Selective Development of One Cone Photoreceptor Type in Retinal Organ Culture

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Purpose. The authors have established an organ culture method in which the postnatal development and the structural integrity of the mouse retina can be maintained for at least 6 weeks. Additionally, they have examined the emergence and in vitro morphogenesis of the photoreceptors and the development of insoluble components of the interphotoreceptor matrix.

Methods. Neural retinas and retinal pigment epithelia from 48-hour-old C3H ++/++ mice were cultured. At various ages, the tissues were fixed and cryosectioned or wholemounted. Photoreceptor development was studied by immunocytochemistry with visual pigment antibodies and by lectin cytochemistry. The ultrastructure of the photoreceptors was studied by electron microscopy.

Results. Immunopositive rods and short-wave sensitive cones were detectable as early as 3 days after explantation. From this time on, matrix domains around cones were also identifiable and labelled with peanut agglutinin lectin. However, the antibody specific to the middle-wave sensitive cone pigment failed to recognize any cones throughout the 6-week culture period.

Conclusions. Both basic photoreceptor types appeared and developed in this organ culture system according to a timetable comparable to normal in vivo development. Surprisingly, under these circumstances, one of the two cone pigments was not expressed by any photoreceptors. Invest Ophthalmol Vis Sci. 1994;35:3910-3921.

Much information on retinal development has been obtained from retinal cell cultures, a model that has proved useful for studying the in vitro development of dissociated cells and for allowing direct experimentation on the cultured cells.7-9 The inevitable disadvantages of the method are, however, the loss of the three-dimensional cytoarchitecture and the lack of normal cell-cell contacts. In addition, the failure of photoreceptor outer segment development has also been reported.8 It is likely that the absence of essential developmental factors is responsible for these problems. Some of these difficulties can be overcome by organ culture, another in vitro method. The first retinal applications of this method were described by Hild and Callas10 and by LaVail and Hild.11 Since then, various modifications of the tissue culturing technique have been introduced in the retinal research.12-14

The greatest advantage of the organ culture is that the overall three-dimensional structure of the retina is retained. Earlier, we established an organ culture method in which newborn mouse intact neural retina and retinal pigment epithelium (RPE) were explanted and cultured together.13 This method preserves not only cell-cell connections in general but also photoreceptor cell-RPE interactions, which have been shown...
to be of importance for normal outer segment formation. Recently, we have reported the expression of certain photoreceptor-specific proteins occurring either in rods only or in rods and cones. However, the expression of cone-specific markers has not yet been investigated.

Electroretinography shows that the mouse has a dichromatic photopic visual system with middle-wave sensitive (M) and short-wave sensitive (S) cone classes. Peanut agglutinin lectin (PNA) selectively labels the cones. Wheat germ agglutinin (WGA) and PNA can distinguish between the rod and cone domains of the interphotoreceptor matrix (IPM). Lectin cytochemistry, however, does not discriminate between the two cone classes of the mouse retina. The two cone types can be identified by antibodies specific to the mammalian red–green and blue cone visual pigments, respectively. In the present study, our aim was to follow the development of cone photoreceptors in cultured mouse retinas using lectin cytochemistry and immunocytochemistry with anti-visual pigment antibodies. Some of the results of this study have been published in abstract form.

MATERIALS AND METHODS

The organ culture method has been described in detail. C57H ++/+ mice were sacrificed by decapitation approximately 48 hours after birth. The enucleated eyes were incubated in a tissue culture medium (see below) supplemented with proteinase K (0.025%, Sigma, St. Louis, MO), at 37°C for 15 minutes. The anterior segment, the sclera, and the vitreous body were removed. The neural retina and the RPE were flattened onto a nitrocellulose membrane attached to a polyamide grid. The tissue was placed in R16 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum and cultured at 37°C with 100% humidity and 5% CO₂.

After 3, 5, 12, 19, 26, and 40 days of culturing, two alternative procedures were followed. First, the retina attached to the nitrocellulose membrane was fixed in 4% paraformaldehyde, infiltrated with 25% sucrose in Sörensen's phosphate buffer, and cryosectioned (10 μm). Second, after the above fixation, the neural retina was carefully removed from the underlying RPE and nitrocellulose membrane and floated in buffer. Both the sectioned and the floating whole cultured retinas were processed for light microscopic immunocytochemistry. For comparison, eyes derived from age-matched normal mice of the same strain were also used. Paraformaldehyde-fixed eyes were infiltrated with sucrose and cryosectioned. For simplicity, the different ages are given in postnatal days rather than postsurgery days. Because the retinas were dissected and explanted 2 days after birth, 3, 5, 12, 19, 26, and 40 days of culturing corresponded to postnatal day 5 (P5), week 1 (P7), week 2 (P14), week 3 (P21), week 4 (P28), or week 6 (P42), respectively. Our observations are based on at least six cultures in each age. However, at least 10 individual preparations each were studied for P7, P14, P21, and P28. Two normal eyes of each age were sectioned for the comparison.

Three antibodies were used: anti-rhodopsin recognizing rods (1:10,000) and the anti-cone visual pigment antibodies, COS-1 (1:50, hybridoma supernatant) and OS-2 (1:10,000, ascites fluid) specific to C-terminal epitopes of middle-to-long (M/L) and short (S) wavelength sensitive visual pigments, respectively. The bound antibodies were detected by secondary anti-rat and anti-mouse antibodies conjugated with fluorescein isothiocyanate (FITC) or rhodamin isothiocyanate (TRITC) (Dakopatts, Glostrup, Denmark). Lectin cytochemistry was performed on cryosections with FITC- and TRITC-conjugates of peanut agglutinin lectin and wheat germ agglutinin (WGA) (Dakopatts), respectively. Combined lectin and immunocytochemistry was carried out using FITC-labeled lectin and TRITC-labeled secondary antibodies, respectively. After mounting coverslips with glycerol-PBS, the immunocytochemical and lectin cytochemical reactions were inspected and photographed with an Axioskop (Zeiss, Oberkochen, Germany) microscope using appropriate filter sets for the fluorescent markers.

For electron microscopy, cultured retinal pieces were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde (GA) in 0.1 M sodium cacodylate buffer and postfixed in 1% osmium tetroxide. The tissue was embedded in araldite (Durecapan ACM, Fluka, Buchs, Switzerland), and the sections were contrasted with uranyl acetate and lead citrate.

Animals were studied in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RESULTS

As shown in our earlier reports, the retinal explants remained flattened and attached to the membrane filter throughout the culture period. The progress of histotypic development followed that of the normal in vivo retina, and the histologic appearance of the cultured retinas was comparable to that of their age-matched counterparts, with all retinal layers present (Fig. 1a). Along the edges of the explants, small rosette-like formations regularly occurred possessing all characteristic layers of the neural retina, with the photoreceptors pointing toward the lumen (Fig. 1b). Electron microscopy revealed the presence of photoreceptor outer segments with stacks of characteristic membrane disks (Fig. 2). As shown earlier, the disk membranes were sometimes aligned parallel, rather than perpendicular, to the photoreceptor axis. There
is no doubt, however, that photoreceptors develop viable outer segments in culture. Rods and cones were present, and they expressed visual pigments as revealed by immunocytochemistry.

The AO faintly labeled the whole photoreceptor cell layer at P5. Initially (P5 to P7), the most intensive label was observed at the apical end of the developing rod cells, corresponding to the outer segments (Fig. 3a). The number of immunopositive rods and the staining intensity gradually increased throughout the 6-week examination period (Figs. 3a, 3b, 3c). After the second week, the immunolabel tended to be concentrated to the rod outer segments in form of an intensely fluorescing band, and the individual rods were not distinguishable (Fig. 3c). Rods were not only confined to the flat parts of the cultured retinas, but also the luminal surfaces of the photoreceptor rosettes were heavily loaded with AO-positive elements (not shown).

Wheat germ agglutinin lectin labeled the inner and outer segments of photoreceptors, together with the surrounding interphotoreceptor matrix IPM. The lectin label was identifiable already at P5 (not shown), and the label intensity increased during the examination period (Fig. 3d). Thickening of the labeled photoreceptor segment layer during the development could be well traced with the lectin (not shown). Because of the high frequency of the WGA-positive elements, the lectin label of individual photoreceptors became confluent, forming a bright band scleral to the outer limiting membrane or a strongly fluorescing patch within the rosettes (Fig. 3d).

Another lectin, PNA, known to be selective for cones, was also used. The cone photoreceptors were clearly identifiable after PNA lectin cytochemistry (Figs. 3e, 3f, 3g, 3h). At P5 to P7, the labeled structures appeared round and were positioned close to the outer limiting membrane (Fig. 3e). During the following weeks, the labeled elements showed a continuous elongation perpendicular to the outer limiting membrane, and the characteristic shape of cone photoreceptors could be recognized (Figs. 3f, 3g, 3h). In addition to the strong PNA label of the photoreceptor layer, a less intense PNA positivity was also observable along the entire cone cell (Figs. 3g, 3h). The cone pedicles were always strongly stained in the outer plexiform layer. The frequency of PNA-positive elements was obviously lower than those of the anti-rhodopsin and WGA-positive cells and showed a characteristic spacing. As are rods, PNA-positive cones were present also in the rosettes (not shown).

Cone visual pigments were expressed early in the culture system. Already at P5, positive immunoreaction was observed with the antibody OS-2 specific to the short-wavelength sensitive visual pigment. At P7, the labeled elements were tiny, elongated, outer segment-like structures localized scleral to the outer limiting membrane (Fig. 4a). The number as well as the length of the OS-2 positive elements increased during the following weeks (Fig. 4b). OS-2 positive cones were
FIGURE 2. Electron micrographs of P12 (a) and P21 (b, c, d) cultured retinas. (a, b) Stratification of the outer retina. Pigmented epithelium (RPE), photoreceptor outer (arrows) and inner segments, outer limiting membrane (OLM, dotted line), outer nuclear and plexiform (OPL) layers, and inner nuclear and plexiform (IPL) layers can be identified. The outer segments are often irregular and composed of concentric lamellae of photoreceptor membrane disks (a, arrow), or organized parallel rather than perpendicular to the photoreceptor axis (c, thick arrow). The distance of the neighboring disks is subject to considerable variation (d, thick arrows), and, occasionally, small, membranous globules are pinched off from the edges of the disks (d, thin arrows). Original magnifications: (a) ×16,000; (b) ×4,000; (c) ×26,000; (d) ×26,000.
FIGURE 3. Immunocytochemistry with anti-rhodopsin antibody (AO, a, b, c) and lectin cytochemistry with WGA (d) and PNA (e, f, g, h) carried out on cryostat sections of cultured mouse retinas. The reactions were visualized with FITC or TRITC conjugated secondary antibodies and lectins, respectively. The level of the outer limiting membrane is marked with dotted lines. Arrows point to the labeled photoreceptors. Immunopositive rods were present already at P7 (a) seen as small fluorescent dots (small arrows). In addition to the strongly stained outer segment-like structures, the cell bodies were also faintly labeled (large arrows). At P14 (b) and P28 (c), the size and frequency of the immunopositive rod segments grew, and the immunofluorescence formed a confluent, horizontal band (small arrows) rather than discrete dots. WGA lectin label marking the interphotoreceptor matrix around rods and cones was also confluent at P14 (d), both within the rosettes (asterisk) and in the flat part of the explant. PNA-labeled cones always remained discrete (large arrows). The first, brightly fluorescing small cone segments were present already at P7 (e), and their size and length grew in subsequent weeks—P14 (f), P21 (g), P28 (h). Note that in addition to the cone outer and inner segments, the pedicles of the cones were also stained by the lectin. Original magnification, ×650.

found on the surfaces of the explants and within the rosettes (Fig. 4c). In contrast to the lectins, OS-2 did not stain the photoreceptor inner segments, only the apical ends of cones. Because of the outer segment specificity of the antibody and the rarity of cones, the immunolabel was less pronounced than that of the anti-rhodopsin or lectin label (Fig. 4d).

When comparing the staining pattern of rosettes with that of the flat parts of the same cultured retinas, immunocytochemical and lectin cytochemical label intensities were found to be relatively stronger within the rosettes than in other parts of the explant (not shown), as if the rosettes represented a more advanced stage of development both in IPM formation and in visual pigment synthesis.

Surprisingly, no immunostaining was found with COS-1, the antibody specific to the middle-wavelength sensitive visual pigment, at any of the examined ages. Even systematic serial sectioning of numerous cultured retinas from various ages failed to reveal the presence of any COS-1 positive element either in the rosettes or in the flat parts (not shown). When antibodies OS-2 and COS-1 were applied to sections of normal mouse eyes, both cone types were abundant in each age from P5 to P10 on (Figs. 5a, 5b). To confirm the complete lack of the COS-1 positive photoreceptor type, we examined the entire surface of the explants with wholemount immunocytochemistry. Anti-rhodopsin and OS-2 labeled numerous photoreceptors throughout the entire retinal area (Figs. 6a, 6b). COS-1, however, failed to label any cones (Fig. 6c), indicating that the lack of COS-1 positive cones in radial sections can be attributed to the absence rather than the extreme rarity of these elements.

Double label experiments with PNA and OS-2 showed that although in the first two postnatal weeks, numerous PNA-positive elements were found unlabeled by the antibody (not shown), in mature cultures, in which all prospective cones developed outer segments, each lectin-labeled cone was also recognized by antibody OS-2 (Figs. 7a, 7b).

The distribution of immunopositive S cones showed a random heterogeneity over the surface of the cultured retina; the density of cones was subject to considerable variation. Few cones were found at the edges, and, although the cone density increased toward the central parts of the culture, a single area with maximal density was not encountered. Instead, areas displaying higher and lower densities alternated irregularly (not shown).

DISCUSSION

In a retinal tissue culture system based on the explantation of the neural retina with RPE, we found that both major photoreceptor cell types grow, develop, and share morphologic and cytochemical characteristics with their normal counterparts. The formation of outer segments with photoreceptor membrane disks was shown by electron microscopy. The expression of rod and cone visual pigments was demonstrated with anti-rhodopsin and anti-cone pigment antibodies, respectively. The presence of cones was also established with PNA lectin cytochemistry. Further, lectin cytochemistry indicates that, at least partially, IPM components are also present. These observations confirm that our retinal culture method can be considered a reliable approach to study photoreceptor development.

An intriguing feature of our cultures was the complete lack of COS-1 positive cones throughout the culture period. Antibody COS-1 stains the green or red-green cones in all mammalian species studied so far, including the mouse. In developing rodent retinas, COS-1 positive green (M) cones appear later than the other, short-wave sensitive cone type recognized by...
FIGURE 4. OS-2 immunoreaction carried out on cryostat sections of cultured retinas. The bound antibodies were visualized by an FITC-conjugated secondary antibody. (a) Five-day-old explant (P7). (b, c) Twelve-day-old explants (P14). (d) Derived from a cultured retina 26 days after the explantation (P28). The outer limiting membrane is marked with dotted line. Some of the immunopositive cones are shown by arrows. Note that immunopositive elements appear as early as 1 week, and the labeled cones become elongated with time (a, b, d). OS-2 positive cones are found in the flat parts of the explants, and they are also abundant in the photoreceptor rosettes (c). Original magnification, ×700.

OS-2 antibody. In the mouse, the first S cones can be identified at P4 to P5, whereas the first immunopositive green cones appear only after P10. A similar sequence has been observed in the rabbit retina in which blue cones also precede the green cones by 5 to 6 days (Szél and Juliusson, unpublished data; 1994). Because S cones in the cultures appear on time according to the developmental timetable (P5), the lack of M cones might indicate important differences between the differentiation pathways of the two cone types. A certain retardation in photoreceptor development might obviously be attributed to the unfavorable conditions of explantation and culturing. However, because the green cones do not emerge even after several weeks, the absence of this cone type cannot be considered a delay.

As shown by PNA-OS-2 double label experiments, each PNA-labeled cone photoreceptor in mature (P21 to P28) cultures is recognized by antibody OS-2 specific for the S pigment. Therefore, the presence of cones containing altered visual pigments and undetectable for the green pigment-specific antibody is highly unlikely. Some of the lectin-labeled cone cells in younger (P5 to P14) explants are immunonegative, but, because PNA stains the IPM ensheathing outer and inner segments alike, these elements obviously represent cones in which the outer segment formation, visual pigment synthesis, or both, has not been

FIGURE 5. Immunofluorescence carried out on normal mouse retina (C3H). Vertical sections were taken from the eye of a P21 mouse and reacted with OS-2 (a) and COS-1 (b) to show outer segments (arrows) of S cones and M cones, respectively. Note that both antibodies labeled numerous elements in the normal retina. Original magnification, ×1100.
FIGURE 6. Immunoreactions carried out on whole cultured retinas. The preparations were made 19 days after the explantation (P21) and were reacted with anti-rhodopsin (a), OS-2 (b), and COS-1 (c) antibodies. Note that whereas numerous immunopositive rods and S-cones can be identified (a, b), the M-cones are totally missing from the wholemounts (c). Many of the stained elements are slender, elongated structures resembling outer segments. Among the anti-rhodopsin positive elements, round structures with processes can also be found in great number. These correspond to the cell bodies of rods. Original magnification, x600.

started yet. The selective death of the presumptive M cones is also unlikely, because degenerating or dying photoreceptors were not encountered in the cultures during the examination period and the frequencies of all identifiable photoreceptor markers showed a steadily increasing tendency up to P28 to P35. Because of a hypothesized greater vulnerability, the primitive green cones that are committed but immunocytochemically not yet identifiable might fall victim to the explantation procedure that inevitably disturbs normal development. The hypothesis of selective green cone damage, however, contradicts the well-known higher vulnerability of the adult and aging blue cones.30

Although visual pigment expression is the first detectable sign of commitment to a certain phenotype, the fate of photoreceptors is obviously determined earlier. The three main photoreceptor phenotypes of the mouse retina are manifested, in the following order: rods, blue cones, green cones.2 It is logical to assume that the three photoreceptor types become committed in the same sequential order as the emergence of their characteristic visual pigments. In contrast, it is known that cone cells are generated earlier than rods.1 Our interpretation is that cones differentiate from other cell types very early (embryonic days 13 to 14).1 However, from one another they differentiate relatively late, probably after the generation of rods. We think many rods are already committed at the time of explantation and, therefore, are not disturbed by the manipulation. The separation of the two cone classes, however, might have just started. Presumably, the explantation coincides with an event crucial for the differentiation of the green cones but indifferent for the rods or blue cones.

One possible scenario is that during the early postnatal days, when retinas are explanted, blue cones are already committed but green cones just start to undergo the differentiation process. If this assumption is correct, it seems plausible that explantation disturbs this process and only those cells already committed...
will grow and develop further, whereas those cones that are uncommitted fail to produce a phenotype characteristic for green cones. Experiments with transgenic mice carrying human red–green opsin promoter genes showed that the product of the reporter gene was first detectable on P4. Although the timetable of the transgene expression does not necessarily coincide with that of the endogenous green opsin gene, this observation indicates that at the time of explantation, the green cones may not yet be differentiated.

Another possible scenario is that initially each cone expresses short-wave pigment, and only later, as a result of an inducing effect, will some of these precocious S cones shift to the expression of the M pigment. During metamorphosis, fish photoreceptors undergo a visual pigment shift as revealed by microspectrophotometry. Because the retina is explanted well before the onset of the M pigment synthesis, it is conceivable that, as a result of the disturbance caused by the explantation, all cones in the cultured retina follow the default pathway and always carry the S pigment.

There is another observation demonstrating a possible selective disturbance in the green cone differentiation pathway. It is known that similarly to most mammals, in the normal rabbit retina, green cones outnumber the blue cones with a factor of approximately 6 to 7. Interestingly, when embryonic rabbit retinas are transplanted into the subretinal space of adult rabbits, the number of green cones in the transplants remains considerably lower than that of blue cones. Despite the obvious differences between the two experimental paradigms, a common feature of both the cultured and transplanted retina is the deprivation of the appropriate age-specific environment. Although it is difficult to draw a parallel between the mouse and the rabbit, the emergence of S-cones precedes that of the green cones in both animals. It is, therefore, likely that the differentiation of the photoreceptor types is governed by common factors in these mammals.

It is reasonable to suppose that a factor necessary to green cone development is present only in reduced amount in adult retinas, causing the partial loss of green cones in transplants. If such a promoting factor does indeed exist and is essential for the specification of the correct phenotypes, it might be missing from the culture medium, leading to the complete drop-out of one photoreceptor cell type. The explanted retina does not grow within the eye; therefore, permissive factors that might normally originate from extraretinal ocular sources (e.g., vitreous body, lens, choroid) are not acting. Long distance influences through the optic nerve or blood circulation are also not exerted. Retinal explants are cultivated in total darkness. Thus, developing photoreceptors are also deprived of normal illumination that might have an impact on the specification of cones.

As a result of dilution in the culture medium, even intraretinally produced factors are probably present at an insufficiently low concentration. The dilution of these factors is probably weaker in the relatively closed rosettes, leading to the apparently more intensive expression of the detectable markers (AO, WGA, PNA, OS-2) in these structures, but the lack of COS-1 immunoreactivity in rosettes indicates the absence of a potential green-cone promoting factor. Because the other markers are present both in the rosettes and in the flat parts of the explants already at the earliest age examined, P5 (5 days after explantation), whether photoreceptor differentiation starts earlier in rosettes than elsewhere remains to be resolved.

Several investigations provided evidence that cellular interactions exerted through temporally regulated diffusible signals are crucial for the determina-
tion of phenotypes in the developing retina. Little is known, however, about the identity of potential factors influencing the specification of photoreceptors. Growth factors are among the most likely candidates to promote cellular differentiation in the central nervous system and in the retina. That photoreceptor development might indeed be influenced by growth factors is confirmed by the presence of fibroblast growth factor (FGF) and binding sites for FGF in the outer retina. In vitro studies showed elevated opsin levels after treatment with acidic FGF, and the administration of basic FGF led to prolongation of cell survival in a culture of newborn retinal cells. Intraocular application of bFGF could retard photoreceptor cell loss in retinal degeneration. Interestingly, the expression of the endogenous aFGF starts at P7 in the normal rat retina, just a few days before the emergence of the first green cone outer segments in the mouse or rat (P9 to P11).

There is a growing body of information indicating that the extracellular matrix might also play an important role in the proliferation, differentiation, and phenotypic stabilization in a variety of tissues, including the nervous system and the retina. An as yet unidentified photoreceptor survival-promoting activity has been found in IPM preparations. S-laminin, another component of the matrix, was shown to promote photoreceptor morphogenesis. The relatively undisturbed global photoreceptor development in our study can at least partly be assigned to the presence of insoluble IPM components. Interphotoreceptor retinoid binding protein (IRBP), however, is released into the culture medium, leading to a low tissue concentration. The role of IRBP is to shuttle retinoids and possibly fatty acids between the RPE and photoreceptors. As suggested earlier, a reduced amount of IRBP and a disturbance of retinoid and/or fatty acid transport might be responsible for the in vitro developmental abnormalities. The coincidental lack of IRBP immunostaining and the paucity of green cones in rabbit retinal transplants might also refer to a possible link between IRBP and photoreceptor differentiation.

Milam has reported on a patient with retinitis pigmentosa whose retina contained photoreceptor rosettes with cones identified by anti-blue opsin but not by anti-red–green opsin antibody. Because in this condition the very same cone class is missing as was missing in our paradigm, this finding might be relevant to our results. In primates, however, the emergence of color-specific photoreceptors seems to occur in a sequence different from that of rodents and the rabbit. Little is known about the pathomechanism of the formation of photoreceptor rosettes. Yet, the lack of red–green cones might reflect common mechanisms in the specification or maintenance of cone phenotypes whether they develop in cultured retinas of nonprimate mammals or in rosettes of patients with retinitis pigmentosa.

An interesting aspect of photoreceptor topography in the mouse retina is the regional heterogeneity of cone classes with an S field where M cones are excluded. Because M cones are missing from the cultures and the S cone distribution is irregular, the complementary cone fields are not identifiable in vitro. In fact, the whole retina in culture resembles the S field of the normal mouse, inasmuch as the entire surface is populated by S cones only. The loss of the separation of cone classes and the absence of M cones indicate that the positional determination of cone phenotypes is not maintained in vitro. Further studies are needed to establish the nature and source of the potential factor(s) directing the differentiation of color-specific photoreceptors and to identify the markers necessary to provide positional clues for individual cones.

Acknowledgments

The authors thank Kataryna Rydén and Richard Hawkins for their expert technical assistance.

Key Words

mouse, retinal organ culture, immunocytochemistry, lectin cytochemistry, cone photoreceptors

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