Research article

Cholinergic and GABAergic pathways in fly motion vision

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Published: 9 February 2001

BMC Neuroscience 2001, **2**:1

Received: 1 December 2000
Accepted: 9 February 2001

This article is available from: http://www.biomedcentral.com/1471-2202/2/1

Abstract

Background: The fly visual system is a highly ordered brain structure with well-established physiological and behavioral functions. A large number of interneurons in the posterior part of the third visual neuropil, the lobula plate tangential cells (LPTCs), respond to visual motion stimuli. In these cells the mechanism of motion detection has been studied in great detail. Nevertheless, the cellular computations leading to their directionally selective responses are not yet fully understood. Earlier studies addressed the neuropharmacological basis of the motion response in lobula plate interneurons. In the present study we investigated the distribution of the respective neurotransmitter receptors in the fly visual system, namely nicotinic acetylcholine receptors (nAChRs) and GABA receptors (GABARs) demonstrated by antibody labeling.

Results: The medulla shows a laminar distribution of both nAChRs and GABARs. Both receptor types are present in layers that participate in motion processing. The lobula also shows a characteristic layering of immunoreactivity for either receptor in its posterior portion. Furthermore, immunostaining for nAChRs and GABARs can be observed in close vicinity of lobula plate tangential cells. Immunostaining of GABAergic fibers suggests that inhibitory inputs from the medulla are relayed through the lobula to the lobula plate rather than through direct connections between medulla and lobula plate.

Conclusions: The interaction of excitatory and inhibitory pathways is essential for the computation of visual motion responses and discussed in the context of the Reichardt model for motion detection.

Background

The fly visual system is characterized by its repetitive, retinotopic organization of four layered structures: the lamina, the medulla, the lobula and the lobula plate. Every layer is composed of thousands of columns each of which contains the same number and types of neurons [1,2]. In contrast to our detailed knowledge about the anatomy of these columnar elements, not much is known about their visual response properties except for the large lamina monopolar cells [3,4] [5]. Due to the small diameter of the fibers, intracellular recordings are hard to accomplish in most cases [6,7,8,9]. Thus, most data on columnar neurons arise from 2-deoxy-glucose activity staining which, however, cannot easily be assigned to individual cell types [10,11,12,13]. Nevertheless, there exists anatomical evidence for at least three major parallel processing streams in the fly optic lobes [14,15]: the first two pathways arise from receptor cells R1-6, which are

connected through lamina cells L1 and L2 and transmedulla neurons to the lobula plate. These two pathways are thought to be involved in motion processing. The third pathway receives input from retinula cells R7 and R8, and, by way of lamina cells L3, projects mainly to the lobula. This pathway is supposed to be involved in the processing of form and color.

Amongst the best-studied cells of the fly visual system are the large lobula plate tangential cells (LPTCs), which, due to their large diameter axons (about 8-10 microns) are relatively easy to record from intracellularly. LPTCs also possess a large dendritic arbor on which they receive input from numerous columnar elements arising presumably from the medulla and the lobula (for review see: [1,16] Many of these LPTCs do not produce regular action potentials but rather respond to excitatory or inhibitory stimuli by a graded shift of membrane potential [17,18,19,20]. Typically, LPTCs respond to visual motion in a directionally selective way: They depolarize when stimulated by preferred direction motion, and become inhibited by motion along the opposite or null direction. According to our current view, their direction selectivity is produced by the antagonistic action of local elements tuned to opposite directions of motion [21]. These input elements are thought to be only weakly selective for the direction of motion. The direction selectivity of the LPTCs is enhanced to such a high degree as it is observed in the electrical responses solely through the subtractive inhibition taking place on the dendrites of the LPTCs. Evidence for this type of input arrangement comes from pharmacological experiments where the inhibitory input is blocked by PTX. Under these conditions the preferred direction response is enlarged and the response to null direction is inverted resulting in an excitation [22]. However, all conclusions pertaining to the response properties of the input elements to the tangential cells are based on indirect evidence only, since, for the reasons outlined above, only few intracellular recordings exist from them.

Another line along which to identify these input elements could be their transmitter system. Here, the tangential cells have been shown physiologically to possess at least two different transmitter receptors on their dendrite: a cholinergic receptor with a typical nicotinic pharmacological profile, and a γ-aminobutyric acid (GABA) receptor [23,24]. Antibodies against the ARD subunit of nicotinic acetylcholine receptors (nAChRs) [25,26] and the RDL subunit of the GABA receptor [27] in *Drosophila* allowed us to investigate the distribution of these receptors in the fly visual system.

In the following we will present immunocytochemical data of antibody staining against nAChRs, GABA receptors and the inhibitory neurotransmitter GABA itself. The distribution of immunoreactivity in the fly visual system for these receptors and GABA is analyzed and the putative pharmacology and cell types of the motion pathway are discussed.

Results

We examined the distribution of nicotinic acetylcholine receptors (nAChR), GABA receptors and GABAergic neurons within the higher order visual neuropils (medulla and lobula complex) of the blowfly *Calliphora erythrocepala*.

Distribution of nicotinic acetylcholine receptor immunoreactivity

We tested 3 different monoclonal antibodies, which originally were generated against *Drosophila* nAChR subunits for their cross-reactivity in the blowfly *Calliphora erythrocephala*. Two of these antibodies were directed against ligand-binding subunits (ALS and Dα2) whereas the third was directed against a structural subunit (ARD) of the *Drosophila* nAChR [25].[28] Initial screening of α-ALS and α-Dα2 sera showed no cross-reactivity in western blots when *Drosophila* and *Calliphora* brain homogenates were compared (data not shown). Immunohistochemical staining with the α-ALS serum also failed to show immunoreactivity in brain cryostat sections from *Calliphora* in contrast to positive controls made in *Drosophila* (data not shown). Western blots with the α-ARD antibody mab 3D2 revealed multiple bands in *Drosophila* and *Calliphora* brain homogenates at approx. 42-50 kD (Fig. 1a). These are thought to correspond to the the ARD subunit, which migrates at about 50 kD in denaturing gels, and some proteolytic degradation products [26]. In the *Calliphora* lane an additional band appeared at approx. 100 kD of unknown origin. It might represent a hetero- or homo-dimer of the subunit or cross-reactivity with Dα3, a novel α-subunit which migrates at 105 kDa and is associated with ARD [26] . Cross-reactivity is, however, not observed in *Drosophila* (Fig. 1a) [26]. Successful immunohistochemical staining with the α-ARD antibody in *Calliphora* depended strongly on the fixation method. Fixation with 4% paraformaldehyd prevented positive immunoreactivity in *Calliphora* brain tissue. In contrast, fixation with FAA, a mixture of formaldehyde, ethanol and acetic acid resulted in clear α-ARD immunofluorescence in neuropilar regions of the visual system (Fig. 1b). This staining pattern could be attributed to the binding of the primary antibody as shown by the control without the primary antibody in figure 1c. α-ARD immunoreactivity in the visual system of *Calliphora* was clearly restricted to the synaptic neuropils: the medulla, the lobula and the lobula plate. The inner chiasma, a fiber tract between these three neuropils, and the layer of somata posterior to the lobula plate did not show immunopositve staining for

ARD (Fig. 1b). The medulla revealed a multi-layered pattern of α-ARD immunoreactivity (Fig 1b, 2a), with a concentration of immunopositve signal in the inner medulla. The layers with the terminals of the lamina monopolar cells L1 and L3 (layers 1,3 and 5) showed higher levels of immunoreactivity than the layers containing the terminals of lamina monopolar cells L2 and of photoreceptors R7, R8 (Fig. 2a). Especially layer 1 showed the highest levels of α-ARD immunoreactivity in most of the analyzed sections. The lower level of α-ARD immunoreactivity in layer 4 with the terminals of photoreceptor R8 was not surprising as fly photoreceptors R1-6 and R8 are known to be histaminergic [29,30,31,32]. Layer 6 with the terminals of the R7 photoreceptors, in contrast, shows some α-ARD immunoreactivity. The serpentine layer, which consists mainly of fibers, had also a decreased level of α-ARD immunoreactivity (Fig. 1b, 2a). All three layers of the inner medulla were highly immunoreactive. Layer 10 was somewhat weaker stained, which might be due to a lower cell density in this layer compared to layers 8 and 9. Layer 10 contains the dendritic arborizations of the T4 cells, which are presumed to represent input elements to the lobula plate tangential cells [14].

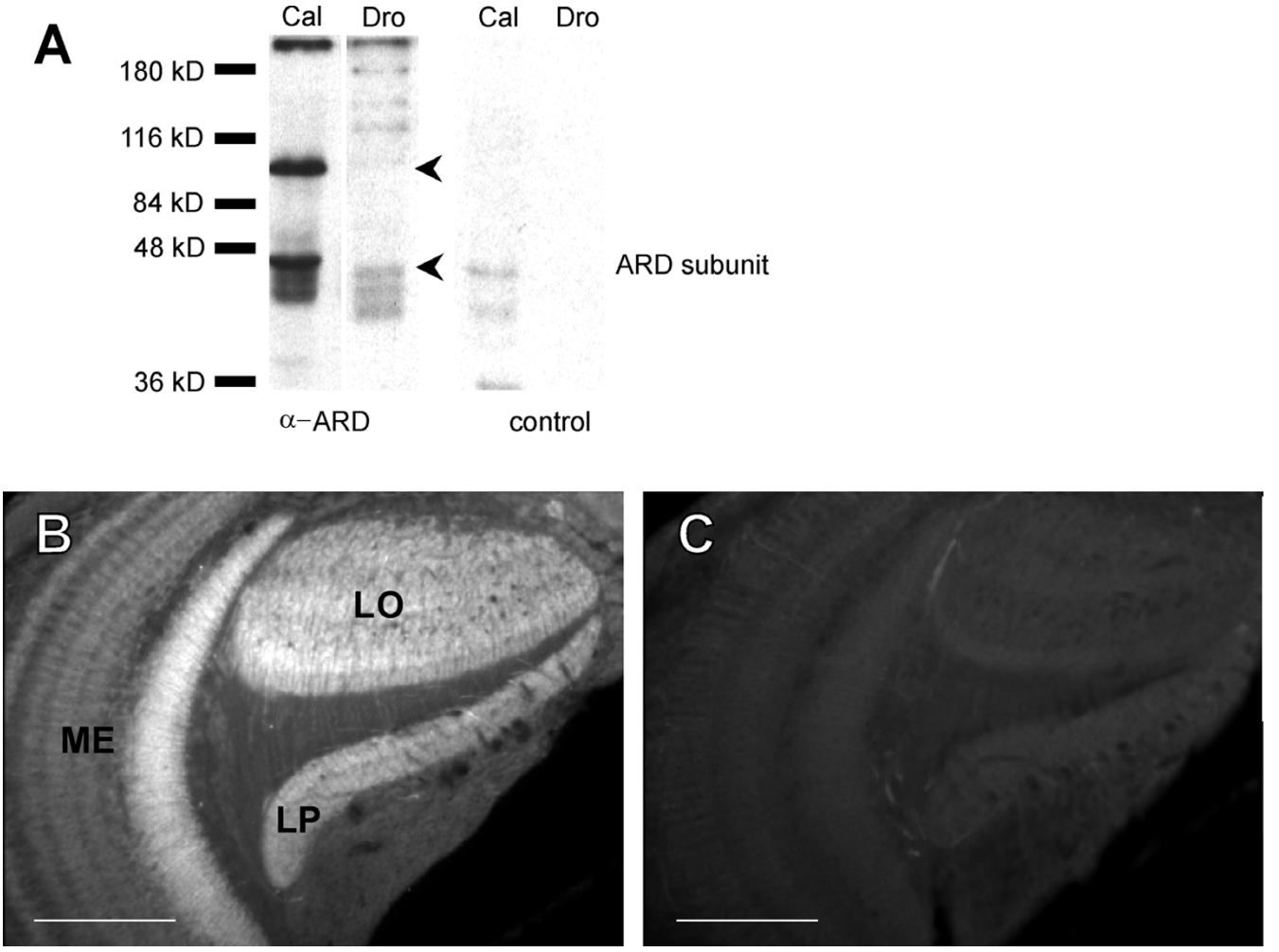

Figure 1
Specificity of the α-ARD antibody in *Calliphora* brain tissue. *A* Westernblot of *Calliphora* brain homogenates and *Drosophila* head homogenates with the α-ARD antibody mab 2D3. In both *Calliphora* and *Drosophila* multiple bands are observed between 42 and 50 kDa. In the *Calliphora* lane a band of twice the molecular weight of the ARD subunit is also visible. *B* Horizontal section of the visual system of *Calliphora* showing fluorescently labeled α-ARD immunoreativity. Immunoreactivity is almost exclusively restricted to the neuropilar regions of the medulla, lobula and lobula plate. The fibers of the inner chiasma, located between medulla, lobula and lobula plate, are immunonegative, as well as the neuronal cell bodies in the soma layer posterior to the lobula plate. *C* Control without the primary antibody in an adjacent section showing that the immunoreactivity is not caused by the secondary antibody. Bottom is posterior, left is lateral. ME: medulla; LO: lobula; LP: lobula plate. Scale bar: 100 μm.

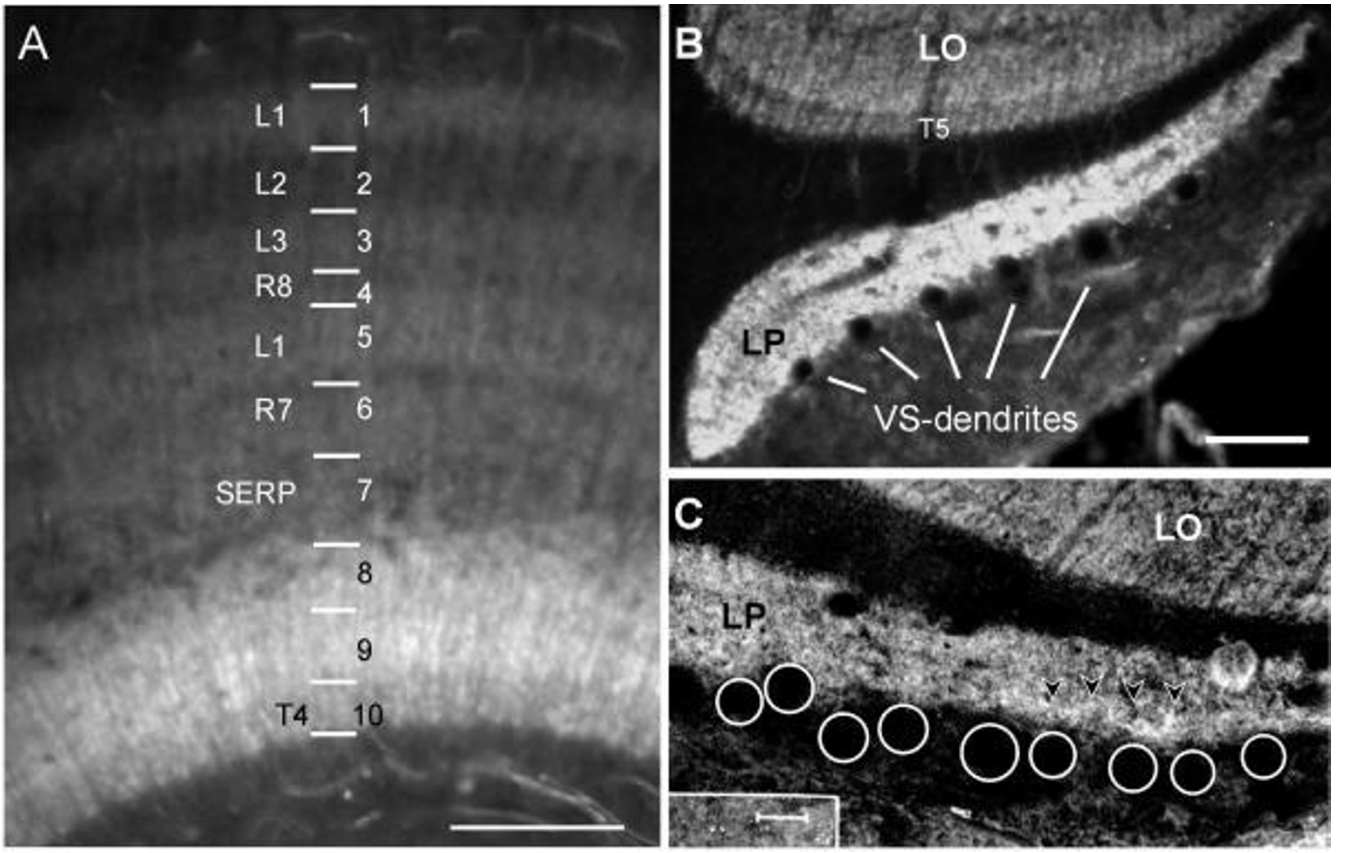

Figure 2
Detailed view of the distribution of α-ARD immunoreactivity in the visual system of *Calliphora*. *A* Layered organization of α-ARD immunoreactivity in the medulla. The medulla layers in this horizontal section are denoted according to the two terminologies used in blowflies and fruitflies. On the left side layers are labeled with Strausfeld's [1,34] terminology, which is based on the termination sites of laminar neurons (L1-L3) and photoreceptor terminals (R7,R8) in *Calliphora*. Labels on the right side reflect the terminology developed by Fischbach & Dittrich [2] for *Drosophila*. The inner medulla shows strong immunoreactivity with layer 9 appearing to have the highest density of nAChRs. In the outer medulla the layers with the terminal arborizations of L1 and L3 lamina monopolar cells are immunopositive. The layers with the terminals of L2 and R8 show lower levels of α-ARD immunoreactivity (bottom is medial, top is lateral). *B* Lobula and lobula plate. The posterior part of the lobula is divided in two α-ARD immunoreactive layers. The peripheral layer of the lobula contains the dendrites of the columnar T5 neurons, which project to the lobula plate and are likely input elements of the lobula plate tangential cells. The lobula plate contains high levels of α-ARD immunoreactivity throughout. In the distal portion of the lobula plate (left in frame) two distinct major layers are apparent, which correspond to those ones labeled in ^{3}H-Deoxyglucose studies [11] after stimulation with horizantal or vertical visual motion. A similar pattern in the lobula plate was also observed in a study with ChAT-immunohistochemistry [48]. Note, the large holes in the sections at the posterior face of the lobula plate mark the main dendrites of the VS-cells. These show no immunoreactivity on their surface, indicating that ARD-containing nAChRs are not present on the main dendrites of VS-cells. C High magnification view of the lobula plate. Immunoreactivity is labeled with DAB. For better comparison with panels A and B, this image was digitally inverted so that positive immunoreactivity is also represented by bright pixels. Note the intense immunoreactivity indicated by arrows close to the main dendrites of VS-cells (white circles). This might indicate a high density of nAChRs on the higher order dendrites of VS cells. Scale bar in A: 40 μm; in B: 50 μm; in C: 16 μm.

Both lobula and lobula plate showed high overall α-ARD immunoreactivity with a less pronounced layering than the medulla (Fig. 1b). The most posterior layer of the lobula, containing the T5 cell dendrites, was clearly distinguished from the rest of the lobula (Fig. 2b). T5 cells project to the lobula plate and are thought to be another type of input elements to LPTCs [8,14]. The lobula plate showed high levels of α-ARD immunoreactivity (Fig. 2b) whereas the fibers in the inner chiasma contained almost no immunoreactivity. The distal part of the lobula plate appeared to be divided into two immunoreactive layers which each might be further subdivided into two more sublayers (Fig 2b left side). These layers would correspond to the functional representation of preferred motion direction, with the LPTC responding to vertical motion in the two posterior layers and LPTCs sensitive to horizontal motion direction in the two anterior layers [11,13]. Several profiles of large LPTCs were visible in the

lobula late. At the posterior edge of the lobula plate, the main dendrites of the VS cells appeared as black and round cross-sectioned profiles in this horizontal section (Fig. 2b). These profiles were not surrounded by α-ARD immunoreactivity on their side facing away from the lobula plate, suggesting that the main dendrites have a lower density of nAChRs than the higher order branches of the dendrites deeper within the neuropile. Figure 2c gives a magnified view of the lobula plate with a clusters of dense α-ARD immunoreactivity (arrow heads) close to the VS cell dendrites. The massive presence of α-ARD immunoreactivity emphasizes the importance of nicotinic cholinergic neurotransmission in the fly visual system.

Distribution of RDL-immunoreactivity

The α-RDL-antibody is directed against the RDL-subunit of the *Drosophila* GABA receptor. In a previous study, this antibody proved useful for the demonstration of GABA receptors in the *Drosophila* central nervous system [27]. Cross-reactivity of the serum could also be shown with GABA receptors in the mushroom bodies of the blowfly, *Calliphora erythrocephala* [33]. In the present study, we were also able to identify significant α-RDL-immunoreactivity in the visual system of *Calliphora* (Figs. 3a,c) when compared with control sections (Figs. 3b,d; α-RDL antibody preabsorbed with fusion protein). α-RDL-immunoreactivity was present in a layered organization in the medulla and the posterior lobula (Fig. 3a). Labeling in the lobula plate was more evenly distributed. Compared to the α-ARD-immunoreactivity, the α-RDL- immunoreactivity was not restricted only to neuropilar regions, but could also be found in perikarya surrounding the neuropils (Figs 3a, asterisks) distal to the medulla, posterior to the lobula plate and in the inner chiasma. The location of the α-RDL-immunopositive somata in the inner (Fig. 3a) and outer chiasma (Fig. 3c, arrows) as well as the layered distribution of α-RDL-immunoreactivity in the medulla matches well with medulla amacrine cells [34], neurons that interconnect multiple columns in one layer.

The most prominently labeled layer in the medulla was L1. In this layer, several GABAergic tangential neurons are found (see below), as well as the presumably GABAergic centrifugal C2 neurons [35]. In addition to L1, there were two more strata weakly labeled in the outer medulla: a very thin portion of layer L3 and perhaps the proximal layer L1. The posterior layers of the lobula showed a similar pattern for α-RDL-immunoreactivity as they do for α-ARD-immunoreactivity (Fig. 3a,e), including the most posterior layer containing the dendritic ramifications of T5 neurons. The lobula plate also showed significant α-RDL-labeling, although distinct layers do not appear as clearly as with the α-ARD antibody. In summary, GABA receptors seem to be widely present in the *Calliphora* visual system.

GABAergic elements in the visual system

With the data presented on the distribution of GABA receptors in the visual system of *Calliphora*, we were interested in the corresponding distribution of GABAergic neurons. Although detailed accounts on GABA immunohistochemistry already exist in the literature [32,35,36,37], we wanted to make direct comparisons in our tissue material. Furthermore, some uncertainty remained in the studies cited above about the identity of columnar GABAergic elements, namely Y-cells [36], connecting the medulla with the lobula and lobula plate.

The monoclonal GABA antiserum, which we used, produced a staining pattern, that was very similar to the results published in previous studies on the visual system of house- and blowflies [35,36,37], indicating a specific binding of the antibody. In particular we confirmed the GABA-immunoreactive somata of putative C2 cells, fine GABA-positive profiles in the vicinity of VS-dendrites, and a bundle of large GABA-positive axons at the proximal border of the lobula plate which were interpreted before as HS axons [37].

GABAergic elements were found in all neuropils of the visual system. In the medulla several layers (distal and proximal L1, L3) contained GABA immunoreactive tangential neurons [36] (Fig. 4a). The arrowheads in figure 4a indicate profiles of such tangential neurons that are shown in figure 4b in a whole mount preparation of the distal medulla. The GABA immunoreactive somata in the inner chiasma probably correspond to the centrifugal C2 neurons. There were also a few GABA immunoreactive somata in the soma cluster posterior to the lobula plate. A cluster of GABA immunoreactive somata could be observed in the distal part of this area between the distal tip of the lobula plate and the medulla (data not shown).

The lobula plate contained a high density of fine GABA immunoreactive profiles, especially in the anterior part. The main dendrites of VS cells were surrounded and contacted by many of these GABA immunoreactive profiles (Fig. 4a).

The inner chiasma contained GABA immunoreactive fibers (Fig. 4a,c). The horizontal section of the inner chiasma in Fig. 4c shows the organization of fiber fascicles containing thick GABA immunoreactive fibers. Although observed before [37] the projection of these fascicles has not been described so far. There were two types of these columnar fiber fascicles. The first type - denoted here as type 1 - projects directly from the medulla into the lobula plate. The second type (type 2) of fascicles contained fib-

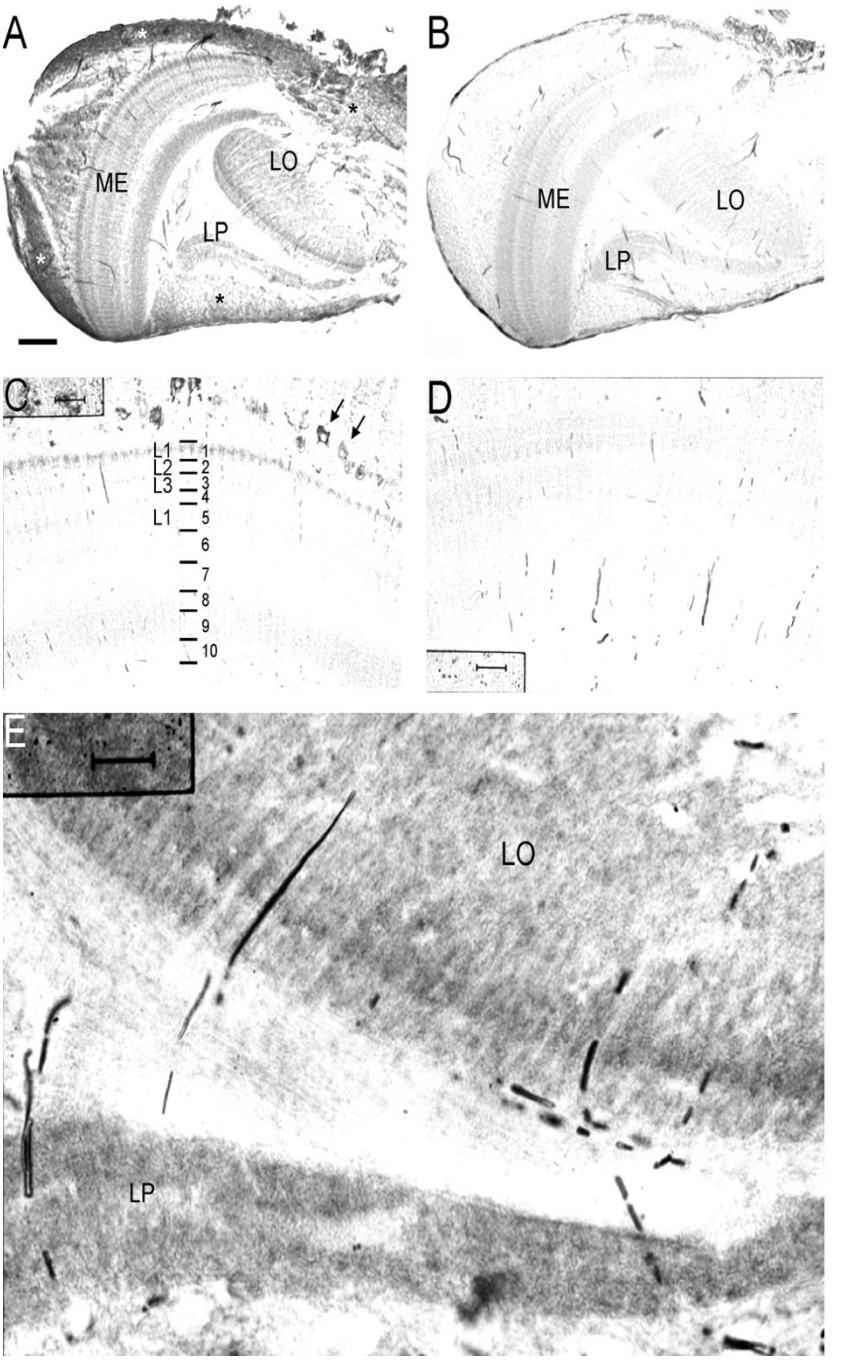

Figure 3

α-RDL immunoreactivity in the visual system of *Calliphora*. A Overview of α-RDL-immunoreactivity in a horizontal section. Besides immunopositive staining (dark labeling) in the neuropilar regions of the medulla, lobula and lobula plate, there is also significant immunoreactivity present in somata located distal to the medulla, inside the inner chiasma and in the soma layers anterior and posterior to the medulla and lobula complex (asterisks). *B* Control in an adjacent section incubated with preabsorbed primary antibody. *C* High magnification view of the medulla. The highest level of immunoreactivity is found in layer L1. There are also 2 more weakly labeled strata in the outer medulla (L3, proximal L1). The inner medulla also shows some α-RDL immunoreactivity. Somata with α-RDL immunoreactivity in the outer chiasma are indicated by arrows. *D* Control in an adjacent section with preabsorbed primary antibody. *E* High magnification view of lobula and lobula plate. The posterior lobula shows a similar pattern with two layers in its α-RDL immunoreactivity, as was also observed in the α-ARD labeling (see figure 2B). α-RDL immunoreactivity in the distal lobula plate also resembles α-ARD immunoreactivity with layered labeling. Dark stripes are artifactual labeling of trachea. Scale bar in A, B: 62.5 μm; in C, D 25 μm; E 16 μm.

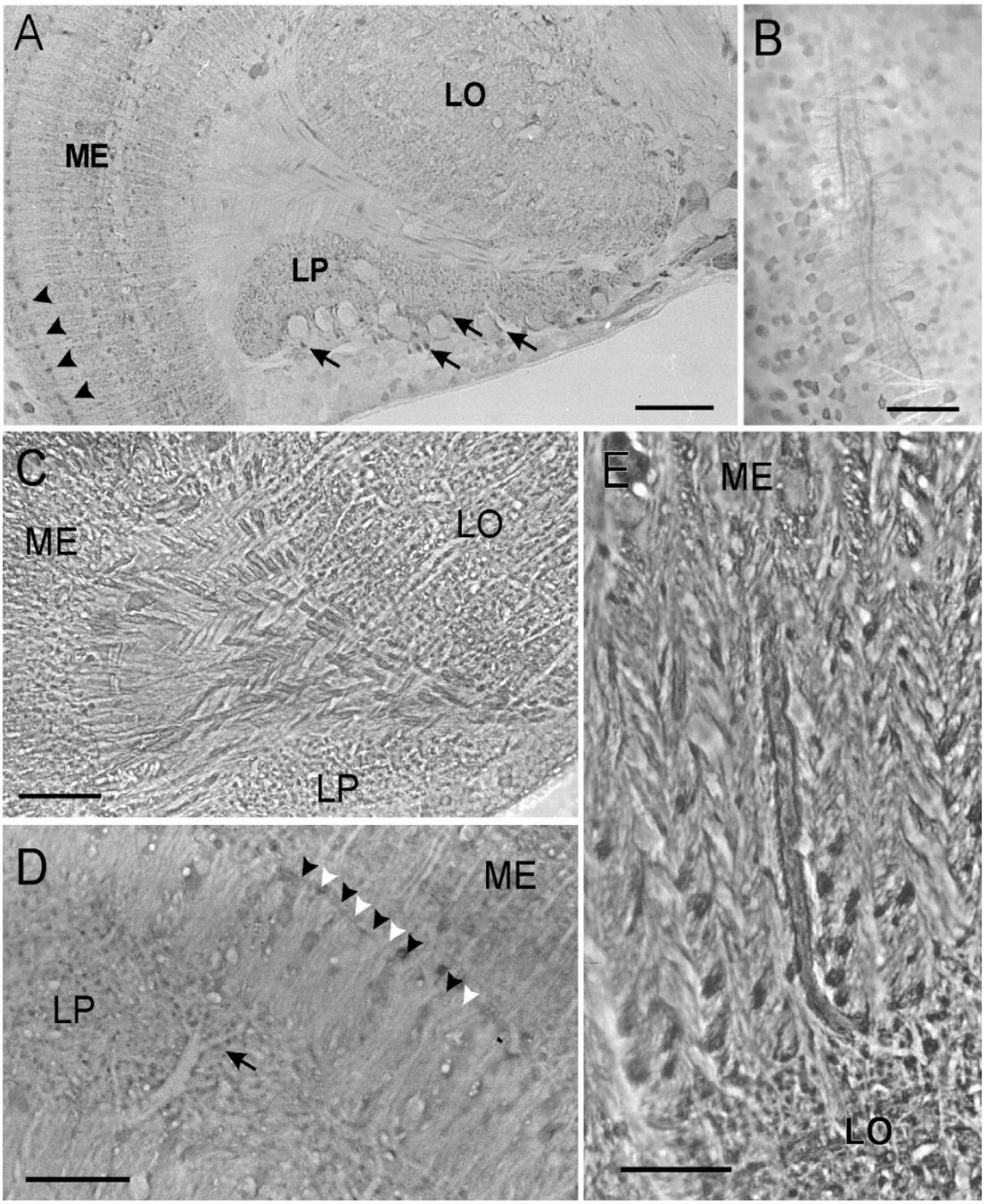

Figure 4
GABA-immunoreactive profiles in the *Calliphora* visual system. *A* Overview in a horizontal section. Note the GABA-like immunoreactivity around the cross-sectioned profiles of the of the VS-cells' main dendrites in the lobula plate (arrows). Arrowheads indicate cross-sections of GABA immunoreactive profiles from tangential neurons in the medulla, shown also in B. *B* Perikarya and tangential fibers in the medulla showing GABA-like immunoreactivity in a whole mount preparation (frontal view). *C* Detailed view of the inner chiasma in a horizontal section observed with phase contrast optics. *D* Detailed view of a frontal section of the inner chiasma between medulla and lobula plate. Fascicles with fibers projecting directly from the medulla to the lobula plate do not contain profiles with GABA-like immunoreactivity (white arrowheads). Fascicles running perpendicular to the plane of section contain thick GABA-immunoreactive profiles (black arrowheads). These fibers project mainly from the medulla to the lobula. In the lobula plate the profile of a LPTC (black arrow) is associated with GABA immunoreactive varicosities. E Thick profile projecting from the medulla to the lobula. The apparent bifurcation in the lower part of the fiber most likely originates from a different fiber pertaining to another fascicle. C, E: Phase contrast optics. Scale bar in A, B: 50 μm; in C, D: 30 μm; in E: 20 μm

ers of columnar neurons projecting from the medulla to the lobula. Figure 4d illustrates these two types of fascicles in a frontal section from the posterior part of the brain at the level of the lobula plate. Type 1 fascicles, which run parallel to the plane of section at this location, project from the posterior medulla to the distal lobula plate. Type 2 fascicles from the same part of the medulla run orthogonal to the plane of section to connect to the retinotopically corresponding part of the lobula and appear as cross-sectioned profiles. From this section it is clear that only type 2 fascicles contained GABA immunoreactive profiles. Thus, the pathway between medulla and lobula contained GABAergic elements, while the pathway between the medulla and the lobula plate most likely did not. Fig. 4e depicts a thick axonal element in type 2 fascicles almost in full length in the inner chiasma connecting the medulla and the lobula. According to Meyer et al. [36] these thick axon profiles presumably represent Y-cells. However, we were unable to positively identify any bifurcations in these axons projecting to the lobula plate. Although the fiber in figure 4c seemed to have a side branch, close inspection revealed that this branch originates from a fiber in a different fascicle. Figure 4d shows the large profile of a LPTC probably of an HS cell. As seen for the VS cell dendrites in figure 4a, this profile apparently had contacts to fine GABA immunoreactive blebs, too.

Discussion

In the present study we have assessed the distribution of nicotinic acetylcholine receptors, GABA receptors and GABAergic neurons in the visual system of the blowfly *Calliphora erythrocephala*. We will now compare these distribution patterns with other immunocytochemical studies using antibodies directed against similar or different targets, with ^{3}H-deoxyglucose labeling results and with the known anatomy of columnar neurons. We will finally discuss our results with respect to their relevance for our understanding of the cellular basis of visual motion processing in the fly.

Distribution of nicotinic receptors

Our staining pattern for nicotinic acetylcholine receptors as demonstrated with the α-ARD antibody resembles very closely the pattern observed originally in *Drosophila* [25]. Before the availability of antibodies against nicotinic acetylcholine receptors of dipteran flies, cholinergic pathways have been analyzed by more indirect methods, mainly by immunocytochemistry against choline acetyltransferase (ChAT), an enzyme of the acetylcholine metabolism [38,39] or autoradiography of ^{125}I-α-bungarotoxin binding [40,41]. The ARD subunit was shown to be associated with a distinct class of α-bungarotoxin binding sites in the *Drosophila* CNS [42]. ChAT-positive neurons would act complementarily as input elements of α-ARD immunoreactive elements. However, because of the wide abundance of acetylcholine in the insect CNS, it is likely that ChAT-positive neurons also possess nAChRs. In fact, the distribution patterns of ChAT in the lobula plate, the lobula and the inner medulla are virtually identical with the pattern we observed with the α-ARD antibody. In the outer medulla most layers match for the pattern of immunoreactivity to ChAT and ARD with exception of the terminal layer of L2 lamina monopolar cells, which shows no α-ARD immunoreactivity but is reported to be ChAT immunopositive in *Drosophila* [38]. In accordance with the ARD data there is also no labeling with ^{125}I-α-bungarotoxin in the L2 layer of the *Drosophila* medulla [40]. It should be noted, however, that layer 10 in the medulla with the dendrites of T4 neurons and the posterior layer of the lobula with the dendrites of T5 neurons are slightly weaker in their α-ARD immunoreactivity than their adjacent neuropil layers. In contrast, the ChAT-labeling in these two layers is much more intensive than that of the adjacent layers [38].

The dense α-ARD labeling in the lobula plate did not allow the mapping of nAChRs to individual LPTCs. However, the concentration of α-ARD immunoreactivity in the region of the VS cell dendrites at the posterior border of the lobula plate is in accordance with our earlier findings of nicotinic receptor responses in these neurons [23].

Distribution of GABA receptors and GABAergic neurons

In our study, the strongest GABA receptor immunoreactivity is in layer 1 (L1) of the medulla. The most likely columnar GABAergic element in this layer is the C2 cell type [36,37]. The C2 cells have a presumably centrifugal projection back to the lamina and also arborizations in the medulla layers 5 (L1), 8 and 10 (T4). We found α-RDL immunoreactivity in all these layers, however, to a lesser extent than in layer 1. Another possible source of GABA in the outer medulla are cells that form horizontal connections between columns: GABA positive tangential neurons, which are also found in layer 1, and amacrine cells, which spread multiple columns. The location of the cell bodies of deep medulla amacrine cells matches with the GABA immunoreactive somata in the inner chiasma. The stratification patterns of these amacrine cells in turn matches with the α-RDL immunoreactivity pattern in the medulla. GABAergic medulla tangential and amacrine cells are of special interest for considerations of cross-column inhibitory interactions in the processing of visual motion information (see below).

As outlined earlier, lobula plate tangential cells (LPTCs) respond to cholinergic and GABAergic stimulation. Our work and that of others show the presence of GABAergic profiles in the direct vicinity of LPTC dendrites. Howev-

er, the identity of the columnar GABAergic cell types providing GABAergic input to LPTCs remains still elusive. So far, Y-cells were assumed to be the most likely columnar GABAergic cell type [36,37]. These statements, however, have to be evaluated with some caution because neither of these references presents direct evidence for GABAergic Y-cells. In *Drosophila*, Y-cells are characterized by thick fibers [2], which is in accordance with our observations of GABA immunoreactive fibers in the inner chiasma. However, we were not able to identify any bifurcated GABA immunoreactive fibers, as one would expect in the case of Y-cells. T4 cells, which are direct columnar input elements to some LPTCs [14], are unlikely to be GABAergic because the fascicles connecting directly between medulla and lobula plate did not contain any GABA immunoreactive fibers. The other columnar cell type that is most likely to provide input to LPTCs are T5 cells originating in the lobula [14]. From our results we cannot eliminate T5 cells as candidates of GABAergic inputs to LPTCs. The Y-cells though also remain likely candidates.

Possible role of GABA in motion detection

In the original model of motion detection, direction selectivity was proposed to arise in several consecutive processing stages [43,44]: In the first step, the retinal luminance levels of adjacent photoreceptors interact with each other in a nonlinear way after one of them is temporally low-pass filtered. This process is repeated in mirror symmetrical fashion. In a second step, the output signals of both operations become subtracted from each other. Finally, the outputs from all these local units are spatially pooled to result in a directionally selective wide field response. With respect to our present picture of the cellular implementation, the second and third processing step is thought to be realized on the dendrites of the lobula plate tangential cells, while the first step of motion detection, i.e. the nonlinear interaction, probably takes place in small columnar neurons of the medulla and/or lobula.

The presence of GABA receptors in layers of the medulla that take part in motion detection argue for a role of GABAergic transmission in the input pathway of lobula plate tangential cells. Therefore, GABA seems not only to play a role in the subtraction stage of the motion detection process [22,23] but is probably also involved in the nonlinear interaction between neighboring retinal locations. In model simulations, such a nonlinearity was accomplished by e.g. a multiplication [21] or a logical AND NOT gate [45]. As has been shown such a computational task could be implemented at the cellular level by shunting inhibition [46].

Comparison of immunostaining with activity-labeling studies in Drososphila

Our understanding of motion processing on the cellular level in the medulla is, compared to the lobula plate, very incomplete. In particular the location and implementation of the nonlinear multiplicative interaction between the input channels remains elusive as outlined above. Few attempts have been made to resolve this problem with intracellular recordings from medulla neurons [6,7,8,9]. Another approach that has been taken was to label the activity in neuropils by means of ^{3}H-deoxyglucose [11]. Visual motion stimuli, i.e. rotating bars around the fly, lead to a characteristic labeling pattern in the medulla. Under these conditions, layers 1 (L1), 2 (L2) and 5 (L1) showed increased ^{3}H-deoxyglucose uptake. Layers 9 and 10 of the proximal medulla also were radioactively labeled [13]. This demonstrates the participation of these layers in the transmission of relevant information for the processing of visual motion. Comparing this to our results from the cholinergic and GABAergic receptor immunocytochemistry, the L1 pathway (layers 1 and 5) is the most likely to carry the relevant input signal for the non-linear interaction through the medulla.

Conclusions

Putative cellular constituents of the fly motion pathway

In earlier studies two main candidates were proposed to constitute the elementary motion detector of the fly visual system: the T4- and the T5 cells, both types also called the 'bushy T-cells' [2,14,15]. The reason why these cells are so suggestive candidates is that i) both of these cells come in 4 different subtypes, each of which ramifies in a different layer of the lobula plate [2], ii) ^{3}H-deoxyglucose measurements using 4 cardinal directions of motion (up, down, left, right) revealed a specific staining pattern of exactly these 4 layers in the lobula plate [11]. Furthermore, LPTCs occupy with their large dendrites preferentially those layers in the lobula plate, which correspond to these different directions of motion [16]. In one case, a direct synaptic contact has been demonstrated at the EM level between a T4-cell and the dendrite of an HS-cell [14]. Taken the available immunocytochemical evidence presented in this paper together with what is known about the anatomy of columnar elements and the physiology of LPTCs, the following picture about the cellular implementation of motion detection in the fly visual system can be drawn in the most parsimonious way: The first major step of motion detection, i.e. the non-linear interaction between input channels, is realized on the dendrites of T4-cells and the input elements of T5 cells in the proximal layer of the medulla by a combined cholinergic-GABAergic mechanism. This results in weakly directional signals for each of the four cardinal directions of motion split into a cholinergic pathway, providing direct excitatory input onto the LPTC den-

drites through T4-cells, and an indirect GABAergic pathway, relayed through the posterior layer of the lobula via T5-cells, providing inhibitory input to LPTC dendrites. Thus, the second step in the computation of direction selectivity, i.e. the subtraction, is mediated by the opponent interaction between T4- and T5 cell input on the dendrites of each LPTC. Finally, these signals become integrated by the LPTC dendrite. At present, this proposal, is highly speculative, but may proof useful to be challenged in the future by electrophysiological or optical recordings from several of the putative constituents of the motion detection circuit.

Material and Methods

In this study we used 2 to 5 day old female blowflies, *Calliphora erythrocephala,* and for control purposes fruitflies, *Drosophila melanogaster* (Berlin WT), both from the laboratory stock of the Max-Planck-Institute for Biological Cybernetics, Tübingen, Germany. If not stated otherwise all chemicals were obtained from Sigma, Deisenhofen, Germany.

Antibodies

We used several different antibodies to map the distribution of nAChRs, GABA receptors and GABAergic neurons respectively. The monoclonal α-ARD antibody mab 3D2 was raised against a structural subunit of the nAChR from *Drosophila* [26]. In the *Drosophila* nervous system mab 3D2 produces exactly the same staining pattern as that observed with polyclonal antisera against the ARD nAChR subunit [25]. We also used the monoclonal antibody α-ALS (mab D4) and a polyclonal serum α-Dα2, both directed against α-like ligand-binding subunits of the *Drosophila* nAChR [25,28]. The polyclonal α-RDL antibody is directed against a *Drosophila* GABA receptor subunit [27]. To detect GABAergic neurons we used a commercially available monoclonal antibody against GABA from Affinity Research Products Ltd. (#GA 1160, Exeter, UK).

Western blot

Western blotting was used to demonstrate cross-reactivity of the α-ARD antibody with *Calliphora* nAChR subunits. For the preparation of membrane proteins, brains from *Calliphora erythrocephala* and heads of *Drosophila melanogaster* were used. About 30-35 *Calliphora* brains were dissected under ice-cold saline and immediately frozen with liquid nitrogen. Similarly 20 *Drosophila* heads were collected under ice-cold saline and frozen in liquid nitrogen. 5 µl of 2x Laemmli buffer with 8 M Urea [47] was added per brain or head, respectively. Brains and heads were homogenized 3 × 20 sec with a sonicator (Branson). Tubes were cooled on ice for 20 s between homogenizations. The homogenate then was boiled for 5 min at 100°C and debris was removed by centrifugation for 10 min at 15000 rpm. Supernatant was divided into aliquots of 20 µl and stored at -70°C until further processing. 10% SDS-PAGE gels were prepared with a minislab gel system (LBK Midget Gelsystem). Up to 10 µl of membrane protein preparation per lane were loaded onto the gel. After SDS-PAGED, proteins were blotted onto nitrocellulose membrane (PVDF Immobilon P Ø 0.45 µm, Millipore). Immunglobulin binding sites were blocked by 2-3 h incubation in PBS with 10% dried milk powder. Primary ARD antibody mab 3D2 was diluted in PBS to a final concentration of 1:200 to 1:1000. The membrane was incubated overnight at 4°C on a shaker, washed 4 × 15 minutes with PBS at room temperature (RT), incubated with a horseradish peroxidase-coupled anti-mouse antibody (1:5000, Dianova, Hamburg) for 3 h at and then washed again 4 × 15 minutes with PBS at RT. Antibody binding was visualized by enhanced chemiluminescence (ECL, Amersham) according to manufacturers directions.

Immunocytochemistry

Antibody stainings were performed on cryostat sections of *Calliphora erythrocephala*. *Drosophila melanogaster* (Berlin WT) was used as a positive control for immunoreactivity of the mab 3D2 α-ARD antibody. *Calliphora* brains were removed from the head capsule under ice-cold standard fly saline [24] and fixed immediately in FAA (Formaldehyd 3,7%, ethanol 50%, acetic acid 5%) for 30 min. Brains were rehydrated through a descending alcohol chain (50%, 40%, 30%, 20%, 10%, 5%; 10 min each) and then transferred to fly saline containing 25% sucrose for cryoprotection overnight. For GABA immunocytochemistry *Calliphora* brains were fixed for 3-4 h in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, 4°C). *Drosophila* heads were harvested and prepared according to a slightly modified protocol described in Buchner et al. [38]. Briefly, flies were attached with head and thorax to a small plastic stick and submerged into ice-cold *Drosophila* saline. Proboscis and ventral air sacs were removed to allow quick access of the fixative. Heads were put into 4% paraformaldehyd for 3-4 h for fixation. Subsequently, heads were washed in *Drosophila* saline with 25% sucrose and left there overnight after changing the saline once.

15-23 µm thick sections of *Calliphora* brains and *Drosophila* heads were cut on a cryostat microtome (2800 Frigocut, Reichert-Jung, Nuβloch). Sections were allowed to warm up to RT and washed for 30 min in PBS + 0.1% Triton X100. Sections were blocked depending on the secondary antibody with either 4% bovine serum albumine (BSA), or horse serum (Vectastain Elite Kit, Vector) for 2 h at RT to prevent unspecific binding. The primary antibody was diluted in PBS + 0,1% Triton or optionally in addition with the previously used serum

(dilutions: α-ARD, α-ALS, α-Dα2 1:10-1:100; α-RDL 1:200; α-GABA 1:500). Sections were incubated overnight at 4°C. Immunoreactivity of the primary antibody was then labeled either with the chromogen diamino benzidine (DAKO Diagnostika, Hamburg) or with a fluorophore-coupled secondary antibody.

For labeling of immunoreactivity with the diaminobenzidine (DAB), the Vectastain Elite ABC-Kit (Vector Laboratories, Burlingame, CA) was used according to the suggested protocol. Sections incubated with the primary antibody were allowed to warm up to RT for 45 min and then were washed 2 × 15 min with PBS + 0.1% Triton. Sections were incubated with the secondary antibody (biotinylated horse anti-mouse IgG) for 1 - $1^1/_2$ h at 37°C. After washing 2 × 15 min with PBS + 0.1% Triton, slides were treated with Vectastain Elite reagent (avidin-peroxidase-complex) for 1 - $1^1/_2$ h at 37°C. Again sections were washed twice for 15 min before they were preincubated with DAB for 1 h at 4°C. DAB-solution was exchanged before reacting the section with diamino-benzidine (30% DAB in 5 ml PBS plus 2 µl 30% H_2O_2) under visual control for 5-10 min. Reaction was stopped by several changes of PBS and sections were washed with water. Alternatively, immunoreactivity of nAChR was labeled with a N,N'-biscarboxypentyl-5,5'-disulfonatoindodicarbocyanine (Cy5) coupled fluorescent secondary antibody. Sections for fluorescent labeling also were allowed to warm up to RT for approximately 1 h and washed twice for 15 min with PBS + 0.1% Triton. Sections were incubated for 2 h at RT in the dark with donkey anti-mouse Cy5 secondary antibody (1:10 in PBS + 0.1%Triton, Jackson Immunochemicals). After washing 2 × 15 min sections were embedded in Mowiol (Hoechst, Frankfurt) with DABCO (1,4-diazobicyclo-2,2,2-octan, Merck) to prevent photobleaching.

DAB-stained sections were photographed with Kodak Ektachrome 64T film and digitized using a Nikon Coolscan slidescanner. Cy5-labeled specimen were imaged with a cooled CCD-camera (CH-250, Photometrics) and acquired into a Macintosh AV computer with IPLab software (Signal Analytics, Vienna, VA). Digitized images were adjusted for brightness and contrast in Corel Photopaint v. 4.0 (Corel Corp.). Images of control sections were processed in the same way as images of the corresponding immunostained sections.

Acknowledgment

We thank H.-E. Stöffler and A. Bergman for help with the Western blots. We are grateful to the Max-Planck-Institute for Biological Cybernetics for the supply of flies and the use of the Cryostat microtome, to Kate Aronstein and Richard ffrench Constant for the RDL antibody, and to Birgit Bochenek for excellent technical assistance. We also thank Thomas Oertner and Mike Defreitas for critically reading earlier versions of the manuscript.

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