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GABA-immunoreactive photoreceptors in the retina of an anuran, *Pelobates fuscus*

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Abstract

We have recently started to unravel the retinal neurochemistry of an anuran species, the spadefoot toad (*Pelobates fuscus*), because of its unique lifestyle. The immunolabelling experiments included tests to localize the major inhibitory transmitter, γ-aminobutyric acid (GABA) to subsets of retinal neurons, using commercially available antibodies. Apart from the regular GABA-immunoreactive pattern observed formerly in other anurans, certain structures in the photoreceptor layer were also regularly labeled for GABA. 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. If these cells release GABA as a transmitter, it may act on the second order cells, from which certain horizontal and bipolar cells have functional GABA receptors. Alternatively, GABA may influence the cones themselves through autoreceptors.

Keywords: Immunocytochemistry; Rods and cones; Horizontal cells; Bipolar cells; Amacrine cells; Frog

Gamma-aminobutyric acid (GABA) is a major inhibitory transmitter in the retina of all vertebrate species studied to date [13,14,30], where all three major receptor subclasses for this substance are present [3,10,25]. One or more of the necessary elements of the GABAergic transmission has been localized to several types of amacrine and at least one type of horizontal cells [19,27,30,31]. In some species, bipolar and ganglion cells also contain GABA or its synthesizing enzyme, GABA-decarboxylase (GAD) [1,7,12,20,21,34]. It seems that GABA containing bipolar cells are regular features of the amphibian retina [1,7,32], and that in lower vertebrates GABAergic amacrines are more numerous than in mammals [30]. The above description leaves only one major retinal neuron type, the photoreceptors which seems to be devoid of GABAergic components. Indeed, it is generally accepted that photoreceptors use glutamate as transmitter [26]. Thus, far only few studies performed in the monkey and lizard retina provided some evidence for the presence of a unique GABA-containing photoreceptor population [6,15]. We have recently started to unravel the retinal neurochemistry of an anuran species, the spadefoot toad (*Pelobates fuscus*) [22] because of its secretive nocturnal fossorial lifestyle. This species dwells most of the time in the dark in its burrow, and active mostly at night [16]. This behaviour might have a bearing on the structure of the nervous system including the retina. In a series of experiments, we have discovered that apart from the regular GABA-immunoreactive pattern observed formerly in other anurans, certain structures in the photoreceptor layer (PRL) were also regularly labeled for GABA. In this paper, we make an attempt to identify them.

Four adult spadefoot toads (*P. fuscus*) from both sexes were 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. In some experiments, these eyecups were incubated with 1 mM GABA dissolved in frog Ringer solution for 30 min under constant bubbling with 95%/5% O_2/CO_2 mixture. The preparations were fixed in 4% paraformaldehyde and

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Table 1
Schedule of the experiments

Preabsorption with	Primary antibody(ies) to	Secondary antibody(ies)	Main results
None	None	Donkey anti-rabbit FITC	No labelling
		Donkey anti-mouse TR	No labelling
None	GABA (raised in rabbit; Sigma; 1:1000–5000)	Donkey anti-mouse TR	No labelling
None	GABA (raised in mouse; Sigma; 1:3000–10,000)	Donkey anti-rabbit FITC	No labelling
None	GABA (raised in rabbit; Sigma; 1:1000–5000)	Donkey anti-rabbit FITC	Strong labelling
None	GABA (raised in mouse; Sigma; 1:3000–10,000)	Donkey anti-mouse TR	Strong labelling
GABA-glutaraldehyde-BSA	GABA (raised in rabbit; Sigma; 1:3000)	Donkey anti-rabbit FITC	No labelling
	GABA (raised in mouse; Sigma; 1:3000)	Donkey anti-mouse TR	No labelling
None	GABA (raised in rabbit; Sigma; 1:3000) and GABA (raised in mouse; Sigma; 1:3000)	Donkey anti-rabbit and donkey anti-mouse	Strong labelling through both filters

FITC: fluorescein isothiocyanate; TR: Texas Red.

0.2% glutaraldehyde dissolved in PBS at 4 °C overnight. Eyecups were then vigorously washed in PBS, cryoprotected in 30% sucrose overnight and cut in a Reichert cryostat at 14–16 μm. Sections were mounted on chrome–alum–gelatin coated subbed slides and stored at −20 °C until use. The immunolabelling experiments, performed by a schedule presented in Table 1 and included several controls, were executed using commercially available primary antibodies to GABA (rabbit polyclonal, 1:1000 dilution and mouse monclonal, 1:400 dilution; both from Sigma). The primary antibodies were applied overnight at room temperature. Fluorophore-coupled secondary antibodies were purchased from Jackson Immunocytochemicals and used in a dilution 1:200 for 2 h. Sections were then washed in PBS and mounted in Vectashield (Vector Laboratories). Digital photographs were taken with a Nikon Eclipse 80 i 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.

Our results show that GABA-immunoreactivity is present in all major layers of the *Pelobates* retina (Fig. 1a). This immunolabelling can be fully eliminated when the primary antibody is preabsorbed with GABA–glutaraldehyde–BSA complex overnight (10 × excess of the antigen; Fig. 1b), regardless if the antibody preparation used in this experiment was monoclonal or polyclonal. Double-labelling experiments clearly proved that both antibodies labelled the same structures (Fig. 1c and d). Horizontal (Fig. 1e), amacrine (Fig. 1f) and bipolar (Fig. 1g) cells, as well as neurons in the ganglion cell layer (GCL; Fig. 1e) could be clearly distinguished, conforming well with the general GABA-immunoreactivity pattern of the anuran retina. Besides the above structures, GABA-immunoreactivity is also localized to a subset of photoreceptor cells in the *Pelobates* retina (Fig. 1c, d and h). The soma diameter of the labeled cells is 5–6 μm. In some cases, the inner segment was labelled more strongly than the cell body (Fig. 1c and j). Photoreceptor cell terminals were also seen occasionally (Fig. 1j and k). In general, preloading with GABA (Fig. 1h, k and l) increased the strength of immunoreactivity in photoreceptor cell bodies (Fig. 1h and l). In general, preloading with GABA neither changed the pattern of immunoreactivity nor the number of labeled photoreceptors. The GABA-positive photoreceptor cells were sparsely distributed, amounting to about 10% of the photoreceptor cell populations (Fig. 1l) which is in register with the ratio of cones among photoreceptor cells this frog species (published in abstract form [2]). In normal light microscopic immunocytochemical preparations the GABA-positive photoreceptors proved to be cones, based on their morphology (not illustrated).

In this paper, we confirm that GABA-immunoreactivity in *Pelobates* retina conforms well with the general anuran pattern (for a review see Ref. [30]). Besides, evidence was found for the presence of GABA-immunoreactive cones. This finding strengthens our view that, although the general morphology of the *Pelobates* retina is similar to those of other anurans, the neurochemistry of certain cell types may substantially differ. One such evidence has recently been published regarding the tachykinin-containing amacrine cells [22] and another difference in the NPY-positive structures has also been noted (published in abstract form [23]).

It is generally accepted that photoreceptors store and release glutamate as neurotransmitter [4,11,26]. Other transmitter candidate substances ever localized to photoreceptors include histamine [5], carnosine [18] and GABA [6,15]. To our knowledge, there has been only two paper published in the literature in which GABA-immunoreactivity was localized to photoreceptor cells. While the former two substances have never been confirmed to exert any discernible action on retinal information processing, GABA is known to be a major inhibitory transmitter both in the outer and inner retina, acting through all three known GABA receptor types (A: [3], B: [25], C: [10]). One can only speculate on the possible function of a GABA-releasing photoreceptor cell. It has been shown that glutamate release is decreased by light stimulation and with the exception (the ON-bipolar cells), second order neurons respond with hyperpolarization to this stimulus. If these cells release GABA as a transmitter, it may act on the second order cells, from which certain horizontal and bipolar cells have functional GABA receptors [3,8,10,25]. GABA may even be depolarizing in the case of the horizontal cells [29]. The chloride equilibrium potential (−20 mV) falls within the operating voltage range of these neurons [8]. This surprisingly high equilibrium potential is supported by the presence of the sodium/potassium/chloride cotransporter in the membrane of these cells [28]. Alternatively, GABA may influence

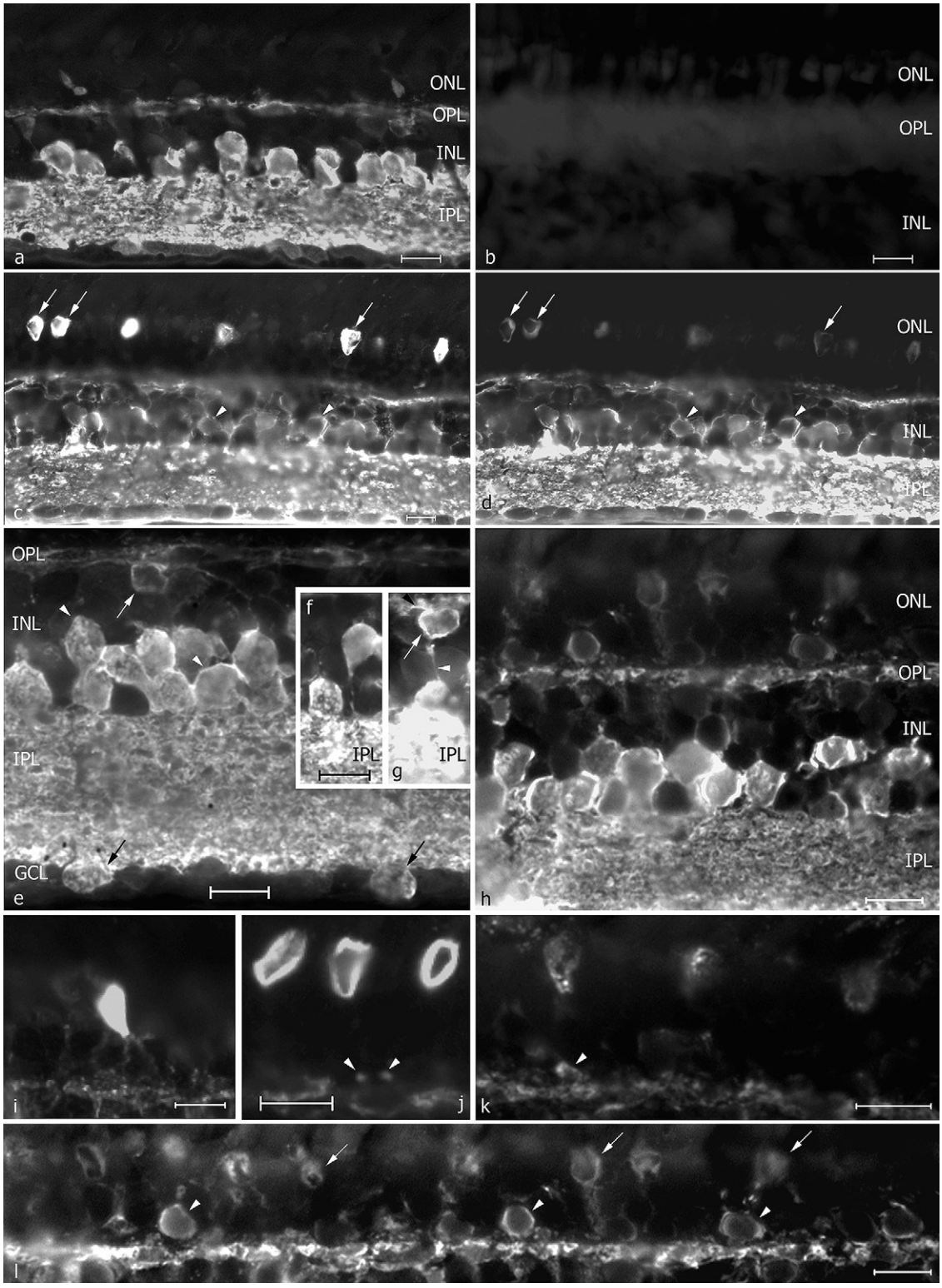

Fig. 1. GABA-immunoreactivity in the *Pelobates* retina. ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars: 10 μm in all photographs. (a) Distribution of GABA-immunoreactivity in retinal layers. (b) After preabsorbtion no immunoreactivity could be detected. (c and d) Monoclonal (mouse) and polyclonal (rabbit) anti-GABA antibodies label the same structures (arrows: cells in the ONL, arrowheads: cells in the INL). (e) Horizontal (white arrow) and amacrin (white arrowheads) cells and also some neurons in the GCL (black arrows) show GABA-immunoreactivity. (f) Two GABA-immunoreactive amacrin cells in different positions of the INL. (g) GABA-immunoreactive bipolar cell (soma: white arrow, dendrite: black arrowhead, axon: white arrowhead). (h) GABA-positive structures in the outer layers of the retina after preloading with 1 mM GABA. (i–k) Labeled cells in the photoreceptor layer without (i and j) and with (k) preloading. In some instances the inner segments are strongly labeled (j). Immunoreactive puncta adjacent to the OPL (arrowheads in j and k) are suspected to be the terminals of the GABA-positive photoreceptors. (l) Both cell bodies (arrowheads) and inner segments (arrows) can be labeled after preloading.

the cones themselves through autoreceptors [9,17,33] since all three main GABA receptor types have been localized to cones in different species [33].

Another intriguing question if GABA is synthetised in *Pelobates* cones or it is produced purely for metabolic purposes as a by-product of the Krebs cycle. In double-labelling experiments for GABA and glutamate the immunoreactivity for glutamate was weak or totally absent in GABA-labelled cones (not shown in this paper). Based on these observations we infer that GABA synthesis in cones is initiated from glutamate as is usual in other GABAergic neuron in the nervous system. The possible effects of GABA on second-order cells, however, are likely to be independent from the ability of GABA synthesis in the photoreceptor cells, since it has been shown that retinal neurons are able to release accumulated substances even if they do not synthesize them [24]. Further experiments are needed to verify if these cones indeed synthetise and/or release GABA, and only GABA, as their neurotransmitter.

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