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Histochemical demonstration of GABA-like immunoreactivity in cobalt labeled neuron individuals in the insect olfactory pathway

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Summary. Individual olfactory neurons in the antennal lobes of *Periplaneta americana* were investigated for their morphological and neurochemical properties by means of intracellular cobalt injection and indirect peroxidase-antiperoxidase immunohistochemistry. GABA-like immunoreactivity was demonstrated in many local interneurons but not in uniglomerular projection neurons.

Introduction

Combined recording of responses and subsequent dye injection of individual neurons has proven a powerful tool for the analysis of neuronal networks especially in invertebrates such as insects (Hoyle 1977). Such methods have even revealed fine structural details and synaptic contacts of identified neurons (Watson and Burrows 1981; Peters et al. 1986). Further progress has been achieved when intracellular labeling was combined with histochemical tests for certain transmitters in the identified neurons (Reaves and Hayword 1979). This approach was applied also in the olfactory pathway of the sphinx moth *Manduca sexta,* where a fluorescent dye, Lucifer yellow (LY; Stewart 1978) was used to label individual neurons, and subsequently fluorescent transmitter-specific rhodamine-conjugated secondary antibodies were applied (Hoskins et al. 1986). However, the application of fluorescent markers met difficulties in the search for GABA-like immunoreactivity (GLIR) in deutocerebral neurons of *Periplaneta americana.* Firstly, and most important, LY seemed to interfere with the immunohistochemical test for GLIR. Secondly, even with highly selective monochromatic filters it was difficult to detect significant differences between light emitted from the LY-stain on the one hand, and from the rhodamine label (TRITC, Dako Co.) of the secondary antibody on the other hand (cf. discussion). In order to avoid such difficulties, cobaltous ions were used as markers for a neuroanatomical identification of neurons in the cockroach deutocerebrum (cf. Ernst and Boeckh 1983). GLIR was demonstrated by immunohistochemical staining according to the indirect peroxidase-antiperoxidase method (Sternberger 1979). This method was used in the present study for a neurochemical characterization of identified multiglomerular interneurons as well as uniglomerular projection neurons in the deutocerebrum of *Periplaneta americana* which have been thoroughly investigated for their morphology and function by previous investigation (for summary see Boeckh et al. 1984; Boeckh and Ernst 1987; cf. Figs. 1 and 2).

Materials and methods

Recording and dye injection. Adult male *Periplaneta americana* from the institute's colony were prepared for single cell injection in the deutocerebrum accorcing to standard methods (Waldow 1975; Burrows et al. 1982). Microelectrodes were filled with $Co(NH_3)_6CL_3$ sat. aeq. sol. Resistances were from 50–200 MΩ. They were introduced into the ventral-anterior soma group of the exposed deutocerebrum where local interneurons are located as well as glomerular projection neurons (cf. Ernst and Boeckh 1983). In order to avoid damage of the electrode tips during penetration of the tough brain sheath, the latter was treated with a few granules of pronase dissolved in Pringle's saline (ca. 0.5% w/v) for ca. one minute (cf. Burrows et al. 1982). Merely to test whether the neurons were olfactory, a few odorants (female pheromone, aromas of foodstuffs) were blown to the ipsilateral antenna, and the responses recorded previous to the injection (cf. Sass 1976; Waldow 1975). The dye was ejected by DC of 2–5 nA for 5–10 min under continuous control of the impulse activity. Then the cobalt was allowed to diffuse for further 30–45 min without current before the brain was excised, and the cobalt precipitated in 2% $(NH_4)_2S$ in Pringle's saline. The brains were then washed in saline, and fixed for 1–2 h in a mixture of picric acid and glutaraldehyde according to Boer et al. (1980). After dehydration in ethanol of increasing concentrations, the brains were cleared in toluene, and infiltrated with paraplast at 59° C. Serial sections of 15 µm were cut, and dried on slides coated with egg albumine/glycerine mixture at 45° C.

Immunohistochemistry. For staining according to the indirect "peroxidase-antiperoxidase" (PAP) method (Sternberger 1979), the wax was removed, and the sections transferred into distilled water via a decreasing concentration series of ethanol. After transfer into TBS (0.3 *M* NaCl-0.1 *M Tris* HCL buffer pH 7.4, Hoskins et al. 1986), the sections were inspected for presence of *visible* cobalt sulfide precipitate, which would mask the diaminobenzidine (DAB) precipitate necessary for the detection of GLIR in the photographic document. However, sufficient submicroscopic cobalt nuclei had to be present for the subsequent silver intensification to reveal the neuron's shape and position after the localization of GLIR. In order to reduce nonspecific deposit of IgG at potential charge carriers, the sections were incubated for 60 min in TBS containing 0,5% Triton-X-100 and 10% fetal calf serum (FCS, Biochrom). The TBS-buffer, used for rinsing the sections and dilution of antisera contained 0,5% Triton-X-100 and 1% FCS. Three GABA antisera were used. Two GABA antisera against GABA conjugates to bovine serum albumin (BSA): *1.* prepared and characterised (Hoskins et al. 1986) by Dr. Kingan (Columbia University, NY); *2.* prepared and characterised (pers. communication) by Dr. Wulle

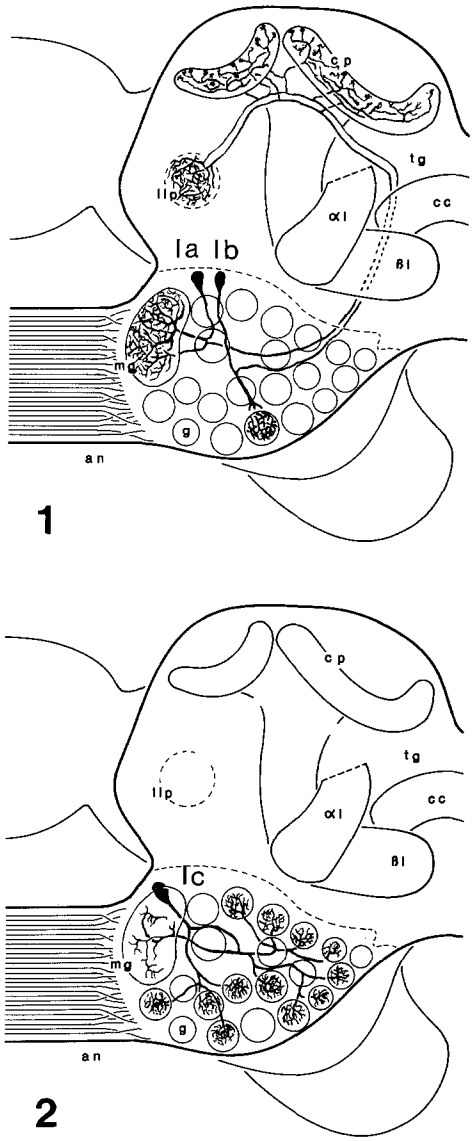
and Dr. Schnitzer (Max-Planck-Institute for Brain Research, Frankfurt). One GABA antiserum against GABA conjugates to keyhole-limpet hemocyanin (KLH), prepared and characterised (Hoskins et al. 1986) by Dr. Kingan. In control experiments all antisera labeled the same proto- and deutocerebral neurons. Under identical conditions and with optimal dilution of the specific primary GABA antiserum, labeling with antiserum against GABA-KLH conjugates was more intensive than with the antisera against GABA-BSA conjugates. Unspecific background staining was low with all three antisera.

Specificity of primary GABA antisera was controlled by the following procedures: *1.* Overnight preincubation at 8° C of the primary GABA antiserum with the corresponding antigen and in parallel with L-glutamic acid-, β-alanine- and L-glutamine conjugates (Hoskins et al. 1986). Preincubation with GABA-BSA conjugate prevented immunohistochemical staining. *2.* Immunohistochemical staining was not successful if primary GABA antiserum was omitted and sections were exposed to normal rabbit serum. The GABA-BSA antiserum was used at a dilution of 1:1000, GABA-KLH antiserum at 1:8000. Subsequently the sections were rinsed in TBS for 1 h and exposed to a swine antirabbit IgG diluted 1:40 (Dako Co.) for 1 h at room temperature. After rinsing again in TBS for 1 h, the sections were incubated in the PAP-complex (Dako, diluted 1:100) at room temperature for 1 h. They were then washed in TBS (containing 0,5% Triton-X-100) and immersed in 3,3′-diaminobenzidine (Sigma, 0,2 mg/ml) and hydrogen peroxide (0,015%) (Hoskins et al. 1986). Finally the sections were rinsed in distilled water and mounted in a mixture (9:1) of glycerine/phosphate buffer (pH 7,4). At this stage, the sections were photographed with a Zeiss photomicroscope at low magnification (20×) in order to localize GLIR in individual neurons with a minimum of marking by cobalt precipitate.

Silver intensification. After carefully removing the cover slides, sections were incubated in distilled water for 20 min to remove excess embedding medium and exposed to the preincubation solution of the intensification process (Tyrer et al. 1980) for 1 h at 50° C and then exposed to intensification solution at 50° C. Precipitation of silver was controlled periodically in the microscope, and the reaction stopped at the proper stage. After washing, the sections were incubated in 5% sodium thiosulfate for 5 min, dehydrated and mounted in DePeX. Then the deutocerebral area with the cobalt-silver-labeled neuron was photographed and eventually the neuron identified as a certain type of local interneuron or projection neuron. The comparison with the exposures taken after the GLIR test but previous to the intensification process permits a reliable test for presence or absence of GLIR in the labeled neuron.

Results

GABA-like immunoreactivity (GLIR) was present in several neuropilar regions of the brain including the deutocerebral glomeruli and also some larger tracts and bundles like the marginal zones of the tractus olfactorio-globularis (TOG). Certain groups or individual somata appeared distinctly stained. Investigation for presence or absence of GLIR in cobalt labeled and morphologically identified neurons was restricted to cells which belong to the two well defined types of either the multiglomerular local interneurons (INs) or uniglomerular projection neurons (PNs, cf. Ernst and Boeckh 1983; Figs. 1 and 2), and which had responded to odor stimulation in the preceding electrophysiological test. The 19 investigated INs had their somata within the deeper (dorsal) portion of the prominent anterior and ventral group of somata. A subgroup of these, the Y-INs, give rise to the characteristic Y-shaped tract within the deep glomerular deutocerebrum (Fig. 3). Each such neuron innervates many glomeruli but does not send a process



Figs. 1 and 2. Diagrams of right half of brain in fronto-horizontal section (after Ernst and Boeckh 1983)

Fig. 1. Camera-lucida drawings of two output neurons (*Ia; Ib*), each of which innervates one glomerulus, the macroglomerulus (*mg*) in case of *Ia*. Their axons run in olfactorio-globular tract (*tg*) to calyces of corpora pedunculata (*cp*), where they branch abundantly. Collaterals reach lateral lobe of protocerebrum (*llp*); an antennal nerve; α1, β1 α and β lobes of corpus pedunculatum; *cc* central body; *g* glomerulus

Fig. 2. Camera-lucida drawing of local interneuron (*Ic*) innervating many glomeruli, with no axon; an antennal nerve; α1, β1, α and β lobes of corpus pedunculatum; *cc* central body

into regions outside the deutocerebrum (Fig. 4c). The somata of 13 out of 19 cobalt labeled INs clearly exhibited GLIR (Fig. 4a and b), while the rest remained unstained before the intensification. These latter neurons were found impregnated to a considerable degree after their intensification, and thus might contain a larger amount of cobalt (cf. discussion).

23 injected neurons were identified as PNs. Their somata are found in either a more medial and superficial (i.e. ventral) group or in a more lateral group in the deutocerebrum (Fig. 5a and b). Each such neuron sends a process

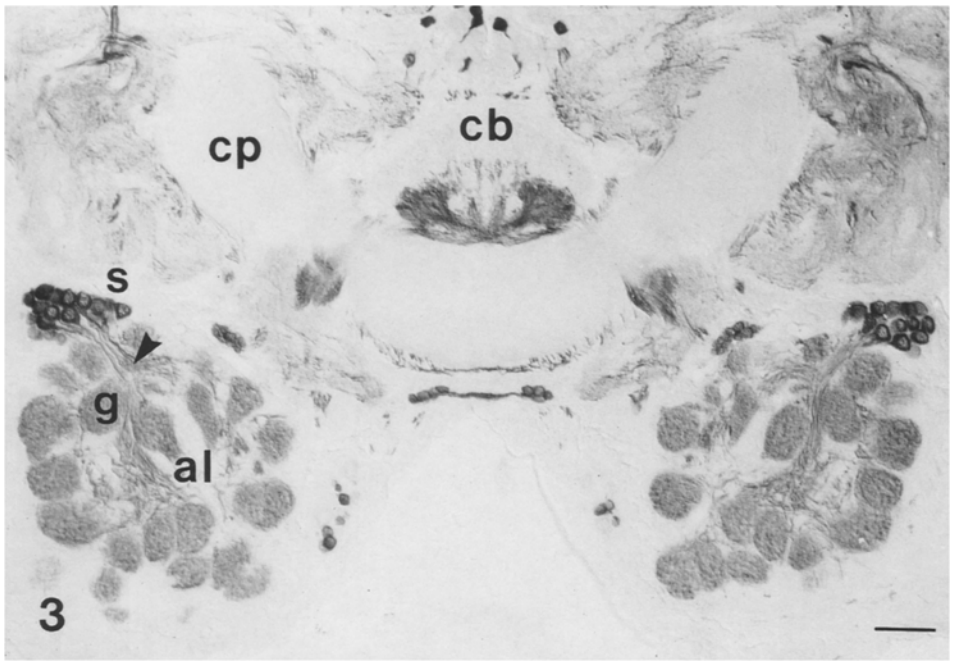


Fig. 3. GLIR in the brain of *Periplaneta americana*. Fronto-horizontal section through a male brain. GLIR staining is dense in the glomeruli (*g*) of the antennal lobe (*al*). Many of the somata of the ventro-lateral group of somata (*s*) show GLIR. Their processes run together into the deutocerebral neuropil, forming characteristic tracts like the "y-shaped tract" (*arrowhead*). *cb*, central body; *cp*, corpora pedunculata. Scale *bar* = 100 μm

to only one glomerulus, and an axon into the medial branch of the TOG (Fig. 5b and c), which connects the deuto- to the protocerebrum (cf. Ernst and Boeckh 1983). None of these injected neurons showed any sign of GLIR in the soma (Fig. 5a and b), and most somata of this region had been left free of PAP-reaction product in the non-injected preparations.

GLIR was found in somata of other areas of the deutocerebrum (Fig. 3), but there is no further information about structure and function of these neurons and they were not studied in more detail. Marginal portions of the TOG contained GLIR while the major and inner portion of the tract remained free of reaction product (Fig. 5c).

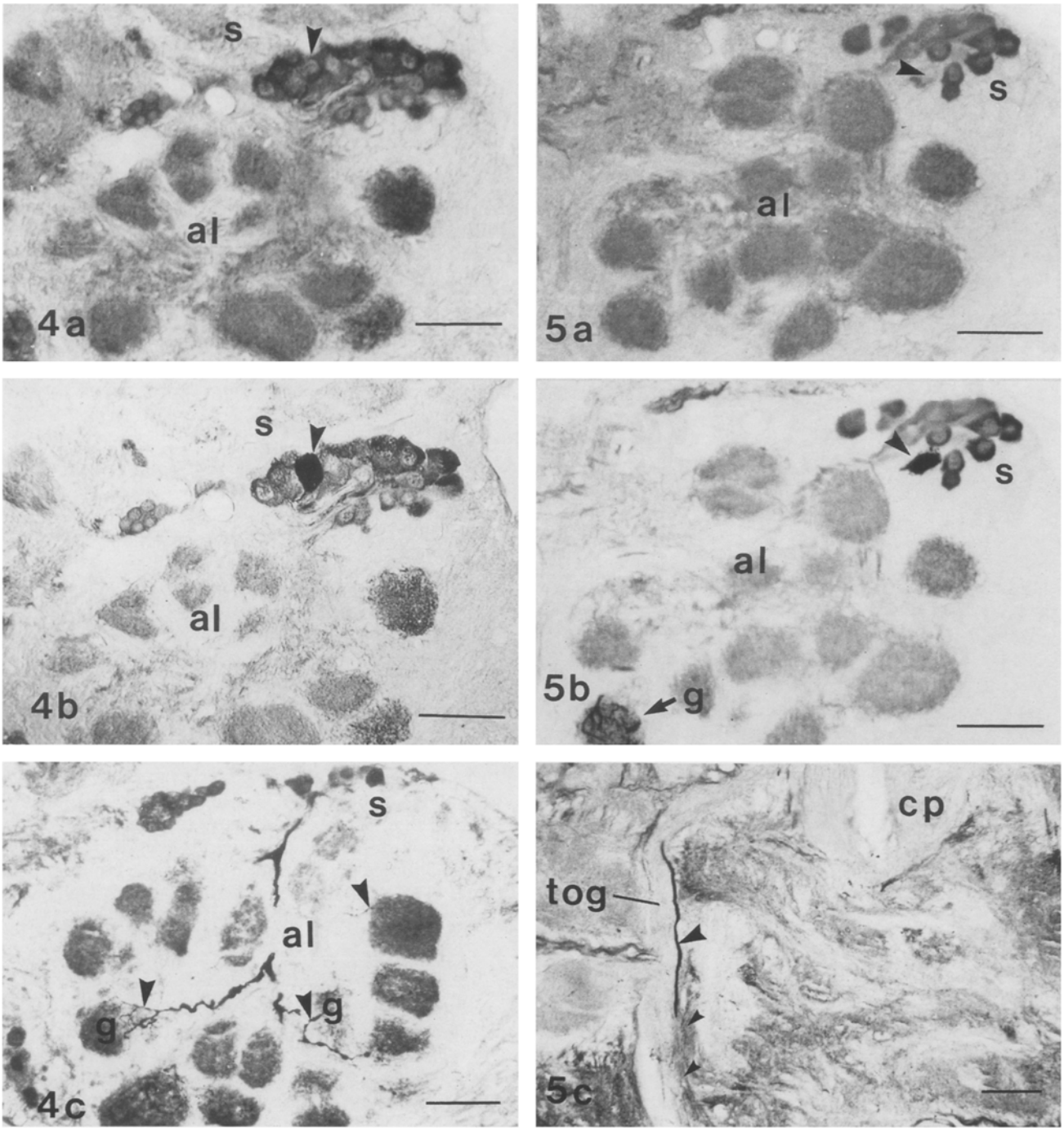
Discussion

Methods

The combination of cobalt injection and immunohistochemical test for GLIR was primarily used because of the difficulties which were met with the application of Lucifer yellow (LY) as an intracellular marker for the morphological identification of neurons. This became apparent when LY injected members of the Y-IN soma group (Fig. 3) failed to display GLIR while in the non-injected preparations they had shown immunoreactivity. Whether this was due to a block of the binding of the antibody or whether other steps of the GLIR test became impeded is not clear. In combination with LY, application of the rhodamine labeled secondary antibody met additional difficulties. LY emitted enough light at wavelenghts in the neighbourhood of the rhodamine spectrum to prevent a clear discrimination when a narrow band pass filter was used for the excitatory light (Leitz, 530–560 nm), and a longpass filter (Leitz, 580 nm) for cutoff. Thus it was difficult to distinguish between neurons which solely contained LY on the one hand, and the ones containing antibody in addition on the other hand. Similar difficulties were met by Voigt (1986) during his studies of the rat retina. The relatively small amounts of cobalt, just sufficient to provide nuclei for the silver precipitate, seem to interfere to a lesser degree, and the DAB reaction product was clearly detected in several such INs especially if the latter were not too intensely cobalt stained. The impregnation of such cells was not complete and their appearance rather brownish. More intensely stained INs appeared black and completely filled after the intensification, but were free of visible DAB reaction product before the intensification. Thus a larger amount of injected cobalt might indeed have impeded the GLIR test. The indirect PAP method was used because of its high sensitivity and contrast in the histological sections. Both labels in combination gave sufficient information about presence of GLIR in INs and its absence in the group of uniglomerular TOG(I)-PNs (Kraus et al. 1988).

GLIR in central olfactory neurons of insects

First reports on the presence of GABA in the cockroach's deutocerebrum were published by Frontali and Pierantoni (1973), who demonstrated localization of tritiated GABA also in cell bodies of the antennal lobes. While at that time further identification of single neurons was beyond the technical possibilities, recent combined morphological, functional, and chemical characterization of such units in the insect deutocerebrum was accomplished by Hoskins et al. (1986). By tandem intracellular staining with LY and immunohistochemical staining for GLIR they identified a number of local INs as well as certain multiglomerular PNs of the antennal lobes of *Manduca sexta* as GABAergic. On the basis of these and other results (Waldrop et al. 1987) a general picture of the distribution of GLIR in the deutocerebrum of this insect was discussed together with the role of GLIR-exhibiting neurons in the signal processing in the antennal pathway. The present study revealed presence of GLIR in certain groups of deutocerebral INs (and its absence in the ventral uniglomerular PNs) of *Periplaneta*, and it seems interesting to discuss also their function in terms of the above-mentioned context. However, this would be



Figs. 4 and 5. Double-labeling experiments involving tandem intracellular cobalt staining and immunohistochemical staining for GABA-like immunoreactivity. Scale *bar* = 100 μm

Fig. 4a–c. Characteristic details of double-labeled local interneurons (Y-INs) from two brains. Labeling for GLIR in the soma-group (**a and b,** one neuron) and typical branching pattern in the olfactory neuropile (**c,** other neuron). **a** Immunohistochemical staining for GLIR of a cobalt stained neuron (*arrowhead*) of the ventro-lateral group of somata (*s*). *al,* antennal lobe. **b** Silver intensification of the same section shows the soma (*arrowhead*) of a local interneuron (Y-IN) which in (**a**) contained GLIR. **c** Characteristic processes of another double-labeled interneuron (Y-IN) in the glomeruli (*g*) of the antennal lobes

Fig. 5a–c. Characteristic details of double-labeled projection neurons (PNs) with absence of GLIR in the soma group (**a** and **b**, one specimen), and axon process in the olfactorio-globular tract (**c,** other specimen). **a** Immunohistochemical staining for GLIR of a cobalt stained neuron (*arrowhead*) of the lateral PN-group (*s*). *al,* antennal lobe. **b** After silver intensification of the same section, a soma appears stained (*arrowhead*) which in (**a**) had not shown GLIR. **c** Axon (*large arrowhead*) of a cobalt stained PN in the olfactorio-globular tract (*tog*). Marginal portions of the tog with GLIR (*small arrowheads*). *cp,* corpus pedunculatum

premature because of the lack of physiological and pharmacological data about connections, and also about transmitters of other neurons in the cockroach's deutocerebrum. Moreover, the primary purpose of this study was to adapt methods for a first combined neurochemical and morphological identification of especially well defined subgroups of INs and PNs whose innervation domaines and sensory functions are known to a considerable extent (Boeckh et al. 1983; Ernst and Boeckh 1983; Boeckh et al. 1984; Boeckh and Ernst 1987). The direction of impact is to introduce an additional label for certain types of neurons into the neuroanatomical study of synaptic connections in the deutocerebral glomeruli, where double labeling experiments have already revealed certain types of connections between antennal receptor axons and identified neurons (Selsam 1987; Boeckh and Ernst 1987).

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