Abstract Visual pigments in the regressed eye and pineal of the depigmented neotenic urodele, the blind cave salamander (Proteus anguinus anguinus), were studied by immunocytochemistry with anti-opsin antibodies. The study included light- and electron-microscopic investigations of both the eye and the pineal organ. A comparison was made with the black pigmented subspecies Proteus anguinus parkei (black proteus), which has a normal eye structure. In the retina of the black proteus, we found principal rods, red-sensitive cones and a third photoreceptor type, which might represent a blue- or UV-sensitive cone. Photoreceptors in the regressed eye of the blind cave salamanders from the Planina cave contained degenerate outer segments, consisting of a few whorled discs and irregular clumps of membranes. The great majority of these outer segments showed immunolabelling for the red-sensitive cone opsin and only a few of them were found to be positive for rhodopsin. An even more pronounced degeneration was observed in the photoreceptors of the animals derived from the Otovec doline, which are completely devoid of an outer segment, most of them not even possessing an inner segment. Even in some of these highly degenerate cells, the presence of rhodopsin could be detected in the plasma membrane; however, immunoreactions with antibodies recognizing cone visual pigment were negative. In the pineals of all studied animals, the degenerate photoreceptor outer segments were recognized exclusively by the antibody against the red-sensitive cone opsin. The presence of immunopositive visual pigments indicates the possibility of a retained light sensitivity in the blind cave salamander photoreceptors.

Keywords Retina · Opsins · Immunocytochemistry · Eye · Regressive evolution · Proteus anguinus (Urodela)

Introduction

The blind cave salamander, Proteus anguinus anguinus Laurenti, inhabits subterranean waters of the Dinaric Karst (Sket 1997). Similarly to most cave animals, the strongly reduced eyes and depigmented skin are characteristic features. The small eye rudiments, seen as black dots, lie deeply buried under the skin. The retina, lens and vitreous body are strongly reduced in size and complexity in comparison to non-cave-dwelling salamander species. Although the eye development begins normally in the embryos, in larval life it is retarded and the degeneration soon becomes evident (Durand 1971, 1973).

The retinal photoreceptor cells show degenerative signs of various degrees with outer segments completely missing or represented only by small irregular structures (Durand 1971, 1973). Although the rudimentary structure of the photoreceptors suggests that they are non-functional, the possibility cannot be excluded that at least some of them retained traces of light sensitivity. Efficient photoreception requires an intact phototransduction molecular pathway, the first and most important member of which is the light-sensitive receptor molecule, the visual pigment. The main goal of the present study was to see whether visual pigments are expressed in the degenerate photoreceptors of the blind cave salamander and, if so, whether they are rod or cone visual pigments.

The same interesting question can be posed in the case of the other photoreceptive organ, the pineal body. This neuroendocrine organ situated in the roof of the diencephalon is directly sensitive to light in lower vertebrates (Hamasaki and Eder 1977; Collin and Oksche...
1981; Vigh and Vigh-Teichmann 1999). The light sensitivity is based on the presence of typical photoreceptor cells having well-developed outer segments with visual pigments similar to those in the retina (Vigh and Vigh-Teichmann 1999). Through secretion of pineal hormones, the organ influences skin pigmentation, metamorphosis and gonadal development, and controls circadian rhythms (Bagnara 1963; Underwood 1990; Filadelfi and Castrucci 1996). In the blind depigmented neotenic mones, the organ influences skin pigmentation, metapigments similar to those in the retina (Vigh and Vigh-Teichmann 1999). The light sensitivity is based on the presence of typical photoreceptor activity (Kos and Bulog 1996a, 2000). Since nothing is known about their photosensitivity, in the present study we wanted to check whether visual pigment(s) is (are) present in the pineal photoreceptors.

For the evaluation of structural and functional reductions in the light-sensitive organs, which have evolved in the blind cave salamander during a period of probably not more than 10,000 years of cave life (Sket 1997), a comparison has been made with the closest relative having normal eyes. A recently discovered small population from southeastern Slovenia, described as a subspecies P. a. parkelj (Sket and Arntzen 1994), is black pigmented and has eyes that can be regarded as normal. Although this black proteus is also a cave animal, it has retained a mostly unchanged retinal structure (Kos and Bulog 1996b) that can be considered as ancestral.

In the present study, we investigated visual pigments in the retinal and pineal photoreceptors of P. a. anguinus and P. a. parkelj using immunocytochemistry with various antibodies against visual pigment opsins. The analysis revealed the presence of visual pigments both in retinal and pineal photoreceptors of the blind cave salamander. Since our previous light-microscopic observations (unpublished) indicated that individuals of P. a. anguinus significantly differ in the extent of structural reduction of the eye, we studied animals of different ages and from different locations and found a variability in both eye structure and immunoreactivity of its photoreceptors.

Materials and methods

Animals

Specimens of Proteus anguinus anguinus were collected in the Planina Cave (Planina, southwest Slovenia, four adult animals, with body lengths 220-280 mm) and in the Otovec doline (Crmomelj, southeast Slovenia, two adult animals, with body lengths 280–290 mm, and two young animals with body lengths of 130–160 mm). In the first location, the animals were caught in their usual habitat, in the depth of the cave. In contrast, animals from the Otovec doline were captured in a water spring outside the subterranean system, where they regularly come out during the night. The animals from the Planina cave were collected in summer and autumn, and the animals from the Otovec doline were caught in summer. One specimen of the black P. a. parkelj (l=170 mm) was caught in the Jelskevnik spring (Crmomelj, southeast Slovenia). The animals were collected with the permission of the Ministry of Environment and Physical Planning of the Republic of Slovenia. Since P. anguinus, and especially the black subspecies, are rare and endangered animals, the number of animals in our study was highly limited. A further difficulty in obtaining a sufficiently high number of animals was that breeding proved to be impossible under our laboratory conditions.

The animals, fed with amphipods, were kept in aquaria in the speleobiological laboratory, in permanent darkness at 12°C for 1–10 days, before being put to death.

Specimen preparation

All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were anaesthetized in 0.3% 3-aminobenzoic acid ethyl ester methanane sulphonate (MS-222) and decapitated. The head was immersed in the fixative containing 0.5% paraformaldehyde and 1.1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4). The eyes were enucleated and the midportion of the brain containing the pineal was excised under a dissecting microscope. The tissues were fixed for 3 h at 4°C, then washed overnight with a mixture of 0.05 M cacodylate buffer and 0.25 M sucrose at 4°C. One eye of each animal and the majority of the pineals of P. a. anguinus, as well as the pineal of P. a. parkelj, were used for immunocytochemistry. Other eyes and one pineal from each group of P. a. anguinus were used for transmission electron microscopy: after fixation they were postfixed with a mixture of 2% osmium tetroxide and 3% potassium ferrocyanide for 1 h at 4°C. All tissues were then dehydrated in ethanol series, infiltrated with propylene oxide and embedded in Durcupan ACM (for immunocytochemistry) or Epon 812 (for electron microscopy).

Antibodies used

OS-2

This is a mouse monoclonal antibody recognizing the C-terminal portion of the blue cone opsin in mammals (Szel et al. 1988; Röhlich and Szel 1993) and most opsins in submammalian species. The lyophilized ascites fluid was used at a concentration of 5 µg/ml.

COS-1

This is a mouse monoclonal antibody recognizing the C-terminal portion of the red/green cone opsins, from amphibia to primates.

Fig. 1 Light micrographs of the eyes (a, c, e) and electron micrographs of rudimentary photoreceptor cells (b, d, f) in Proteus individuals from different locations. a Proteus anguinus parkelj (the black proteus). Individual layers of the retina can be distinguished; photoreceptors are present (L lens). b A part of a rod photoreceptor cell in the retina of the black proteus showing a fairly normal outer (OS) and inner segment (IS). c The rudimentary eye of the blind cave salamander, P. a. anguinus, from the Planina cave. The eye is strongly reduced but layers of the retina can still be recognized. The vitreous is practically missing and the lens (L) is represented only by a small group of cells. Photoreceptor inner segments protrude into the space between the outer limiting membrane and the pigment epithelium. d A disorganized outer segment of a photoreceptor cell of the same proteus, composed of whorled disks and clumps of membranes. e Light micrograph of the strongly degenerated eye in a young P. a. anguinus from the Otovec doline. The retina has lost its characteristic stratification and photoreceptor cells are not seen. The lens is replaced by a cell mass (M) proliferating from the borders of the retina and the pigment epithelium (5 criculate cartilage). f Electron micrograph of a region near a pigment epithelial cell (PE). Photoreceptor cells cannot be recognized by their morphology; outer and inner segments and even sensory cilia are missing. Accumulations of mitochondria reminiscent of inner segment ellipsoids are occasionally seen (arrow).×120 (a), ×3220 (b), ×150 (e), ×15,000 (d), ×220 (e), ×4000 (f)