GABA-IMMUNOREACTIVE PHOTORECEPTORS IN THE RETINA OF AN ANURAN, *PELOBATES FUSCUS*

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Introduction

Over the last decades, many species of amphibians (frogs, toads, salamanders and newts) throughout the world have declined markedly in numbers from natural habitats because of habitat degradation and environmental changes. Common spadefoot (*Pelobates fuscus; Anura; Pelobatidae*) is a lowland species (fig. 1. a) found throughout much of the plains and hilly regions of Central and Eastern Europe. Distribution of this species in Hungary is only partly mapped (fig. 1. b). Common spadefoot have congeneric species in Europe (*P. cultripes, P. syriacus*) and in North America (*Scaphiopus sp., Spea sp.*). Common spadefoot lives in burrows and actively searches for prey mostly at night and dawn.
In spite of this unique behaviour most of the vertebrate prey of the diurnal red-footed falcon (*Falco vespertinus; Aves; Falconidae*) is *P. fuscus*. We are interested in studying the retina and later other parts of the visual system of this species and to compare that with well-known frog species.

Figure 1. The common spadefoot toad, *Pelobates fuscus* **(a)**, and the distribution of common spadefoot toad on the 10x10 km UTM grid map of Hungary and the natural habitat of the species **(b)** .

Materials and methods

Twelve adult spadefoot toads (*Pelobates fuscus*) from both sexes were used in this study. The animals were collected with the permission of the Danube-Drava National Park. They were kept in terraria and fed twice weekly with mealworms. The NIH and ARVO animal care guidelines were considered during the experimental procedures. After urethane anesthesia the animals were decapitated, the eyes were quickly dissected and placed in ice-cold phosphate-buffered saline (PBS, pH 7.4), where the cornea and the lens were removed.

Light microscopy

To study the general structure of the *Pelobates* retina eyecup preparations were fixed in 4% paraformaldehyde dissolved in phosphate buffer saline (PBS) at 4˚C overnight. The specimens were embedded in Durcupan ACM resin and sectioned in an MT 7000 ultramicrotome at 2 µm (see details at electron microscopy). The sections were contrasted by toluidine blue. PR cells were counting in resin embedded cross sections from all major part of the retina. The minor rods and the cones were identified based on their unique morphology.

Electron microscopy

Small pieces of eyecup preparation was fixed with 2.5% glutaraldehyde in 2% paraformaldehyde dissolved in phosphate buffer (PB) at 4˚C overnight. Subsequently the specimens were washed six times with PB and incubated in 0.5% OsO4 for 1 h. Specimens were then dehydrated with increasing concentration of ethanol, treated twice with propylene oxide for 15 minutes and incubated in a 1:1 propylene oxide-Durcupan ACM resin mixture for 2 h. Subsequently, the specimens were transferred into pure resin at 4˚C for overnight and again put into fresh resin for polymerization at 56˚C for 36 h. The resin blocks were cut using an MT 7000 ultramicrotome. The electron microscopy was performed using a JEOL 1200 EX electron microscope.

Immunolabeling experiments

Eyecup and wholemount preparations were fixed in 4% paraformaldehyde and – only for detection of GABA-immunoreactivity – 0.2% glutaraldehyde dissolved in PBS at 4˚C overnight. Eyecups were then washed in PBS, cryoprotected in 30% sucrose overnight and cut in a Reichert cryostat at 16 µm. Sections were mounted on chrome-alum-gelatin coated subbed slides and stored at -20˚C until use. The immunolabeling experiments were executed using primary antibodies to GABA (rabbit polyclonal, 1:1000 dilution and mouse monoclonal, 1:400 dilution; both from Sigma) and to cone opsin (mouse monoclonal (COS-1), 1:1000 dilution, from Ágoston Szél's laboratory) overnight at room temperature. Fluorophore-coupled secondary antibodies were used in a dilution 1:200 for 2 h. Sections were then washed in PBS and mounted in Vectashield (Vector Laboratories). For normal light microscopic analysis small pieces of wholemount preparations and whole retina preparations were done and GABA and COS-1 immunoreactivity was detected in them with the avidin-biotin method. Small pieces of wholemount preparations were embedded in Durcupan ACM resin and sectioned at 2 µm while the whole retina preparations were covered in slides with glycerol.
Digital photographs were taken with a Nikon Eclipse 80i microscope equipped with a cooled CCD camera. Images were taken with the Spot software package. Photographs were further processed with the Adobe Photoshop 7.0 program. Images were adjusted for contrast only, aligned, arranged and labelled using the functions of the above program.

Aims of the study

In our previous works we performed a series immunolabeling experiments. It showed that many features of the general organization of the *Pelobates* retina (calretinin-, tyrosine hydroxylase-, serotonin-, calbindin immunoreactivities) conforms well with the general anuran pattern. Only minor differences were observed in the distribution on neurochemical markers between *Pelobates* and other anuran species studied formerly by other laboratories. The most conspicuous results are the NPY-like positive centrifugal fibers in sublamina 1 (fig. 2) and that two major tachykinins, substance P and substance K are localize to distinct subsets of retinal amacrine cells (fig. 3.). The results from the light microscopy studies showed that general structure of the Pelobates retina is similar to other anurans. Only one major difference was observed: the lack of oil droplets from cones. Accordingly in the present study we have focused on the photoreceptors (PRs) of the *Pelobates* retina. Aims of the study were the following:

- to determine the rod/cone distribution
- to analyse the ultrastructure of the PRs
- to find neurochemical differences from other anuran species
- to compare the results with anuran species studied formerly.

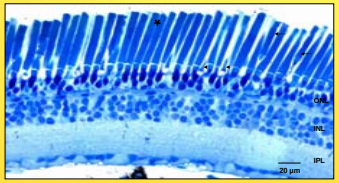

Figure 4. General structure of the *Pelobates retina*. (arrowheads: cones, arrows: minor rods, star: rod)

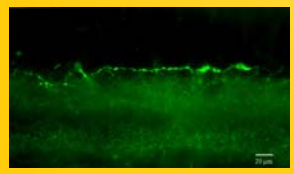

Figure 2. NPY-like positive centrifugal fibers in sublamina 1 of the inner plexiform layer of *Pelobates* retina.

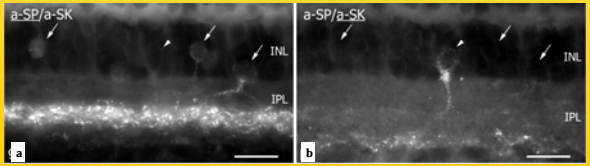

Figure 3. Double labelling between SP **(a)** and SK **(b)** in the *Pelobates* retina. Positions of SP-positive cells are marked with arrows while the SK-positive cells with arrowheads. None of the SP-positive cells show SK-immunoreactivity and vice versa. (Schäffer & Gabriel (2005) Neurosci. Lett. 386, 194-198.)

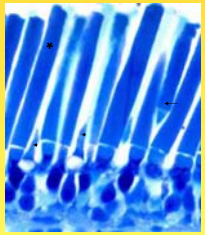

Figure 5. The three main photoreceptor types in *Pelobates* retina. Signs are the same as in Fig 4.

Results

The general structure of spadefoot toad's retina is similar to other anurans studied formerly. We observed three main photoreceptor types in the *Pelobates* retina: rods, „minor" rods and cones (Fig. 5.). Based on morphology we find only one cone type unlike *Xenopus*, *Bufo* and *Rana* where there are at least two types. The cone inner segments of the above anuran species contain an oil droplet (Fig. 6.) that is absent from the *Pelobates* cones. To confirm the results mentioned above we executed electron microscopic analysis. This supported the lack of oil droplet – which is clearly visible in *Xenopus* (Fig. 8.) – and revealed the presence of a dense body in the inner segment (Fig. 7.). Insted of the expected two rod types we have found three. Besides the generally known major and minor rod we have found a rod with unusual inner segment and nucleus structure (Fig 7.).

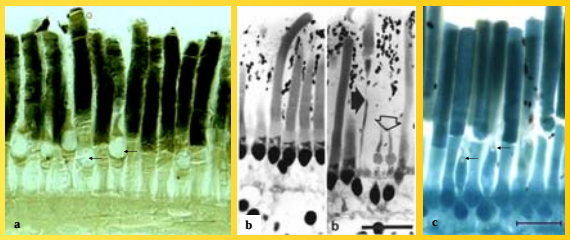

Figure 6. Transverse sections of the *Xenopus* **(a)**, *Bufo* **(b)** and *Rana* **(c)** retina. The oil droplets (thin arrows) are clearly visible. Figure **(b)** were taken from Zhang & Straznicky (1991) Anat. Embryol. 183, 97-104.

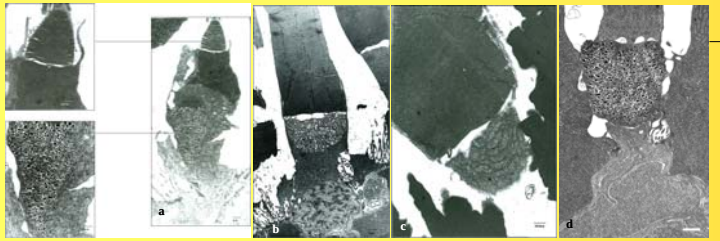

Figure 7. Micrographs from ultrathin sections of *Pelobates* photoreceptors. The cones **(a)** do not comtain oil droplet only a dense body in the inner segment. Besides the generally known major **(b)** and minor rod **(c)** we have found a rod with unusual inner segment structure **(d)**.

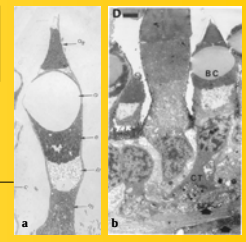

Figure 8. Micrographs from ultrathin sections of *Xenopus* photoreceptors. The ultrastructure of major rods **(b)** are similar to *Pelobates* but that of the cones **(a)** are not. Figure **(a)** were taken from Röhlich & Szél (2000) Microscopy Research and Technique 50, 327-337. while Figure **(b)** from Wilhelm & Gabriel (1999) Cell Tissue Res. 297, 35-46.

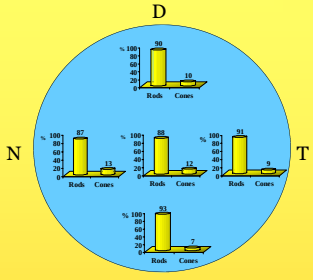

Figure 9. Photoreceptor distribution in the *Pelobates* retina.

Based on observations made on the semithin sections contrasted with toluidine-blue we defined the rod/cone ratio from several locations of the *Pelobates* retina. The highest ratio was detected in the central and the nasal part of the retina while the lowest in the ventral part (Fig 9.). It is similar to *Bufo* (Fig 11.) but different from *Xenopus* (Fig 10.) where the rod/cone ratio did not change from center to periphery. The overall rod/cone ratio was 9.64:1 in the *Pelobates* retina which means that only approximately 10 percent of the photoreceptors were cones. The overall rod/cone ratio is much higher in *Xenopus* (1.13:1) and *Bufo* (2:1).

The distribution of photoreceptors was not even in the *Pelobates* retina and it was similar to *Bufo* inasmuch as a peak of rod and cone density was found in the central retina and lowest cone density was detected in the ventral part, unlike *Xenopus* where the photoreceptor distribution were even in all part of the retina.

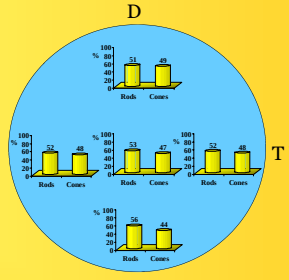

Figure 10. Photoreceptor distribution in the *Xenopus* retina. Based on Wilhelm & Gabriel (1999) Cell Tissue Res. 297, 35-46.

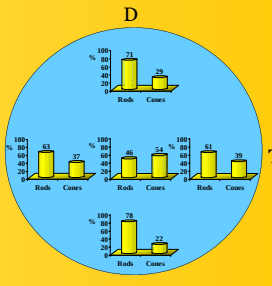

Figure 11. Photoreceptor distribution in the *Bufo* retina. Based on Zhang & Straznicky (1991) Anat. Embryol. 183, 97-104.

We also localized the major inhibitory transmitter, γ-aminobutiric acid (GABA) to subsets of retinal neurons. Apart from the regular GABA-immunoreactive pattern observed formerly in other anurans, certain structures in the photoreceptor layer were also regularly labeled for GABA (Fig. 12. a). The soma diameter of the labeled cells is 5-6 µm and the outer segment seems to be unlabeled. In resin-embedded preparations GABA-positive photoreceptor cells were identified as cones based on their sparse distribution and short outer segments (Fig 12. b).

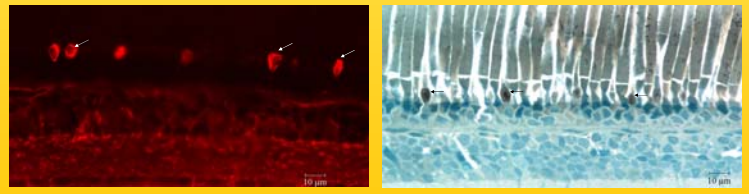

Figure 12. GABA immunoreactivity in the *Pelobates* retina. Structures in the photoreceptor layer were regularly labeled for GABA (arrows).

Conclusions

1. The general structure of the *Pelobates* retina is very similar to that of other anuran species

2. Our work showed some difference in the photoreceptor morphology and distribution of *Pelobates* retina compared to *Bufo*, *Rana* and *Xenopus*.

3. The presence of GABA containing photoreceptors seems to be a unique feature of *Pelobates* retina. Further work is required to clarify their role in retina information processing.

Acknowledgement

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