UNIVERSITY OF PÉCS

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Life Conditions or Taxonomic Relationship: The Evaluation of Morpho-functional Features of the Retina in Spadefoot Toads

Theses of PhD Dissertation

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PÉCS, 2012

1. Introduction

The three orders of the class of ampibians (Anura, Caudata and Gymnophiona) include more than 6700 species worldwide. According to the most recent survey by IUCN (International Union for Conservation of Nature and Natural Resources), about 30% of these species are threatened. Population dynamical and fauna studies having been carried out in recent decades confirm this fact, making clear that population sizes and species numbers of amphibians are declining globally, less amphibians are found in natural habitats, as is reported in Hungary by a number of publications. The spadefoot toad (*Pelobates fuscus*), the subject of our investigations is distributed commonly in South-Transdanubia, occuring with high abundance in sandy areas of the Barcs juniper grove. Due to its secretive, nocturnal behaviour, little is known about its exact distribution pattern in Hungary. Relying on literature data, public collections and data from researchers, we have prepared the Hungarian distribution map of the spadefoot toad. The 800 occurrence data were located in 312 squares of the UTM grid, meaning a coverage rate of 29,6% for Hungary. The majority of spadefoot toad distribution data (~80%) are from studies after 1970, thus the produced map can be considered as up-to-date. Irrespective of the rate of coverage, the distribution of occurrence locations suggests that spadefoot toad individuals are found throughout entire Hungary.

The body length of the spadefoot toad is 6-8 cm. Its skin is smooth, featuring olive, light brown or grayish drab colouration on the back, with longitudinal markings. The limbs are short, with spade-shaped metatarsal tubercules on the hind feet, which are used as an aid when digging in the soil. The eyes are large, the pupils are vertical. According to our current knowledge, the daily activity of *P. fuscus* starts in the hours after sunset, and lasts until a few hours after midnight. Accordingly, spadefoot toads are nocturnal, spending the greatest part of the day burrowed in the soil. Under experimental conditions we have been able to confirm such a circadian activity pattern. Movements of the animals were observed and videotaped using infrared cameras. In addition to daily activity patterns, these video sequences also clearly showed that the tongue of spadefoot toads is not anchored at the front and is not flicked out like in the case of most terrestrial toad species, therefore its feeding behavior is also found to be significantly different from that of other anurans living in Hungary. In order to catch its prey, the animal first needs to get closer, then strikes with its whole body. In many cases, the forelimbs also assist in forwarding the prey into the oral cavity. Based on all these features described above, it was anticipated that the behavioural pattern of *P. fuscus* is reflected in the anatomical organisation of the visual system, particularly in the structure of the retina. Since such data were not found in earlier literature, we believe that the description of the visiual system of terrestrial toads, and in particular, of the general structure of the retina, can serve as basis for comparative studies.

Neurons found in largest numbers in the retina of toads are photoreceptors. There are two basic types of photoreceptors (cones and rods): in darkness both types release their glutamate transmitters tonically. A minimum of two types of cones are present in almost all of the toad species having been studied in detail so far, even in species where the role of colour perception is minimal. About 10 variations of bipolar cells are known to occur in vertebrates, and this is true for toads as well. In the majority of vertebrates, including frogs and toads,

ganglion cells are categorised in 10-20 types based on their anatomical features (dendritic field size, orientation and ramification number, connections with bipolar and amacrine cells. The main route of information flow in the retina is photoreceptor ⇒ bipolar cell ⇒ ganglion cell, with glutamate being the transmitter substance in almost all of the cells of the chain. Retinal information processing integrates also signals from horizontal and amacrine cells in the two synaptic layers. These cells, too, have their input from photoreceptors and bipolar cells, whereas their output is either a feedback to the cell type from where the input arrived or a forwarded signal to the dendrites of bipolar cells and ganglion cells. The functioning of horizontal and amacrine cells is that forms the system of centre-surround organisation.

Amphibians lack cerebral cortex, and their major visual centre is tectum opticum in the midbrain. In addition to the tectum, the retina sends out fibres to the thalamic nuclei (nucleus Bellonci and corpus geniculatum thalamicum) located in the anterior part of the diencephalon, to area pretectalis and to nucleus basalis opticus. These visual fibres end in the neuropil region located near the surface of the aforementioned structures. The visual tract is composed of axons of the retinal ganglion cells which cross over almost completely at the base of the diencephalon (this area is called as chiasma opticum), and only then enter the central nervous sytem. The projection of retinal ganglion cells into the visual centres of the brain were tested using anterograde tracers. These centres have incoming optic nerve fibres from both eyes, but the number of ipsilateral projections is much lower (cca. 4%) than the number of projections arriving from the contralateral retina. The most important functions of the visual system include the recognition of prey, co-ordination of catching prey, as well as the identification of predators and initialising escape behaviour processes.

2. Objectives

According to our hypothesis, the special life conditions and adaptation to the environment in *Pelobates* are reflected in the organisational and physiological features of the retina of this toad, therefore we anticipated to reveal a retina structure that is substantially different from that of other species. During the analysis of the retina we focused on two distinguished cell types: photoreceptor cells and the most diverse cells of the retina, the amacrine cells. With regard to these, our objectives are summarised as follows:

- comparison of neurobiological features of the spadefoot toad with those of the much better researched species (*Bufo*, *Xenopus* and *Rana*) in respect of their retinal structure and neurochemistry
- investigation of the photoreceptor cells, looking at their distribution within the retina, their morphology and neurotransmitter content
- investigation of the morphology and neurotransmitter content of amacrine cells.

To be able to answer our questions, microscopic anatomical observations and immunohistochemical stainings were applied, with the additon of routine electron microscopic examinations.

3. Material and methods

Adult spadefoot toads (*Pelobates fuscus*) were used in our studies. Permission for collecting the animals was obtained from Duna-Drava National Park Directorate, and the toads were used in our experiments following the animal care ethics guidelines of NIH (National Institutes of Health) and ARVO (Association for Research in Vision and Ophthalmology). Following euthanasia with urethane, the eyes were dissected and eyecup preparations were made. In some of the investigations aiming at GABA-labeling, these preparations were incubated in 1mM GABA solution for 30 minutes.

For studying general retinal structure, eyecup preparations were fixed in 4% paraformaldehyde solution overnight, at a temperature of 4˚C. Tissue pieces were embedded in Durcupan ACM solution, and semi-thin sections were cut and stained with 1% toluidin-blue. For electron microscopy, tissue pieces were fixed overnight in a mixture of 2,5% glutaraldehyde and 2% paraformaldehyde, dissolved in phosphate buffer (PB), at a temperature of 4˚C. Further procedures were executed based on standard histochemical protocols. First flushing was done for 6x10 minutes in PB, then aditional fixation in 0,5% OsO_4 for one hour. The samples were then dehydrated in an ascending ethanol series, then kept in propylene-oxide and then in a 1:1 mixture of propylene-oxide and Durcupan ACM resin for two hours. New resin was mixed for embedding, and the blocks were left to polimerise at 56˚C for a duration of 36 hours. Sectioning was done using a MT 7000 ultramicrotome (cutting 70-80 nm ultrathin sections), and the samples, after lead-citrate counterstaining, were examined in a JEOL 1200 EX electron microscope.

The eyecup and wholemount preparations were fixed in 4 % paraformaldehyde and – only the ones used for testing GABA immunoreactivity – in 0,2 % glutaraldehyde, for 4 hours at 4 ^{o}C, then rinsed in PBS. Preparations were kept in 30 % sucrose solution for at least 4 hours, then 14-16 μm thin cross-sections were cut using cryostat at a temperature of -20 ^{o}C. The sections were pre-incubated for 30 minutes with antibody solvant (ABS), and then incubated with primary antibodies. For fluorescent staining, secondary antibodies linked to fluorophore were used for 4 hours, dissolved in ABS at 1:200. Sections were then rinsed with PBS, covered in VectaShield (Vector Laboratories). In some cases whole retina preparations (wholemount) were made. On these, GABA, COS-1 and Neuropeptid Y (NPY) immunolabelling was applied, using the avidin-biotin method. Wholemounts were covered and stored in glycerol, and some pieces were embedded in resin for cutting 2 μm thin sections, then covered in Permount. Digital photographs were made using a Nikon Eclipse 80i microscope equipped with a cooled CCD camera. Images were captured using the Spot software and processed in Adobe Photoshop 7.0. Image processing included only adjustments of contrast; the images were then arranged, organised in tables and given identifications. The spatial organisation of all the NPY positive cells in a wholemount preparation was reconstructed with the Neurolucida 3.23 (MicroBrightField, Inc.) computer programme. For determining the distribution pattern of cells, we used the Neuroexplorer 3.03a programme and calculated the mean nearest neighbour distance.

4. Results

The general retinal structure of *Pelobates fuscus* is not substantially different from other tetrapod vertebrates having been studied so far. All of its tissue layers are like in mammals, and regarding fine histological structure, the *Pelobates* retina is close to what is found in *Xenopus*, a *Bufo* and *Rana* species. Histological layers of the retina are distinct, and their relative thickness is also similar to that in the aformentioned anurans.

The position of photoreceptor cells and their basic morphology as seen in the cross-sections, are like in other anurans. However, their detailed analysis revealed some deviations from the general picture, typical for this species. Two types of rods (minor és major) and a single cone type were differentiated. Minor rods were identified based on their shorter outer segment, connected to the soma by the narrowed inner segment. The presence of cones was confirmed using immunocytochemical investigations, applying antibodies produced against cone-opsin (COS-1). Altogether 1975 photoreceptors were counted in semi-thin toluidin-blue stained sections from 5 different (dorsal, nasal, ventral, temporal, central) areas of the retina. The relative proportion of rods and cones was determined. Cones represented only about 10 % of all the photoreceptor cells, which is a very low figure compared to other anuran species studied so far. According to our findings, the proportion of cones relative to rods is higher on the central and nasal side of the retina than on its ventral side. In order to be able to obtain an accurate picture about the retinal distribution of cones, wholemount preparations stained with COS-1 antibody were also made. When we investigated the GABA-immunoreactive elements of *Pelobates* retina, we found that, in addition to the GABA-distribution pattern observed in other amphibians, there were some structures regularly marked by anti-GABA antibodies in the layer of photoreceptors (ONL) too. The diameter of soma marked in the layer of photoreceptors was 5-6 μm. If the number of GABA-positive cones is compared with toluidin-blue stained and fluorescent samples, it appears that all of the cones are GABA-positive. None of the rods with cylindrical outer segments were found to be GABA-immunoreactive. When the ultrastructure of photoreceptors was analysed, it was found that cones are clearly identifiable but are different from *Xenopus* cones. Instead of oil droplets, a granular, electron-dense matrix is present. The electron microscopic analysis of rods also revealed a surprising finding. Besides the major and minor rods identified in light microscopic sections, it was possible to identify a photoreceptor cell type which resembled major rods in all of its features, except that the nucleus featured irregular, loose chromatine structure. In all of the cases, these cells were located in the outermost cell layer of photoreceptors. Their proportion was found to be low when all ultrathin section surfaces were analysed, ranging around 1-3% of total rod numbers.

Besides the photoreceptor marking described above, there were also horizontal, amacrine and bipolar cells in the inner nuclear layer that were marked with anti-GABA antibodies. Based on morphology, it was possible to identify at least two types of amacrine cells. One was characterised with a long dendrite, its oval soma located further away from IPL, and the other type had large, round soma sitting right on the IPL. About half of the amacrine cells were found to be GABA-immunoreactive.

Many cells of *P. fuscus* retina were marked with anti-calretinin antibodies. Well-marked structures were found in all five sublayers of the inner plexiform layer. Cones, possible ”displaced” bipolar cells (assumed from their morphology) and ganglion cells were marked in large numbers, especially in periphery of the retina. Based on morphology and location, we could differentiate two calretinin-positive amacrine cell populations. The soma of the first type was located right on the boundary of the inner nuclear and the inner plexiform layers, whereas the other had its cell body outwards from this boundary, sending a prominent dendrite towards the inner plexiform layer, often ramifying already in the first sublayer.

In anurans, too, tyrosine-hydroxylase is a marker molecule used for indicating dopaminerg cells. It was possible to indicate such cells in the inner nuclear layer of *P. fuscus* retina, and the dendrites in the first sublamina of the inner plexiform layer. We found large (12-15 μm) serotonine-containing cells in the inner nuclear layer, whose dendrites ramified in the entire width of the inner plexiform layer. The most diversly ramified structure was observed in the second and third sublaminae. It was possible to reveal Substance P-immunoreactivity in sublaminae 1, 3 /4 and 5 of the inner plexiform layer. Two cell types were clearly identifiable in these preparations. The dendrites of one type run down into the third sublamina without ramifications, whereas dendrites of the other type radiate already in the first sublamina.

Neurons showing neuropeptid Y-immunoreactivity were amacrine cells. Morphologically, two types were differentiated. One had its soma laying right on the proximal surface of the inner plexiform layer (IPL), sending fibres into its first sub-layer, whereas the cell body of the other type was located further away from the IPL, sending its dendrites mostly into sublaminae 1 and 2. Both cell types had round soma and a broad field dendritic. Besides amacrine cells, centrifugal fibres were also present which ran mostly in the first sublamina of the inner plexiform layer, but smaller sections were found in sublaminae 2 and 5 as well. We could observe a few poorly immunoreactive Müller-cells too, but it was not possible to eliminate this marking even with preabsorption experiments, thus we regarded it as aspecific. The two types of amacrine cells and centrifugal fibres observed in immunofluorescent analyses were identified in the wholemount preparations as well. The dendrites of amacrine cells cover a distance of about 200 μm. Varicose centrifugal fibres run a long way and interweave the entire retina surface. We also analysed the spatial distribution of Neuropeptide Y-positive amacrine cells in a wholemount preparation. In this analysis we evaluated the cells as one population. In the preparation with 43,9 mm^2 total area, 964 pieces of NPY-positive amacrine cells were found, giving a density value of 22/mm^2 in the retina. The nearest neighbour analysis showed that the pattern of cells was regular (Rn=1,59), the individual cells being spaced at nearly equal distances, evenly in the entire retina.

Because of the diversity of amacrine cell types, it can be important to provide their neurochemical code system, and to indicate co-localisation of neurochemical markers. Like in all other studied anuran species, spadefoot toads, too, are characterised with the similarity of the distribution patterns of tyroxine hydroxylase and Neuropeptide Y-immunoreactive elements. We could not find double-labelled structures which confirms the findings of other laboratories. Nevertheless, our results indicated that in a certain group of amacrine cells of

Pelobates retina, Substance P immunoreactivity is present, and even Substance K could be identified in a rare population of amacrine cells with broad dendrite field. The somas of the neurons were found in the innermost layer of the INL, whereas the Substance K immunopositive dendrites were located in sublamina 1 and at the boundary of sublamina 4/5, meaning that they partly overlapped with the dendrite area of Substance P immunopositive dendrites. Displaced amacrine cells showing Substance K immunopositivity were not found. Our double-labelling analyses showed that there are at least 6-8 times more Substance P immunoreactive cells in *Pelobates* retina than Substance K positive amacrine cells. Based on this evidence, the above two cell populations are in clearly distinct, even if the dendritic fields of Substance P and Substance K immunopositive cells were found to partially overlap.

5. Discussion

As concluded from our investigations we can establish that the histological structure of spadefoot toad retina is highly similar, regarding both retina thickness and the relative proportions of its layers, to the retina of other studied anuran species (e.g. *Rana catesbiana, Bufo marinus, Xenopus laevis*). However, a few differences were noted in the morphology and distribution of photoreceptors, in comparison with what has been described for the genera of *Bufo*, *Rana* and *Xenopus*.

For *Bufo marinus*, *Rana pipiens* and *Xenopus laevis*, earlier investigations revelaed two (minor vs. major) types of rods. In the spadefoot toad, an additional rod type was found, appearing morphologically quite similar to major rods, differing from normal rods only in their nucleus structure. Unlike in the well-studied *Rana* and *Xenopus* species, *Pelobates fuscus* cones had, instead of an oil droplet, a dense structure. Looking at the density of cones in various retina areas, it was found that their distribution was uneven, and the measured values were much lower than what had been recorded for the other studied anurans. Earlier investigations of mammalian and reptile species as well as *Bufo marinus* later on, cast light to the fact that besides a high density of photoreceptors (mostly cones) in the central areas of the retina, the location of acute vision is distinctly identifiable in the layer of ganglion cells, in the form of a visual streak or fovea centralis. In the toad *Bufo marinus* the highest density of cells in the ganglion cell layer was measured along the nasotemporal meridian of the retina, and similar topographic neuron distribution was noted for the outer and inner nuclear layers too. The difference of cell densities between central and peripheric retina areas in *Bufo* is nearly 6-fold. Because this difference in *Pelobates* was found to be only 1 to 1.5, we evaluated this as insufficient for being qualified as a visual streak, but instead was categorised as a centrally increased density of cells. Unlike in other anurans having been studied earlier, the proportion of cones relative to rods in *Pelobates* retina was very low (1:9). This proportion in *Bufo marinus* was 1:2, whereas in *Xenopus laevis* it was almost 1:1. From this we can conclude that cones have lesser significance in the spadefood toad in visual information processing, possibly related with its nocturnal behaviour.

We have confirmed the presence of GABA immunoreactive cones. GABA has been proved to be the main inhibitory transmitter in both the inner and the outer retina areas where it acts through all three known GABA receptor types (A, B, C). If the aforementioned

photoreceptor cells release GABA as transmitter, it can affect horizontal and bipolar cells which have functioning GABA receptors. GABA can evoke depolarisation in horizontal cells, and through autoreceptors it may an affect the cones themselves, as all three GABA receptor types have been indicated in the cones of different species.

We have found that the distribution of neurochemical markers of *Pelobates* retina was only slightly different from the retina of anurans studied earlier in other laboratories.

Our results indicate that GABA immunoreactivity in *Pelobates* retina has a similar pattern to that in other anurans. About half of the amacrine cells of spadefoot toad retina were GABA positive, which is in line with the results that have been reported in studies of other amphibian species. In addition to amacrine, horizontal and bipolar cells, we found GABA positive cells in the layer of ganglion cells, too. These can be either displaced amacrine cells or ganglion cells. Investigations of *Bufo marinus* individuals have shown that a small proportion of neurons in the layer of ganglion cells are GABA immunopositive ganglion cell, and some immunoreactive axons have also been identified in the layer of optic fibres. This might suggest that GABA-containing ganglion cells send inhibiting input to the visual centre of the anuran brain.

Studies having been performed on amphibians earlier have come up with contradictory results regarding the distribution of calcium-binding proteins in retinal neurons, these contradictions probably arising from the difference in the antibodies used in the studies. In the spadefoot toad we were not able to detect the presence of calbindin 28 kDa, a protein that was confirmed by earlier studies to exist in the cones of anurans and toads. In our case, based on the markings recorded in the photoreceptor layer and the inner retinal layers it seems possible that the main calcium-binding protein in the spadefoot toad is calretinin. During our studies, calretinin immunoreactivity were more common in cell types that play a role in the vertical information processing, i.e. in bipolar cells, ganglion cells and photoreceptors. Similarly to what has been described for *Rana esculenta* and *Xenopus laevis*, we found no evidence for calretinin immunoreactive horizontal cells in the retina of the spadefoot toad. The occurrence of calretinin immunoreactive amacrine cells was rare. These cells had two morphological types in *Pelobates* retina. In the ganglion cell layer many calretinin positive cells were found in amphibians. In our studies we also found several calretinin immunoreactive cell bodies, and observed a number of fibres in the GCL, based on which it is likely that at least some of these cells are ganglion cells. In order to confirm this assumption, retrograde cell marking is necessary. The high number of calretinin immunopositive fibres and the variable size of immunoreactive cell bodies suggest that several ganglion cell types can be calretinin-positive.

Dopaminergic amacrine cells are found generally in the retina of vertebrate species, its density always being low. The marker of dopaminerg cells in amphibian retina is tyrosine-hydroxylase. In *Bufo* and *Xenopus* species, dopaminergic neurons have uniform dendrite morphology, whereas in *Rana* two sub-types (thin and thick cells) are differentiated. They represent is approximately 0,5% of all amacrine cells. Certain dopaminerg cells send fibres towards the outer plexiform layer, while their dendrites ramify in sublaminae 1 and 5. In our experiments we found that the TH-positive cells detected in spadefoot toad retina sent their fibres to sublamina 1 of the IPL, and their number was low. No dendrites towards the OPL

were observed, which is somewhat different from patterns in other anurans where dendrites towards the OPL were present, although in low numbers.

In the retina of amphibians having been studied so far, most of the serotonin immunoreactive cells are amacrine cells, nevertheless in *Xenopus laevis* bipolar neurons, and in *Rana pipiens* ganglion cells were found. Based on their morphology, serotonin-containing amacrine cells are categorised in two groups. One contains neurons with large soma and broad dendrite field, sending fibres to sublaminae 1 and 2. The second group has cells with small soma and diffuse dendrite distribution. Serotonine-containing cells in *Pelobates* show no morphological difference; they all appear to be serotonine-synthetising cells with large diameter. However, to prove this, further studies are needed.

Substance P-immunopositive cells are typical cell types of anuran retina, which was confirmed by our studies too. We were able to prove the presence of two, anatomically different Substance P-containing amacrine cells in the retina of the spadefoot toad. Earlier micro-anatomical studies of *Bufo* retina reported only one Substance P-positive cell, but this population of cells appeared to be heterogeneous regarding its GABA immunoreactivity. It is possible that among all anurans, at least the terrestrial species have two Substance P-immunopositive cell types in their retina, one of which possibly uses GABA as a transmitter. Based on the results of the Substance P/Substance K double labelling study, Substance P and Substance K immunoreactivity, respectively, are localised in distinctly separated cell populations of *Pelobates*, which finding might mean the discovery of a new, neurochemically identifiable cell type. Because it is generally known that most of the peptide-containing amacrine cells contain other transmitter(s) too, we assume that at least one additional neuro-active transmitter material is present in Substance K-containing cells as well. Based on morphological features, however, it is unlikely that this cell is identical with any of the formerly described amacrine cells characterised with a wide dendritic field. Further analyses of co-localisation are required to be able to confirm if these cells contain one of the major inhibiting transmitters glycine or GABA.

There is evidence in literature that tachykininerg cells in the nervous system of toads are able to produce both Substance P and Substance K. Our studies have provided evidence that both peptides are produced in the retina. Based on these findings it is reasonable to think about how Substance P and Substance K are synthesised in these separate populations of amacrine cells. According to the results of an earlier study dealing with tachykinin synthesis in the ocular tissues of guinea-pigs, the molar concentration of Substance P was higher than that of Substance K in all types of eye tissues, except for the retina. From chromatographic analyses, the same study concluded that the transcription of tachykinin-1 gene, together with different precursor processes and/or post-translation modifications can result in a new neurochemical phenotype, and can influence the physiology of vision. Truly enough, results of the only physiological paper available about the influence of Substance K on visual processes show that its effects are just the opposite of those of Substance P.

Our studies have confirmed the presence of two types of Neuropeptide Y-positive amacrine cells with broad dendrite field. Similarly to what was observed in *Bufo marinus*, the spatial distribution of these cells was quite even in the retina of the spadefoot toad, but their

density was lower (22/mm^2) than what was observed in *Bufo marinus*. The most striking result was the appearance of Neuropeptide Y-immunopositive fibres in the first sublamina of the inner plexiform layer. Earlier immunocytochemical investigations in amphibians have indicated the presence of FMRFamide- and Substance P immunoreactive centrifugal visual fibres in the optic nerve and the retina. Studies of fish proved the FMRFamide and luteinizing hormone-releasing hormone (LHRH) content of the terminal nerve. These fibres reached the inner nuclear layer via the optical fibre layer of the retina and the inner plexiform layer, where they form a plexus of long and straight fibres. Their axon terminals end on the dopaminerg interplexiform and horizontal cells and GABA-erg amacrine cells, i. e. on their dendrites. This allows the assumption that through the horizontal and/or amacrine cells they modify the visual signal processing activites of the retina. In order to confirm whether Neuropeptide Y-immunopositive fibres can have similar role in the retina of the spadefoot toad, further studies are necessary.

Similarly to earlier investigations of anurans, we did not find Neuropeptide Y/tyrosine-hydroxylase double labelled stuctures in our experimental subjects, although in the first sublamina of the inner plexiform layer, the dendrites of the mentioned cells are found to be in close anatomic proximity. No evidence is known in other vertebrate species of the co-localisation of these neurochemical markers in retinal neurons, but it is likely that there is a mutual stimulatory connection between the two cell types, exactly because of the aforementioned close spatial proximity. To confirm this, electron microscopic analyses are required.

6. Conclusions

The general histological and structural composition of the retina, as well as the morphology of the observed neurons are greatly similar to those found in other studied ampibians, yet there are certain fundamental differences:

1. The number and relative proportion of cones compared to rods are substantially smaller than in other amphibians having been studied so far.
2. The density of cones is highest in the central-nasal region of the retina. However, the increase in density relative to its values in the peripheric areas is much more moderate (cca. 1,5x) than in *Bufo marinus* (cca. 6x), a species with a distinct visual streak, which finding suggests the possible absence of visual streak in the spadefoot toad.
3. Our electron microscopic investigations revealed that in addition to the morphologically separated formerly known two rod types, another photoreceptor also exists which is similar to ordinary rods but is different in its loose nuclear chromatine structure.
4. The neurochemistry of certain studied cell types was also found to be somewhat different from that of other amphibian taxa. Our investigations proved the presence of GABA-immunoreactive cones.
5. During our experiments we were able to confirm occurrence of Substance K-immunoreactive amacrine cells in which Substance K was not co-localised with Substance P.
6. We identified Neuropeptide Y-immunopositive centrifugal fibres.
7. Only slight difference was found in the distribution of neurochemical markers in the case of calretinin, tyrosine-hydroxylase, serotonine, Substance P and Neuropeptide Y, whereas it was not possible to confirm presence of somatostatin.

The low number of cones, the difference in neurochemical markers, and the possible absence of a visual streak justify the assumption that there is relationship between the structure of the visual system and the life conditions of the spadefoot toad, but in order to provide firm evidence of this, it is necessary to performe the detailed analysis of the cerebral structures of the visual system, too.

7. The author's own publications

7.1 Scientific publications forming the basis of the PhD dissertation

1) **Schäffer D.A.**, Gábriel R. (2005): Two major tachykinins, Substance P and Substance K, are localized to distinct subsets of amacrine cells in the anuran retina. *Neurosci. Lett.* 386: 194-198.

2) **Schäffer D.A.**, Purger J.J. (2005): A barna ásóbéka (*Pelobates fuscus*) elterjedése Magyarországon. [angol összefoglalóval]. *Állattani Közl.* 90: 25-39.

3) **Schäffer D.A.**, Gábriel R. (2007): GABA-immunoreactive photoreceptors in the retina of an anuran, *Pelobates fuscus*. *Neurosci. Lett.* 416: 202-205.

7.2 Conference participations forming the basis of the PhD dissertation

1) **Schäffer D.**, Purger J.J., Gábriel R. (2003): A barna ásóbéka (*Pelobates fuscus*) magyarországi elterjedése az eddigi kutatások alapján. VI. Magyar Ökológus Kongresszus, Gödöllő. [abstract]

2) **Schäffer D.**, Dénes V., Gábriel R., Purger J.J. (2003): Distribution of chemical markers in the retina of the spadefoot toad (*Pelobates fuscus fuscus*). A MITT IX. Konferenciája, Balatonfüred, *Clinical Neurosci.* 56(2): 77-78. [abstract]

3) Gábriel R., **Schäffer D.**, Purger J.J., Wilhelm M. (2003): Neurochemical analysis of retinal cell types in *Pelobates fuscus*. 6th IBRO World Congress of Neuroscience, Prága. [abstract]

4) **Schäffer D.**, Purger J.J. (2004): Kétéltű-szaporodóhelyek felmérése terelőkerítések és vödörcsapdák alkalmazásával. II. Szünzoológiai Szimpózium, Budapest. [előadás]

5) Babai N., **Schäffer D.**, Gábriel R. (2005): Photoreceptor distribution in retina of the spadefoot toad (*Pelobates fuscus*). A MITT XI. Konferenciája, Pécs, *Clinical Neurosci.* 58(S1): 9. [abstract]

6) **Schäffer D.**, Wilhelm M., Gábriel R. (2005): Neuropeptide-Y like immunoreactive elements in the retina of the spadefoot toad (*Pelobates fuscus*). A MITT XI. Konferenciája, Pécs, *Clinical Neurosci.* 58(S1): 83. [abstract]

7) Gábriel R., **Schäffer D.A.** (2006): GABA-immunoreactive photoreceptors in the retina of an anuran, *Pelobates fuscus.* FASEB Summer Research Conferences, Retinal Neurobiology and Visual Processing, Indian Wells, California. [abstract]

8) **Schäffer D.A.**, Pirkhoffer E., Horváth Gy., Purger J.J. (2006): Distribution of common spadefoot toad (*Pelobates fuscus*) and soil types in Hungary. 1st European Congress of Conservation Biology, Eger. [abstract]

7.3 Other scientific publications

1) Horváth Gy., Hamburger K., **Schäffer D.** (2002): Újabb adatok a Dráva felső szakaszának kisemlős faunájához. *Nat. Somogy.* 3: 111-130.

2) Horváth Gy., Pogány Á., Hamburger K., **Schäffer D.** (2004): A védett csalitjáró pocok, *Microtus agrestis* (Linnaeus, 1761) országos elterjedése az 1999-ig gyűjtött adatok alapján. [angol összefoglalóval]. *Természetvédelmi Közl.* 11: 607-611.

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7.4 Other conference participations

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2) Horváth Gy., **Schäffer D.**, Hamburger K., Molnár D., Pogány Á. (2001): Kisemlősök populáció és közösségi vizsgálata két ártéri erdőtípusban. II. Dráva Konferencia, Pécs. [előadás]

3) Csete S., **Schäffer D.**, Horváth Gy. (2001): The impact of vegetation structure on the microhabitat preferences of three rodentia in patchy habitat. 44th IAVS Symposium, Vegetation and Ecosystem Function, Freising-Weihenstephan. [abstract]

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5) **Schäffer D.A.**, Végh B., Voigt A., Türmer K., Horvai V., Purger J.J. (2006): A barna ásóbéka szaporodásbiológiai vizsgálata a Barcsi-borókás területén. VII. Magyar Ökológus Kongresszus, Budapest. [abstract]