Mole tissue sections showed normal progression of prenatal retinal development (figure 1a). Early differentiation of the retina commences properly, with inner neuroblastic layer (INBL) and outer neuroblastic layer (ONBL) defined before birth (s5b and s7 in figure 1a). In newborn moles (s9), the ONBL segregates into inner nuclear layer (INL) and outer nuclear layer (ONL). Later in development, an outer plexiform layer (OPL) becomes identifiable (s11 and s14). However, cell nuclei from the INBL remain randomly distributed along the inner side of the retina even in adults. As a consequence, unlike the mouse, neither a discrete ganglion cell layer (GCL) nor an acellular inner plexiform layer (IPL) are established at any time.

The morphological ultrastructure of retinal cells was studied (figure 1b; Carter-Dawson & LaVail 1979; Jeon et al. 1998; Cernuda-Cernuda et al. 2002). In the Iberian mole, the retina is characterized by a prominent photoreceptor layer. Müller cells were distinguishable by their characteristic electron-dense irregular nuclei. Large, round, electron-lucent nuclei were identified as amacrine cells. Horizontal cells showed less electron-dense and more spherical nuclei than Müller cells. Bipolar cells were identified based on their round nuclei with prominent nuclei and location (close to the OPL). OLM, outer limiting membrane; a, amacrine cell; b, bipolar cell; h, horizontal cell; m, Müller cell; r, rod; c, cone.

Figure 1. Morphological characterization of retinal cells types in *T. occidentalis*. (a) Haematoxylin and eosin staining of mole and mouse tissue sections. The optic cup (OC) is established at stage s3 in *T. occidentalis*. Inner neuroblastic layer (INBL) and outer neuroblastic layer (ONBL) are identifiable at s5b–s7. Inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL) differentiate during postnatal stages (s9–s14). No discrete ganglion cell layer (GCL) or inner plexiform layer (IPL) are formed. Mouse P2 and adult retinal sections represent normal mammalian retinal development. (b) Ultrastructural study of the mole retina. (i) Toluidine-blue-stained semithin section of an adult Iberian mole retina. (ii–vii) TEM photomicrographs of adult (ii–iv) mole and (v–vii) mouse retinas. (ii) GCL- and IPL-like regions in *T. occidentalis*. (iii) INL and ONL in *T. occidentalis*. (iv) Photoreceptor layer in *T. occidentalis*. (v) GCL and IPL in the mouse. (vi) INL in the mouse. (vii) Photoreceptor layer in the mouse. Müller cells were distinguishable by their characteristic electron-dense irregular nuclei. Large, round, electron-lucent nuclei were identified as amacrine cells. Horizontal cells showed less electron-dense and more spherical nuclei than Müller cells. Bipolar cells were identified based on their round nuclei with prominent nuclei and location (close to the OPL). OLM, outer limiting membrane; a, amacrine cell; b, bipolar cell; h, horizontal cell; m, Müller cell; r, rod; c, cone. (a) Scale bar represents 200 μm in s3–s14 images and 60 μm in mole adult and mouse P2 and adult images. (b) Scale bars: (i) 30 μm, (iv) 15 μm and (ii, iii, v–vii) 20 μm.
cells were found in the ONBL (arrows). As in the mouse occasional ectopic location in the INL (figure 2), randomly distributed across the inner retina, with an figure 2). In the adult mole, BRN3a-positive cells were less numerous after birth than in prenatal stages (s9 in stages (figure 2)). In the mouse, although some single positive cells were detected in the outer retina during developmental stages (figure 2b), all BRN3a-expressing cells were located exclusively in the GCL in three adult mouse samples analysed (figure 2).

In adult mice, about 1 per cent of the RGC population expresses melanopsin (Hattar et al. 2002). We studied melanopsin immunoreactivity in T. occidentalis (figure 3a–f). First evidence of expression was seen in newborn moles at s9, but not before (n = 3 samples per stage). In adult moles (n = 5), a very high proportion of cell bodies in the inner retina was melanopsin-positive (figure 3a) compared with the mouse (figure 3c). Melanopsin-containing RGCs showed normal morphology with axons projecting widely throughout the retina (figure 3a–c), directed towards the optic disc and contributing to the optic nerve (figure 3f).

Histology and DiI labelling showed that axons from RGCs contralaterally traverse the optic chiasm (figure 3g,h). No ipsilateral projections were detected in any of the five mole samples analysed. The study showed that moles have at least two classes of molecularly specified, though morphologically disorganized, RGCs that make projections to the CNS visual pathway.

In the adult mole, Pax6 immunofluorescence was observed in the GCL and in the inner sublayer of the INL (figure 4). Ap2α-positive amacrine cells occupied the distal region of the INL (figure 4).

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(ii) Amacrine and horizontal cells
Amacrine and horizontal cells were characterized by double sequential immunostaining of Pax6 and AP2α proteins (figure 4, top). Pax6 is expressed in retinal progenitors and differentiates RGCs, amacrine cells and horizontal cells (Walther & Gruss 1991; Hitchcock et al. 1996; Philips et al. 2005). AP2α is a marker of amacrine cells (Bisgrove & Godbout 1999; Bassett et al. 2007). Hence, cells immunopositive for both anti-Pax6 and anti-AP2α were identified as amacrine cells.

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In the Iberian mole, the INL of retinas from infant moles of s10 (n = 3) showed a pattern of AP2α/Pax6 expression similar to that in the adult mouse, with an inner band of AP2α-positive/Pax6-positive amacrine and a outer zone of AP2α-negative/Pax6-positive horizontal cells (top row of figure 4). Nevertheless, this pattern was disrupted later in development, with the adult mole showing a disorganized distribution of horizontal and amacrine cells in both the GCL/PL-like regions and INL (top row of figure 4, centre).

(iii) Müller glia
Glia fibrillary acidic protein (GFAP) expression in the apical endplates of retinal Müller cells is a common feature of vertebrates (Sassoe Pognetto et al. 1993). GFAP was detected from s10 to adulthood in T. occidentalis (figure 4). In infant moles at s10, cell bodies in the inner periphery of the retina were GFAP-positive (arrowhead in second row of figure 4, left). However, in adult moles (n = 5), GFAP immunosignal of the Müller cells was more extensive throughout the whole depth of the retina, with GFAP-positive processes reaching the ONL (figure 4). In other mammals such as mice, this extension of GFAP localization throughout the Müller glia is a feature of reactive gliosis associated with damage.

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Bipolar cells
Protein kinase C alpha (PKC\(\alpha\)) robustly identifies rod bipolar cells (Greferath et al. 1990; Osborne et al. 1991; Gloßmann et al. 2008). PKC\(\alpha\)-immunopositive cells were present at s10 in \textit{T. occidentalis}\(\) (third row of \textit{figure 4}). At this developmental stage, and in subsequent developmental stages, the PKC\(\alpha\)-positive rod bipolar sublayer was located in the outer side of the INL, similar to the mouse (\textit{figure 4}, right). However, in contrast to the mouse retina, some bipolar cell somata were displaced to the ONL (\textit{figure 4}) and their axon terminals were distributed along the whole inner retina.

Photoreceptors
The major rod opsin (rhodopsin) and two cone opsins (a shortwave-sensitive S and a middle-to-longwave-sensitive M opsin) were present in the photoreceptor layer of the Iberian mole retina (bottom rows of \textit{figure 4}), showing that the normal range of rod and cone photoreceptors was present. High levels of rhodopsin expression were observed from stage s9 until adulthood (\textit{figure 4}). S opsin-positive cones first appeared at s10, while M opsin was first detected one stage later, at s11 (fourth row of \textit{figure 4}, left image). Displaced rod and cone photoreceptors were observed in the inner retina of the Iberian mole (\textit{figure 4}).

Mean photoreceptor densities, the cone/rod ratio and the percentage of dual cones were assessed in four flat-mounted retinas obtained from four different adult individuals (electronic supplementary material, table S1). \textit{Talpa occidentalis} has a retina with an area of around 0.6 mm\(^2\), which is rod-dominated but contains cone photoreceptors expressing either S or M cone opsin or both S and M cone opsins in a single photoreceptor (dual cones).

Immunostaining on flat-mounted retinas revealed a dorsoventral (DV) gradient of S cones (\textit{figure 5a,e}). In contrast, M cones had a homogeneous distribution across the retina; S cones were more abundant in the ventral pole (\textit{figure 5a,c}). Cone densities were significantly higher in the ventral retina (\textit{figure 5b}), an unusual feature that is discussed below.

\textit{Figure 3. Immunodetection of melanopsin and optic nerve tracing in \textit{T. occidentalis}. In the adult mole, melanopsin-positive cells were found throughout the inner retina (a,b), and their density was much higher than in the adult mouse (c). (d,e) Melanopsin-immunoreactive axons were distributed across the plane of the retina, and incorporated into the optic disc (OD; arrowheads in f). (g) Haematoxylin staining of a transverse section of a head from s5c mole embryo; the optic nerves are observable (arrows). (h) DiI labelling of the optic nerve in an s5c embryo. OC, optic chiasm; E, eye position. Photomicrographs from (d) and (e) were taken from flat-mounted retinas. Scale bars: (b,e) 50 \(\mu\)m, (a,c,d,f) 100 \(\mu\)m, (g) 1.4 mm and (h) 1 mm.}
Figure 4. Molecular characterization of the retinal cell types in *T. occidentalis*. (PAX6/AP2α) The AP2α/PAX6 expression profile of the INL in stage s10 moles (top left) does not differ considerably to that in the adult mouse (right). In adult moles (centre), this distribution pattern becomes disorganized (see inset in mole adult). (GFAP) Moderate levels of GFAP expression were detected in the inner border of the retina in s10 moles (arrowheads in left image), similar to that in adult mouse (right). In adult moles, the GFAP-immunosignal extended the whole depth of the retina (arrows centre) and GFAP-positive cell bodies were distributed across the inner retina (white arrowheads in centre). (PKCα) PKCα-positive bipolar cells were first detected at s10 located in the outermost side of the OPL, their axons and endfeet distributed along the inner retina. Adult mouse retinas exhibited a similar spatial profile (right), although the endfeet were observed exclusively in the GCL. (Rod opsin) High expression levels during all postnatal developmental and adult stages were detected in *T. occidentalis* (left and centre), similar to those in the mouse (right). (M/S opsin) Shortwave-sensitive (S) and a middle-to-longwave-sensitive (M) cone opsins, in the photoreceptor layer of the Iberian mole (left and centre). s11* stage is shown in this row because M opsin is first expressed at this stage, whereas S opsin is expressed at s10. Cone opsin expression was maintained in adulthood (centre), with a pattern very similar to the mouse (right). Displaced cells immunoreactive for rod opsin, and S/M cone opsins were detected across the depth of the retina in *T. occidentalis* (arrows in left and centre). Yellow arrowhead highlights autofluorescent erythrocytes. Scale bar: 50 μm.
Compartmentalized RALDH expression during development of Iberian mole retina

Two members of the retinaldehyde dehydrogenase (RALDH) family, RALDH1 and RALDH3, were used as markers of DV regionalization during retinal development (figure 5e). These enzymes in retinoic acid metabolism are asymmetrically expressed in the developing mammalian retina. Raldh1 is expressed dorsally, whereas Raldh3 shows exclusive restriction to the ventral retina (Schulte & Bumsted-O’Brien 2008). In the retina of E12 mice, as expected, we confirmed dorsal expression of Raldh1/RALDH1 and ventral (V) expression of Raldh3/RALDH3 in E12 mice and s5b moles. D, dorsal; N, nasal. Scale bars, (a,b) 300 μm, (c) 60 μm, (d) 30 μm and (e) 250 μm.

Wavelength-dependent light-avoidance capabilities of T. occidentalis moles

Our morphological and molecular data suggest that, although it is dysgenic to some extent, the retina of T. occidentalis is potentially functional. To test this hypothesis, light-avoidance experiments were performed with four adult moles, using flashing white light in a Y-maze setting in which the animals were offered to approach or avoid a light source during an escape response. The results obtained in this study are summarized in table 1. The choice to approach the dark tunnel was significant in the four animals used. This indicates that T. occidentalis is able to respond to flashing light stimuli, under the experimental conditions employed. In all cases, the animals responded by avoiding the light source, suggesting that in stressful situations they move towards darkness.

3. DISCUSSION

(a) How strong is the selective pressure on the mole retina?

In low-light environments, animals may be under selective pressure for increased photoreceptive sensitivity. However, an alternative evolutionary strategy in conditions where light levels are too low for any ocular function to be possible (e.g. in caves) is for eye regression
with expansion of the other senses. Moles, as fossorial animals, fall into a category of sporadic light exposure for which either evolutionary strategy may be advantageous. There are potentially competing pressures to minimize the energetic cost of eyes in an environment where photic sensory information may be valuable.

The data presented here show that *T. occidentalis* has a surprisingly well-conserved retina. This suggests either that the Iberian mole is on a pathway to further eye regression which is not yet complete (evolutionary lag) or that there is some adaptive advantage to maintain the retina. Our data demonstrate that the eyes are functional, but that some retained aspects of eye patterning could potentially be lost in future.

(b) **Differentiation of the Iberian mole retina**

The pattern of differentiation of the retinal cell types is highly conserved among vertebrates (Lamb et al. 2007). Our results show that *T. occidentalis* has retained an ordered timing of retinal cell differentiation very similar to that of the mouse.

Photoreceptor distribution is patterned across the tangential plane of the retina (Ahnelt & Kolb 2000; Peichl 2005). Surprisingly, both RALDH1 and RALDH3 showed the same robust compartmentalization distribution in *T. occidentalis* as in the mouse (McCaffery et al. 1998; Li et al. 2000), thus suggesting that the mechanisms that establish DV position-dependent cues have been conserved in the Iberian mole. This may be necessary to set up the DV differential in cone photoreceptor frequency discussed below, or it may be atavistic.

(c) **Disrupted lens development could induce the postnatal retinal defects observed in *T. occidentalis***

Our results suggest that prenatal retinal development progresses properly in *T. occidentalis*. However, postnatally, amacrine and horizontal cell organization becomes disrupted in the INL, with overt anomalies observed in the adult.

The vertebrate lens plays a key role in the development of the retina (Coulombre & Coulombre 1964; Breitman et al. 1987; Landel et al. 1988; Kaur et al. 1989; Ashery-Padan et al. 2000; Yamamoto & Jeffery 2000; Kurita et al. 2003; Vihitelic et al. 2005), and in this respect it is interesting that the retinal phenotypes observed in moles occur subsequent to the development of the major lens abnormalities we described previously, resulting from misregulation of the forkhead transcription factor FOXE3 in the lens (Carmona et al. 2008). We speculate that impaired regulation of lens FOXE3 may secondarily initiate retinal dysgenesis.

(d) **Extensive GFAP expression in retinal Müller cells**

Müller cells represent the principal glial cell type of the retina. They extend apically to basally, and GFAP is normally expressed only in the very inner segments of Müller glia in healthy retinas (Sassou Pognetto et al. 1993). However, the whole glial surface becomes GFAP-positive during reactive gliosis following disease or trauma (Guérin et al. 1990; Lewis et al. 1995; Bringmann et al. 2006). In neonatal (1–7 dpp) Iberian moles, GFAP showed a spatial expression pattern very similar to that observed in adult mice. However, from stage s11 (7–12 dpp), GFAP immunoreactivity expanded across the depth of the retina. Müller glia are, in mammals, the major retinal stem cell class (Bernardos et al. 2007), and it may be that in moles, they are responding to some aspect of the defective retinal phenotype by attempting to activate regenerative properties that would ‘repair’ the retina.

(e) **Diurnal nuclear pattern of photoreceptor nuclei in *T. occidentalis***

Solovei et al. (2009) showed that rod nuclei of nocturnal species such as the mouse are small and present an ‘inverted’ pattern of chromatin organization in which a heterochromatic core occupies most of the nuclear volume. The inverted nuclei produce a more efficient transmission of light. We found that *T. occidentalis* exhibits the conventional rod nuclear pattern typical of diurnal species. Analysis of the figures in Lluch et al. (2009) suggests that shrews (Soricidae), which are closely related to moles, have an intermediate rod nuclear pattern that correlates with their polyphasic lifestyle (Genoud 1984). The rod architecture of mole species could be a retained trait of a diurnal common ancestor of moles and shrews, and there is no evidence that moles have been under selective pressure to maximize the light-gathering capacity of their eyes—they do not ‘try’ to see in dim light, and are not evolving to do so.

(f) **Possible implications of the retina in the ecology of moles**

Cone proportions range from low (0.5–3%) in nocturnal species and medium (2–10%) in crepuscular and arrhythmic species to high (8–95%) in diurnal ones. Rod densities generally are higher in nocturnal species, with most mammals showing values ranging from 200 000 to 400 000 rods mm⁻² (Ahnelt & Kolb 2000; Peichl 2005). The percentage of cone photoreceptors varies between species and, in insectivores, seems to relate to their activity pattern (Peichl et al. 2000). For instance, the mostly nocturnal Crocidura spp. show relatively low values (4–6%; Genoud & Vogel 1981; Vogel et al. 1981) and the polyphasic Sorex araneus higher ones (13%; Genoud 1984). Surprisingly, a previous study performed in *T. europaea* (Glössmann et al. 2008) and that reported here for *T. occidentalis* have shown that talpid moles exhibit high cone proportions similar (9–15%) to those of the closely related soricids. This provides further evidence that the eyes of moles are not
adapted to low-light vision. Taking into account that the strictly subterranean African mole-rats of the rodent family Bathyergidae also have high cone proportions (around 10%; Peichl et al. 2004), that the photoreceptor mosaic is not adapted to low-level vision in subterranean mammals may be a common phenomenon.

A common feature between T. occidentalis and the other insectivores studied is the DV gradient of S cones, with higher densities in the ventral retina. This striking patterning, which seems to be conserved among Eu Lipotyphla (Peichl 2005), has been observed in several mouse species (Szél et al. 1996). S cone asymmetry increases the sensitivity of the ventral retina to short wavelengths in the mouse (Calderone & Jacobs 1995). In the case of fossorial moles, there is a hypothetical advantage in having this cone distribution. The ventral half-retina receives light from the upper visual field, and an increased S cone density may allow the mole to have a better perception of breaches in their tunnel network. A possible role of the visual systems in antipredatory behaviour has been previously suggested for subterranean rodents (Némec et al. 2007, 2008).

The random distribution of melanopsin-containing RGCs across the whole depth of the inner retina made an accurate estimation of their relative number in T. occidentalis not feasible. However, immunostaining clearly showed that the percentage of RGCs expressing melanopsin was far higher in the Iberian mole than in adult mice, with melanopsin-positive axons successfully incorporated to the optic nerve. Unusually high proportions of the melanopsin-positive RGC subset (about 20% of the total RGC population) have been reported also in the rodent Spalax (Hamnibal et al. 2002), thus suggesting that this feature may have evolved convergently in unrelated fossorial mammals.

The maintenance of a high proportion of neonatal melanopsin-containing RGCs throughout the whole life of T. occidentalis could have represented an important adaptive acquisition during talpid evolution. Moles show a clear periodicity of activity and rest (Macdonald et al. 1997; Borroni et al. 1999) and are strict seasonal breeders, with a cycle that varies in length according to latitude (Jiménez et al. 1990). Thus, information on photoperiod changes may be an essential requirement for these fossorial insectivores. An increase in the melanopsin-containing cell density could represent a very valuable adaptive trait.

4. MATERIAL AND METHODS

(a) Material analysed

Ninety-seven specimens of T. occidentalis were obtained from animals captured in the wild as described previously (Barrionuevo et al. 2004a) under permission granted by the Andalusian Environmental Council. Developmental stages were determined according to Barrionuevo et al. (2004b) and Carmona et al. (2009).

Positive controls were carried out with mouse samples of the CBA/Ca strain.

(b) Immunohistochemistry and haematoxylin and eosin staining

Tissues were embedded in wax and sectioned following standard procedures. Immunohistochemistry and haematoxylin staining was described previously (Carmona et al. 2008). Electronic supplementary material table S2 summarizes the antibodies used in this study.

(c) Retinal flat-mount immunofluorescence

Retinas were dissected from fixed eyes into TBS, 0.05 per cent Tween-20, 0.1 per cent Triton X-100 for 30 min at RT. After washing with TBS, 0.05 per cent Tween-20 (TBS-T), retinas were exposed to primary antibodies in PBS, 4 per cent BSA overnight at 4°C, washed again with TBS-T and incubated in secondary antibodies diluted in PBS, 4 per cent BSA, for 3 h at RT. Retinas were flat-mounted in medium containing DAPI.

(d) Photoreceptor densities and topography measurements

Densities and topographic distributions of rod and cone photoreceptors were obtained by analysing four adult flat-mounted retinas (from four different moles) after double immunostaining for S and M opsins for analysis of density and in higher sampling windows of 160 × 120 μm for topographic assay. Rods were identified as unlabelled photoreceptors using DIC optics. Shrinkage was evaluated by planimetrically determining the retinal area before and after mounting.

(e) DiI labelling of retinal ganglion cell axons

Five-stage s5b–c mole heads were fixed for DiI staining of the optic nerve. After removing the cornea, the lens was removed from the embryonic eye and the inside of the retina was carefully dried with filter paper. A crystal of DiI (D282; Molecular Probes, Eugene, OR, USA) was adhered to the optic disc and the lens was replaced. Heads were incubated in PBS, 0.02 per cent NaN₃ at 37°C for 4 days. Subsequently, the brain was dissected and placed in PBS for whole-mount photomicrography using a red single bandpass fluorescence mirror unit.

(f) Ultrastructural analysis

Samples were processed for transmission electron microscopy (TEM) by the Electron Microscopy Facility of the Institute of Medical Sciences, University of Aberdeen using a Philips CM10 microscope.

(g) Mole sight study

Behavioural tests were conducted in a Y-maze, made of 50 mm diameter black PVC tubing. Flashing white light bulbs (12 V, 22 mm diameter, 4 flashes s⁻¹) were placed at the ends of the two symmetric arms of the maze. The illumination that these lamps provided at the bifurcation of the Y-maze was 30 lux. For each trial, a dark-adapted mole (kept for 2 days prior to the experiment to get them used to captivity) was released into the maze through the access tube, the light switched on in one branch and the subject allowed to choose the dark or the illuminated arm. The light and any reflection were not visible from the entrance of the Y-maze. Four adult moles (two males, two females) were used. Sets of 10 trials were done with each animal. Left and right Y-maze arms were randomly illuminated. To minimize stress, animals were rested between consecutive sets of experiments. The χ² distribution was determined to test for statistical significance.

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