

Published in final edited form as:

Vis Neurosci. 2004 ; 21(2): 107–117.

Identification of retinal neurons in a regressive rodent eye (the naked mole-rat)

STEPHEN L. MILLS¹ and KENNETH C. CATANIA²

¹ Department of Ophthalmology and Visual Science, University of Texas at Houston—Health Science Center, Houston

² Department of Biological Sciences, Vanderbilt University, Nashville

Abstract

The retina consists of many parallel circuits designed to maximize the gathering of important information from the environment. Each of these circuits is comprised of a number of different cell types combined in modules that tile the retina. To a subterranean animal, vision is of relatively less importance. Knowledge of how circuits and their elements are altered in response to the subterranean environment is useful both in understanding processes of regressive evolution and in retinal processing itself. We examined common cell types in the retina of the naked mole-rat, *Heterocephalus glaber* with immunocytochemical markers and retrograde staining of ganglion cells from optic nerve injections. The stains used show that the naked mole-rat eye has retained multiple ganglion cell types, 1–2 types of horizontal cell, rod bipolar and multiple types of cone bipolar cells, and several types of common amacrine cells. However, no labeling was found with antibodies to the dopamine-synthesizing enzyme, tyrosine hydroxylase. Although most of the well-characterized mammalian cell types are present in the regressive mole-rat eye, their structural organization is considerably less regular than in more sighted mammals. We found less precision of depth of stratification in the inner plexiform layer and also less precision in their lateral coverage of the retina. The results suggest that image formation is not very important in these animals, but that circuits beyond those required for circadian entrainment remain in place.

Keywords

Mole-rat; Regressive evolution; Eye; Vision; Retina

Introduction

Mammalian retinas are remarkable in the extent to which they share a basic plan of construction across their enormous variety of ecological niches. Many cell types are well conserved across the orders, for example, an axon-bearing horizontal cell, a single type of rod bipolar cell, and AII and starburst amacrine cells. The differences that can be found in the densities, morphology and connectivity of various cell types reveal how the basic plan is tailored according to varying environmental demands.

Mole-rats are burrowing rodents with severe deterioration of visual capacity, particularly in image formation. Previous studies of mole-rat eyes have focused on structural abnormalities at the light- and electron-microscopic level, identification of photoreceptor types, and on the reduction in the number of ganglion cells. The blind mole-rat, *Spalax ehrenbergi*, from the

family Spalacidae, has been the best-characterized species, and is sometimes said to have a purely circadian eye (Cernuda-Cernuda et al., 2002). Here we examine naked mole-rats (*Heterocephalus glaber*), which are members of the Bathyergidae family, which also contains the Zambian mole-rats (*Cryptomys anselli* and *C. hottentotus*).

Naked mole-rats retain the ability to expose their eyes to direct light, in contrast to the eye of *Spalax*, which has no pupil, no detectable visual evoked potential, or behavioral response to light stimulation and little, if any, visual cortex (Nevo, 1999). Both species nevertheless maintain both rod and cone photoreceptors, the inner nuclear layer (INL) appears normal, and ganglion cells are maintained, if sparse. The retinal projection to the suprachiasmatic nucleus is disproportionately enlarged, but strongly reduced projections to other visual areas can be found in *Spalax* and an African mole-rat *Cryptomys hottentotus* (Bronchti et al., 1991; Cooper et al., 1993a,b; Negroni et al., 2003). Curiously, the blind mole-rat lacks short wavelength photoreceptors, while the African mole-rats, including *H. glaber* contain almost exclusively short-wavelength cones (David-Gray et al., 2002; Cernuda-Cernuda et al., 2003; Peichl et al., 2004).

This study extends previous studies of mole-rat retinal morphology by closely examining the presence and morphology of well-characterized retinal neurons with common immunocytochemical markers. The goal of this approach is to gauge if any common mammalian cell types have been discarded, and how those that are retained may have been reorganized in response to a subterranean environment.

Materials and methods

Naked mole-rats of the species *Heterocephalus glaber* were obtained from Jenny Jarvis at the University of Cape Town in South Africa. The colony from which they were obtained has been captive for a number of years; all of the obtained animals were born in the colony. Our group included both males and females; two were juveniles, while the remainder were adults. Animals were arterially perfused with 4% paraformaldehyde and the brain removed for examination of cortical specializations (Catania & Remple, 2002) in accordance with institutional animal welfare practices. The remainder of the skull, including the eyes, was stored in 2% paraformaldehyde for periods ranging from a few weeks to a few months.

Retinas were isolated by dissecting the eyes from their orbits, then lightly brushing the retina apart from the sclera and pigment epithelium. They were then rinsed in 0.1 M phosphate buffer and stored in buffer + 3% donkey serum. Both eyes of seven animals were used. We used several common antibodies that label retinal neurons in other species. Suppliers and antibodies were as follows: Sigma Chemical Co., St. Louis, MO (parvalbumin, 1:1000; calbindin-28 kDa, 1:500; tyrosine hydroxylase, 1:1000), Chemicon, El Segundo, CA (choline acetyltransferase, 1:100; calretinin, 1:1000), Calbiochem, San Diego, CA (tyrosine hydroxylase, 1:500), and Santa Cruz (the alpha subunit of protein kinase C, 1:1000). Additionally, crystals of 1,1'-dioctadecyl-3,3',3'-tetramethyl-6-carbocyanine perchlorate (DiI_{C18}(3); DiI; Molecular Probes, Eugene, OR) were applied to the optic nerve of some intact eyes to label ganglion cells and left to diffuse in 0.1% paraformaldehyde at 4°C for 6 weeks.

Two retinas were embedded in 4% agarose (Sigma) and radial vibratome sections were cut at 30-μm thickness. One rat retina was sectioned in the same manner. These sections were stored in 3% donkey serum (Jackson ImmunoResearch, West Grove, PA) and subsequently reacted with primary and secondary antibodies in 0.1 M phosphate-buffered saline + 0.5% Triton + 0.1% sodium azide + 1% donkey serum, each overnight. Whole retinas were incubated in primary antibodies for 7–10 days. Secondary antibodies were incubated overnight at a concentration of 1:200 in the Triton/sodium azide/serum-containing buffer; fluorescent tags

used were donkey IgG fragments of Cy3 and Cy5 (Jackson ImmunoResearch), and Alexa488 (Molecular Probes).

Sections of rat retina are shown for comparison and were incubated in parallel with mole-rat sections. The purpose was to verify the viability of the antibodies and to provide a comparison of staining in a well-characterized species. The evolutionary distance from the mole-rat families to other rodents is fairly large and roughly comparable across the common members of the family. The closest common species is probably the guinea pig (Reyes et al., 2004). The rat was chosen for comparison, however, because of its ready availability and because it has been the best-characterized rodent retina.

Optical sections were made on a Zeiss LSM410 confocal microscope. When scanning wholemounts, sections were usually acquired at 1- μ m distances over the region of interest, although 0.5- μ m sections were occasionally obtained. Vibratome sections were scanned at 0.5- μ m distances. Images were acquired with contrast and brightness settings appropriate for filling the 8-bit coding range and were subsequently adjusted in Adobe Photoshop to further optimize contrast and brightness for printing on a Codonics, Inc. NP-1600 color printer (Middleburg Heights, OH). Tissue was usually reacted with at least three antibodies at a time and visualized with Cy3, Cy5, and Alexa-488 secondaries. Cy3 fluorescence was therefore imaged with a emission filter bandpass of 610–650 nm, to exclude Cy5 fluorescence.

Results

The eyes of the blind mole-rat, *Spalax ehrenbergi*, are subcutaneous, and sometimes further embedded in the Harderian gland (Sanyal et al., 1990; Nevo, 1999). The eyes of the naked mole-rat, however, retain an opening in the skin that will admit light. Some of the older animals appeared to have additional occluding tissue between eyelid and cornea, possibly from an overgrown Harderian gland, and which would degrade spatial vision if present. Fig. 1 shows the head of an captive naked mole-rat (*H. glaber*), demonstrating its exposed pupil.

Gross examination of vertical sections of the naked mole-rat retina were compared with similar sections of rat retina. Interference optics did not highlight the structure of the mole-rat retina (Fig. 2A) in as much detail as the more regular rat retina (Fig. 2B). The major observable differences were in the sparse nature of the ganglion cell layer, the relative thicknesses of the plexiform layers, and the length of the photoreceptors. The nuclear layers were of comparable thicknesses between the two species. The dark descending lines in the naked mole-rat section were probably processes of Müller cells, although possibly axons from rod bipolar cells. As no Müller cell stain was used, this was not confirmed.

Ganglion cell staining

We next investigated the relative heterogeneity of the ganglion cells with a combination of retrograde labeling with DiI and with labeling of ganglion cell types with antibodies. Fig. 3A shows two merged confocal micrographs of DiI labeling. The red portion shows DiI labeling in the ganglion cell layer (GCL), while the green labeling show displaced ganglion cells also stained by DiI, but whose cell bodies are located in the INL. Most ganglion cells are in the GCL, as expected, but some are displaced to the amacrine cell layer. Axons could sometimes, although not always, be observed emerging from these displaced ganglion cells. DiI applied to the optic nerve should stain only ganglion cells; no small somas were stained and no other potentially artifactual DiI staining (e.g. horizontal cells, bipolar cells) was seen. Therefore, we believe all of the DiI-stained cells represented ganglion cells, some of which were displaced to the amacrine cell layer of the INL. Fig. 3B shows DiI labeling in the GCL (red) of the rat retina. Fig. 3C shows a mole-rat retina stained with antibodies to parvalbumin (green) and calbindin (red). There are some moderately large ganglion cells predominantly stained red,

with distinct initial processes. Another larger type appears yellow, as it was stained with both antibodies and a third smaller and less brightly labeled group appears predominantly green. Overall, the diversity of labeling and soma sizes suggests that at least three different ganglion cell types can be found in the naked mole-rat.

The quality of staining was uneven across the mole-rat retinas with both the DiI and immunocytochemical techniques. We did not attempt to estimate the total number of ganglion cells for this reason, nor did we count labeled axons with the DiI treatment.

Parvalbumin staining

Retinal cell types stained by antibodies to the calcium-binding protein parvalbumin differ widely across species. Staining is often found in horizontal cells, ganglion cells, and amacrine cells (Röhrenbeck et al., 1987, 1989), but Hamano et al. (1990) found no staining with an antibody to parvalbumin in the guinea pig. Anti-parvalbumin stains AII amacrine cells in rat and rabbit retina (Wässle et al., 1993; Casini et al., 1995; Massey & Mills, 1999). In the naked mole-rat retina, anti-parvalbumin staining was fairly weak, variable, and nonselective, although ganglion cell staining was reliable. Putative AII amacrine cells were stained with both parvalbumin and calretinin antibodies (not shown); however, anti-calretinin staining was stronger and more specific. More amacrine cell types were stained with anti-parvalbumin and staining was of low contrast. For these reasons, anti-parvalbumin staining was not used further.

Calretinin staining

AII amacrine cells are a ubiquitous cell type in mammalian retinas, from wallaby to primate (e.g. Wong et al., 1986; Wässle et al., 1993, 1995; Mills & Massey, 1999, Massey & Mills, 1999). The AII amacrine cell is well known to serve as an interneuron between rod bipolar cells and cone bipolar cells, having minor or no direct contact with ganglion cells (Famiglietti & Kolb, 1975). The best marker for labeling AII amacrine cells across many species has been anti-calretinin. We stained both rat and mole-rat retinas with anti-calretinin (1:1000). Amacrine and ganglion cells were stained in each species (Fig. 4). The inner plexiform layer (IPL) of the rat retina displayed a tristratified appearance common in many species and the nerve fiber layer showed considerable staining, as well as some ganglion cells. Staining in the IPL of the mole-rat was less clearly demarcated, although relatively thicker staining was observed high in the IPL, which we believe corresponded to the lobular appendages (arrowheads) of mole-rat AII amacrine cells shown both in radial (Fig. 4C) and flatmount view (Fig. 4E). The finer dendrites that contact rod bipolar cells and make gap junctions in sublamina *b* were less distinct. This was partially because they were not concentrated into obvious bands as in more sighted mammals, but instead ramified more diffusely both laterally and vertically in sublamina *b* and beyond. Nevertheless, stout processes that descend below the level of the lobular appendages can be glimpsed in Figs. 4C–4E. The rightmost cell in Fig. 4C, in particular, appears to have a process that descends to the very boundary of the IPL with the GCL. In general, however, processes descending into the putative ON sublamina could not be unambiguously traced to the finer processes that ramified among rod bipolar cell endfeet.

The calretinin antibody also stained cells in the outer portion of the INL in the naked mole-rat. These included horizontal cells (Figs. 4A & 4D, arrow) and probable bipolar cells (Figs. 4A & 4D).

Tyrosine hydroxylase staining

Antibodies to the dopamine-synthesizing enzyme, tyrosine hydroxylase (TOH), are among the most reliable markers across species (Bellesta et al., 1984; Brecha et al., 1984). In the rat retina, each of the two TOH antibodies produced staining in cells resembling those well characterized in a number of species as dopaminergic amacrine cells. In rat retina (Fig. 5B), the more sensitive

Sigma antibody, used at low dilution (1:1000 vs. 1:100,000 in other species), stained some cells in the outer portion of the INL (stars), the TOH-containing amacrine cell (arrowheads), and a population of smaller amacrine cells. Neither antibody at any tested dilution stained cells in the naked mole-rat (Fig. 5A). This panel is shown at high gain to highlight the lack of staining relative to background.

Choline acetyltransferase staining

An antibody to the acetylcholine-synthesizing enzyme, choline acetyltransferase (ChAT), reliably stains starburst amacrine cells in mammalian retina (Eckenstein & Thoenen, 1982; Famiglietti, 1983). In the rat retina (Fig. 6B), a ChAT antibody stained the typical two parallel bands of processes in the inner and outer sublamina of the IPL and the corresponding somas in the GCL and INL, similar to the results of Voigt (1986). Similar, but more diffuse and variable labeling was found in the naked mole-rat. ChAT staining was most distinct in the distal IPL (Fig. 6A) and the number of stained somas was greater in the INL than in the GCL (Figs. 6C & 6D). Varicosities were found throughout the IPL, but declined in density with increasing depth toward the GCL (Fig. 6A). We found considerable heterogeneity of staining and soma size and shape in both nuclear layers, but were unable to determine whether this variation represented any additional cell types or a larger variation in the usual starburst morphology.

Calbindin staining

An antibody to the 28-kDa calcium binding protein (calbindin; CaBP) stains cell types in all retinas reported, with considerable variability across species as to cell types. Horizontal cells are frequently stained (Röhrenbeck et al., 1987, 1989), as are various types of bipolar and ganglion cells (Pasteels et al., 1990; Grünert et al., 1994; Massey & Mills, 1996; Haverkamp & Wässle, 2000). Fig. 7 shows a flatmount view of a CaBP-positive horizontal cell in the naked mole-rat. The same cell is shown in radial view in the inset. The retinas of some other rodents are reported to contain only axon-bearing (B-type) horizontal cells (Peichl & Gonzalez-Soriano, 1994), but we were unfortunately unable to distinguish the processes of these horizontal cells sufficiently well to determine if they were axon-bearing (B-type) or axonless (A-type). Note, however, the large processes marked by arrows in Fig. 7. These resemble the large-caliber processes of axonless horizontal cells in other retinas (e.g. rabbit; Mills & Massey, 1994) and which led to a dim soma (long arrow). Fig. 8 shows a radial section of mole-rat retina stained for CaBP (green) and PKC α (red). The most intense CaBP antibody staining was in ganglion cells, with amacrine cells, horizontal cells, and bipolar cells more weakly stained.

Protein kinase C staining

Antibodies to PKC α consistently stain rod bipolar cells across mammalian species. There are differences between species and between PKC antibodies in terms of what other retinal neurons may be stained, typically cone bipolar cells or amacrine cells. Fig. 8 shows a radial section of PKC α staining (red) in the naked mole-rat. The most brightly stained cells appear to have the morphology of rod bipolar cells (arrowheads), which are also lightly stained with CaBP immunoreactivity (green). It appears likely that some cone bipolar cells may also be stained by the PKC α antibody, as there are red somas lacking the bushy dendritic arbor of rod bipolar cells and also lacking CaBP immunoreactivity in this substructure (large stars). There are also some weakly CaBP-immunoreactive bipolar cells without PKC α immunoreactivity (small stars).

Most remarkable is the degree to which PKC immunoreactivity appears diffusely throughout the IPL, rather than being restricted to the most proximal IPL layer, as is common in other species. This is seen even more distinctly in conjunction with the CaBP label. The PKC α -positive putative rod bipolar cell processes can extend further than the bottom of the IPL (short arrows), sometimes wrapping around ganglion cell somas and even branching into the nerve

fiber layer. Some of these stray processes contain swellings and varicosities which usually mark sites of synaptic contact in other species. This suggests that rod bipolar cells in the naked mole-rat may break the mammalian rule of making exclusive contact with AII amacrine cells and in fact may directly contact ganglion cells. On the other hand, as both AII amacrine cells and rod bipolar cells ramify more broadly and less distinctly than in other mammals, there may be rod bipolar/AII amacrine cell contacts at unusual levels of the retina.

It is possible that some of the IPL staining by anti-PKC α that occurred more distal to the GCL than is usual for rod bipolar cell terminals might arise from cone bipolar cells or amacrine cells. We cannot rule this out, but it appeared that at least some rod bipolar cells branched and produced varicosities at these more distal levels.

Fig. 9 shows a wholemount view of putative AII amacrine cells stained with anti-calretinin and rod bipolar cells stained with anti-PKC α . In rabbit retina (inset), the strong association between rod bipolar cell endfeet and AII amacrine cell processes is obvious. Comparable association of structures is much less apparent in the naked mole-rat. A few calretinin-positive AII processes ramify at this level and do indeed appear to preferentially contact rod bipolar cell endfeet, but most endfeet do not obviously contact a putative AII amacrine cell process. This again suggests that the remaining rod bipolar cell terminals may contact unlabeled ganglion cell processes, in addition to the likely somatic contacts observed in Fig. 8. This issue could only be resolved by examination of the ultrastructure.

Discussion

The purpose of this study was to investigate the fate of well-characterized retinal circuits in an animal whose image-forming capabilities may have deteriorated in response to its almost exclusively subterranean environment. We used the naked mole-rat, a member of the order Rodentia. Rodentia is historically divided into three suborders, Myomorpha (including rats and mice), Sciuromorpha (squirrels), and Hystricognatha (which includes guinea pigs and the African mole-rat family, *Bathyergidae*). The bathyergid family of mole-rats is fairly distant from the blind mole-rat genus, *Spalax*, which apparently evolved independently into a subterranean environment. The closest living relatives of the African mole-rats are cane rats, rock rats, and Old World porcupines, with the caviomorphs (which include guinea pigs) being next most distant.

Mole-rat retinas typically have a sparse ganglion cell population, and projections to targets other than the suprachiasmatic nucleus have been strongly reduced (Bronchti et al., 1991; Cooper et al., 1993a,b; Negroni et al. 2003). If their eyes are primarily circadian in function, have pathways concerned with image formation, color, and motion been discarded?

We investigated this question by staining the eyes of naked mole-rats, *Heterocephalus glaber*, with several antibodies associated with various retinal neurons. A basic finding was that the regularly occurring mammalian neural types we examined can still be found in the naked mole-rat. A possible exception is the dopaminergic amacrine cell, which is stained by an antibody to TOH in all known species, but which was unstained by two TOH antibodies in the naked mole-rat. There are other explanations possible besides complete loss of this cell type. Mutations of the epitope may have rendered it unrecognizable by the standard antibodies, or made the antigenic site less accessible following formaldehyde fixation. Another explanation might be that these cells exist in the mole-rat, but that tyrosine hydroxylase is down-regulated below detection levels, due to the subterranean environment. One might expect that dopamine would still be required for entrainment of the circadian rhythm. On the other hand, the ganglion cells that form the retinohypothalamic (circadian) tract in the blind mole-rat, *Spalax ehrenbergi* (Hannibal et al., 2002) are immunopositive for melanopsin, as is also

the case for more sighted mammals (Berson et al., 2002; Hattar et al., 2002; Panda et al., 2003).

Dopamine has often been cited as a neuromodulator that might underlie mechanisms of light and dark adaptation and circadian rhythmicity, but its exact roles in these processes have remained controversial (Baldrige & Ball, 1991; Weiler et al., 1997; Ribelayga & Mangel, 2003). It is clear that dopamine levels shift many cellular processes, possibly enhancing transition from rod to cone function (Witkovsky & Deary, 1991). The naked mole-rat retains both rods and cones, rod and cone bipolar cells, and AII amacrine cells. Nevertheless, it is not clear that the naked mole-rat is ever exposed to background light over long periods of time. A slow neuromodulator such as dopamine might not be maintained at high levels in these animals, but this is purely speculative.

Horizontal cells

Mammalian retinas are usually distinguished by the presence of two types of horizontal cell (reviewed in Peichl et al., 1998). One contains an axon with terminal processes that contact rods. The other has axonal processes which are much reduced, as in the primate, or completely axonless, as in cat and rabbit. We were able to observe horizontal cells in retinas stained with antibodies to either calbindin or calretinin. The retinas of some other rodents, mouse and rat, contain only the axon-bearing horizontal cell type (Peichl & Gonzalez-Soriano, 1994). As noted by these authors, this may be an attribute of the family *Muridae*, as some other rodents, for example, the guinea pig retain two types. The guinea pig and naked mole-rat both belong to the suborder caviomorpha. (This assignment differs across sources.) It would therefore not be surprising if these two species were similar in their horizontal cell typologies. The naked mole-rat clearly retains at least one horizontal cell type. It is our belief that a second type was also present. It could be differentiated by larger diameter dendrites than those emanating from the more clearly marked horizontal cell. The large caliber of the dendrites (Fig. 7) suggests that this might be the homolog of the rabbit and cat axonless (A-type) horizontal cell. We were unable to discriminate an axon in either type, however, so we cannot conclusively call either type axon bearing.

Bipolar cells

Antibodies to the alpha subunit of PKC reliably stain rod bipolar cells in mammals, although they may also stain other retinal cell types. Which additional types are stained varies widely across species. Anti-PKC α stained the majority of bipolar cells in the naked mole-rat and almost certainly stains the rod bipolar cell, which is distinct with its bushy top and terminal endfeet. Some other bipolar cells were also stained by the PKC α antibody and also by CaBP immunoreactivity. We conclude that some cone bipolar cells can also be found in the naked mole-rat. As these are the target cells of AII amacrine cells, some would be expected to survive even in a purely scotopic retina, or else substantial rewiring must occur. Reliable counts could not be made due to variation in staining intensity in mole-rat wholemounts, but our impression was that rod bipolar cells strongly outnumber cone bipolar cells, contrary to the normal finding in even rod-dominated mammals (Strettoi & Masland, 1996; Jeon et al., 1998).

All amacrine cells

AII amacrine cells are an obligatory portion of the mammalian rod pathway and are believed to exist in every mammalian species. An antibody to calretinin, which we have previously used to stain AII amacrine cells in primate and rabbit retina, stained cells with the general morphology of AII amacrine cells in the naked mole-rat. Calretinin antibodies did not stain AII amacrine cells in either rat or mouse retinas (Veruki & Wässle, 1996; Haverkamp & Wässle, 2000), but the morphology of calretinin-stained amacrine cells in the naked mole-rat suggested that AII amacrine cells were stained by this antibody. Further, these cells were also

staining weakly by an antibody to parvalbumin, which stains AII amacrine cells in rat retina (Wässle et al., 1993). In the calretinin-stained material, the presence of the characteristic lobular appendages (Fig. 4) was the most important determinant of this classification, although processes extending into sublamina *b* were also evident. The pattern of staining in the control rat sections (Fig. 4B) was similar to that previously found (Pasteels et al., 1990; Veruki & Wässle, 1996) and presumed to be an amacrine cell of undetermined type.

AII amacrine cells are typically identified by their distinctive morphology coupled with the presence of TOH-positive “rings” around the soma (Voigt & Wässle, 1987) and strong synaptic association with rod bipolar cell terminals. Each of these criteria is less reliable in the naked mole-rat. TOH staining was always negative. Circular structures resembling lobular appendages appeared in the distal portion of the IPL (Fig. 4) and finer dendrites appeared to course throughout the region where PKC-positive rod bipolar cell endfeet appear (Fig. 9). It was difficult, however, to unequivocally trace processes as they descended, so as to link up the two types of processes. Further, as shown in Fig. 9, the putative AII dendrites are sparse in the area of the rod bipolar cell endfeet. The varicosities that are located on well-described processes do seem to contact rod bipolar cell endfeet preferentially. Based upon these considerations, we feel that the amacrine cell stained by the anti-calretinin antibody were in fact AII amacrine cells

Ganglion cells

The retina of the blind mole-rat, *Spalax ehrenbergi*, contains fewer than a thousand ganglion cells, of which about 20% contain melanopsin and project to the suprachiasmatic nucleus (Hannibal et al., 2002). Therefore, even in this extreme case of regressed sight, there remains some diversity of ganglion cell types, which maintain sparse projections to standard visual areas (Bronchti et al., 1991; Cooper et al., 1993a,b; Negroni et al., 2003). We found that the naked mole-rat also contained a variety of ganglion cells, some of which were displaced to the amacrine cell layer. Further identification or determination of the exact number of types was not possible, although no fewer than three types seems probable. The density of each of these types was also larger than that expected for a melanopsin-containing cell, so that this additional type may also be present, as in *Spalax*. Negroni et al. (2003) injected viral markers into the suprachiasmatic nucleus of the related bathyergid, *Cryptomys hottentotus*. The ganglion cells labeled by that method were much more sparsely distributed than those labeled by DiI in *H. glaber*. We therefore conclude that the ganglion cells responsible for light entrainment are a small subset of the total ganglion cell population, perhaps comparable to the 20% attributed to the *Spalax* retina by Hannibal et al. (2002).

The structure of the IPL

Given the extreme disorganization of the IPL, one might question whether the ON/OFF subdivision of the IPL is maintained. Our evidence suggests that it is. First, ChAT-positive cells are found in both GCL and INL. These are well known to correspond to ON and OFF starburst amacrine cells in more sighted mammals (Eckenstein & Thoenen, 1982; Famiglietti, 1983). The morphology of AII amacrine cells found in the naked mole-rat provides further support. The presence of both lobular appendages in sublamina *a* and finer processes in sublamina *b* indicates that the naked mole-rat retina has retained both ON and OFF sublaminae function.

The most compelling feature of the naked mole-rat, however, was not in the loss of cell types, especially in the INL, but in the reduction in organizational specificity throughout the mole-rat retina. Strict segregation of dendrites from any cell type was never seen across the depth of the IPL. In the lateral dimension, processes were inclined to wander in a meandering fashion without either tiling the retina effectively or forming strict associations with observed

postsynaptic targets. The implication was that spatial information is not likely to be gathered in any but the coarsest manner.

The indication that the finer dendrites of AII amacrine cells are not exclusively associated with rod bipolar cell endfeet, and that rod bipolar terminals may even wrap around and potentially contact ganglion cell somas, suggests that the naked mole-rat may differ from other mammals, where rod bipolars and AII amacrine cells do not directly contact ganglion cells, but contact only AII amacrine cells and cone bipolar cells, respectively. The output synaptic contacts made by AII amacrine cells in other species are via gap junctions to ON cone bipolar cells and inhibitory synapses to OFF cone bipolar cells (Famiglietti & Kolb, 1975). Therefore, to avoid inappropriate reversal of light response, these putative contacts with ganglion cells would presumably have to occur *via* gap junctions with ON ganglion cells and *via* inhibitory synapses with OFF ganglion cells. Similarly, any rod bipolar cell contacts should occur onto only ON ganglion cells.

It is notable that cell types associated with directional sensitivity, the ChAT-positive amacrine cells, were retained. We could not determine if their normal targets, the ON and ON-OFF directionally selective ganglion cells, were present in the mole-rat. It would be reasonable to conjecture that some sensitivity to gross movement might be retained so that an animal could sense movements of potential predators in those rare cases where they are exposed. The pattern of immunoreactivity suggests that the naked mole-rat has retained a more viable OFF response than ON.

Roughly 15–20 parallel pathways are found in mammalian retinas (Kolb et al., 1992; Boycott & Wässle, 1999; Rockhill et al., 2002; Marc & Jones, 2002), as deduced from the number of ganglion cell types. Mole-rat retinas have a reduced number of ganglion cells, but it was not possible to determine how many types are still present. The variation in morphology and immunocytochemistry suggest at least 3–5 types, but it is not clear whether the reduced absolute number of ganglion cells is due to reduction in number of types, or reduction in density due to lack of need for spatial detail.

Our findings suggest that elements of retinal circuits do not disappear as their functions are devalued. Instead, retinal architecture becomes extremely disorganized, both in the vertical and lateral domains. Projections remain to all major structures in the blind mole-rat, albeit strongly reduced and disorganized in some cases. It would be interesting to know if circuits normally dedicated to acuity, color, and fine motion detection merely provide reduced information of that type, or whether they have been retooled for other purposes.

Acknowledgements

This research was supported by NIH (EY10121 to S.L. Mills and core grant EY10608) and Research to Prevent Blindness (an unrestricted award to the Department of Ophthalmology and Visual Science).

References

- Baldrige WH, Ball AK. Background illumination reduces horizontal cell receptive-field size in both normal and 6-hydroxydopamine-lesioned goldfish retinas. *Visual Neuroscience* 1991;7:441–450. [PubMed: 1764414]
- Bellesta J, Terenghi G, Thibault J, Polak JM. Putative dopamine-containing cells in the retina of seven species demonstrated by tyrosine hydroxylase immunocytochemistry. *Neuroscience* 1984;12:1147–1156. [PubMed: 6148714]
- Berson DM, Dunn FA, Takao M. Phototransduction by retinal ganglion cells that set the circadian clock. *Science* 2002;295:1070–1073. [PubMed: 11834835]
- Boycott BB, Wässle H. Parallel processing in the mammalian retina: The Proctor lecture. *Investigative Ophthalmology and Visual Science* 1999;40:1313–1327.

- Brecha NC, Oyster CW, Takahashi ES. Identification and characterization of tyrosine hydroxylase immunoreactive amacrine cells. *Investigative Ophthalmology and Visual Science* 1984;25:66–70. [PubMed: 6142028]
- Bronchti G, Rado R, Terkel J, Wollberg Z. Retinal projections in the blind mole rat: A WGA-HRP tracing study of a natural degeneration. *Developmental Brain Research* 1991;58:81–84. [PubMed: 2015657]
- Casini G, Rickman DW, Brecha NC. AII amacrine cell population in the rabbit retina: identification by parvalbumin immunoreactivity. *Journal of Comparative Neurology* 1995;356:132–142. [PubMed: 7629307]
- Catania KC, Remple MS. Somatosensory cortex dominated by the representation of teeth in the naked mole rat brain. *Proceedings of the National Academy of Sciences of the USA* 2002;99:5692–5697. [PubMed: 11943853]
- Cernuda-Cernuda R, DeGrip WJ, Cooper HM, Nevo E, Garcia-Fernández JM. The retina of *Spalax ehrenbergi*: Novel histologic features supportive of a modified photosensory role. *Investigative Ophthalmology and Visual Science* 2002;43:2374–2383. [PubMed: 12091440]
- Cernuda-Cernuda R, Garcia-Fernández JM, Gordign MCM, Bovee-Guerts P, DeGrip WJ. The eye of the african mole-rat *Cryptomys anelli*: To see or not to see? *European Journal of Neuroscience* 2003;17:709–720. [PubMed: 12603261]
- Cooper HM, Herbin M, Nevo E. Visual system of a naturally microphthalmic mammal: The blind mole rat, *Spalax ehrenbergi*. *Journal of Comparative Neurology* 1993a;328:313–350. [PubMed: 8440785]
- Cooper HM, Herbin M, Nevo E. Ocular regression conceals adaptive progression of the visual system in a blind subterranean mammal. *Nature* 1993b;361:156–159. [PubMed: 7678449]
- David-Gray ZK, Bellingham J, Munoz M, Avivi A, Nevo E, Foster RG. Adaptive loss of ultraviolet-sensitive/violet-sensitive (UVS/VS) cone opsin in the blind mole rat (*Spalax ehrenbergi*). *European Journal of Neuroscience* 2002;16:1186–1194. [PubMed: 12405979]
- Eckenstein F, Thoenen H. Production of specific antisera and monoclonal antibodies to choline acetyltransferase: Characterization and use for identification of cholinergic neurons. *EMBO Journal* 1982;1:363–368. [PubMed: 6821323]
- Famiglietti EV. ‘Starburst’ amacrine cells and cholinergic neurons: Mirror-symmetric on and off amacrine cells of rabbit retina. *Brain Research* 1983;261:138–144. [PubMed: 6301622]
- Famiglietti EV, Kolb H. A bistratified amacrine cell and synaptic circuitry in the inner plexiform layer of the retina. *Brain Research* 1975;84:293–300. [PubMed: 1111833]
- Grünert U, Martin PR, Wässle H. Immunocytochemical analysis of bipolar cells in the macaque monkey retina. *Journal of Comparative Neurology* 1994;348:607–627. [PubMed: 7530731]
- Hamano K, Kiyama H, Emson PC, Manabe R, Nakauchi M, Tohyama M. Localization of two calcium-binding proteins, calbindin (28 kD) and parvalbumin (12 kD) in the vertebrate retina. *Journal of Comparative Neurology* 1990;302:417–424. [PubMed: 2289978]
- Hannibal J, Hindersson P, Nevo E, Fahrenkrug J. The circadian photopigment melanopsin is expressed in the blind subterranean mole rat, *Spalax*. *Neuroreport* 2002;13:1411–1414. [PubMed: 12167764]
- Hattar S, Liao HW, Takao M, Berson DM, Yau KW. Melanopsin-containing retinal ganglion cells: Architecture, projections, and intrinsic photosensitivity. *Science* 2002;295:1065–1070. [PubMed: 11834834]
- Haverkamp S, Wässle H. Immunocytochemical analysis of the mouse retina. *Journal of Comparative Neurology* 2000;424:1–23. [PubMed: 10888735]
- Jeon CJ, Strettoi E, Masland RH. The major cell populations of the mouse retina. *Journal of Neuroscience* 1998;18:8936–8946. [PubMed: 9786999]
- Kolb H, Linberg KA, Fisher SK. Neurons of the human retina: A Golgi study. *Journal of Comparative Neurology* 1992;318:147–187. [PubMed: 1374766]
- Marc RE, Jones BW. Molecular phenotyping of retinal ganglion cells. *Journal of Neuroscience* 2002;22:413–427. [PubMed: 11784786]
- Massey SC, Mills SL. A calbindin-immunoreactive bipolar cell type in the rabbit retina. *Journal of Comparative Neurology* 1996;366:15–33. [PubMed: 8866843]
- Massey SC, Mills SL. An antibody to calretinin stains AII amacrine cells in the rabbit retina: Double label and confocal analysis. *Journal of Comparative Neurology* 1999;411:3–18. [PubMed: 10404104]

- Mills SL, Massey SC. Distribution and coverage of A- and B-type horizontal cells stained with Neurobiotin in the rabbit retina. *Visual Neuroscience* 1994;11:549–560. [PubMed: 7518689]
- Mills SL, Massey SC. AII amacrine cells limit scotopic acuity in central macaque retina: An analysis with calretinin labeling, confocal microscopy and intracellular dye injection. *Journal of Comparative Neurology* 1999;411:19–34. [PubMed: 10404105]
- Negroni J, Bennett NC, Cooper H. Organization of the circadian system in the subterranean mole rat, *Cryptomys hottentotus* (Bathyergidae). *Brain Research* 2003;967:48–62. [PubMed: 12650965]
- Nevo, E. Mosaic evolution of subterranean mammals: regression, progression, and global convergence. Oxford, UK: Oxford University Press; 1999.
- Panda S, Provencio I, Tu DC, Pires SS, Rollag MD, Castrucci AM, Pletcher MT, Sato TK, Wiltshire T, Andahazy M, Kay SA, Van Gelder RN, Hogenesch JB. Melanopsin is required for non-image-forming photic responses in blind mice. *Science* 2003;301:525–527. [PubMed: 12829787]
- Pasteels B, Rogers J, Blachier F, Pochet R. Calbindin and calretinin localization in retina from different species. *Visual Neuroscience* 1990;5:1–16. [PubMed: 2125465]
- Peichl L, Gonzalez-Soriano J. Morphological types of horizontal cell in rodent retinæ: A comparison of rat, mouse, gerbil, and guinea pig. *Visual Neuroscience* 1994;11:501–517. [PubMed: 8038125]
- Peichl, L.; Sandmann, D.; Boycott, BB. Comparative anatomy and function of mammalian horizontal cells. In: Chalupa, L.; Finlay, B., editors. *Development and Organization of the Retina*, NATO ASI Series A. 299. New York: Plenum Press; 1998. p. 147–172.
- Peichl L, Némec P, Burda H. Unusual cone and rod properties in subterranean African mole-rats (Rodentia, Bathyergidae). *European Journal of Neuroscience* 2004;19:1545–1558. [PubMed: 15066151]
- Reyes A, Gissi C, Catzeflis F, Nevo E, Pesole G, Saccone C. Congruent mammalian trees from mitochondrial and nuclear genes using Bayesian methods. *Molecular Biology and Evolution* 2004;21:397–403. [PubMed: 14660685]
- Ribelayga C, Mangel SC. Absence of circadian clock regulation of horizontal cell gap junctional coupling reveals two dopamine systems in the goldfish retina. *Journal of Comparative Neurology* 2003;467:243–253. [PubMed: 14595771]
- Rockhill RL, Daly FJ, MacNeil MA, Brown SP, Masland RH. The diversity of ganglion cells in a mammalian retina. *Journal of Neuroscience* 2002;22:3831–3843. [PubMed: 11978858]
- Röhrenbeck J, Wässle H, Heizmann CW. Immunocytochemical labelling of horizontal cells in mammalian retina using antibodies against calcium-binding proteins. *Neuroscience Letters* 1987;77:255–260. [PubMed: 3302765]
- Röhrenbeck J, Wässle H, Boycott BB. Horizontal cells in the monkey retina: Immunocytochemical staining with antibodies against calcium binding proteins. *European Journal of Neuroscience* 1989;1:407–420. [PubMed: 12106128]
- Sanyal S, Jansen HG, DeGrip WG, Nevo E, DeJong WW. The eye of the blind mole-rat, *Spalax ehrenbergi*. Rudiment with hidden function? *Investigative Ophthalmology and Visual Science* 1990;31:1398–1404. [PubMed: 2142147]
- Strettoi E, Masland RH. The number of unidentified amacrine cells in the mammalian retina. *Proceedings of the National Academy of Sciences of the USA* 1996;93:14906–14911. [PubMed: 8962154]
- Veruki ML, Wässle H. Immunohistochemistry localization of dopamine D1 receptors in rat retina. *European Journal of Neuroscience* 1996;8:2286–2297. [PubMed: 8950093]
- Voigt T. Cholinergic amacrine cells in the rat retina. *Journal of Comparative Neurology* 1986;248:19–35. [PubMed: 2424943]
- Voigt T, Wässle H. Dopaminergic innervation of AII amacrine cells in mammalian retina. *Journal of Neuroscience* 1987;7:4115–4128. [PubMed: 2891802]
- Wässle H, Grünert U, Röhrenbeck J. Immunocytochemical staining of AII-amacrine cells in the rat retina with antibodies against parvalbumin. *Journal of Comparative Neurology* 1993;332:407–420. [PubMed: 8349840]
- Wässle H, Grünert U, Chun MH, Boycott BB. The rod pathway of the macaque monkey retina: Identification of AII-amacrine cells with antibodies against calretinin. *Journal of Comparative Neurology* 1995;361:537–551. [PubMed: 8550898]

- Weiler R, Baldridge WH, Mangel SC, Dowling JE. Modulation of endogenous dopamine release in the fish retina by light and prolonged darkness. *Visual Neuroscience* 1997;14:351–356. [PubMed: 9147486]
- Witkovsky P, Deary A. Functional roles of dopamine in the vertebrate retina. *Progress in Retinal Research* 1991;11:247–292.
- Wong RO, Henry GH, Medveczky CJ. Bistratified amacrine cells in the retina of the tammar wallaby—*Macropus eugenii*. *Experimental Brain Research* 1986;63:102–105.



Fig. 1. A captive naked mole-rat (*Heterocephalus glaber*) investigating an opening in its tunnel system. Although its visual system is greatly reduced, the naked mole-rat retains an eyelid allowing the small eye to be directly exposed to light. Note also the tiny ear (raised area, behind the eye).

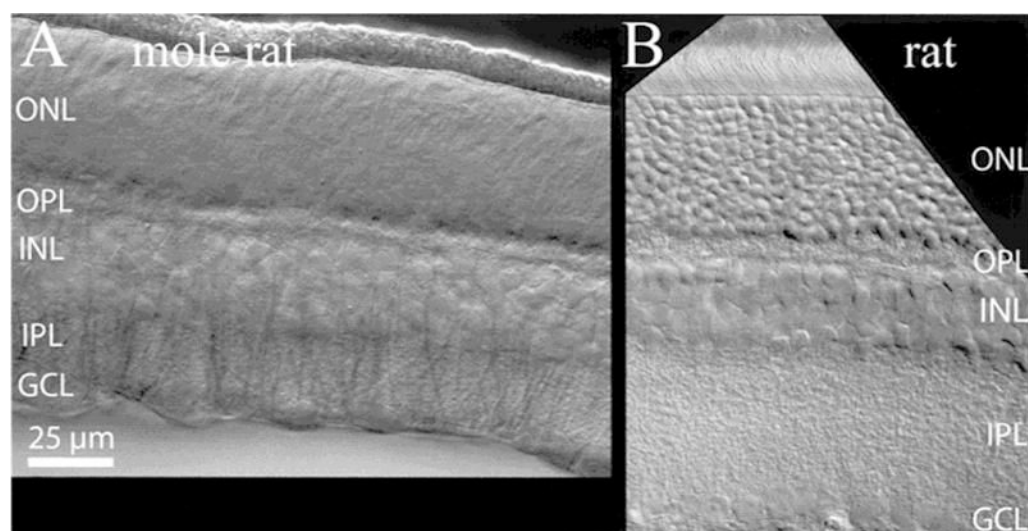


Fig. 2. The general structure of the mole-rat (A) and rat (B) retinas are shown with differential interference contrast photography. The structures of the mole-rat retina are less well delineated, but the major differences are the thinner plexiform layers, fewer ganglion cells, and a shortening of the photoreceptor inner and outer segments. OPL: outer plexiform layer; IPL: inner plexiform layer; ONL: outer nuclear layer; INL: inner nuclear layer; and GCL: ganglion cell layer.

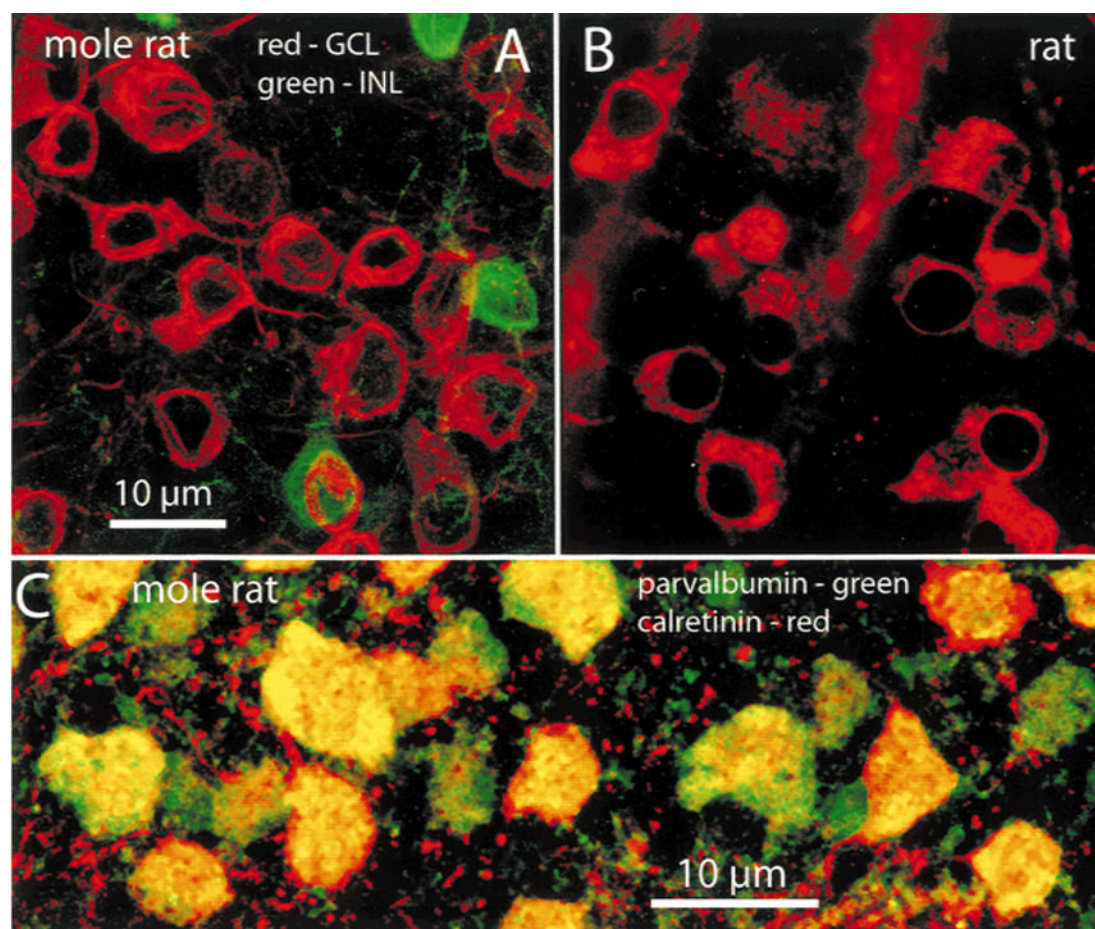


Fig. 3.

A diversity of ganglion cell types is revealed by staining with DiI or with antibodies to calcium-binding proteins. (A) DiI applied to the optic nerve stains cells both in the GCL (red) and INL (green). Stacks of 0.5-μm sections were taken separately from the two layers and pseudocolored for contrast. (B) Retrograde DiI staining of ganglion cells is shown in a rat retina for comparison. (C) Combined staining with antibodies to parvalbumin (green) and calretinin (red) suggest at least three types of ganglion cells exist in the naked mole-rat retina.

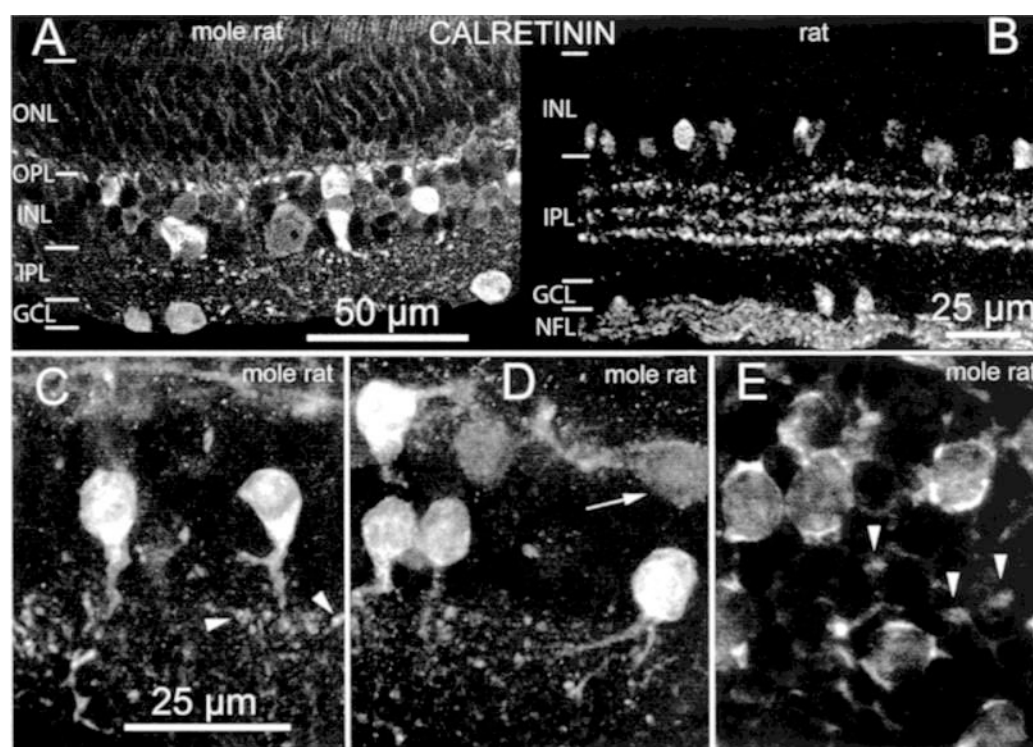


Fig. 4.

An antibody to calretinin stains a variety of cells in mole-rat (A) and rat (B) retina. (A) Anti-calretinin stains some types of ganglion, amacrine, horizontal, and bipolar cell in the naked mole-rat. Calretinin immunoreactivity is primarily found in non-AII amacrine cells in the rat retina, as well as some ganglion cells. The nerve fiber layer is also stained. (C) The primary type of amacrine cell stained in the mole-rat retina has the general morphology of AII amacrine cells, as a stout process descends from the soma, contains apparent lobular appendages (arrowheads) in sublamina *a*, and has processes descending further into sublamina *b*. (D) A horizontal cell (arrow) is stained by anti-calretinin in the mole-rat; likely bipolar cells are also stained (left). (E) A flatmount view of the calretinin immunoreactivity in the naked mole-rat further shows the lobular appendages near the somas of the putative AII amacrine cells (arrowheads). Each micrograph is a stack of 0.5- μ m confocal sections.

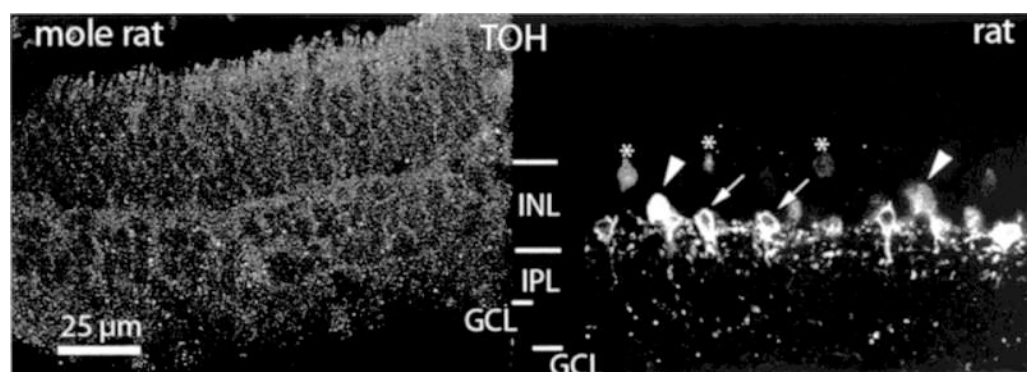


Fig. 5.

An antibody to the dopamine-synthesizing enzyme tyrosine hydroxylase stains no cells in the naked mole-rat retina (A) at 1:500 dilution. In rat retina (B), this dilution stains the dopaminergic amacrine cells (arrowhead) as well as some other types of amacrine cell (arrows) and probable bipolar cells (stars). Higher dilution (1:10,000) in rat retina stains only the dopaminergic amacrine cell (data not shown).

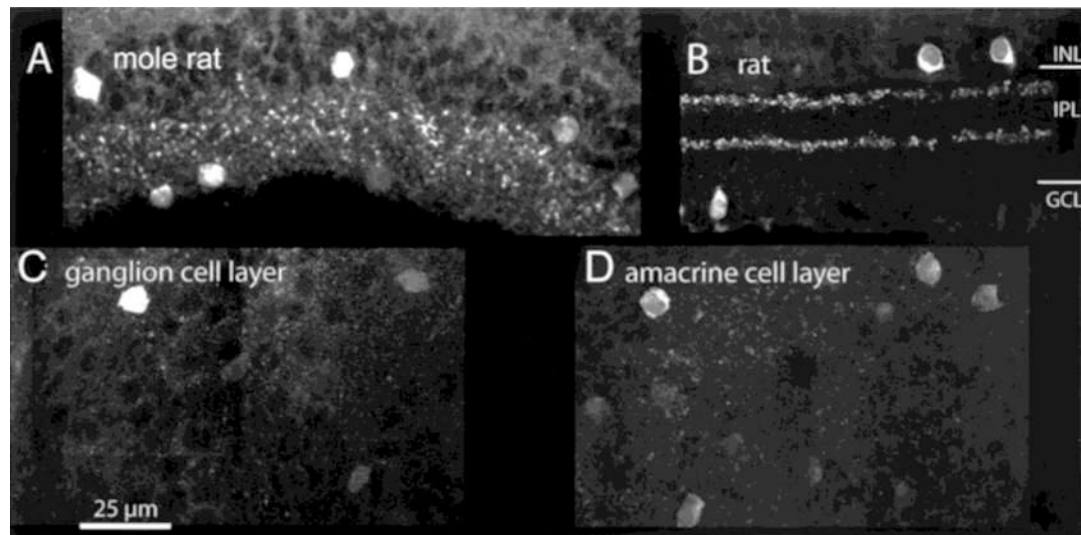


Fig. 6.

An antibody to the acetylcholine synthesizing enzyme, choline acetyltransferase (ChAT), stains amacrine cells in both the INL and GCL. (A, B) Radial sections of the mole-rat (A) and rat (B) retinas. Staining in the mole-rat retina lacks the obvious bands present in other mammalian species. (B) Staining in the rat retina has a more even distribution of cell bodies and produces the narrow, parallel bands typical of mammalian ChAT staining. Wholemount views of the mole-rat INL (C) at the level of the amacrine cells and of the GCL (D) show more numerous and intense staining of cells in the INL than in the GCL, consistent with the more distinct labeling in the distal portion of the IPL.

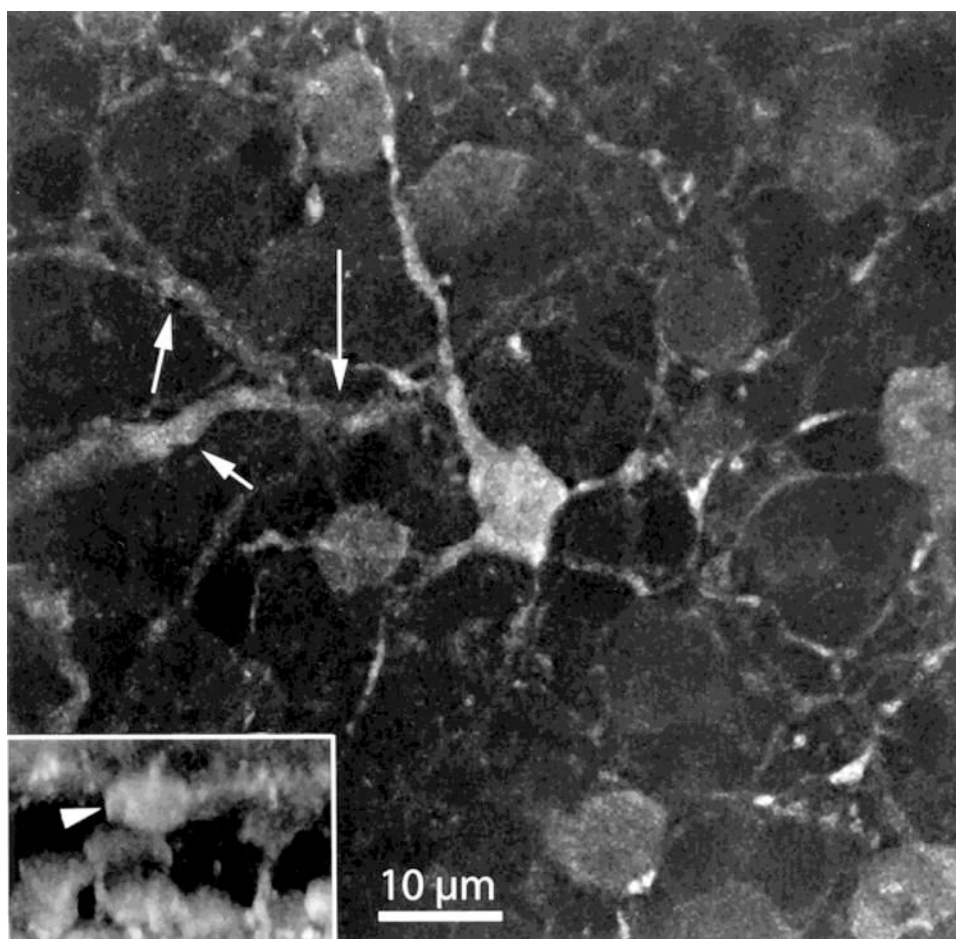


Fig. 7.

An antibody to calbindin stains a horizontal cell in the mole-rat retina. Many other unidentified somas are also stained in the outer portion of the INL. The large caliber dendrites marked by arrows lead to a dim soma (long arrow). The brighter horizontal cell has thinner dendrites. No axon is readily detectable emanating from either cell. Inset: A z-rotation shows a radial view of the same horizontal cell prominent in the wholemount view, as well as some amacrine cells located in the proximal INL.

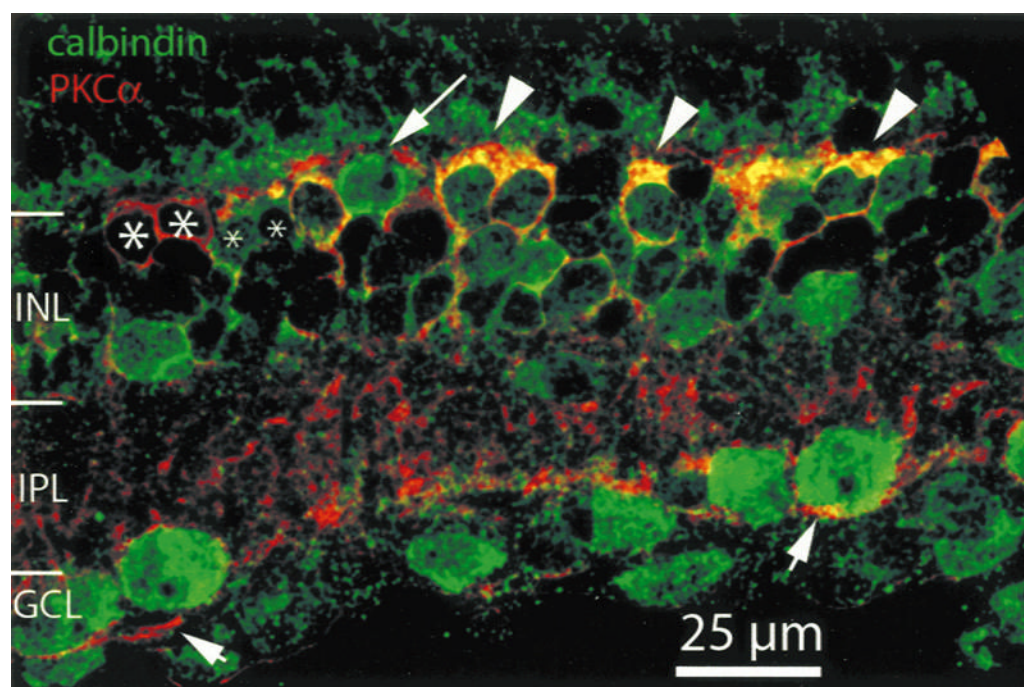


Fig. 8.

A radial section of PKC staining (red) in the naked mole-rat. Rod bipolar cells are most brightly stained (arrowheads), which are also lightly stained with CaBP immunoreactivity (green). Some cone bipolar cells may also be stained by PKC, as there are red somas lacking the bushy dendritic arbor of rod bipolar cells and also stained with CaBP immunoreactivity (large stars). Some weakly CaBP-immunoreactive bipolar cells lacking PKC immunoreactivity (small stars) are also presumably cone bipolar cells. PKC immunoreactivity appears diffusely throughout the IPL. The PKC-positive bipolar cell processes extend further than the bottom of the IPL (short arrows), sometimes wrapping around ganglion cell somas and even branching into the nerve fiber layer. CaBP immunoreactivity also labels a putative horizontal cell (long arrow).

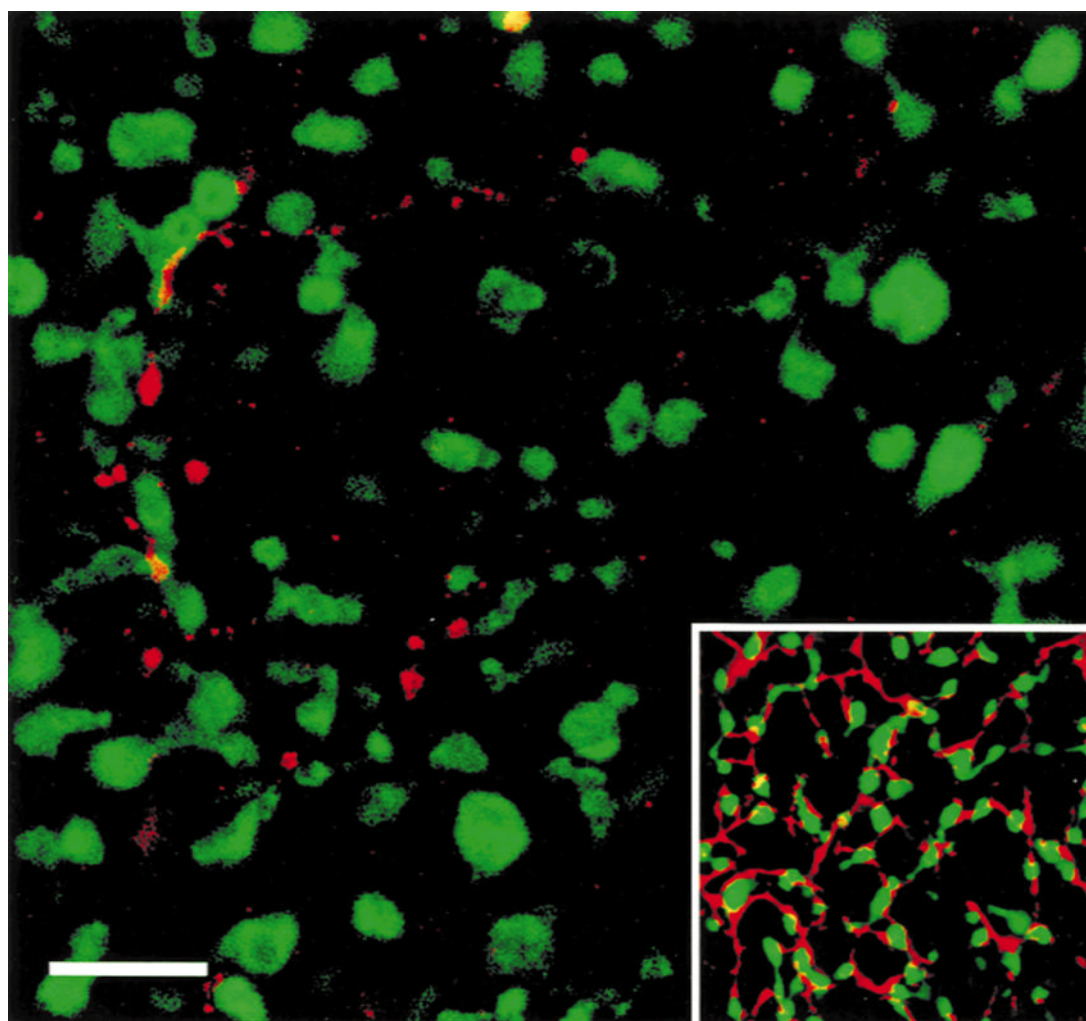


Fig. 9.

A flatmount view of PKC immunoreactivity (green) and calretinin immunoreactivity (red) in the mole-rat retina. The inset shows comparable staining in the rabbit retina, where each PKC-immunoreactive rod bipolar cell endfoot is contacted by an calretinin-immunoreactive AII amacrine cell process. In the mole-rat, the calretinin-immunoreactive AII amacrine cell processes do not cover the entire surface of the retina; many rod bipolar cell endfeet are apparently missed. Some AII varicosities appear to contact (unlabeled) processes other than rod bipolar cell endfeet. The variation in the size of the rod bipolar terminals is large, compared to the rabbit. Scale bar: 12 μm in figure and 25 μm in inset.