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# Immunocytochemical demonstration of γ-amino butyric acid and glutamic acid decarboxylase in R7 photoreceptors and C2 centrifugal fibres in the blowfly visual system

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**Summary.** Neurons within the compound eye of the fly *Calliphora erythrocephala,* suspected of containing gamma-aminobutyric acid were revealed immunocytochemically, using antibodies directed against gamma-aminobutyric acid (GABA) and glutamic acid decarboxylase (GAD). The GABA content within putative GABAergic neurons was increased by high affinity uptake of GABA and selective blocking of GABA metabolism with Gabaculine. Only neuronal populations which were labelled with the GABA as well as the GAD antibodies were presumed to be GABAergic. The first optic neuropil (lamina) exhibited two distinct GABAergic fibre populations amongst a larger population comprised of fourteen cell classes. One fibre population was formed by the axons of the photopic photoreceptors R7 which pass through the lamina and terminate in the second optic neuropil (the medulla). The identity of R7 was established from longitudinal and transverse sections of the retina where R7 can be unequivocally distinguished from the six scotopic photoreceptors R1–6 and the other photopic receptor, R8.

The other fibre population matched the profiles in the lamina of terminals of efferent C2 neurons. These neurons project distally from beneath the medulla out to the lamina ganglionaris where each retinotopic unit (cartridge) contains a characteristic hook-like terminal arbor distally. We propose from these data that the photoreceptors R7 and the efferent C2 neurons use GABA as a neurotransmitter.

## Introduction

The optic lobes of dipteran insects are one of the most thoroughly studied neuropiles in arthropods. There is a volume of information about both the cellular morphologies and synaptic connections of a number of neurons (Strausfeld and Nässel 1981) and electrophysiological data provide information about the functional role of many cell types (DeVoe 1985).

However, our knowledge about transmitters mediating the numerous synaptic interactions is comparatively small. Only a few studies deal with the neuronal localization of neurotransmitters in dipteran optic neuropil. These include histochemical and immunocytochemical studies of the monoaminergic neurons (Klemm and Axelsson 1973; Nässel and Klemm 1983; Nässel et al. 1983) and autoradiographic studies on the GABA localization in the lamina, employing GABA uptake (Campos-Ortega 1973). The drawback of this approach is that it relies on the hypothesis that GABA is inactivated at the synaptic site by a high affinity uptake into the presynaptic terminal (Iversen 1971). However, it has been shown that such an uptake can also be into postsynaptic terminals and into glial cells (Iversen et al. 1975).

A more reliable indicator of GABAergic neurons, which is widely used, is the immunocytochemical localization of the GABA biosynthetic enzyme, glutamic acid decarboxylase (GAD). The presence of GAD within a neuron is considered

to be one of the best indicators for GABAergic action by this neuron (Oertel et al. 1984). In addition it has now become possible to directly detect endogenous GABA with an anti-GABA antibody (Storm-Mathisen et al. 1983).

The aim of the present study was to reveal GABAergic neurons in the peripheral part of the fly's visual system. Possibly, such neurons may use GABA as a neurotransmitter, as indicated by the immunocytological demonstration of endogenous GABA, and its synthesizing enzyme , GAD.

## Materials and methods

Experiments were carried out with the blowfly *Calliphora erythrocephala*, reared in the authors' laboratory. Light or dark adapted flies were used.

Immunocytochemistry was done with three different antibodies:

1. GABA-antibody (Immuno Nuclear Company; raised in rabbit);
2. GAD (glutamic acid decarboxylase) I antibody (gift from Chr. Brandon, Chicago; raised in rabbit);
3. GADII antibody (gift from D. Oertel, Munich; raised in goat).

Animals were decapitated and the back of the head opened to allow penetration of fixative. For GABA or GADII immunocytology tissue was fixed at 20 °C for 1 h with a solution of 4% paraformaldehyde, 0.05% glutaraldehyde and 3% sucrose in 0.1 *M* Sörensen phosphate buffer. For GADI immunocytology the tissue was fixed with 2% paraformaldehyde, 0.03% glutaraldehyde in 0.05 *M* Sörensen phosphate buffer (pH = 6.5) for 20 min, followed by 2% paraformaldehyde, 0.03% glutaraldehyde in 0.05 *M* phosphate buffer (pH = 8.5) for 1 h and then 2% paraformaldehyde in 0.05 *M* phosphate buffer (pH = 8.5) for 0.5 to 1 h.

After fixation tissue was washed twice for 15 min in 0.1 *M* Sörensen phosphate buffer and soaked overnight in 30% sucrose in 0.1 *M* Sörensen phosphate buffer at 4 °C.

The tissue was then embedded in cryomatrix (Shandon). Frozen 18 µm–25 µm sections were cut horizontally or frontally and mounted on gelatine precoated slides. These were placed for 2 h on a hot plate (37 °C) and then washed 20 min in PBS (for GABA- and GADII antibody) or KPBS (PBS with elevated potassium concentration; for GADI antibody). Sections, determined for immunocytology with GABA or GADI antibody were incubated in 10% normal goat serum in PBS or KPBS, at 20 °C for 1 h. Afterwards, 18–24 h incubation with the primary antibody was done at 4 °C. The antibodies were diluted as follows: GABA-antibody in PBS with 0.3% Triton X and 5% NGS at dilutions, ranging from 1:1000 to 1:3000; GADI antibody in KPBS with 0.05% Triton X and 5% NGS at dilutions of 1:750 to 1:1000; GADII antibody in PBS with Triton X at a dilution of 1:1000. After incubation with GADI antibody, sections were washed in KPBS and then gently transferred into PBS by adding PBS to KPBS. All the following steps were interrupted by washes (two times, 15 min) in PBS and carried out at 20 °C.

Immunoreactivity was detected with either the peroxidase-antiperoxidase method (PAP) or the avidin-biotin binding method (ABC) with diaminobenzidine (DAB) as the chromogen.

In case of GABA immunocytology the GABA immunoreactivity of putative GABAergic neurons was enhanced by incubating the fly's head in a solution of 100 µ*M* GABA and 100 µ*M* Gabaculine hydrochloride (a gift from Ciba Geigy, Basel). There is evidence that Gabaculine is a potent inhibitor of the GABA degrading enzyme GABA transaminase (Löscher 1980), thus resulting in GABA accumulation.

In order to check the specificity of the antibodies used, control experiments were preabsorbtion of the primary antibodies with their corresponding antigenes; and, incubation with the second antibody (for PAP also for the third antibody) in absence of the primary antibody.

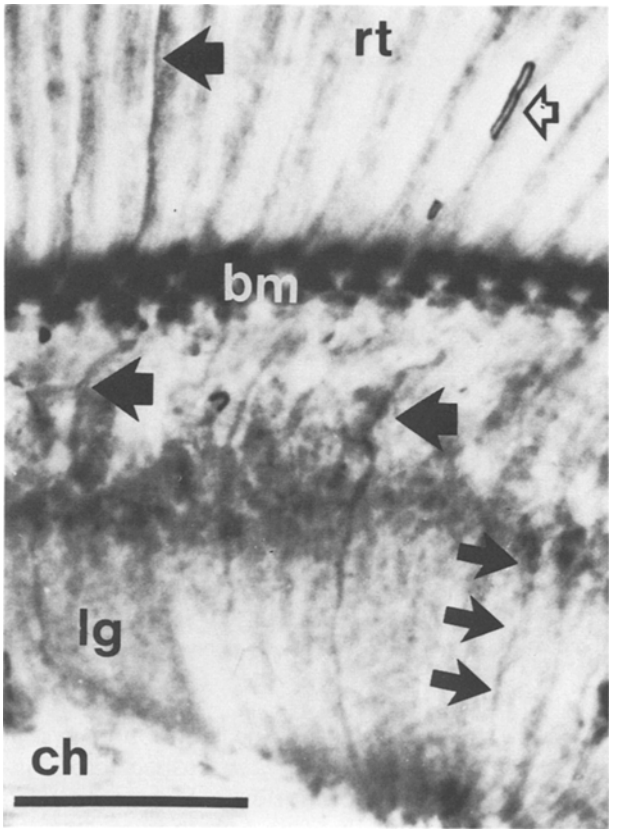



**Fig. 1.** Frozen sections (20 µm) containing part of the retina (*rt*), the lamina (*lg*) and the first optic chiasma (*ch*). The section was incubated with GABA antiserum. Immunoreaction was revealed using the PAP-method. Large black arrows indicate immunoreactive fibres in the retina which cross the basement membrane (*bm*) and enter the lamina. Thin black arrows indicate an immunoreactive fibre which enters the lamina from the chiasma and terminates at its distal border with a beaded structure. Several similar structures are visible within the lamina and retina. Open arrows indicate tracheal structures. Scale = 50 µm

## Results

### *Staining intensities*

Frozen sections labelled with any of the three antibodies revealed a great number of neuronal elements containing immunoreactive material and also some labelled non-neuronal elements (Fig. 1). A comparison of the staining pattern of the three

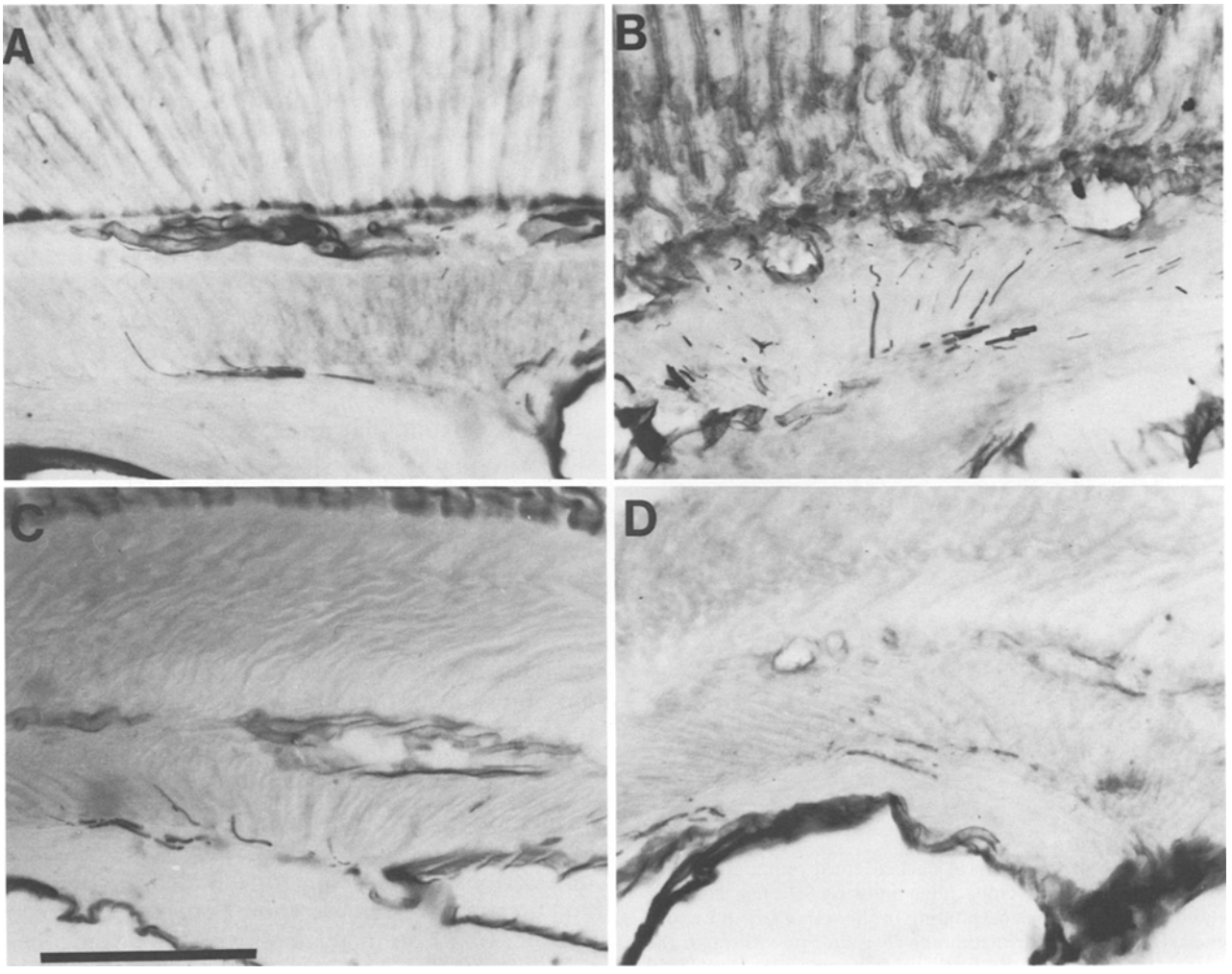



**Fig. 2. A** Horizontal section treated with the ABC-method, omitting the primary antibody. Unspecific labelling of trachea network and airbag membranes is pronounced and unspecific labelling of the basement membrane and the pigment cells is weak. **B** Section incubated with anti-GABA preabsorbed with 1 m*M* GABA prior to incubation. Compared to the labelling without primary antibody additional labelling of the rhabdomeres of the photoreceptors is visible. **C** Section incubated with anti-GADII, preabsorbed with 300 μ*M* GAD prior to incubation, shows no unspecific labelling. **D** Section incubated with anti-GAD I, preabsorbed with 300 μ*M* GAD prior to incubation, shows unspecific labelling of some monopolar cell bodies. Scale = 125 μm

antibodies showed slight differences in the number and types of labelled neuronal and nonneuronal elements. On the other hand all three antibodies labelled prominent and morphological defined neuronal elements. These are described below.

The following control experiments distinguish labelling of elements other than neurons, as well as unspecific labelling, from specific neuronal labelling. When the staining procedures are carried out in the absence of the primary antibody, networks of trachea and airbags were still labelled as is, to a lesser extent, the basement membrane and the pigment cells of the retina (Fig. 2A). This type of labelling is possibly due to endogenous peroxidase. The same pattern of staining could be demonstrated with the DAB reaction alone. Unspecific (as well as the specific) labelling showed differences in intensity in consecutive sections. The reason for this is not yet clear and no systematic variability was found. The variability was observed for background labelling, which we could reasonably well suppress using normal goat serum and high dilutions of primary antibody. In addition the ABC method prooved slightly superior to the PAP method.

In preabsorbtion experiments where the GABA antibody was preabsorbed with 1 m*M* GABA, neuronal fibre labelling was absent, but the rhabdomeres of the photoreceptors still remained labelled (Fig. 2B). Preabsorption of the GADII anti-

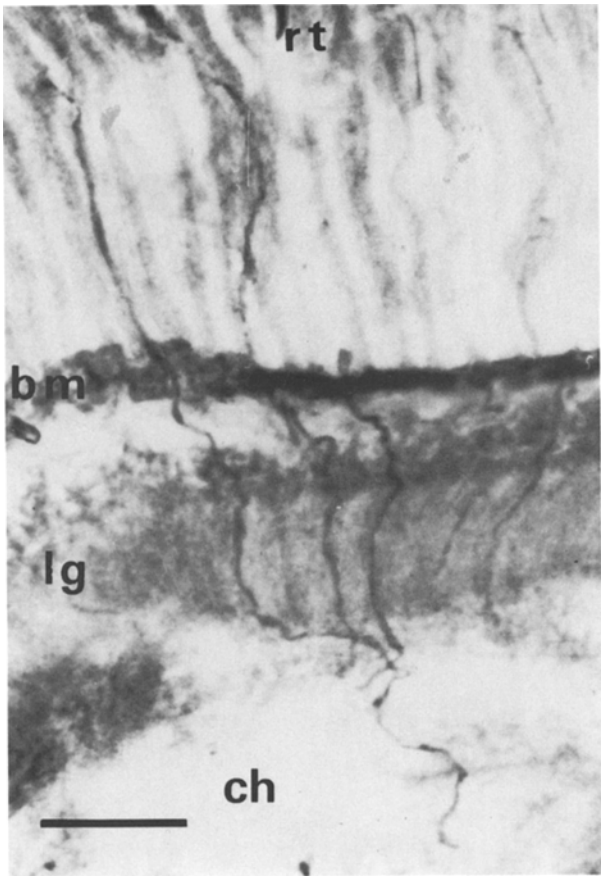



**Fig. 3.** Anti-GABA staining of dark adapted retina after incubation in GABA and Gabaculine showing selective labelling of photoreceptor axons originating in the retina (*rt*). R7 axons penetrate the basement membrane (*bm*) and pass through the lamina (*lg*) and enter the first optic chiasma (*ch*). Scale = 50 μm

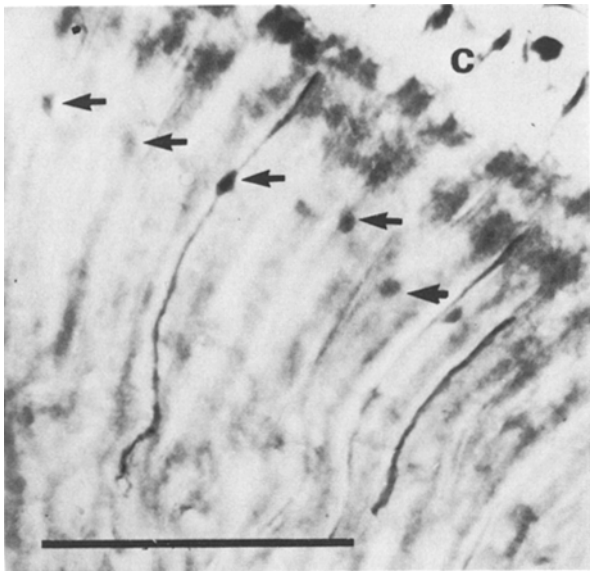


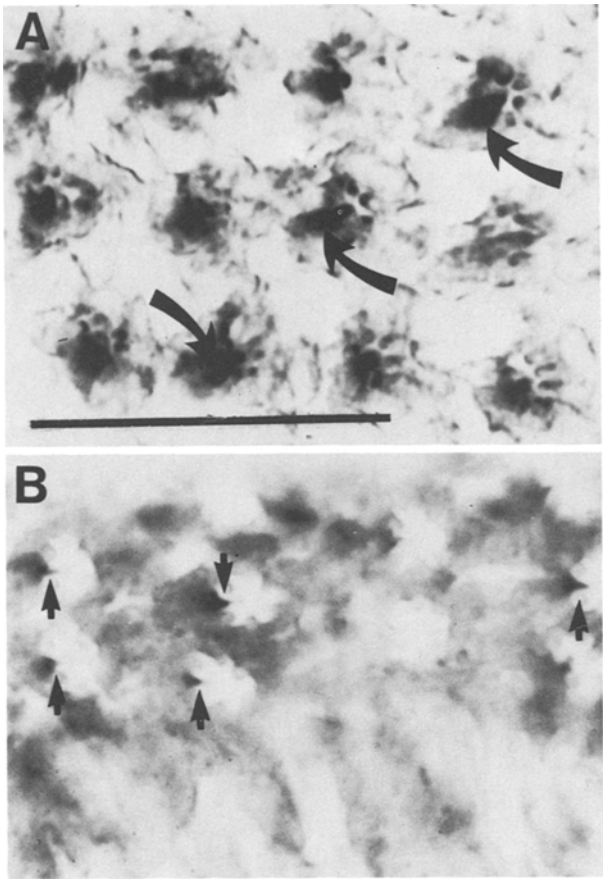

**Fig. 4.** R7 photoreceptors in the distal part of the retina stained with the GABA antibody, after dark adaptation and preincubation in GABA and Gabaculine. Arrows indicate R7 nuclei. Cornea (*c*); Scale = 125 μm

body with 300 μM GAD (Sigma, Type II) prevented all neuronal labelling (Fig. 2C). Preabsorption of the GADI antibody did not prevent labelling of some somata of monopolar cells (Fig. 2D). These somata were also occasionally labelled by GABA antisera. Due to their unspecific labelling by anti-GAD such cells are here assumed not to be GABAergic.

Reproducible labelling of specific profiles, using all three different antibodies, finally revealed only two fibre systems in the lamina. One system comprises axons that enter the lamina from the retina, pass through the lamina and then entering the first optic chiasma (Fig. 3). The other system comprises axons that apparently terminate at the distal border of the lamina, entering the lamina from first optic chiasma (Figs. 6, 7). The two oppositely projecting systems were not evenly labelled in all experiments, however. Certain conditions did, though, result in enhanced staining the first system. Dark adaptation of the retina enhanced GABA labelling of centripetal fibres from the retina, but partially supressed labelling of the centrifugal fibres out to the lamina. Incubation of the living head in Ringer solution containing GABA and Gabaculine had similar effects. In comparison GADII always showed stronger labelling of fibres terminating in the lamina from the medulla, and labelling was further improved by reducing fixation periods. The reasons for these selectivities are not understood, but we took advantage of them in order to get optimal staining of one or the other system.

### *GABAergic profiles*

Figure 3 shows intense GABAergic fibres, labelled with anti-GABA, originating in the retina and projecting through the lamina in the first optic chiasma. The only fibres projecting in this fashion are the long photoreceptors R7 and R8 (Campos-Ortega and Strausfeld 1972). The outer segments of R7 and R8 are arranged in tandem in each ommatidium (Melamed and Trujillo-Cenoz 1968). R8 somata are always situated in the proximal half of the retina, thus facilitating the identification of GABAergic labelled photoreceptors. These lie in the distal part of the retina (Fig. 4) and thus must belong to R7. Sections cut through the retina perpendicular to the long axis of photoreceptor cells




and labelled with anti-GABA (Fig. 5A) demonstrate that only one photoreceptor is labelled in each ommatidium at the R7 position (Dietrich 1909). Immunoreactivity using the GAD antibody on sections cut parallel to the axis of photoreceptors also labelled R7 photoreceptors. However, these were less intense than with GABA antibody. In cross sections, however, the same antibodies clearly distinguished R7 photoreceptor cells in the distal part of the retina (Fig. 5B), thus confirming the results obtained with GABA antibody. The GABAergic R7 photoreceptors are possibly distributed throughout the entire retina. In five examined flies no region was detected were R7 labelling was constantly lacking.

The second fibre system, labelled by all three antibodies is shown in Fig. 6. This system is strongly GAD positive in comparison to R7 axons which always appear much fainter. The fibres of the second system terminate at the distal edge of the lamina and obviously arise from intensely labelled axons that cross the first optic chiasma. The

**Fig. 5. A** Cross-section showing twelve ommatidia each with a stained R7 photoreceptor (arrows). GABA immunoreaction; preincubation with GABA and Gabaculine. **B** Oblique section of the distal retina viewed just beneath the cornea. R7 photoreceptor cells are labelled in several ommatidia (arrows) with GADI antibody. Scale = 50 μm

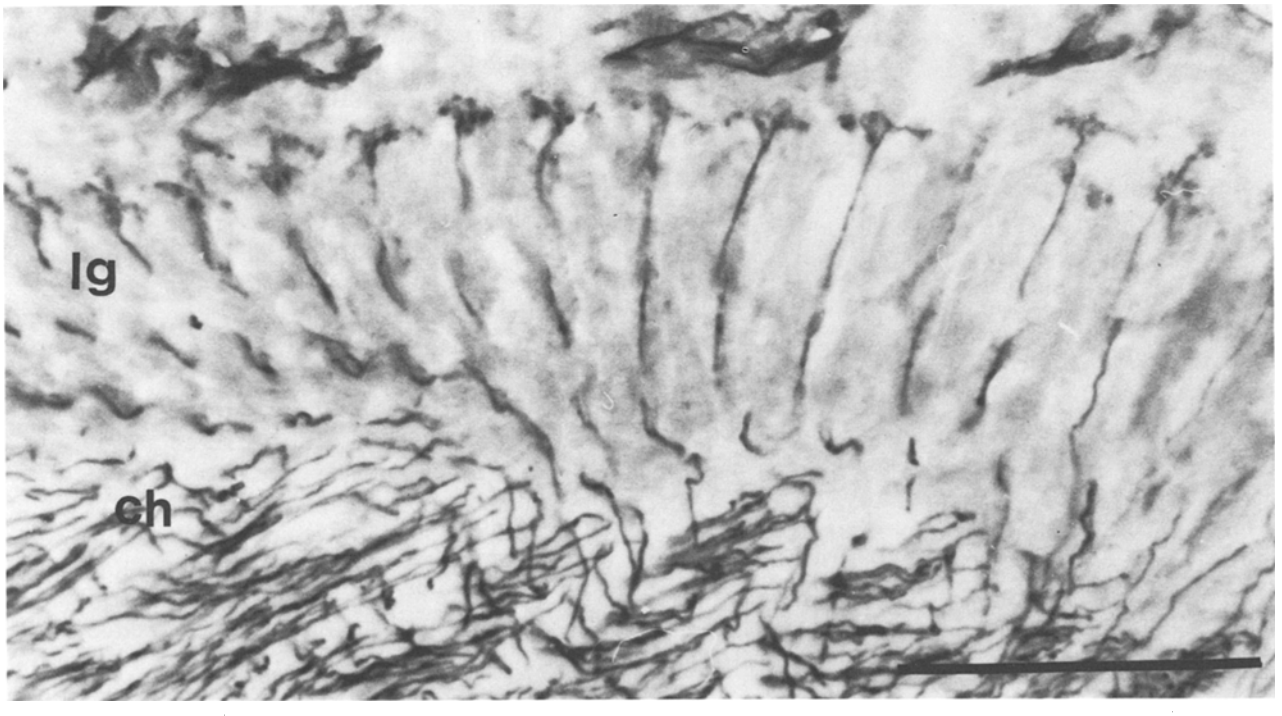



**Fig. 6.** Horizontal section showing C2 terminals within the lamina (*lg*) arising from the first optic chiasma (*ch*). Note labelling in almost every cartridge. GADII antibody immunoreaction; scale = 50 μm

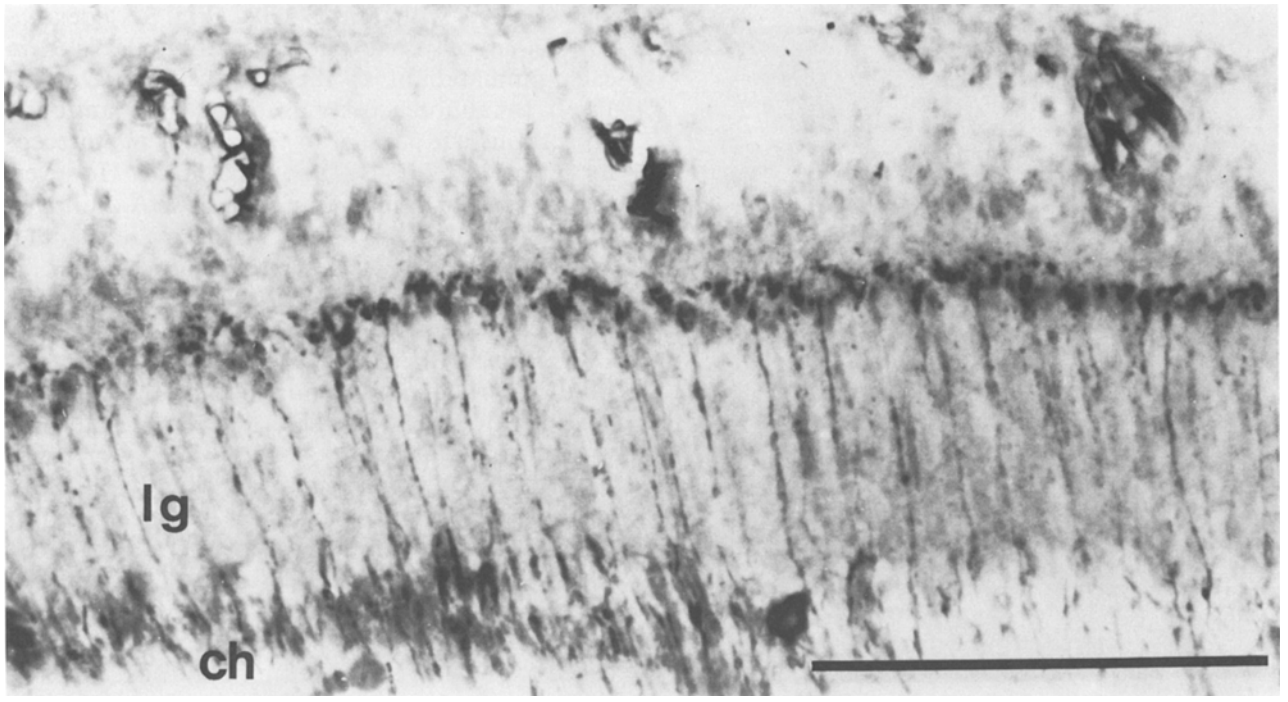



**Fig. 7.** Frontal section showing C2 terminals within the lamina (*lg*) revealed with anti-GABA. Scale = 50 μm

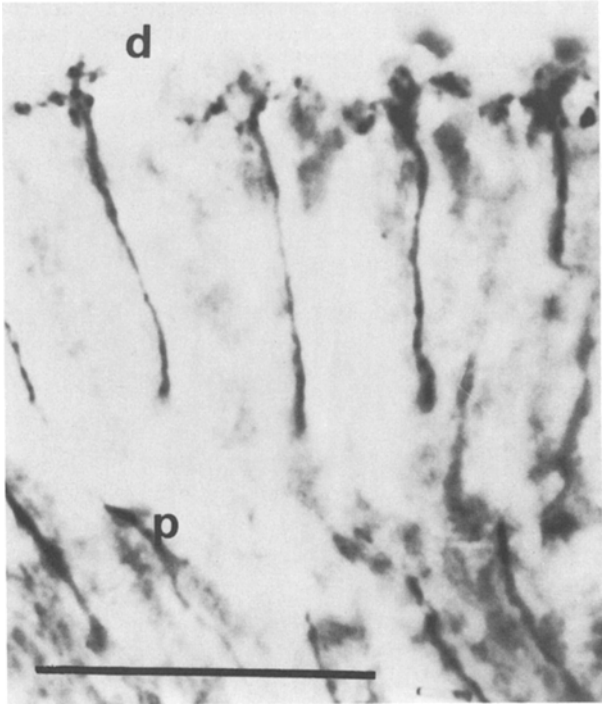



**Fig. 8.** Horizontal section showing C2 terminals within the lamina, demonstrating their palmate specializations at the distal border (*d*). GABA immunoreaction; preincubation with GABA and Gabaculine; scale = 50 μm

same pattern of staining can be seen with GABA antibody although the labelling is paler (Fig. 7).

Within the lamina, elements of the second system are arranged as a palisade, and are retinotopic, having a 1 to 1 ratio with the ommatidia. Their axons terminate at the distal border of the lamina with palmate like specializations (Fig. 8). Axons ascending through lamina occasionally show bleb-like specializations.

Golgi studies on the dipteran lamina (Strausfeld 1971, 1976) show identical profiles within the lamina, arising from a characteristic neuron that originates in the medulla and which was termed the C2 cell. Perikarya of this cell type are located central to the proximal border of the medulla and should thus be detectable there with anti-GAD and anti-GABA.

Indeed GABA immunoreaction labels cell bodies in the appropriate region. In general only few putative C2 cell bodies were located posteriorly beneath the medulla. Anteriorly, nearer the outer edge of the lobula, putative C2 cell bodies were encountered more frequently. Many putative C2 perikarya were found between the anterior edge of the medulla and the outer proximal edge of the lobula, arranged in clusters (Fig. 9). This gave rise to fibres that were directed to the medulla. In one case the medullary C2 fibre part could be labelled (Fig. 10).

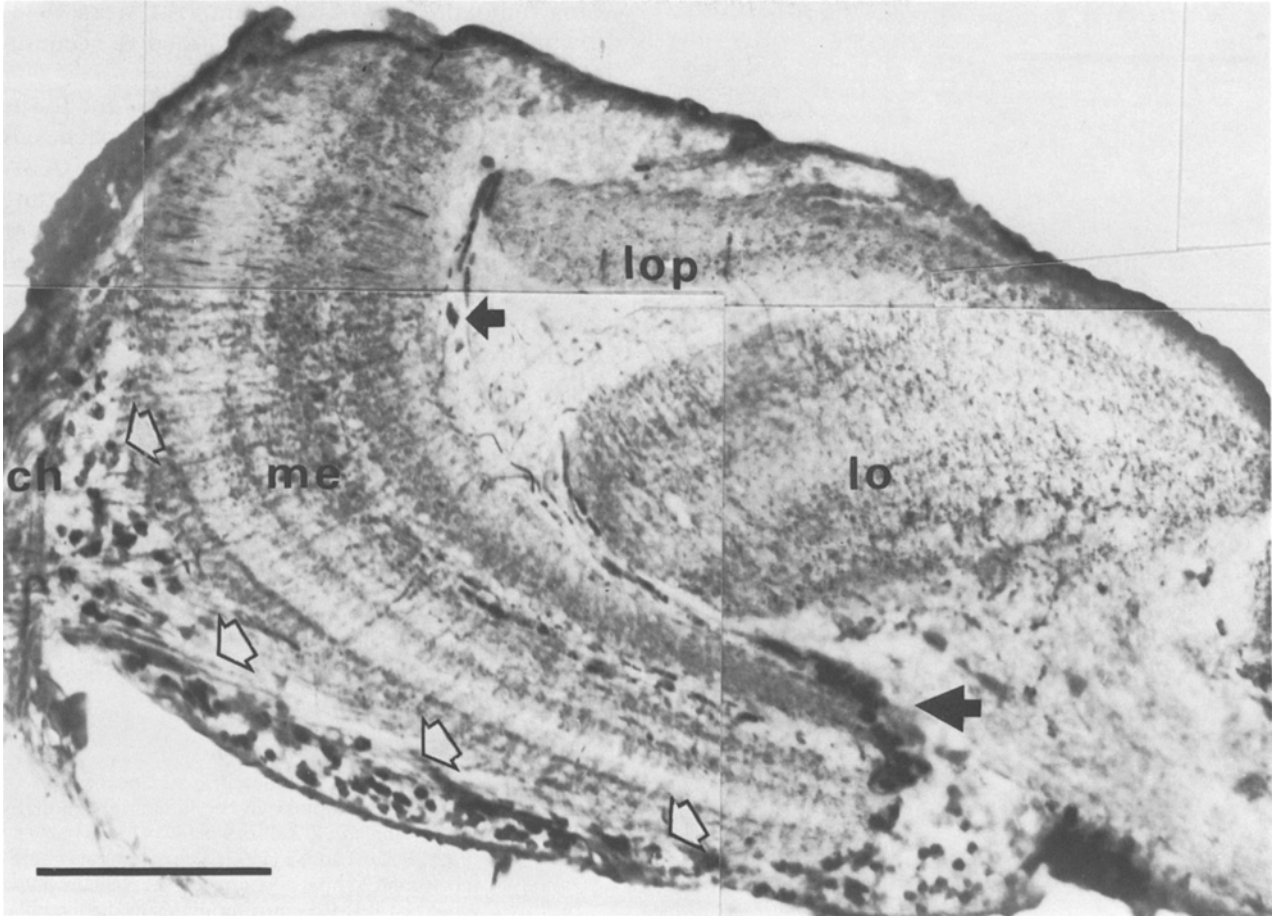



**Fig. 9.** Photomontage of a horizontal section through the medulla (*me*), lobula (*lo*) and lobula plate (*lop*). The medulla is well characterized by the densely labelled strata. Many cell bodies, lying distal to the medulla within the cell body rind, (open arrows) are labelled by anti-GABA. A cluster of putative C2 cell bodies (large black arrows) is labelled proximal to the medulla behind the lobula. An isolated labelled cell body (small black arrow) presumably represents another more centrally disposed C2 cell body. Scale = 125 μm

Numerous cell bodies were also labelled distal to the medulla amongst axons of the first optic chiasma. This is the area in which arise the great majority of retinotopic neurons that make up the bulk of the medulla neuropil. Such cells include transmedullary cells, and amacrines. Centrifugal fibres linking the medulla and lamina (T1) also originate from cell bodies at this location. However, since we could not find any fibres in the lamina, which belong to these elements we must conclude that immunostained somata belong to neurons supplying the medulla only.

It is worth remarking that anti-GABA and anti-GAD staining reveals a complex pattern of immunoreactive elements in the medulla. In general, fibres organized as strata show a more intense labelling than fibres running perpendicular to these strata. This would suggest that the immunoreactive cell bodies in the first optic chiasma possibly belong to stratified amacrine cells rather than retinotopic relay neurons.

## Discussion

Our results suggest that R7 photoreceptors contain GABA. The small number of observations (5 eyes of 5 flies), however, does not allow us to decide whether all R7, or a subpopulation of them, are GABAergic. So far, the putative neurotransmitters of dipteran photoreceptors are completely unknown. Our demonstration that some, if not all R7 contain GABA is the first reasonable evidence for the identity of a photoreceptor transmitter. Our results strongly suggest that GABA is not a transmitter of the R1–6 receptor system.

GABA content in R7 photoreceptors could be increased by dark adaptation, high affinity uptake of GABA and blocking of GABA transaminase.

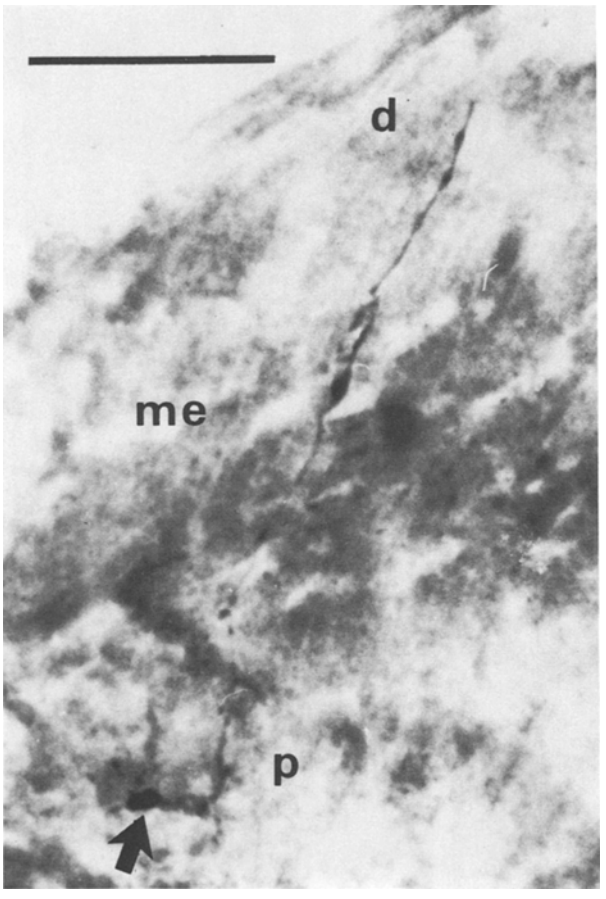



**Fig. 10.** Medullary component of a C2 cell stained by anti-GABA, following preincubation with GABA and Gabaculine. The cell body (open arrow) lies at the proximal border (*p*) of the medulla (*me*). The fibre shows swellings along its course and has a typical hook-like branch in the outer third of the neuropil (*d*); scale = 50 μm

This supports the idea of R7 as a GABAergic neuron releasing its transmitter upon depolarization in response to illumination (Iversen 1971).

C2 neurons are clearly GABAergic, staining most intensely at their terminals. However, little is known about the physiological role of C2 neurons, and the only hint as to their function are their synaptic relationship with the monopolar neurons L1, L2 and L3 (Strausfeld and Nässel 1981; Trujillo-Cenoz 1985). There are as yet no reports of electrophysiological recordings of centrifugal efferent fibres and at most we may suggest that GABAergic C2 neurons provide inhibitory input to the major centripetal outputs from each cartridge.

Finally we have convincingly demonstrated that epithelial and marginal glia cells are not GABAergic using our experimental conditions. This contradicts the interpretation of an uptake study using radiolabelled GABA (Campos-Ortega 1973) in which epithelial glia were suggested to accumulate the transmitter.

This discrepancy might, however, be due to the completely different methodological approaches. It is known that glial elements do take up GABA (Iversen et al. 1975) and in two recent papers using uptake of radiolabelled GABA as a marker for GABAergic neurons has been seriously questioned (Zucker et al. 1984; Yazulla et al. 1986). More emphasis is given to the immunocytochemical localization of GAD. Significantly, our neuron identification is based both on their immunoreactivity to anti-GABA and the antibody of its synthesizing enzyme, GAD. This is probably the most reliable identification available at present.

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**Note added in proof.** Just recently two works were published containing results of GABA labelled neurons in visual systems, which are similar to our findings.

The one reports about GABA labelled neurons in insect compound eye, the other about the GABA content of vertebrate photoreceptors.

Meyer EP, Matute C, Streit P, Nässel DR (1986) Insect optic lobe neurons identifiable with monoclonal antibodies to GABA. Histochemistry 84:207–216

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