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The larval chemosensory system of *Drosophila melanogaster*: neuroanatomy and neurotransmitter distribution

THESE

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Table of contents

SUMMARY

The goal of my Ph.D. thesis was to study the larval chemosensory system of the fruit fly *Drosophila melanogaster*, both in terms of its organization and neurotransmitter content. My studies were based on the enhancer trap technique, which provides useful neuronal marker lines and allows ectopic expression of any transgene of interest in the labeled cells. Four enhancer trap lines selected for their chemosensory-specific expression pattern – together with neuron-specific and neuropil-specific antibodies – served as markers for olfactory and gustatory receptor neurons and their target neurons in the brain. Laser confocal microscopy in the third instar larva allowed me to establish (1) the neuronal organization of smell and taste organs, (2) the nerves carrying the chemosensory axons from the larval head into the central nervous system and (3) the organization of their central target regions, the larval antennal lobe (LAL) and the tritocerebral-suboesophageal region. My data suggest an adult-like complexity of the LAL structure, despite drastically reduced numbers of odorant receptor neurons. To extend the description of the larval chemosensory system, I then examined the cellular distribution of the classical neurotransmitters acetylcholine – studied as the expression of choline acetyltransferase – γ-aminobutyric acid, histamine, octopamine and serotonin. My data showed an essentially similar cellular distribution of these neurotransmitters as in the adult chemosensory system, suggesting shared mechanisms of chemosensory information processing. In conclusion, based on this neuroanatomical and neurochemical description, I propose the larval chemosensory system of *D. melanogaster* as an alternative model system for studying smell and taste.

RESUME

L'objectif de mon travail de thèse a été d'étudier le système larvaire chémosensoriel de la mouche du vinaigre *Drosophila melanogaster*, à la fois sur le plan de son organisation et de son contenu de neurotransmetteurs. Mes études ont été basées sur la technique "enhancer trap", qui fournit des lignées utiles pour le marquage neuronal et permet l'expression ectopique d'un gène transgénique digne d'intérêt dans les cellules désignées. Quatre lignées "enhancer trap" sélectionnées pour leur pattern d'expression spécifique au système chémosensoriel – combinées avec des anticorps reconnaissant le neuropile et les neurones – ont servi comme marqueurs pour les neurones olfactifs et gustatifs et les neurones cibles dans le cerveau. La microscopie confocale à laser dans la larve de troisième stade m'a permis d'établir (1) l'organisation neuronale des organes de l'odorat et du goût, (2) les nerfs portant les axones chémosensoriels de la tête de la larve jusqu'au système nerveux central et (3) l'organisation de leurs régions cible centrales, appelé le lobe antennaire larvaire (LAL) et la région tritocérébrale-suboesophagiale. Mes données suggèrent une complexité de la structure du LAL semblable à celle de l'adulte, malgré des nombres drastiquement réduits de neurones récepteurs de l'odorat. Pour élargir la description du système larvaire chémosensoriel, j'ai ensuite examiné la distribution cellulaire de neurotransmetteurs classiques tels que l'acetylcholine – étudiée par l'expression de la choline acetyltransferase – l'acide γ-aminobutyrique, l'histamine, l'octopamine et la sérotonine. Mes données ont montré principalement une distribution cellulaire de ces neurotransmetteurs similaire au système adulte chémosensoriel. Ceci suggère des mécanismes partagés dans le traitement de l'information chémosensorielle. Sur la base de cette description neuroanatomique et neurochimique, je peux conclure que le système larvaire chémosensoriel de *D. melanogaster* est également un système modèle pour l'étude de l'odorat et du goût.

INTRODUCTION

The fruit fly *Drosophila melanogaster* is certainly one of the major model systems in animal genetics, for a number of reasons. It has a small body size and a short generation time, and each female produces hundreds of progeny. Recently, the approximately 120-megabase euchromatic portion of the genome has been sequenced (Adams et al., 2000). It encodes about 13'000 genes, compared to 30'000 genes for the human genome (Lander et al., 2001; Venter et al., 2001). Lately, *Drosophila* has become a focus of interest in neurobiology as well. Whereas the human brain is composed of approximately 10^{14} neurons, the fly brain contains only 10^{5} neurons, many of which are identifiable. Various behavioral assays have been established both in the adult and in the larva. Moreover, hundreds of mutants have been isolated showing behavioral or neurological defects (Pflugfelder, 1998). Finally, the fruit fly disposes of powerful new genetic and molecular tools (Rubin, 1988; Greenspan, 1996), in particular the enhancer trap technique (O'Kane and Gehring, 1987). The P[GAL4] variant of this technique is a versatile method allowing selective expression of any transgene in the cells of interest (Fischer et al., 1988; Brand and Perrimon, 1993).

P[GAL4] enhancer trap flies are generated initially by the injection of a transposable P element, the P[GAL4] construct into embryos. The construct integrates randomly in the genome and can be remobilized subsequently in order to generate new insertion strains. The P[GAL4] construct carries the yeast GAL4 transgene as a "primary reporter gene". Depending on the precise insertion site, the GAL4 gene may trap the activity of an enhancer located in its vicinity, by expressing GAL4. In other words, the expression of the GAL4 gene reflects the spatio-temporal expression of a nearby *Drosophila* gene, because both are controlled by the same enhancer. When crossing a given P[GAL4] strain with a second transgenic strain containing a P[UAS] construct, the GAL4 protein will bind to the GAL4 binding site UAS (Upstream Activating Sequence). Any gene or transgene fused to UAS will then be activated ectopically according to the trapped enhancer (Figure). Such target genes ("secondary reporter genes") may allow visualization of the expression pattern, e.g. the bacterial ß-galactosidase (*lacZ*) gene or the green fluorescent protein (GFP) gene from the jellyfish *Aequora victoria* (O'Kane

and Gehring, 1987; Yeh et al., 1995). Other transgenes are helpful tools for functional studies. For example, expression of the tetanus toxin light chain gene (Sweeney et al., 1995) leads to block of synaptic transmission. The functional defects thus produced will result in measurable behavioral changes. In summary, the enhancer trap technology permits selection of P[GAL4] lines exhibiting reporter expression in selected cell types or tissues. Such lines are thus attractive markers that allow to follow selected cells/neurons through development, or to impair their function via toxin expression (Reddy et al., 1997; Stocker et al., 1997; Tissot et al., 1998; Baines et al., 1999; Heimbeck et al., 1999; Heimbeck et al., 2001).

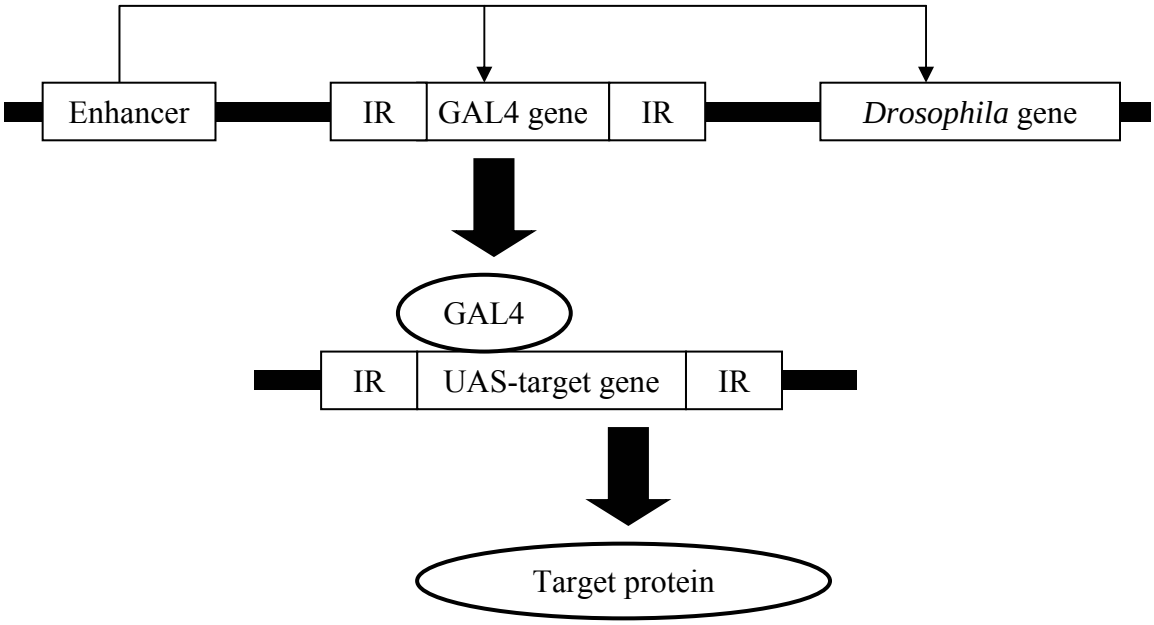

Figure. Schematic representation of the enhancer trap technique. The P[GAL4] construct contains the GAL4 "primary reporter gene" located between inverted terminal repeats (IR). A genomic enhancer in the vicinity of the P[GAL4] insert may activate at the same time a Drosophila gene and the GAL4 gene. When crossed to a P[UAS] strain, the GAL4 protein, a transcription factor from yeast (GAL4 encircled), binds to the UAS sequence, activating the expression of any "secondary reporter gene" fused to UAS (Target protein encircled).

The neuroanatomical basis of the adult chemosensory system in *Drosophila* has been well described (Nayak and Singh, 1983; Stocker, 1994; Singh, 1997; Laissue et al., 1999; Shanbhag et al., 1999; Shanbhag et al., 2001). Compared to vertebrate systems, it exhibits a tremendous reduction of cell numbers, despite surprising parallels in terms of organization (Hildebrand and Shepherd, 1997). This renders the fly an attractive model system for analyzing olfaction and gustation. In contrast, few studies have focused on the larval chemosensory system (Singh and Singh, 1984; Tissot et al., 1997; Cobb, 1999; Heimbeck et al., 1999; Stocker, 2001;). Considering its even further reduced cellular complexity, the goal of my work was to explore the potential of the larval system as an alternative, equally attractive chemosensory model system.

In my thesis work, I used the GAL4/UAS expression system as a tool for studying in the confocal microscope the neuroanatomy and neurotransmitter content of the larval chemosensory system. I chose four P[GAL4] lines that show specific reporter expression in the olfactory and/or gustatory system, both in the periphery and in the central nervous system (CNS). By simultaneously expressing a fluorescent GFP reporter and applying neuronal or neuropil antibody markers, I initially focused on the organization of the larval chemosensory system. I investigated (1) the neuronal composition of the larval olfactory and gustatory organs, (2) the peripheral nerves used by their afferent axons on their way to the CNS, and (3) the organization of the larval antennal lobe (LAL), the primary target of olfactory afferents. Special attention was given to putative subunits of the LAL, resembling adult glomeruli, and to a corresponding compartmentation of afferent terminals and of dendritic arborizations of target neurons. The major types of target neurons are local interneurons – intrinsic to the LAL – and projection neurons, providing links to higher brain centers. The most significant observation of this study was an adult-like structural complexity of the LAL despite drastically reduced numbers of odorant receptor neurons. The paper will be published in the “Journal of Comparative Neurology” (in press).

In the second part of my work, I extended the description of the larval chemosensory system by analyzing in the same P[GAL4] lines the distribution of choline acetyltransferase (ChAT) – the acetylcholine-synthesizing enzyme – and of the neurotransmitters γ-aminobutyric acid (GABA), histamine, octopamine and serotonin. I

provide evidence that subsets of the olfactory and gustatory afferents as well as many of projection neurons are strongly ChAT immunoreactive, and that perhaps the entire set of local interneurons contain GABA as a neurotransmitter. In addition, I identified a putative serotonergic interneuron that arborizes in the LAL neuropil. Taken together, the cellular distribution of these classical neurotransmitters is similar as in the adult chemosensory system, suggesting shared mechanisms of chemosensory information processing. In summary, my thesis work proposes the larva of *D. melanogaster* as an alternative model system for studying smell and taste at the functional, molecular and developmental level.

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Chapter 1

Adult-like complexity of the larval antennal lobe of *Drosophila melanogaster* despite markedly low numbers of odorant receptor neurons

This chapter will be published in *The Journal of Comparative Neurology* (2002), vol. 445.

ABSTRACT

We provide a detailed analysis of the larval head chemosensory system of *Drosophila melanogaster*, based on confocal microscopy of cell-specific reporter gene expression in P[GAL4] enhancer trap lines. In particular, we describe the neuronal composition of three external and three pharyngeal chemosensory organs, the nerve tracts chosen by their afferents, and their central target regions. With a total of 21 olfactory and 80 gustatory neurons, the sensory level is numerically much simpler than that of the adult. Moreover, its design is different than in the adult, showing an association between smell and taste sensilla. In contrast, the first order relay of the olfactory afferents, the larval antennal lobe (LAL), exhibits adult-like features both in terms of structure and cell number. It shows a division into approximately 30 subunits, reminiscent of glomeruli in the adult antennal lobe. Taken together, the design of the larval chemosensory system is a 'hybrid', with larval-specific features in the periphery and central characteristics in common with the adult. The largely reduced numbers of afferents and the similar architecture of the LAL and the adult antennal lobe, render the larval chemosensory system of *Drosophila* a valuable model system, both for studying smell and taste, and for examining the development of its adult organization.

Abbreviations: AL antennal lobe, AN antennal nerve, DO dorsal organ, DOG ganglion of the DO, DPS dorsal pharyngeal sensilla, LAL larval antennal lobe, LBN, labial nerve, LI local interneuron, LN labral nerve, mAb monoclonal antibody, MN maxillary nerve, PN projection neuron, PPS posterior pharyngeal sensilla, SOG suboesophageal ganglion, TO terminal organ, TOG ganglion of the TO, TR tritocerebrum, VO ventral organ, VOG ganglion of the VO, VPS ventral pharyngeal sensilla

INTRODUCTION

Since the identification of putative olfactory and gustatory receptors in *Drosophila melanogaster* (Clyne et al., 1999, 2000; Gao and Chess, 1999; Vosshall et al., 1999) and the subsequent demonstration of receptor-specific afferent connections in the brain (Vosshall et al., 2000; Scott et al., 2001), the fruitfly has become an attractive focus in chemosensory research (reviews: Vosshall, 2000, 2001; Stocker, 2001; Warr et al., 2001). The neuroanatomical basis required for the interpretation of these data has been well established in the adult fly (Nayak and Singh, 1983; Stocker, 1994; Singh, 1997; Laissue et al., 1999; Shanbhag et al., 1999; Shanbhag et al., 2001). In contrast, apart from a few isolated reports (Singh and Singh, 1984; Tissot et al., 1997), the larval chemosensory system lacks a sound structural description. This is a drawback considering the potential attractiveness of the larval system for functional and molecular studies, due to its extremely reduced cellular complexity (see below) (Cobb, 1999; Heimbeck et al., 1999; Stocker, 2001).

The chemosensory apparatus of the larval head is formed during late embryogenesis (Frederik and Denell, 1982; Campos-Ortega and Hartenstein, 1997). It consists of three organs located on the cephalic lobe, the dorsal organ (DO), the terminal organ (TO), the ventral organ (VO), and three sets of pharyngeal sensilla (Singh and Singh, 1984). The fine structure of the DO, TO and VO in *Drosophila* (Singh and Singh, 1984) is very similar to that of the housefly larva *Musca domestica*, which has been meticulously described (Chu and Axtell, 1971; Chu-Wang and Axtell, 1972a; Chu-Wang and Axtell, 1972b). In both species, 21 putative olfactory receptor neurons in the DO establish profuse dendritic arbors in a central dome sensillum whose wall is perforated by thousands of pore tubules. The dome is surrounded by six sensilla with large distal pores suggesting a gustatory function. Apical pores are present also in most of the TO sensilla, which consist of at least six different types clustered in a distal and a dorsolateral group, as well as in one of the four sensilla comprising the much simpler VO. Hence, the DO appears to be a mixed smell and taste organ, whereas the TO, VO and pharyngeal sensilla may be exclusively gustatory (Chu and Axtell, 1971; Chu-Wang and Axtell, 1972a; Chu-Wang and Axtell, 1972b; Singh and Singh, 1984; Singh, 1997).

This is supported by recent toxin inactivation and electrophysiological studies in *Drosophila* (Heimbeck et al., 1999; Oppliger et al., 2000) which assigned an olfactory function to the DO and a gustatory one to the TO.

Although fruitfly larvae respond to a large variety of chemicals (Ayyub et al., 1990; Cobb et al., 1992; Cobb and Dannet, 1994; Cobb, 1999; Heimbeck et al., 1999; Cobb and Domain, 2000; Oppliger et al., 2000), their chemosensory system is remarkably simple in cellular terms. For example, it comprises no more than the 21 odorant receptor neurons of the DO, compared to 1300 in the adult fly. The 21 olfactory afferents converge onto the larval antennal lobe (LAL), the precursor of the adult antennal lobe (AL) (Tissot et al., 1997). The LAL is no bigger than a single glomerulus of the adult lobe, and input and output fibers of the LAL – when studied as populations – did not seem to display any obvious glomerular-like arborization (Stocker et al., 1997; Tissot et al., 1997), which is typical for the adult AL. These observations suggest simplicity at the central level as well. However, no description of the LAL structure or of afferent and target neuron arborizations is available at cellular resolution. Moreover, apart from a few casual data (Tissot et al., 1997), nothing is known about gustatory target regions in the larval CNS, in particular for pharyngeal sensilla. This motivated us to study the organization and connectivity of the larval chemosensory system of *Drosophila*, both at the peripheral and central level. Specifically, we wanted to determine whether and in which sense the larval system is indeed "simpler" than the adult system. This report focuses on the larval head and will not include putative chemosensory organs on the rest of the body.

In the present study, we made use of P[GAL4] enhancer trap lines (Brand and Perrimon, 1993) that show expression in larval chemosensory neurons and/or their target neurons in the brain. Analyzing their expression patterns in the confocal microscope, in combination with neuronal or neuropil markers, allowed us to determine the cellular composition of the larval chemosensory organs, the peripheral nerves used by their afferents and their central target regions. In particular, we were interested in the architecture and neuronal organization of the LAL. We show that the LAL consists of numerous subunits, which in terms of afferent and target neuron morphology are reminiscent of typical antennal lobe glomeruli.

MATERIALS AND METHODS

The P[GAL4] lines 4551 and 189Y (Osborne et al., 1997) were provided by J.-F. Ferveur (Université de Bourgogne, Dijon) and K. Kaiser (University of Glasgow), respectively. The lines GH146 and GH86 were isolated by G. Heimbeck (Stocker et al., 1997; Heimbeck et al., 1999). As secondary reporter strains, we used UAS-GFP (Yeh et al., 1995) and UAS-Tau-GFP (Murray et al., 1998), both kindly provided by A. H. Brand (Wellcome/CRC, Cambridge). The CantonS (CS) strain served as a wildtype reference. All strains were raised on standard cornmeal medium at 18°C or 25°C.

Antibody staining was adapted from Laissue et al. (1999). In brief, dissected tissues from the third larval instar were fixed for 2 hours on ice in 4% paraformaldehyde (Merck) (4 hours for the adult head), dissolved in phosphate buffered saline (pH 7.2) containing 0.2% Triton X-100 (PBS-T, pH 7.2). Subsequent reactions took place with gentle shaking on ice. After 3 washes of 20 minutes each in PBS-T, they were blocked for 1-2 hours in the blocking solution (BS) containing 3% normal goat serum (Jackson ImmunoResearch) in PBS-T. Tissues were then incubated overnight in the primary antibody diluted in BS. Washes in PBS-T (3x 20 minutes) were followed by the incubation of the secondary antibody diluted in BS for 5 hours. Samples were again rinsed 3x 20 minutes in PBS-T and finally mounted in Vectashield medium (Vector Laboratories). As primary antibodies we used mouse mAb nc82 (dilution 1:10; a gift from A. Hofbauer, University of Regensburg), mouse anti-Synapsin (1:100; provided by E. Buchner, University of Würzburg) and mouse anti-Elav 9F8A9 (1: 200). The anti-Elav antibody developed by G.M. Rubin was obtained from the Developmental Studies Hybridoma Bank developed under the auspices or the NICHD and maintained by The University of Iowa, Dept. of Biological Sciences, Iowa City, IA 52242. The secondary antibody utilized was Cy3-coupled goat anti-mouse IgG (Jackson ImmunoResearch), diluted 1:100 in BS.

Preparations were studied with a BioRad MRC 1024 confocal microscope equipped with a Kr/Ar laser. Z series of pictures were taken at intervals of 0.5 to 2 μm.

Image analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Color selection of images was done by the Adobe PhotoShop program.

RESULTS

The four enhancer trap lines 4551, 189Y, GH86 and GH146 which express GAL4 in subsets of sensory neurons and their target interneurons allowed us to study the organization of the major chemosensilla in the third larval instar, to identify the peripheral pathways and central target regions of their afferents and to analyze the structure and interneuron composition of the LAL (cf. Fig. 8 for a summary diagram). The expression patterns were studied in whole mounts by confocal microscopy. The patterns visualized by the two reporters GFP and Tau-GFP were similar, in particular with respect to afferent paths and terminals in the CNS. However, Tau-GFP was superior for demonstrating axonal pathways (Fig. 1A,B), due to the microtubular association of the Tau protein. GFP and Tau-GFP patterns were confirmed by ß-Galactosidase reporter staining in whole mounts and by Tau immunostaining in cryosections (cf. Heimbeck et al., 1999; data not shown). Neuronal identity of cells was established by means of the mAb anti-Elav, and the neuropil structure of the LAL was dissected by applying the mAbs nc82 (Laissue et al., 1999) and anti-Synapsin (Klagges et al., 1996).

Chemosensory Organs of the Larval Head

Reporter gene expression driven by line 4551 revealed the entire neuroanatomy of the DO and TO (Figs. 1A,C, 2A). Their bipolar sensory neurons are assembled in two separate ganglia, which are located well below the epidermis, in close contact to each other. As noted previously, a small subset of neurons in the DO ganglion send their dendrites into the TO (Kankel et al., 1980; Frederik and Denell, 1982; Tissot et al., 1997) (Figs. 1C, 2A; see below).

Labeling with anti-Elav revealed an average of 37 sensory neurons in the dorsal ganglion of CS wildtype larvae (n=6; males: 37-39; females: 35-37) (Table 1), which is similar to the 35-41 neurons counted in the housefly larva (Chu and Axtell, 1971). Likewise, the dorsal ganglion of the line 4551 comprises 34-38 neurons (n=5) (Fig. 2A), 24 of which on an average express GAL4 (n=6; 22-25) (Figs. 1C, 2A). The expression pattern confirms that the central dome of the DO – the exclusive olfactory organ of the larva by fine structural criteria (Singh and Singh, 1984) – is innervated by seven bundles of dendrites (cf. Singh and Singh, 1984; Campos-Ortega and Hartenstein, 1997) (Fig. 1C). According to these reports, each bundle consists of a triplet of dendrites, yielding a total of 21 odorant receptor neurons, whereas additional neurons of the dorsal ganglion innervate the six putative taste sensilla surrounding the dome. Interestingly, none of the GAL4-positive neurons of line 4551 send their dendrites into one of these six sensilla. On the other hand, three labeled dendrites extend toward a putative gustatory sensillum of the TO (Figs. 1C, 2A, see below). This suggests that the 24 GAL4-positive neurons in the DOG comprise the 21 odorant receptor neurons and three taste neurons associated with the TO (Table 1).

In the terminal ganglion of CS larvae we counted an average of 32-33 neurons (n=6; males: 33-35; females: 30-34) (Table 1), four of which – perhaps associated with mechanosensory scolopidia – are located more distally than the rest (Fig. 2A). This corresponds to the 32 neurons observed in the terminal ganglion of the housefly larva (Chu-Wang and Axtell, 1972a) and to the 30-34 neurons found in line 4551 (n=5). The TO consists of a distal group of 11 individual sensilla and a dorsolateral group of three sensilla, one of which – a putative gustatory papillum – is innervated by three dendrites

(Kankel et al., 1980; Singh and Singh, 1984) (Fig. 1C, inset). As mentioned before, GAL4 expression in line 4551 suggests that three dendrites originating from the dorsal ganglion extend toward the dorsolateral group of the TO, most likely into that papillum (Fig. 1C, inset). Apart from that, line 4551 labels about 15 neurons in the terminal ganglion (n=6; 13-17) (Figs. 1C & 2A). Most if not all of them are likely to be gustatory, given that a large majority of the neurons of this ganglion may mediate taste according to fine structural criteria (Chu-Wang and Axtell, 1972a). The remaining neurons may represent mechano-, hygro- or thermoreceptors. In close vicinity to the terminal ganglion we observe an additional cluster of up to ten neurons (Fig. 2A), which may correspond to the "associated organ" described in the embryo (Schmidt-Ott et al., 1994), a sensillum of unknown function.

The VO, the third external chemosensory organ, has its terminal pore located between the third and fourth rows of spinules (Figs. 1D, 2A). Anti-Elav staining revealed up to seven neurons in the VO ganglion (Fig. 2A, Table 1), one of which is labeled by line 4551. Earlier studies in the VO of *Musca* and *Drosophila* reported the presence of seven and eight neurons, respectively (Chu-Wang and Axtell, 1972b; Singh and Singh, 1984).

The expression patterns of the lines 4551 and 189Y together with anti-Elav tagging revealed several groups of pharyngeal sensilla. Two anterior clusters are located in front of the cephalopharyngeal H-piece (Campos-Ortega and Hartenstein, 1997), a dorsal and a ventral one (DPS, VPS) (Figs. 1A, 3). Moreover, a small sensillum (PPS) sits further posterior in the lateral wall of the pharynx, in an area characterized by longitudinal ridges (Campos-Ortega and Hartenstein, 1997) (Figs. 1A,B, 3). Due to developmental rearrangements, the identity of these three groups compared to the embryonic sensillum pattern is not easy to determine. According to the relative location and associated nerve, the DPS is likely to correspond to the labral sense organ of Campos-Ortega and Hartenstein (1997) known also as "epiphysis" (Schmidt-Ott et al., 1994) or "dorsal pharyngeal sensilla D_1-D_6" (Singh and Singh, 1984). The VPS appears to be equivalent to the labial sensory complex (Campos-Ortega and Hartenstein, 1997; synonyms "hypophysis": Schmidt-Ott et al., 1994; "ventral pharyngeal sensilla V_1-V_4": Singh and Singh, 1984), whereas the PPS may correspond to the hypopharyngeal organ

(Schmidt-Ott et al., 1994; Campos-Ortega and Hartenstein, 1997). The ganglia of the DPS comprise 16-17 anti-Elav stained neurons each (S. Crevoiserat, personal communication) (Table 1), substantially more than ten neurons, as counted by Singh and Singh (1984). Fourteen to fifteen of them express GAL4 in line 4551. The PPS ganglia include six neurons, all marked by GAL4 (S. Crevoiserat, personal communication). Moreover, each VPS ganglion contains 17 neurons (Singh and Singh, 1984), none of which is labeled by 4551.

Chemosensory Head Nerves

The dorsal ganglion connects to the CNS by means of the antennal nerve (AN) (Figs. 1A, 8). Hence, the AN is a mixed nerve comprising 21 olfactory afferents from the dome sensillum and 12 putative gustatory afferents from other DO sensilla and the dorsolateral TO papillum (Table 1). At about two thirds of the distance toward the brain, the AN is joined by the labral nerve (LN) (cf. Schmidt-Ott et al., 1994), which carries at most 22 gustatory afferents from the DPS and PPS (Fig. 1A, Table 1). The afferents from both nerves then travel together toward the CNS. However, we were not able to distinguish whether the two nerves have indeed fused or are just closely aligned. In support of the second interpretation, the compound nerve bifurcates again shortly before approaching the CNS. One branch turns toward the midline, joins the brain from laterally and extends toward the LAL (Fig. 1E,F). The second branch enters the CNS closer to the midline, at a site that is presumably of tritocerebral identity (TR) (Fig. 1B inset,E). Tracing of the afferents in the line 4551 and in 189Y – whose sensory expression is restricted to pharyngeal sensilla (Fig. 1A,B, inset, E) – suggests that the fibers in the branch towards the LAL originate in the AN, whereas those in the branch towards the TR derive from the LN. This further argues against a fusion of the two nerves. However, we cannot exclude the possibility that GAL4-negative afferents deriving from the six DO sensilla around the dome may pass into the TR branch. Together with the mixed innervation of the TO by two distinct ganglia (see above), the pairing of AN and LN illustrates the highly modified dipteran neuroanatomy (cf. Strausfeld, 2001).

The ganglia of the TO and the VO are connected to the CNS by means of the maxillary nerve (MN) (Fig. 1A). Hence, in contrast to the AN, the MN lacks an olfactory component. It carries about 23 gustatory afferents from the distal TO sensilla and the remaining dorsolateral sensilla, as well as seven gustatory afferents from the VO (Table 1) (Chu-Wang and Axtell, 1972a; Chu-Wang and Axtell, 1972b; Campos-Ortega and Hartenstein, 1997). The MN joins the CNS more ventrally and posteriorly and closer to the midline than the LN branch of the compound nerve, at a location that obviously belongs to the suboesophageal ganglion (SOG) (Fig. 1E). These two entry sites are consistent with the supra- and suboesophageal identity of the LN and MN, respectively (Campos-Ortega and Hartenstein, 1997). The fourth chemosensory head nerve is the labial nerve which is chosen by the approximately 15 gustatory afferents from the VPS (Singh and Singh, 1984; Schmidt-Ott et al., 1994) (Table 1). It joins the SOG region of the CNS still further back than the MN.

Larval Antennal Lobe: Structure and Afferent Projections

Unlike the adult AL, the LAL is not the most anterior prominent neuropil area of the brain. Rather, it is a small, metameric structure of 20-30 µm diameter, situated between the mushroom bodies and the TR-SOG region (Fig. 2B-D,F) (Stocker et al., 1995; Tissot et al., 1997). The LAL is barely visible in the unstained brain, but becomes manifest upon the application of diverse mAbs or transgenic markers (Fig. 2B-D). Remarkably, the mAb nc82 – which reveals the glomerular architecture of the adult AL (Laissue et al., 1999) (Fig. 2E) – binds in a non-homogeneous manner to the LAL neuropil as well (Fig. 2E, inset). This non-homogeneous pattern is clearly more pronounced than that of other neuropil regions. Intensely stained areas of 5 to 10 µm diameter, termed here subunits, are separated by clefts (Fig. 2G). The total number of subunits may not exceed 30. More precise estimates are hampered by their ambiguous contours, which are less distinct than in adult glomeruli. A similar patterning of the LAL neuropil is also visible with the anti-Synapsin marker (Fig. 4), especially with respect to two particular subunits (see below).

Remarkably, GFP staining in the afferent lines studied reveals also non-homogeneity in the pattern of sensory terminals. Their arborizations occupy small

domains, most of which are of similar size and shape as the subunits mentioned before (Fig. 2G). Inside the domains, smaller structures associated with the afferents are visible, as reported previously for the chemosensory-specific line GH86 (Heimbeck et al., 1999) (Fig. 5A-C). Very likely these smaller particles represent the afferent terminals proper. Three of the sensory domains are more intensely labeled than the others (Figs. 2G, 4, 5A-C). Two large, elongated domains (E, E') extend from lateral to anteromedial in the posterior LAL region. They are the targets of subsets of AN afferents that segregate from the rest of the fibers shortly after entering the LAL (Fig. 5A,B). A smaller and heavily marked domain occupies a more dorsal position (D) and is innervated by a single sensory axon (Fig. 5B,C). Hence, at least in these cases, individual afferents appear to terminate in subregions of the LAL rather than distributing over its entire volume.

Do the neuropil subunits mentioned before match to the afferent domains? Indeed, nc82 labeling in the afferent line 4551 shows a striking correspondence between the two elements (Fig. 2G). This is particularly evident for the intensely stained E and D domains (Figs. 2G at 9 µm & 15 µm, 4), which are labeled by the anti-Synapsin marker as well (Fig. 4). Such an overlap may also apply to other LAL regions, as suggested by conspicuous parallels in the size, shape and arrangement of many sensory domains and neuropil subunits. Thus, we propose that many of the subunits labeled by the neuropil markers may correspond to afferent arborizations.

Larval Antennal Lobe: Interneurons and their Arborizations

Similar to the adult AL, the LAL comprises two major types of interneurons, i.e., local interneurons (LIs) whose arborizations are restricted to the lobe, and projection neurons (PNs), which link the lobe with higher brain centers. Line 189Y labels five to six LIs in the LAL, which represents very likely only a fraction of their total number. As for adult LIs (Stocker et al., 1997), larval LIs have their cell bodies posterolateral to the lobe (Figs. 1B, inset & 2B, inset) and appear to arborize in the entire LAL neuropil. Double labeling with nc82 or anti-Synapsin reveals highest GFP or Tau-GFP reporter expression in the subunits mentioned before (Fig. 6A), suggesting that they are the main sites of LI arborizations.

Adult flies of the line GH146 express GAL4 in about 90 PNs, which may represent about two thirds of their total number (Stocker et al., 1997). PNs that have their cell bodies located anterodorsal or lateral to the AL establish uniglomerular dendritic arborizations, whereas PNs with ventral cell bodies are mostly polyglomerular (Jefferis et al., 2001). Many of the adult PNs derive from differentiated larval PNs (Stocker et al., 1997). In GH146 larvae only an anterodorsal cluster of about 30 and a lateral cluster of about 20 PNs are visible (Fig. 5D), apart from an additional, isolated cell body of unknown identity dorsal to the LAL. As in the adult, larval PNs connect the AL by means of the inner antennocerebral tract (Fig. 7) with the calyx of the mushroom bodies and the lateral protocerebrum.

Careful inspection of the Tau-GFP reporter pattern in confocal stacks from GH146 larvae suggests that individual PNs may arborize in subregions of the LAL rather than in its entire neuropil (Fig. 7A-C and D-F). In particular, we observe that the number of fibers extending from the PN trunks into the LAL neuropil does not exceed the number of PN cell bodies (Fig. 7), and that each of these fibers apparently restricts its dendritic arborization to a single subregion. Again, there is often a correspondence between these arbors and the neuropil subunits labeled by nc82 (Fig. 6B). Hence, regarding their anatomy, larval PNs show striking parallels with adult uniglomerular PNs.

In summary, our data demonstrate the existence of subunits in the LAL of third instar larvae. However, we do not know whether the entire LAL neuropil is organized in this way and whether the correspondence between neuropil subunits, afferent terminals and PN arborizations is a general feature. Yet, based on our evidence at least some subunits may be classified as glomeruli.

Chemosensory Projections in the Tritocerebral-Suboesophageal Region

As shown by line 189Y, many afferents deriving from the LN branch of the compound nerve (see above) terminate in the TR region, very close to the LAL neuropil (Fig. 1B,E), whereas others extend to the SOG region (Fig. 1B, inset). However, we

cannot distinguish whether these two components are due to distinct populations of afferents or represent two branches of the same fibers. In both TR and SOG, the terminals remain ipsilateral. Line 4551 shows that afferents carried by the MN terminate in the SOG as well, though more posterior and closer to the midline (Fig. 1E). They seem to branch into an ascending component which ends in the TR area and a posterior component that extends partially to the contralateral side. A similar connectivity was reported based on Lucifer Yellow injections in the TO ganglion (Tissot et al., 1997). Line 4551 labels also a number of interneurons of unknown identity (Fig. 1E). As shown by the lines 4551 and GH86, AN afferents projecting into the E domain of the LAL (see above) and perhaps other subregions often exhibit processes that descend into the TR-SOG region (data not shown). In contrast, we have no unambiguous evidence of ascending afferents connecting the TR-SOG region with the LAL. This is unlike the adult fly, which is characterized by an important ascending tract of afferents from the maxillary palps toward the AL (Stocker, 1994).

DISCUSSION

The Peripheral Chemosensory System of the Larva

A selection of P[GAL4] enhancer trap strains together with neuronal and neuropil markers were used for dissecting the connectivity of the larval chemosensory system. Thanks to the labeling of different combinations of neurons, these strains allowed us to distinguish between putative olfactory and gustatory elements in three external and in three pharyngeal groups of chemosensilla and to trace their afferents into their central target regions.

Anti-Elav labeling shows that the ganglion of the DO comprises on an average 37 sensory neurons (Table 1), 24 of which express GAL4 in the line 4551. In agreement with previous reports in *Musca* and *Drosophila* (Chu and Axtell, 1971; Singh and Singh, 1984), 21 of the GAL4-positive neurons send their dendrites into the central dome, the olfactory portion of the DO. Those of the remaining three labeled neurons extend into the putative gustatory papillum in the dorsolateral group of TO sensilla (see below). The

GAL4-negative neurons are likely to innervate the six peripheral DO sensilla, which may mediate taste as well (Chu and Axtell, 1971; Singh and Singh, 1984). The average of 32-33 neurons we have determined in the TO ganglion (Table 1) is in the same range as the 35 neurons reported from the housefly larva (Chu-Wang and Axtell, 1972a) and the total of 33 dendrites observed in TO sensilla of *Drosophila* (Singh and Singh, 1984). Similarly, as in two previous studies (Chu-Wang and Axtell, 1972b: *Musca*; Singh and Singh, 1984: *Drosophila*), we have counted a total of seven to eight neurons in the VO ganglion.

Individual measurements in DO and TO ganglia of both CS and 4551 larvae revealed some variation in neuron numbers. Because similar figures were obtained when counted by different persons, we consider the differences to reflect real variability in neuronal number rather than a counting artifact. How the variability relates to individual sensilla is not known. Variations in sensory structures and the underlying neurons are known also from the adult olfactory and gustatory systems (Nayak and Singh, 1983; de Bruyne et al., 1999; Shanbhag et al., 2001). The minor numerical differences observed between males and females appear to be within the range of general variability. A significant sexual dimorphism is not to be expected given the similar feeding strategies of male and female larvae.

Anti-Elav labeling and reporter expression in line 4551 demonstrate the existence of three simple sets of larval pharyngeal sensilla, termed DPS, VPS and PPS. They comprise no more than 16, 15 and 6 putative gustatory neurons, respectively (Table 1). The first two have been studied previously (Singh and Singh, 1984); the small PPS which is located far more posterior, is described here for the first time.

Chemosensory Target Regions in the CNS

The mixed expression of most available P[GAL4] lines in various subsets of larval chemosensilla often prevents a precise assignment between individual receptor neurons and their central target regions. Nevertheless, the afferent pathways shown by the lines used strongly suggest that the fibers from the DO ganglion travelling in the AN extend into the LAL, whereas those from the remaining chemosensilla carried by the

LN, MN and LBN project into successively more posterior regions of the TR-SOG neuropil (Fig. 8). The TR-SOG as a target of putative taste neurons from the TO and pharyngeal sensilla was confirmed by a recent study, in which reporter expression driven by gustatory receptor gene promoters was used to trace the target regions of single larval chemosensory neurons (Scott et al., 2001). Our data essentially confirm Lucifer yellow injections in the DO and TO ganglia of first instar larvae (Tissot et al., 1997), although their study had revealed the TR-SOG region as an additional target of the AN. Judged from our data, these extra projections might either derive from the six gustatory DO sensilla surrounding the dome – which are unlabeled by 4551 – or correspond to the descending processes from the E domain of the LAL which we occasionally observed (Fig. 8) (see below).

Architecture of the Larval Antennal Lobe

Perhaps our most significant observation is that the LAL consists of structural subunits, demonstrating that the LAL architecture is less homogeneous than previously assumed. Morphological subunits can be visualized by the application of the neuropil markers nc82 and anti-Synapsin, and are also shown by the terminal patterns of afferents, the dendritic arborizations of PNs and – to a lesser extent – by the branching pattern of LIs. These different aspects of subunits show considerable overlap regarding their size, shape and position, comparable to the glomerular architecture of the adult AL. Although we regularly observed this type of connectivity, we do not know whether it applies to all afferents and all PNs, and whether the subunits are the exclusive sites of synapses as in adult glomeruli. Yet, the input and output fibers we have seen seem to be associated with subunits of the LAL rather than with its entire neuropil. This is very reminiscent of the adult AL and invites speculations about a functional compartmentation of the LAL (Rodrigues, 1988; Joerges et al., 1997; Galizia et al., 1998).

The afferent-specific LAL domains E, E' and D are remarkable for their prominence and peculiar shape. A domain resembling the E domain is visible also in P[GAL4] lines that label exclusively dome-unrelated neurons in the DO ganglion (unpublished observations), which may be gustatory. Together with the descending

projections of the E domain in the gustatory TR-SOG neuropil (see above), this suggests that apart from its classical role as an olfactory target, the LAL may comprise (a) subregion(s) associated with non-olfactory functions.

The presence of a glomerular-type or nodular-type LAL is not unique for *Drosophila*, but has been reported from other holometabolan larvae, *Danaus plexippus* and *Manduca sexta* (Nordlander and Edwards, 1970; Kent and Hildebrand, 1987; Salecker and Malun, 1999). Structurally homogeneous LALs have been observed in the bee (Masson and Arnold, 1984; Gascuel and Masson, 1991). However, the eventual detection of subunits when applying tools of higher resolution would not be surprising.

What are the relations between LAL subunits and adult glomeruli, if any? The estimated total of subunits – approximately 30 – contrasts with the adult number of 43 glomeruli (Laissue et al., 1999). Also, their average size of 5 to 10 µm diameter is only about 1/5 of that of an adult glomerulus. More important, adult glomeruli in the holometabolous insects studied are formed *de novo* during metamorphosis from an aglomerular neuropil (Tolbert et al., 1983; Oland and Tolbert, 1996). Hence, there is certainly no direct correlation between larval subunits and adult glomeruli. However, it remains possible that larval LAL subunits serve as organizing nuclei for some of their adult counterparts, e.g. by carrying site-specific markers. Attractive cellular candidates for such markers are the larval PNs which seem to persist through metamorphosis (Stocker et al., 1997). Mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999; Jefferis et al., 2001) should allow one to study the metamorphic reorganization of dendritic arbors of individual PNs and to perturb the transformation processes in the antennal lobe.

Functional Implications

An important task of sensory systems is to distinguish among different modalities. Consequently, modality-specific sensory subsystems including their central target regions are often strictly separated. For example, in adult flies the receptor neurons for smell and taste sit on different appendages and their afferent axons project to spatially distinct centers. Surprisingly, the larval DO/TO complex exhibits strong ties between smell and taste sensilla and this mix of modalities may even apply to the target region, the LAL. These links between the two modalities may be related to the predominant short-range orientation of larvae, which is also reflected by the dominance of ≤ 79 gustatory over 21 olfactory receptor neurons (Table 1). For animals that live directly on their food supply, a distinction between smell and taste stimuli may not be very crucial. Alternatively, the links between the two senses can be explained in developmental or evolutionary terms. For example, initially separated cephalic structures may fuse during late embryogenesis (Schmidt-Ott et al., 1994; Campos-Ortega and Hartenstein, 1997), as illustrated by the innervation of the dorsolateral TO papillum from the DO ganglion (Frederik and Denell, 1982). Also, the larval system may reflect a phylogenetically ancient state in which smell and taste systems have not yet become fully independent.

Interestingly, recent functional and molecular data also suggest closer relationships between smell and taste than were previously assumed. For example, locust contact chemoreceptors can respond to certain volatile cues as well (Newland, 1998). Furthermore, the expression patterns of the newly detected family of *Drosophila* gustatory receptors (Gr) (Clyne et al., 2000; Scott et al., 2001) display no clear separation between the two modalities, in particular in the larva. Some of the Gr members appear to be expressed in gustatory neurons, some in olfactory neurons and some even in both. For example, Gr2B1 was found to be expressed in two dome-associated DO neurons and in one TO neuron. These data suggest both functional and evolutionary links between taste and smell.

In situ hybridization and Gr-driven reporter expression suggest that each gustatory neuron expresses only one Gr type (Scott et al., 2001), similar to what had

been proposed before for odorant receptors (Vosshall et al., 1999; Vosshall et al., 2000). The Gr family appears to be composed of at least 56 genes (Scott et al., 2001). So far for seven Gr genes, promoter-driven transgene expression was detected, for five among them in larval chemosensilla. If the remaining Gr genes are in fact expressed in the chemosensory system, extrapolation from this 5/7 ratio suggests a total of about 40 larval Grs, i.e., functional types of neurons. This figure is compatible with the total of about 100 chemosensory neurons determined in the larval head (Table 1) and the observation that each of the five larval Grs is expressed in 1-3 neurons (Scott et al., 2001). It further supports the idea that each neuron expresses a single receptor only. This is in contrast to mammalian gustatory neurons (Hoon et al., 1999; Adler et al., 2000) and chemosensory neurons in *C. elegans* (Bargmann et al., 1993; Troemel et al., 1997) which express multiple receptors.

The Larval Olfactory System of *Drosophila*: a Model System?

There is emerging evidence that vertebrate and insect olfactory systems may be organized according to common principles, in spite of largely different cell numbers (Hildebrand and Shepherd, 1997). Hence, it is not surprising that *Drosophila* comprising 1300 odorant receptor neurons and less than 50 glomeruli has become a focus of chemosensory research. Another olfactory model system, *C. elegans*, is attractive for an even smaller set of 16 chemosensory neurons (Chou et al., 1996). However, expression of multiple receptors per neuron and a unique brain organization suggest that the worm system may operate according to different rules. Here we study the usefulness of the larval olfactory system of *Drosophila* as a model, a system whose complexity in terms of sensory neuron numbers exceeds the *C. elegans* system only by a factor of five. We ask whether its central circuitry is in fact as simple as suggested by the highly reduced number of odorant receptor neurons (Stocker, 2001). Three organizational patterns are possible: (A) the larval system is a miniature version of the adult system, comprising similarly reduced numbers of sensory neurons, target neurons and glomeruli, (B) the design of the two systems is totally different, both at the peripheral and central level, or (C) despite the reduced numbers of sensory neurons, the central target organization is as complex as in the adult.

Possibility A can clearly be rejected, on the following arguments: Based on extrapolations from the nc82 pattern and the GH146 expression pattern, the numbers of neuropil subunits and of PNs appear to be only slightly reduced in the larva, perhaps by a factor of 2 or 3. This is in large contrast to the reduction of olfactory receptor neurons, which is almost two orders of magnitude lower than in the adult. In addition, the low number of receptor neurons is not accompanied by a simpler neuroanatomy, neither in the periphery nor in the LAL, as shown e.g. by the larval-specific association between smell and taste. Possibility B is unlikely as well, due to the striking similarities in the architecture of the larval and adult antennal lobe. Rather, our data support interpretation C which states that the markedly low number of sensory neurons is not accompanied by simplicity at the central level.

These data imply that the design of the larval chemosensory system includes larval-specific elements in the periphery and elements shared with the adult system at the level of the LAL. This 'hybrid' organization is certainly related to the fact that during metamorphosis the sensory component of the nervous system undergoes a radical transformation, whereas many of the central elements persist (Tissot and Stocker, 2000). Any interpretation of functional data in the larval chemosensory system has to take into account its specific design. Nevertheless, the observed parallels in the architecture of the larval and adult antennal lobe may render the larval chemosensory system a very valuable model system. The genetic and molecular tools available in *Drosophila* will certainly allow to fully exploit its potential.

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Table 1. Neuronal composition and putative modality of larval head chemosensory organs and their nerves

	sensilla	olfactory	gustatory	other	total	ganglion	nerve
dorsal organ (DO)	1 dome	21	–	–	21	DO	antennal
	6 others (4 types)	–	9	2	11	DO	antennal
terminal organ (TO), dorsolateral	1 papillum	–	3	–	3	DO	antennal
group	1 scolopidium [2]	–	–	2?	2?	DO	antennal
	2 others (2 types)	–	2	–	2	TO	maxillary
terminal organ (TO), distal group	2? scolopidia [2]	–	–	4	4	TO	maxillary
	11 others (4 types)	–	21	5	26	TO	maxillary
ventral organ (VO)	5 (2 types)	–	7	–	7	VO	maxillary
dorsal pharyngeal sensilla (DPS) [1]	6 (3 types)	–	16 [3]	? [3]	16	DPS	labral
posterior pharyngeal sensilla (PPS)[1]	2	–	6	–	6	PPS	labral
ventral pharyngeal sensilla (VPS) [1]	4 (3 types)	–	15	2	17	VPS	labial
DO ganglion - antennal nerve		21	12	3	36		
TO & VO ganglia - maxillary nerve		–	30	9	39		
DPS & PPS ganglia - labral nerve		–	22 [3]	? [3]	22		
VPS ganglion - labial nerve		–	15	2	17		
total neurons / afferents		21	≤ 79	≥ 14	114		

Synthesis of own and previous data in *Drosophila* (Singh and Singh, 1984; Schmidt-Ott et al., 1994; Campos-Ortega and Hartenstein, 1997) and *Musca* (Chu and Axtell, 1971; Chu-Wang and Axtell, 1972a; Chu-Wang and Axtell, 1972b). Data obtained by the different studies are in general agreement, except for DPS in which only 10 neurons were counted before (Singh and Singh, 1984).

[1] For synonyms of DPS, PPS and VPS, see text

[2] The presence of scolopidia – reported so far only from *Musca* – is assumed here by the distinctive positions of certain neurons in the DO and TO ganglia

[3] Some of the 16 neurons of the DPS may be non-gustatory, suggesting that the total of gustatory neurons might be less than the 79 indicated in the bottom row

Table 1

FIGURE LEGENDS

Fig.1. Confocal images showing the chemosensory circuitry in third instar larva of *D. melanogaster*. **A:** Reporter expression in the P[GAL4] line 4551 reveals the dorsal and terminal organs (DO, TO) including their ganglia (DOG, TOG) as well as dorsal and posterior groups of pharyngeal sensilla (DPS, PPS). Afferent axons from the DOG travel by means of the antennal nerve (AN), those from the TOG by means of the maxillary nerve (MN), and pharyngeal afferents by means of the labral nerve (LN). Arrowhead: contact between LN and AN. Strong expression is present in the larval antennal lobe (LAL), the tritocerebral-suboesophageal neuropil (TR-SOG) and the mushroom bodies (MB). **B:** Line 189Y labels afferents from the DPS and PPS in the LN, as well as local interneurons (LI) of the LAL. Arrowhead: junction of nerves from the DPS and PPS. Inset: Higher magnification of the LAL, the entrance of the LN in the TR-SOG area and LN-derived afferent terminals (arrowheads). **C:** Close-up of DO and TO expression in line 4551. Most of the labeled dendrites of the DOG extend in bundles (BU) into the central dome (DM), except three dendrites which invade the TO (arrowhead). They end in a papillum (P) of the dorsolateral group of the TO (DLG, inset) (see text for details). DIG: distal group of TO sensilla. **D:** Cuticular autofluorescence reveals TO, DM and ventral organ (VO) (wildtype CS). **E:** Line 4551 showing the entries of the AN (double arrowheads), the LN (large arrowhead) and the MN (small arrowhead) into the CNS. Expression includes several central elements, e.g., an interneuron with a contralateral process (CI). **F:** Line GH86 reveals a characteristic loop of the AN before joining the CNS (large arrowhead) and its separation into three branches (small arrowheads). A-C & E: Tau-GFP reporter; C (inset) & F: GFP reporter. The numbers of optical sections and the section thickness vary in different panels. The CNS is oriented with anterior on top. Bars = 100 µm in A,B; 25 µm in C-F.

Fig. 2. Confocal images of the DO, TO and VO (A) and of the larval CNS (B-G) in the line 4551 (except B: 189Y). **A:** Anti-Elav labeling (red) displays neurons in the DOG, TOG and the VO ganglion (VOG). A VO neuron expressing Tau-GFP (arrowhead) sends its dendrite (DE) toward the VO opening. AG: associated ganglion (see text for details). **B:** Tau-GFP expression in 189Y in afferents (cf. Fig. 1B) and in local interneurons (LI) (green) and anti-Synapsin immunostaining (red) show overlap in the LALs (encircled, inset). **C:** GFP expression shows the LAL (encircled), the TR-SOG region and the MB Kenyon cells (KC). **D:** Overlap of Tau-GFP (green) and neuropil-specific nc82 staining (red) in the LAL, TR-SOG region and MBs. **E:** Single sections of adult AL and LAL (inset) at the same magnification, labeled by nc82. **F:** Double staining of GFP (green) and nc82 (red) yields overlap in the LAL and the MB calyx (CX). **G:** Serial sections of the LAL (encircled in F) at 3 µm intervals. Afferent GFP label (green) and neuropil nc82 label (red) and their overlap (third row) are shown. Small arrowheads indicate LAL subunits labeled by both markers. The pattern similarities between the two markers are particularly obvious for two subunits, E and D. Anterior is on top. Bars = 10 µm in E,G; 25 µm in A,F; 50 µm in C; 100 µm in B,D.

Fig. 3. Entire set of pharyngeal sensilla shown by anti-Elav (**A**) and Tau-GFP reporter pattern (**B**) in line 4551. DPS, PPS, VPS: dorsal, ventral and posterior pharyngeal sensilla, respectively. Anterior is to the right. Bar = 100 µm.

Fig. 4. Single confocal sections of the LAL in line 4551 shown by afferent GFP reporter labeling (left panels) and anti-Synapsin (B,F) or nc82 neuropil markers (D,H). Left and right panels represent the same section each. Panels E,F and G,H are 6 µm apart from the sections A,B and C,D, respectively. The subunits D and E visualized by GFP labeling are also stained by the anti-Synapsin and anti-nc82 markers. The arrowhead indicates a seemingly identical subunit labeled by GFP (A,C) and the two neuropil markers (B,D). Bar = 10 µm.

Fig. 5. The LAL visualized by lines GH86 (A), 4551 (B,C) and GH146 (D). **A:** The AN splits into a major and two minor branches (arrowheads). The minor branches project into the more intensely labeled elongated domains E and E'(encircled). Inset: LAL at

lower magnification. **B,C:** Intensely labeled domains E and D including an axon projecting into the latter (arrowheads in C). **B**: LAL at the entire depth. **C**: Assembly of 3 optical sections of the same LAL. Inset: same LAL at lower magnification. **D:** Projection neurons comprise a lateral cluster (LC) and an anterodorsal cluster (AC) of about 20 and 30 cell bodies, respectively. CB: isolated cell body of unknown identity. A-C: GFP reporter; D: TAU-GFP reporter. Anterior is on top. Bars = 10 μm in A-C; 25 μm in D.

Fig. 6. Local interneurons shown by line 189Y (**A**) and projection neurons shown by GH146 (**B**) both display arborizations in small domains of the LAL (green, arrowheads). These domains overlap the neuropil subunits shown by nc82 (red) labeling, as shown in the third column. GFP reporter. Bars = 10 μm.

Fig. 7. Successive sections through the LAL in line GH146 show two small subregions (stippled in A-C and in D-F) which are innervated by a dendritic process (arrowheads) extending from the PN trunk. Tracing of confocal stacks suggests restriction of individual dendritic arbors to single subregions. The number of processes does not exceed the number of PN cell bodies suggesting that each PN may innervate a single subregion. iACT inner antennocerebral tract. Tau-GFP reporter. Anterior is on top. Bar = 10 μm.

Fig. 8. Schematic diagram of head chemosensilla in the third instar larva of *D. melanogaster* including peripheral nerves and central target regions. Olfactory dendrites are shown as a W-symbol, gustatory dendrites as black boxes. Gray circles in the LAL denote subunits, and the gray rectangle indicates the E domain (see text for details). The dashed line represents projections from gustatory DO sensilla to the TR-SOG region (Tissot et al., 1997) which are unlabeled by the lines used here. DIG/DLG distal/dorsolateral group of TO sensilla, DM dome, LPR lateral protocerebrum, MB mushroom bodies, PH pharynx. For other abbreviations, see list.

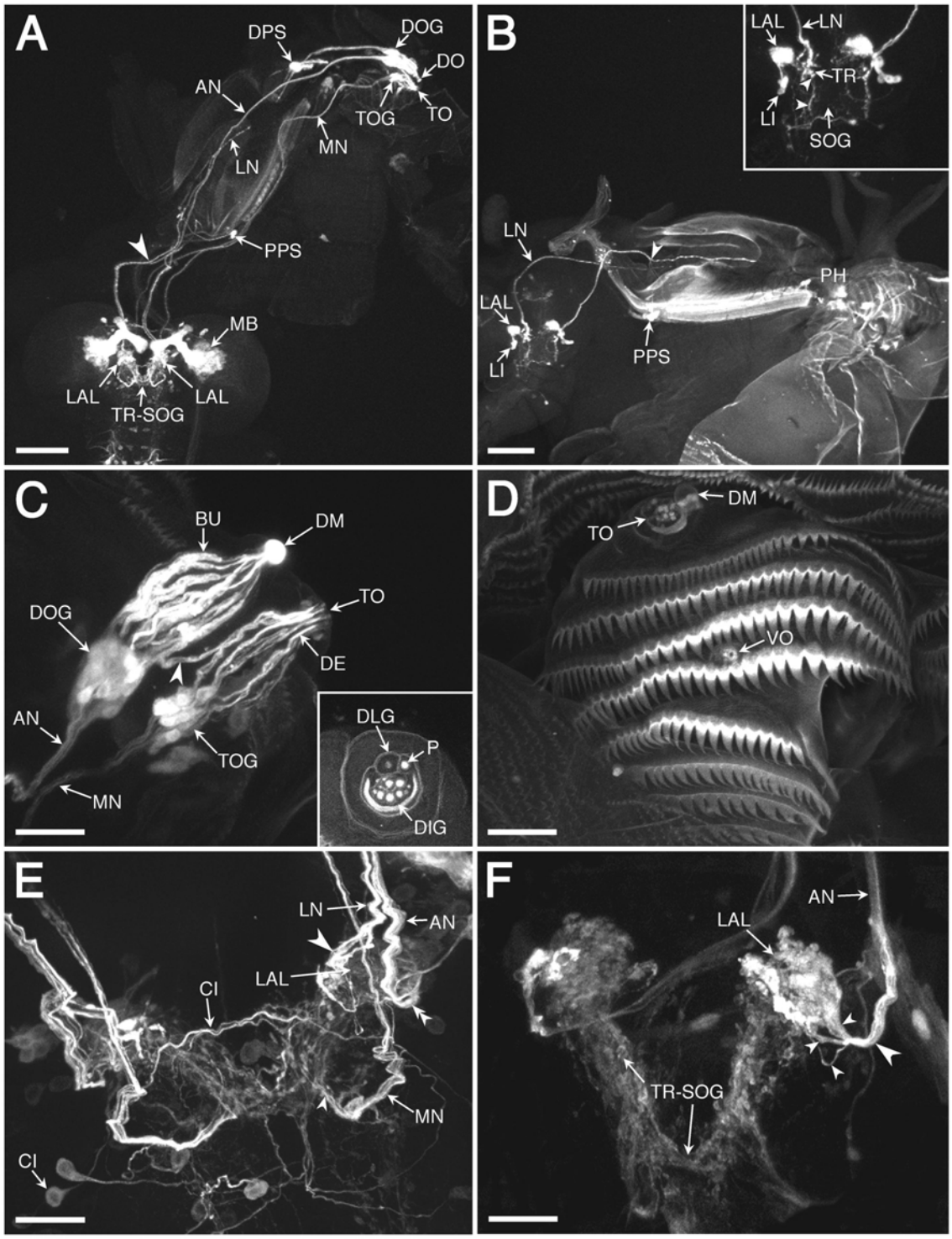
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Fig. 1

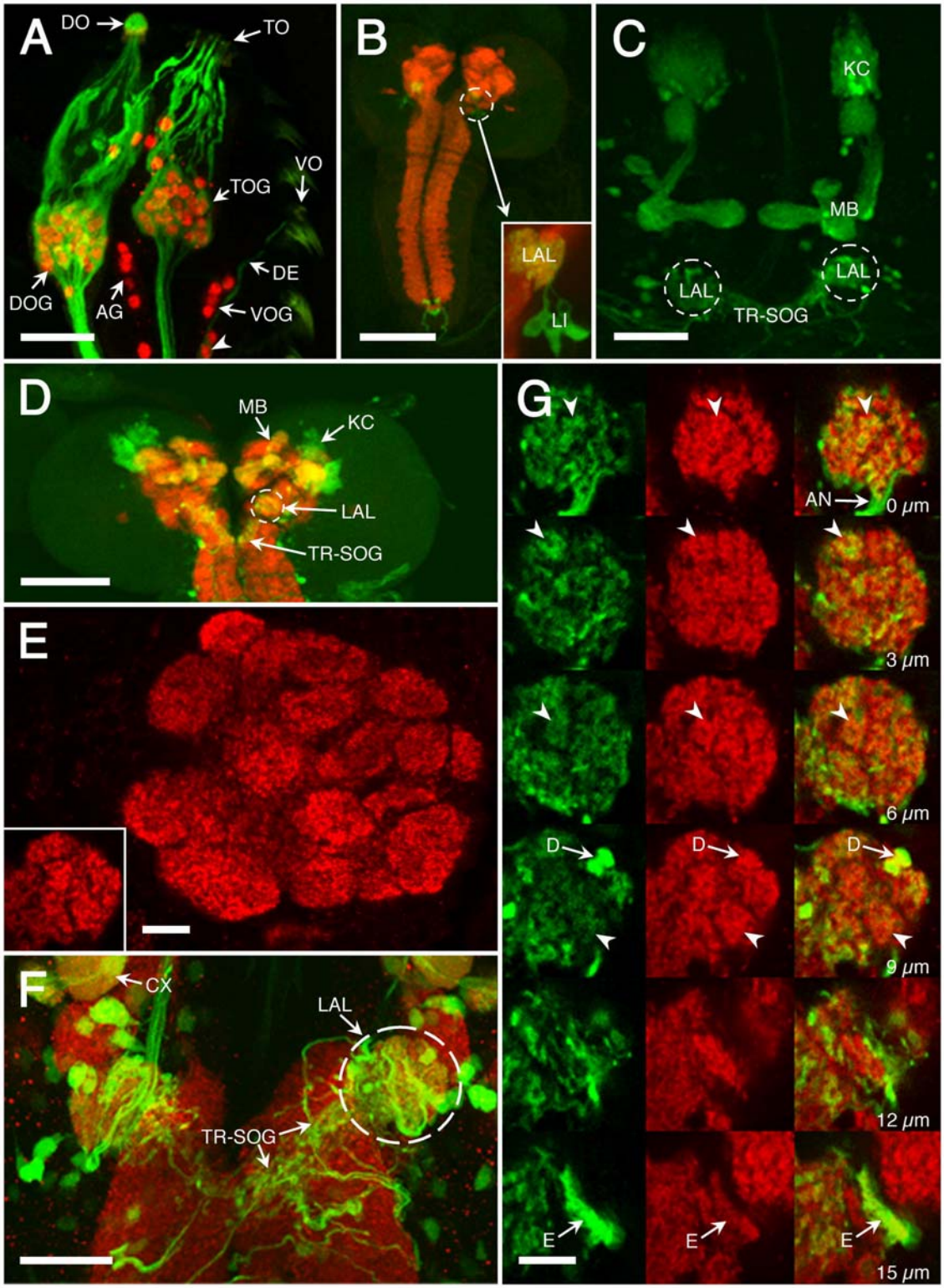
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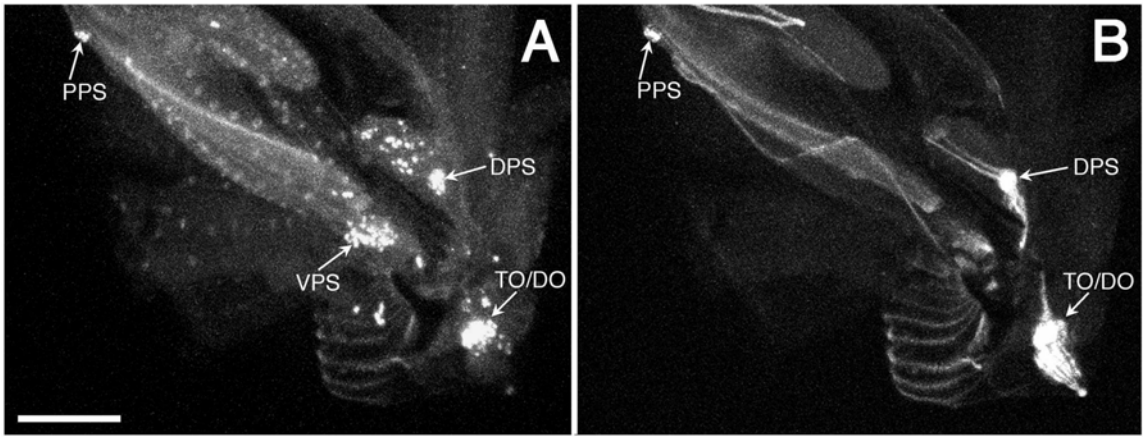
Fig. 2



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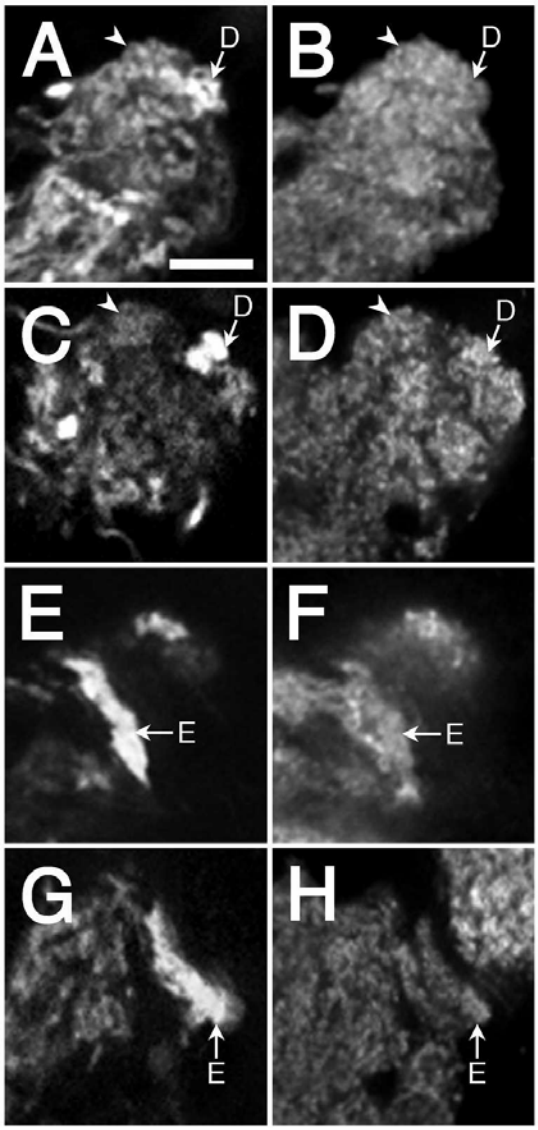

Fig. 4

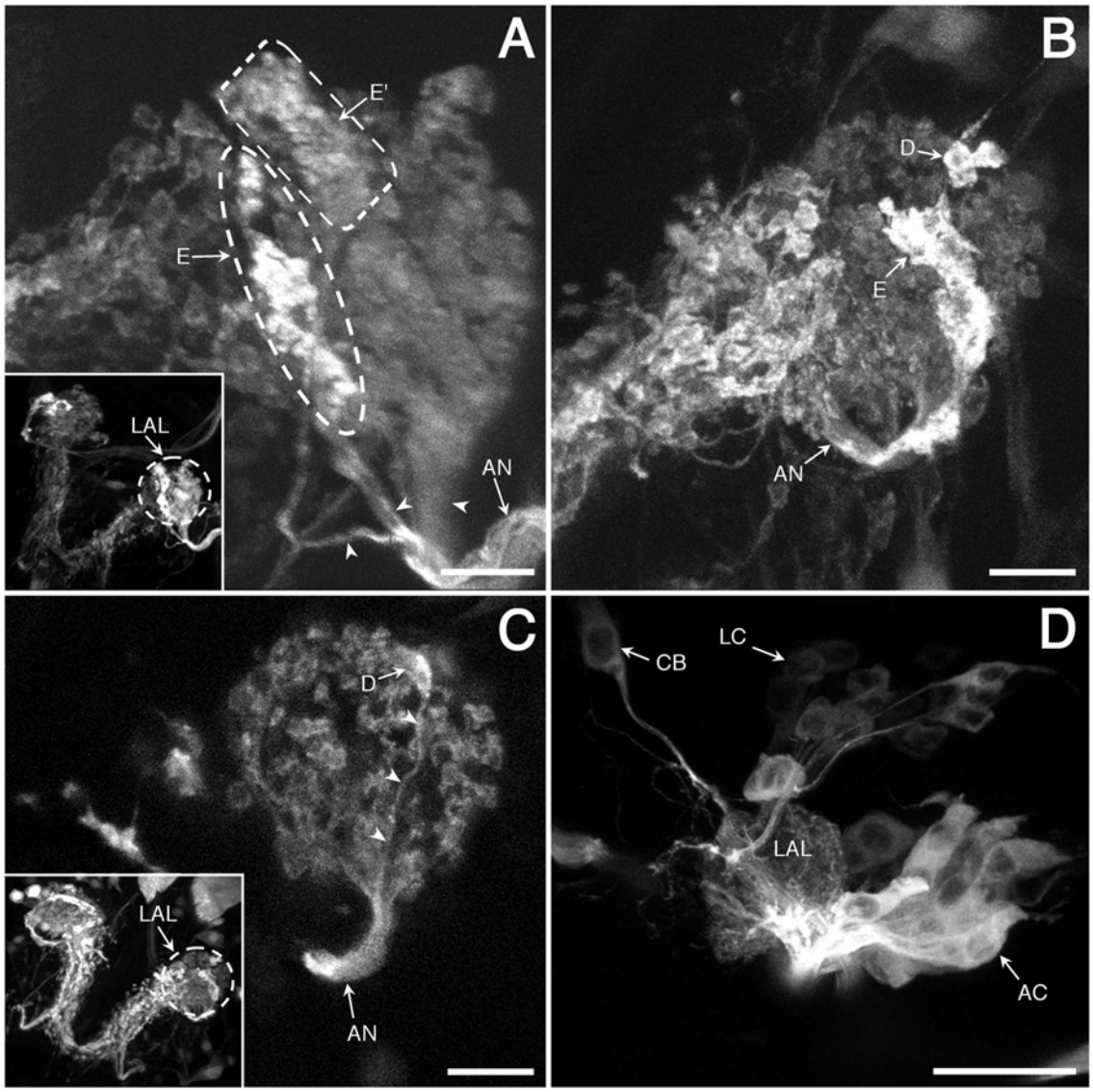

Fig. 5

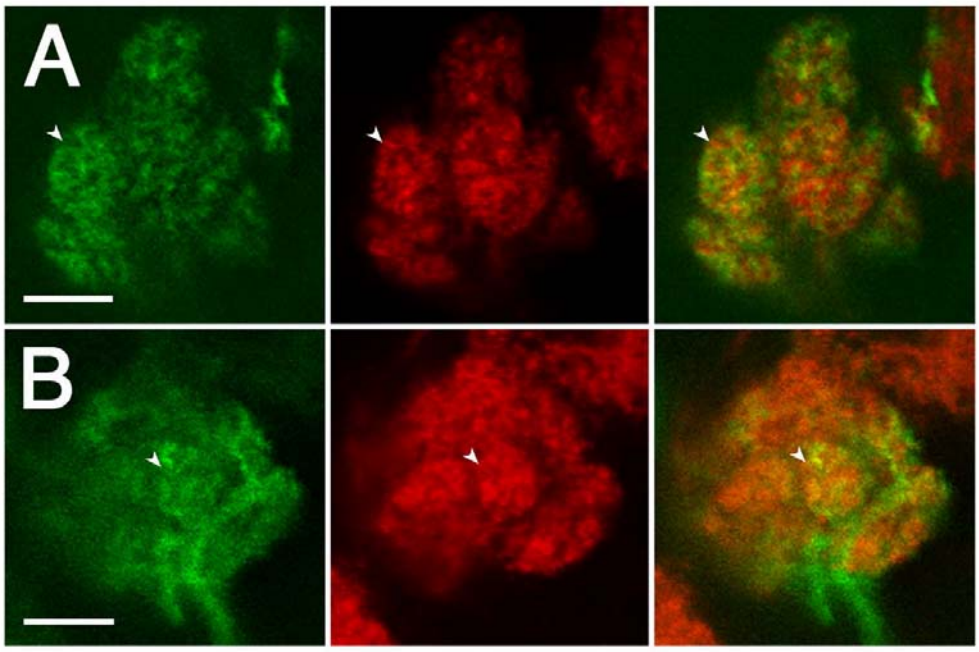

Fig. 6

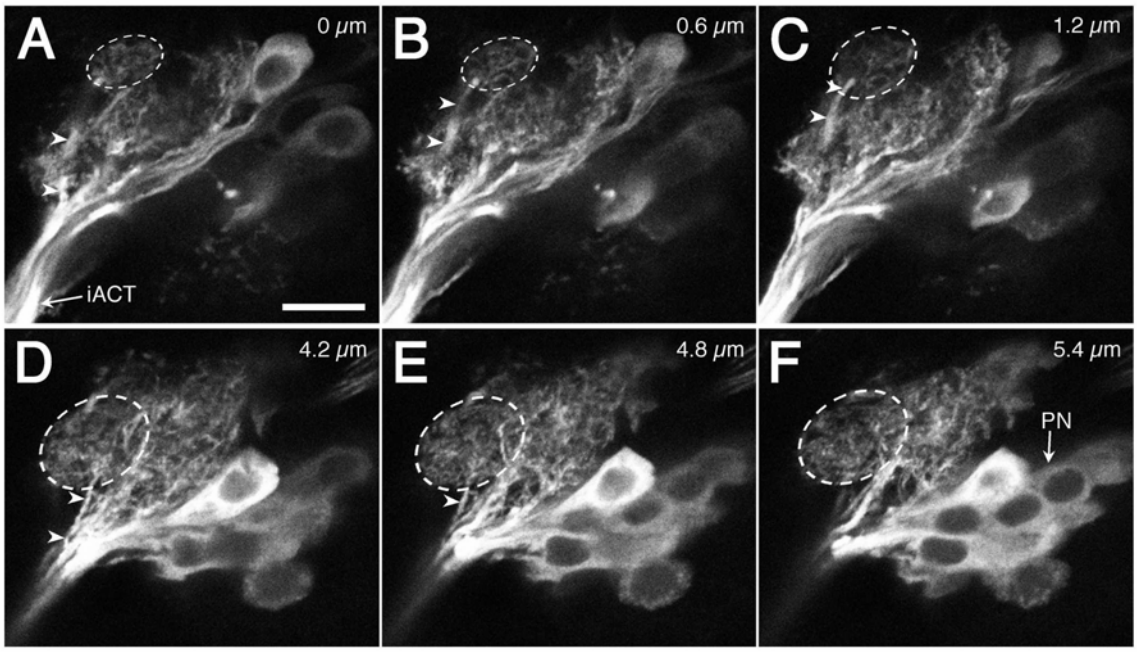

Fig. 7

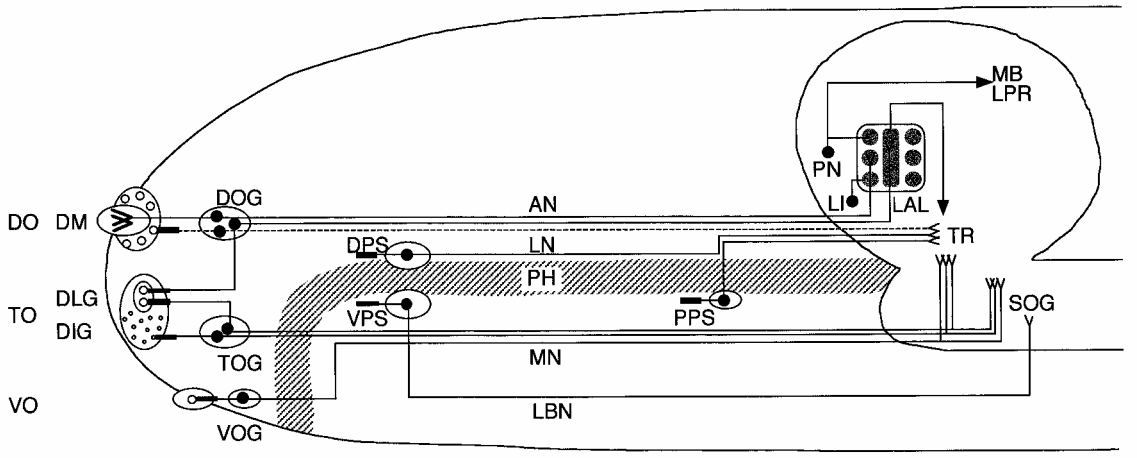
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Fig. 8

Chapter 2

Immunoreactivity against choline acetyltransferase, GABA, histamine, octopamine and serotonin in the larval chemosensory system of *Drosophila melanogaster*

This chapter has been submitted to *The Journal of Comparative Neurology*.

ABSTRACT

We study the distribution of choline acetyltransferase (ChAT), γ-aminobutyric acid (GABA), histamine, octopamine and serotonin in the larval chemosensory system of *Drosophila melanogaster*. Colocalization at the confocal level with GFP or Tau-GFP reporters, expressed in selected P[GAL4] enhancer trap lines was used to identify the cellular localization of these neurotransmitters. As in the adult fly, larval olfactory afferents project into the (larval) antennal lobe (LAL), where they synapse onto local interneurons and projection neurons, while gustatory afferents terminate essentially in the tritocerebral-suboesophageal (TR-SOG) region. We demonstrate that the neuropils of the LAL and the TR-SOG are immunoreactive to ChAT and GABA. In addition, serotonin- and octopamine immunoreactive fibers are present in the LAL. ChAT immunoreactivity is localized in subsets of olfactory and gustatory afferents and in many of the projection neurons. In contrast, GABA is expressed in most, perhaps all of the local interneurons. Finally, serotonin immunoreactivity in the LAL derives from a single neuron that is situated close to the LAL and arborizes in additional neuropil regions. These findings resemble the situation in the adult fly. Hence, considering the highly reduced numbers of odorant receptor neurons in the larva as shown in our previous study (cf. Python and Stocker, 2002), the larval system may become an attractively simple model system for studying the roles of neurotransmitters in olfactory processing.

Abbreviations: ACh acetylcholine, AL antennal lobe, ChAT choline acetyltransferase, DO dorsal organ, GABA γ-aminobutyric acid, IR immunoreactive, IRy immunoreactivity, LAL larval antennal lobe, LI local interneuron, PN projection neuron, SOG suboesophageal ganglion, TO terminal organ, TR tritocerebrum

INTRODUCTION

Drosophila melanogaster has become a major focus of attraction as a chemosensory model system, because of the recent discovery of odorant receptors (Clyne et al., 1999; Vosshall et al., 1999) and the mammalian-like connectivity of the odorant receptor neurons (Ressler et al., 1994; Vosshall et al., 2000; Scott et al., 2001). In addition, the fly olfactory system is remarkable for its small numbers of sensory neurons and interneurons. Compared e.g. to rodents, the odorant receptor neurons are reduced by three orders of magnitude. Thus, no more than 1300 olfactory afferents project to the antennal lobe (AL) where they synapse with two types of target interneurons, local interneurons (LIs) and projection neurons (PNs). The former establish 'horizontal' connections within the AL, while the latter convey olfactory information 'vertically' to higher centers, i.e., the mushroom body calyx and the lateral protocerebrum. The AL is therefore the primary relay center for the processing of olfactory information (Homberg et al., 1989; Boeckh et al., 1990; Stocker, 1994, 2001; Hildebrand, 1996; Hansson, 1999). In contrast to the olfactory system, the gustatory system is less well known, apart from the fact that gustatory target regions are scattered over the tritocerebrum (TR), the suboesophageal ganglion (SOG) and the ventral nerve cord (Stocker and Lawrence, 1981). For example, very few data are available on gustatory target interneurons (Nayak and Singh, 1985).

A number of studies have shown that acetylcholine (ACh) and γ-aminobutyric acid (GABA) are two major neurotransmitters involved in olfactory processing of the insect AL (reviews: Bicker, 1999; Homberg and Müller, 1999). Moreover, the biogenic amines dopamine, histamine, octopamine and serotonin, which have been implicated as neurotransmitters or modulators in the insect CNS, seem also to play a role in the olfactory system (reviews: Homberg and Müller, 1999; Monastirioti, 1999; Nässel, 1999; Blenau and Baumann, 2001). Finally, there is increasing evidence that a number of neuropeptides as well as nitric oxide may act as neurotransmitters in the ALs

(reviews: Müller, 1997; Homberg and Müller, 1999; Nässel, 1999; Taghert, 1999; Nässel, 2000; Bicker, 2001).

In contrast to the adult fly, only few studies have dealt with the chemosensory system of the larva (Singh and Singh, 1984; Tissot et al., 1997;Heimbeck et al., 1999; Oppliger et al., 2000; Scott et al., 2001; reviews: Cobb, 1999; Stocker, 1994, 2001). Moreover, although neurotransmitter distribution has been studied in the larval CNS (Vallés and White, 1986, 1988; Gorczyca and Hall, 1987; Pollack and Hofbauer, 1991; Monastirioti et al., 1995; Nishikawa and Kidokoro, 1999), reference was not given to the chemosensory system. To do so seems however interesting because of an extremely reduced number of odorant receptor neurons, yet a seemingly adult-like organization of the larval antennal lobe (LAL) (Python and Stocker, 2002). This suggests the larval olfactory system of *Drosophila* as a promising and simple adult-like model system. Briefly, 21 odorant receptor neurons localized in the so-called dorsal organ (DO) – the larval antenna – send their afferents into the LAL, whereas an estimated 80 gustatory afferents from the terminal organ (TO) and four other sensilla on the head and on the pharynx terminate in the TR-SOG region (Tissot et al., 1997; Heimbeck et al., 1999; Python and Stocker, 2002).

To expand the description of the larval chemosensory system, we investigated using confocal microscopy the distribution of choline acetyltransferase (ChAT), an established marker of ACh activity, and of the other classical neurotransmitters / modulators GABA, histamine, octopamine and serotonin in the chemosensory system of the third larval instar. Due to the absence of a reliable marker for dopamine in the third instar larva and the reported lack of immunostaining against this neurotransmitter in the fly antennal lobe (Nässel and Elekes, 1992; Lundell and Hirsh, 1994; Homberg and Müller, 1999), we have not included the search for dopamine expression in the present study. We made use of three P[GAL4] enhancer trap lines (Brand and Perrimon, 1993), described in previous work (Python and Stocker, 2002) to examine the cellular localization of neurotransmitter immunoreactivity (IRy), both at the sensory and central level. These three lines are very useful markers of chemosensory afferents, of LIs and

PNs. We observe strong ChAT IRy and GABA IRy in the olfactory and gustatory target regions, the LAL and the TR-SOG, as well as IR varicosities for octopamine and serotonin in the LAL. Our data suggest that subsets of olfactory and gustatory afferents and many PNs are cholinergic, and that all LIs labeled by the marker line used are GABAergic. Finally, a serotonergic interneuron – independent of LIs or PNs – was found to project into the LAL.

MATERIALS AND METHODS

The P[GAL4] line *GH146* was generated by G. Heimbeck (Stocker et al., 1997; Heimbeck et al., 2001), line *4551* was obtained from J.-F. Ferveur (Université de Bourgogne, Dijon) and line *189Y* (Osborne et al., 1997) was provided by K. Kaiser (University of Glasgow). As UAS-reporter strains, we used UAS-GFP (Yeh et al., 1995) and UAS-Tau-GPF (Murray et al., 1998), both kindly provided by A. H. Brand (Wellcome/CRC, Cambridge). Animals were raised between 18°C and 25°C on standard cornmeal medium. P[GAL4]/UAS-reporter heterozygotes were collected as feeding third instar larvae.

The antibody directed against choline acetyltransferase (ChAT, # 4B1; a generous gift from P. M. Salvaterra; Beckman Institute, City of Hope) was applied at 0.6µg/ml, anti-Histamine (kindly provided by E. Buchner, University of Würzburg, Germany) at 1:500 or 1:1000, anti-Octopamine (Chemicon) at 1:500, anti-serotonin (Sigma) at 1:1000, and anti-GABA (# 4TB, provided by H. Dircksen, University of Bonn, Germany) at 1:500 or 1:1000. Cy3-coupled goat anti-mouse or anti-rabbit IgGs were used as secondary antibodies (1:100, Jackson ImmunoResarch).

For antibody staining, the larvae were essentially processed as previously described (Python and Stocker, 2002). However, for anti-Histamine and anti-Octopamine, we used a fresh solution of 4% EDAC (Sigma) dissolved in phosphate

buffered saline containing 0.2% Triton X-100 (PBS-T, pH 7.2). Moreover, for anti-GABA staining (Homberg et al., 1999), larval tissues were fixed for 2 hours on ice in a fresh solution of 1 part (by volume) 25% glutaraldehyde (Fluka), 3 parts saturated picric acid (Fluka) and 1% acetic acid (Merck). After blocking with goat serum (Python and Stocker, 2002) and applying the anti-GABA antibody, the tissues were treated with a solution containing 0.13M NaBH4, 0.1M Tris/HCl (pH 7.5) and 0.3M NaCl. Incubation with the secondary antibody was followed by mounting in Vectashield medium (Vector Laboratories).

Whole-mount preparations were viewed on a Biorad MRC 1024 confocal microscope equipped with a Kr/Ar laser. Optical sections were imaged at intervals of 0.5 to 2 μm. Image analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The Adobe PhotoShop program was used for image pseudocoloring.

RESULTS

We have used the P[GAL4] enhancer trap lines *4551*, *189Y* and *GH146* as neuroanatomical markers for analyzing the IRy pattern against ChAT and the neurotransmitters / modulators GABA, histamine, octopamine and serotonin in the chemosensory system of the third instar larva. IRy to ChAT – the ACh-synthesizing enzyme – is commonly used in *Drosophila* to establish the presence of ACh, due to the lack of specific antibodies against cholinergic neurons (Homberg, 1994; Yasuyama et al., 1995; Takagawa and Salvaterra, 1996). Double labeling using fluorescently tagged antibodies against ChAT or the neurotransmitters, together with the GAL4-driven GFP or Tau-GFP reporters allowed us to study at the confocal level a possible cellular colocalization. Line 4551 shows expression essentially in all of the 21 olfactory afferents from the DO and in a subset of gustatory afferents from the TO, including their

terminals in the LAL and TR-SOG region, respectively (Python and Stocker, 2002). Line *189Y* labels chemosensory afferents of the labral nerve as well as a subset of five to six LIs, whereas line *GH146* identifies about 50 PNs from an unknown total. Neither for ChAT nor for the neurotransmitters studied here, IRy was observed in the cell bodies of the sensory neurons. However, staining was seen in afferent terminals and in central elements of the chemosensory pathway. Yet, pharyngeal sensilla were not studied here.

Choline acetyltransferase

Previous reports have shown strong ChAT IRy in the entire larval neuropil including the LAL (Gorczyca and Hall, 1987; Yasuyama et al., 1995). A considerable proportion of this IRy appears to be due to the terminals of cholinergic afferents (Yasuyama and Salvaterra, 1999). In the present work we tried to test this notion in the larval brain. Interestingly, the terminals of the 21 olfactory afferents which all express GFP in line *4551* (Python and Stocker, 2002) often overlap in the LAL with ChAT-IR elements in the LAL (Fig. 1A,B). In addition, two recently discovered subunits 'D' and 'E' inside the LAL neuropil, which are visualized by the neuropil-specific monoclonal antibody nc82 (cf. Python and Stocker, 2002) coincide perfectly with the structures labeled by anti-ChAT (Fig. 1C-F). These results suggest that ChAT may indeed be expressed in afferent terminals. ChAT staining is also seen in the antennal nerve at its entrance in the LAL (Fig. 1A,B), but neither in the peripheral parts of the nerve nor in the sensory neurons of its origin, the DO. Lack of anti-ChAT staining in sensory neurons was also reported from the adult olfactory system (Yasuyama et al., 1995). In contrast, odorant receptor neurons on the antenna and maxillary palps were readily labeled by ChAT/lacZ transformants (Kitamoto et al., 1995; Yasuyama and Salvaterra, 1999). Hence, the absence of ChAT IRy in the peripheral part of the larval olfactory system may not be conclusive.

Colocalization between GFP expressing afferent structures in line *4551* and elements labeled by anti-ChAT occurs also in the gustatory target region, i.e. the TR-SOG area (Fig. 1A,B). Minor IRy is seen in the labral nerve (not shown), but none in the

maxillary nerve or in its origin, the TO (Fig. 1A,B). Again, since gustatory neurons in the adult were labeled in ChAT/lacZ transformants (Kitamoto et al., 1995) (but probably not by anti-ChAT staining), the lack of ChAT IRy does not exclude the cholinergic nature of larval gustatory neurons.

In addition to the colocalization of ChAT with chemosensory afferents, we observe ChAT IRy in target neurons of the LAL. Overlap with reporter expression in interneurons is clearly seen in the PN marker line *GH146* (Fig. 1I,J), but not in the LI marker line *189Y* (Fig. 1G,H). According to their shape and their output processes in the inner antennocerebral tract, the labeled neurons *in GH146* are obviously PNs. From the two clusters of PNs (cf. Python and Stocker, 2002), only the anterodorsal one carries IRy, but not the lateral one (Fig. 1I,J). Moreover, only approximately half of the anterodorsal PNs show anti-ChAT staining. In the PN region, we also observed ChAT-positive cell bodies that do not overlap with GFP-positive PNs of the *GH146* line, indicating that the number of PNs labeled by this line is incomplete (Fig. 1I,J). In agreement with our data, subsets of PNs in the adult olfactory pathway of *Drosophila* (Buchner et al., 1986; Gorczyca and Hall, 1987), *Manduca sexta* (Homberg et al., 1995) and the honeybee (Kreissl and Bicker, 1989; Bicker, 1999) were supposed to be cholinergic. In summary, the presence of ChAT IRy in olfactory and gustatory terminals and in subsets of PNs suggests that ACh may play a major role in larval olfactory and gustatory processing.

GABA

Despite some background staining displayed by the anti-GABA antiserum, it has proven useful to reveal GABAergic neurons in the locust, being identical to the IRy pattern for the GABA-synthesizing enzyme glutamic acid decarboxylase (Homberg et al., 1999). In the larval CNS of *Drosophila* we observe GABA IRy in the LAL and the TR-SOG, but the staining pattern does not coincide with the terminals of olfactory or gustatory afferents visualized by line *4551* (Fig. 2A,B). Moreover, neither the chemosensory neurons in the DO and TO nor the nerves carrying their afferents show

any anti-GABA staining. In contrast, all the LIs labeled by line *189Y* exhibit strong GABA IRy (Fig. 2C,D). Numerous additional GABA-IR somata are present in the brain, many of them even in close vicinity to the LAL. Yet, none of them seems to correspond to a PN, as no colocalization was observed with any of the 50 PNs labeled by the line *GH146* (Fig. 2E,F) (Python and Stocker, 2002). This is unlike *M. sexta* where subsets of PNs were reported to be GABA-IR (Hoskins et al., 1986). It is conceivable that some among these additional GABA-IR neurons represent LIs that do not express GAL4 in the *189Y* line. However, the majority of them remain unknown. Their abundance also in the TR-SOG cortex region even suggests that some of the gustatory interneurons – yet unidentified – might be GABAergic as well.

These data for the first time assign GABA IRy in *Drosophila* positively to LIs. Previous studies had reported higher-than-background GABA IRy in the glomeruli of the adult AL (Aronstein and ffrench-Constant, 1995; Harrison et al., 1996). The presence of GABA in LIs is well known from a variety of species, such as honeybees, *M. sexta, Periplaneta americana* and *Schistocerca gregaria* (review: Homberg and Müller, 1999). These parallels suggest that the role played by GABA in olfactory processing might be shared in different species as well as in adult and larval systems.

Histamine

Based on the IRy of an antibody directed against histamine, this biogenic amine was proposed as a major neurotransmitter in photoreceptor and mechanosensory bristle neurons of adult *Drosophila* (Pollack and Hofbauer, 1991; Buchner et al., 1993). For example, afferents from antennal mechanosensory bristles were labeled, but neither olfactory afferents (nor presumably gustatory afferents from the legs). Consistent with this view, we did not observe any histamine IRy in the chemosensory system of the larva when applying the same antibody, neither in sensory neurons of the DO and TO nor in the LAL and its cellular components LIs and PNs (Fig. 3A-D). This is in contrast to a number of hemi- and holometabolous insects in which histamine IRy was detected in subsets of LIs (Homberg and Müller, 1999). Yet, we found anti-histamine label in a

cluster of three neurons and two additional separate neurons in each brain hemisphere. Most or all of them establish arborizations in the SOG region but do not show any obvious overlap with putative gustatory terminals labeled by line *4551* (Fig. 3A,B). Thus, histamine does not seem to be involved as a neurotransmitter in fly larval primary chemosensory processing. We also note that none of the chemosensory head nerves – antennal, labral or maxillary nerve – exhibits distinct histamine IRy. Hence, if histamine in the larva shares its adult function, then neither the DO nor the TO appear to comprise mechanosensory components.

The adult brain was reported to contain about 12 histamine-IR pairs of cells (Pollack and Hofbauer, 1991). However, the relations between them and the five pairs we observe in the larval brain remain unknown. Likewise, possible links between an additional 20 histamine-IR neurons in the ventral nerve cord (Fig. 3A) and 18 histamine-containing neurons in the thoracico-abdominal ganglia of adult *Drosophila* (Nässel et al., 1990) remain in the dark.

Octopamine

The general pattern of octopamine IRy in the larval brain and SOG has been described previously (Monastirioti et al., 1995). Accordingly, a cluster of 10-14 octopamine-IR cells in the ventral midline of the SOG exhibits profuse IR varicosities throughout the neuropil. We confirm this pattern, despite elevated background staining of the anti-octopamine antibody used (Fig. 3E,I). Moreover, we note that the nerves carrying olfactory and gustatory afferents as well as their cell bodies in the DO and the TO are devoid of labeling. Although low-density varicose IR fibers are present in the LAL, there is no coincidence with afferent terminals as shown by line *4551* (Fig. 3E). Similarly, there is no overlap of octopamine IRy and reporter labeling in the LI and PN marker lines (Fig. 3F-J). This is in contrast to the adult fly in which octopamine-IR cells were found found in the AL cortex, close to the midline (Monastirioti et al., 1995). We believe that one of the octopamine-IR cell bodies in the ventral midline gives rise to the arborization in the LAL but are not able to exactly identify the neuron. Very likely the

adult octopamine-IR cells near the midline derive from the larval midline cluster (Monastirioti et al., 1995). Finally, no evidence of octopamine IRy is present in the terminal region of gustatory afferents shown by line *4551*. These data indicate that octopamine might at most be involved indirectly in larval olfactory processing, perhaps as a general neuromodulator.

Serotonin

The general pattern and development of serotonin IRy in *Drosophila* was described previously (Vallés and White, 1988). In the larva, four IR clusters comprising a total of 22 neurons were observed, whose arborizations cover most of the central brain neuropil. However, their relations with chemosensory pathways were not studied. Using the *4551* marker line, we note the absence of serotonin IRy in chemosensory neurons and their afferents in the peripheral nerves. Also, we see no IR overlap with olfactory afferents in the LAL, and no staining at all in the TR-SOG region (Fig. 4A), suggesting that serotonin is not involved in afferent chemosensory processing. Moreover, neither the LIs nor the PNs visualized by the two marker lines show colocalization of serotonin IRy (Fig. 4B-D). On the other hand, the LAL displays a network of varicose serotonin-IR fibers which derive from a single cell body close to the anterodorsal PN cluster (Fig. 4C,D). This neuron does not extend a process in the inner antennocerebral tract, argueing against its PN identity. Instead, it appears to establish arborizations in other neuropil regions of the brain (not shown), but the documentation of its morphology is hampered by two accompanying neurons which are even stronger serotonin-IR. They exhibit a contralateral branch, but do not innervate the LAL (Fig. 4C,D). Comparison with the study of Vallés and White (1988) suggests these three neurons belong to the SP2 cluster. Interestingly, all serotonin-IR larval neurons including the SP2 neurons appear to persist through metamorphosis and become integrated into the adult brain (Vallés and White, 1988). Moreover, serotonin-IR varicosities have been observed in the adult AL, although the corresponding cell body/bodies remain(s) unknown (Vallés and White, 1988). In *M. sexta*, a larval serotonergic AL interneuron is remarkable for its persistence through metamorphosis (Kent et al., 1987). Although its anatomy is different

from the neuron we observe, they might share similar functions, perhaps as modulatory neurons in the larval and adult olfactory pathway.

DISCUSSION

Here we determine the cellular localization of ChAT, GABA, histamine, octopamine and serotonin in the chemosensory system of the third larval instar, by applying the corresponding antibodies to three *Drosophila* P[GAL4] enhancer trap lines that show reporter expression in olfactory and gustatory afferents, and/or in their central target neurons (Python and Stocker, 2002). As shown before (Python and Stocker, 2002), the larval chemosensory system lends itself as an attractive model system, due to a surprisingly low number of sensory neurons, and an adult-like organization of the LAL, the primary olfactory center. Studying the neurotransmitter distribution in its cellular elements represents an important next step in the description of this system, which will improve our understanding of olfactory and gustatory information processing in the larva. In the following paragraphs we discuss our data by comparison with the system in the adult fly and with the larval and adult chemosensory systems of other insects.

To demonstrate the distribution of ACh, we employed a monoclonal antibody against ChAT, the enzyme catalyzing ACh synthesis (Gorczyca and Hall, 1987; Yasuyama et al., 1995), because of the lack of useful immunoprobes against this neurotransmitter. As recent studies have shown, the patterns of ChAT IRy, ChAT gene transcripts and ChAT-driven transformants overlap but are not identical, the latter yielding perhaps the highest resolution (Kitamoto et al., 1995; Yasuyama et al., 1995; Yasuyama and Salvaterra, 1999). The lack of ChAT IRy has therefore to be interpreted with caution. In contrast, positive labeling can be considered a reliable marker for cholinergic neurons. For the other neurotransmitters, we used antibodies against the

neurotransmitters themselves providing the most straightforward way to describe their expression pattern.

Olfactory and gustatory afferents

According to the exhaustive study by Yasuyama and Salvaterra (1999), a large proportion of the adult sensory neurons in *Drosophila* appear to be cholinergic. This seems to be true also of *Manduca* (Homberg and Müller, 1999) and the honeybee (Bicker, 1999). In adult *Drosophila*, possibly all the olfactory and gustatory neurons as well as certain mechanoreceptors were shown to express either ChAT or the ChAT-driven reporter lacZ, or to exhibit a hybridization signal with a ChAT mRNA probe (Buchner et al., 1986; Yasuyama et al., 1995; Yasuyama and Salvaterra, 1999). The cholinergic nature of odorant receptor neurons is further supported by ^{3}H-choline uptake studies (Buchner and Rodrigues, 1983). In the embryo, ChAT IRy was observed in the olfactory DO and the visual Bolwig's organ, while in the larva expression in these organs was seen only in ChAT/lacZ transformants (Gorczyca and Hall, 1987; Yasuyama and Salvaterra, 1999). We confirm and extend these data and show that subsets of afferents in the larval antennal nerve and their central target, the LAL (Tissot et al., 1997; Python and Stocker, 2002), display ChAT-IRy. The staining pattern we observe in the LAL neuropil is reminiscent of the reporter expression of the afferent-specific line *4551* and the monoclonal antibody nc82, which highlights the division of the LAL into subunits (Python and Stocker, 2002). However, similar to the adult system (Yasuyama et al., 1995), the ChAT antibody did not reveal staining of sensory cell bodies in the DO. It is conceivable that ChAT expression is strongest in the olfactory arborizations and diminishes in the axons at short distance from the terminals. Potential target neurons of cholinergic olfactory afferents may be LIs, which in the adult have been shown to express nicotinic ACh receptors (Jonas et al., 1994). In addition, subsets of adult AL glomeruli exhibit an enriched density of muscarinic ACh receptors (Blake et al., 1993; Harrison et al., 1995), although their cellular localization remains unknown.

We also note the presence of ChAT IRy in the labral nerve and its colocalization in the TR-SOG region with afferent terminals. In contrast, the exclusively gustatory maxillary nerve did not reveal any ChAT staining. Again, this lack of label could be due to technical limitations. The positive evidence in the gustatory target region renders the cholinergic nature of at least part of the taste neurons very likely.

While the specific ChAT-IR pattern described here in the LAL is likely to be linked to olfactory afferents, other pattern components may be related to putative cholinergic interneurons of the LAL (see below). Finally, we note that none of the other immunoprobes applied showed any staining overlapping with the terminals of olfactory or gustatory afferents – despite of occasional staining in the LAL (see below).

Local interneurons

The LI-specific line *189Y* allowed us to clearly colocalize GABA IRy in all LIs labeled by this line. Moreover, GABA IRy was present in other neurons close-by, which did not overlap with PNs labeled by line *GH146* (see below). This suggests that they might represent additional, GAL4-negative LIs. Considering data from *M. sexta* (Hoskins et al., 1986), the presence of GABA IRy in the entire set of LIs would not be unusual. None of the other immunoprobes tested here showed colocalization in LIs.

While GABA immunostaining of LIs has been demonstrated in a number of holometabolous and hemimetabolous insects (see below), the only relevant data available in *Drosophila* refer to higher-than-background GABA IRy and strong IRy against a GABA receptor subunit in the glomeruli of the adult AL (Aronstein and ffrench-Constant, 1995; Harrison et al., 1996). However, due to the lack of cell body staining, it was not possible to determine the cellular localization of this neurotransmitter and its receptor. Our data suggest for the first time a GABAergic function of LIs in *Drosophila*, at least at the larval stage.

In the honeybee, *M. sexta, P. americana* and *S. gregaria*, the GABAergic nature of LIs has been well demonstrated both anatomically and functionally (reviews: Bicker, 1999; Homberg and Müller, 1999). In the cockroach, most of the synaptic input of uniglomerular PNs was shown to derive from GABA-IR LI profiles (Malun, 1991), suggesting GABAergic effects of LIs on PNs. Indeed, GABA-mediated inhibition was shown to affect odorant-evoked synchronous activity across PNs, a process which seems to be involved in the discrimination of similar – but not of dissimilar – odorants (Stopfer et al., 1997).

In contrast to data from adult bees and cockroaches, we cannot confirm histamine IR in subsets of LIs (Homberg and Müller, 1999). In fact, no obvious histamine IRy was observed in the entire larval chemosensory system, consistent with data from its adult counterpart (Pollack and Hofbauer, 1991; Buchner et al., 1993).

The fully-developed morphology of the LIs in the *Drosophila* larva (Python and Stocker, 2002) and their neurotransmitter expression suggest that they are mature and functional. Interestingly, GABAergic larval LIs are also known from *M. sexta* (Homberg and Hildebrand, 1994). Yet, whether larval and adult LIs share the same function remains to be shown. Interestingly, the LI marker line *189Y* which we have used here was reported to be mutant for larval foraging behavior (Osborne et al., 1997; Shaver et al., 1998; de Belle and Kanzaki, 1999), which may suggest hidden relations between this behavioral phenotype and a functional defect in LIs.

Projection neurons

Double-labeling in the PN marker line *GH146* shows anti-ChAT IRy in many of the PNs, suggesting that they are fully functional at the larval stage. In contrast, all other immunoprobes tested, proved negative in PNs. The observed anti-ChAT staining is compatible with data from the adult olfactory system of several species. In adult *Drosophila*, the inner antennocerebral tract carrying the PN output fibers and the PN terminals in the mushroom body calyx exhibit strong acetylcholinesterase staining and

ChAT IRy (Buchner et al., 1986; Gorczyca and Hall, 1987; Yusuyama et al., 2002). In addition, cell bodies associated with the AL express the ChAT gene and are ChAT IR (Yasuyama et al., 1995, 1996). Both in *M. sexta* and the honeybee, subsets of PNs reveal acetylcholinesterase activity (Kreissl and Bicker, 1989; Homberg et al., 1995). Interestingly, our data show that only PNs belonging to the anterodorsal (but not the lateral) cluster of these neurons may be cholinergic. According to recent reports in adult flies, the two clusters are associated with different AL glomeruli and exhibit different output branching patterns in the mushroom body calyx and the lateral protocerebrum (Jefferis et al., 2001; Marin et al., 2002). However, it is not known whether they differ with respect to their ChAT IRy. Nevertheless, the neurochemical difference we observe in the larval system strongly suggests that the two clusters may represent two functionally distinct subpopulations.

In contrast to *M. sexta* (Hoskins et al., 1986; Homberg et al., 1989) and the honeybee (Schäfer and Bicker, 1986), we did not observe GABA IRy in PNs. However, we cannot exclude that PNs which are GAL4-negative in the *GH146* line may in fact be anti-GABA positive.

Other neurons involved in the chemosensory pathway

Apart from chemosensory afferents, LIs and PNs, at least another cellular element appears to be involved directly in larval olfactory processing, i.e., a putative serotonergic interneuron that establishes varicose IR terminals in the LAL and in other brain areas. Unfortunately, serotonin IRy in two adjacent neurons prevented us from verifying its entire morphology. According to a previous report (Vallés and White, 1988), most if not all larval serotonin-IR neurons may persist into adulthood. Indeed, their study showed serotonin IR varicosities in the adult AL. However, whether the adult IR element is identical to larval neuron described here remains unknown. Persistence through metamorphosis has been demonstrated for a serotonin-IR neuron in the LAL of *M. sexta* (Kent et al., 1987). Yet, a suspected role of this neuron as a template for glomerular reorganization of the AL has not been confirmed (Oland et al., 1995). Apart

from *M. sexta*, serotonin-IR interneurons with extensive arborizations throughout the AL have been observed in the adult honeybee and in *P. americana* (Rehder et al., 1987; Salecker and Distler, 1990; Homberg and Müller, 1999). In both *M. sexta* and *P. americana* these neurons appear to provide centrifugal input from protocerebral brain centers, suggesting a modulatory function of olfactory processing in the AL (Homberg and Müller, 1999).

Regarding the abundance of GABA-IR cell bodies in the TR-SOG region, we consider it not unlikely that GABA may act as an (inhibitory) neurotransmitter in subsets of larval gustatory target interneurons. However, evidence for this will have to await colocalization in identified neurons. Finally, the sparse innervation of the LAL and other neuropil areas by octopamine-IR processes, deriving from ventral midline neurons, suggests a very general role of this biogenic amine, perhaps as a neuromodulator. An octopaminergic neuron in the bee AL, called VUMmx1 (Kreissl et al., 1994), has become very famous for its role in gustatory-reinforced olfactory conditioning (Hammer, 1993).

Conclusions

We have provided evidence about the identity of some of the neurons in the *Drosophila* larval chemosensory system expressing classical insect neurotransmitters. Our data suggest that subsets olfactory and gustatory afferents and many PNs may be cholinergic, that perhaps all the LIs may be GABAergic, and that serotonin might be involved as a neuromodulator in a single identified neuron of the LAL. These patterns are very similar as in the adult olfactory system of the fly and other insects, suggesting that these neurotransmitters share similar roles in the processing of olfactory (and perhaps gustatory) information at the larval and adult stage. Hence, together with a number of recent studies (Cobb, 1999; Heimbeck et al., 1999; Oppliger et al., 2000; Scott et al., 2001; Python and Stocker, 2002), these data propose the *Drosophila* larva as an attractive alternative chemosensory model system.

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FIGURE LEGENDS

Fig. 1. Larval central chemosensory pathways, showing the distribution of ChAT-immunoreactivity (IRy) (B,D,F,H,J) and GFP (A,C,E) or Tau-GFP (G,I) reporter gene expression, visualized by three P[GAL4] enhancer trap lines. Panels **A**/**B**, **C**/**D**, **E**/**F**, **G**/**H**, and **I**/**J** represent identical confocal sections each. **A-F:** Single sections of line *4551*. Most of the terminals of chemosensory afferents (arrowheads) marked by GFP in the larval antennal lobe (LAL, encircled) and the tritocerebral-suboesophageal neuropil (TR-SOG) express ChAT. Colocalization between GFP and ChAT is particularly obvious in the structural subunits E and D of the LAL. **G,H:** Assembly of confocal sections displays that local interneurons (LI) of the LAL, visualized by the *189Y* marker line, do not exhibit ChAT IRy. **I,J:** In contrast, subsets of projection neurons (PNs) shown by the line *GH146* are anti-ChAT-IR (asterisks) (confocal assembly). Note that IRy is present only in about half of an anterodorsal cluster (AC) of PNs – including their output fibers in the inner antennocerebral tract (iACT) – but not in a lateral cluster (LC). The arrowheads in J indicate ChAT-positive cell bodies that do not overlap with GFP-labeled PNs in *GH146*. Anterior is on top. Bars = 25 µm in A-J.

Fig. 2. Detection of GABA IRy (B,D,F) in the LAL, visualized in confocal images by GFP (A) or Tau-GFP reporter labeling (C,E). The **A**/**B**, **C**/**D**, **E**/**F** panels show the same section(s) each. **A,B:** Single confocal section in which the LAL and the TR-SOG (**A**: line *4551*) show GABA IRy (**B**). The small arrowheads in **A** indicate afferent subunits E and D of the LAL, which lack GABA IRy (cf. Fig. 1C-F). **C,D** (assembly): Black asterisks in **D** denote GABA-IR cell bodies corresponding to the entire set of LIs marked in line *189Y* (white asterisks). **E,F** (assembly): The numerous additional GABA-IR somata around the LAL (**F**: arrowheads) do not coincide with the PNs shown by the *GH146* line (**E**). Anterior is on top. Bars = 10 µm in A-F.

Fig. 3. Confocal images (assemblies) showing the distribution of histamine IRy (red: A-D) and octopamine IRy (red: E,G-J) in the larval CNS, visualized by GFP (green: E) or Tau-GFP reporter staining (green: A-D,F,H-J). **A,B:** No overlap of the two labels is observed in the LAL (encircled) and the TR-SOG (line *4551*). However, the brain hemispheres exhibit a cluster of three somata and two separated somata that are all strongly histamine-IR (arrowheads in A). Moreover, an additional 20 histamine-IR neurons are located in the ventral nerve cord (VNC). At higher magnification (**B**), two histamine-IR neurons can be seen to project into the SOG region (arrowheads). MB, mushroom bodies. **C:** Line *189Y* does not show any overlap of histamine IRy in LIs, the LAL and the labral nerve (LN). **D:** Line *GH146* reveals histamine IRy neither in PNs nor in the iACT (inset). Arborizations of histamine-IR neurons (arrowheads) are observed in the VNC and SOG. CX: calyx, OL: optic lobe. **E:** As shown by line *4551*, the LALs (encircled) are slightly octopamine-IR, while the TR-SOG region, the antennal nerve (AN) and maxillary nerve (MN) lack IRy. **F-H** (same sections): LIs stained by line *189Y* (**F,H**) are not octopamine-IR. **I,J** (line *GH146*; **J:** close-up): As shown by the lack of staining in PNs and in the iACT, octopamine IRy in the LAL is not due to its presence in PNs. Anterior is on top, except for D. Bars = 100 µm in A,D; 25 µm in B,C,J; 50 µm in E,I;10 µm in F-H.

Fig. 4. Distribution of serotonin IRy (red) in the LAL, labeled with GFP (green: A) or Tau-GFP reporters (green: B,C)(confocal assemblies). **A:** Line *4551* displays serotonin IRy in the LAL (insets) but lack of IRy in the TR-SOG region and in the antennal and maxillary nerves (AN, MN). **B:** No IRy is seen in LIs (line *189Y*). **C,D:** This pair of panels shows the same confocal image in line *GH146*, i.e. anti-serotonin labeling (**D**) and double labeling with the Tau-GFP reporter (**C**). In the neighborhood of the LAL and the PNs, three serotonin-IR interneurons are recognized, one of which (small arrowhead) sends a process into the LAL, while the other two (large arrowheads) exhibit a contralateral branch (large arrowhead). All the PNs shown by *GH146* and the iACT lack IRy. The yellow color in panel C does not indicate overlap with the PNs. Anterior is on top. Bars = 25 µm in A; 10 µm in B-D.

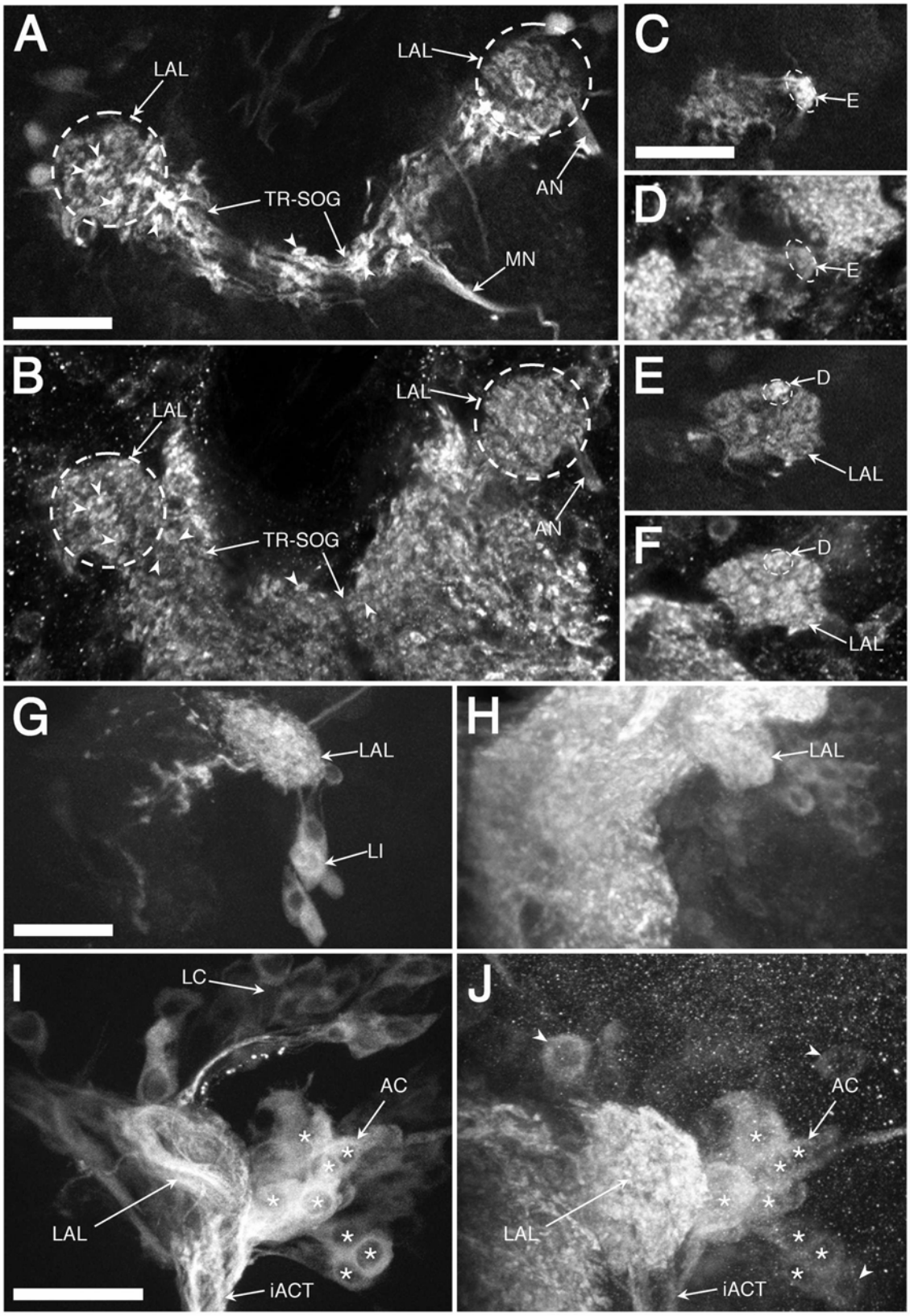
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Fig. 1

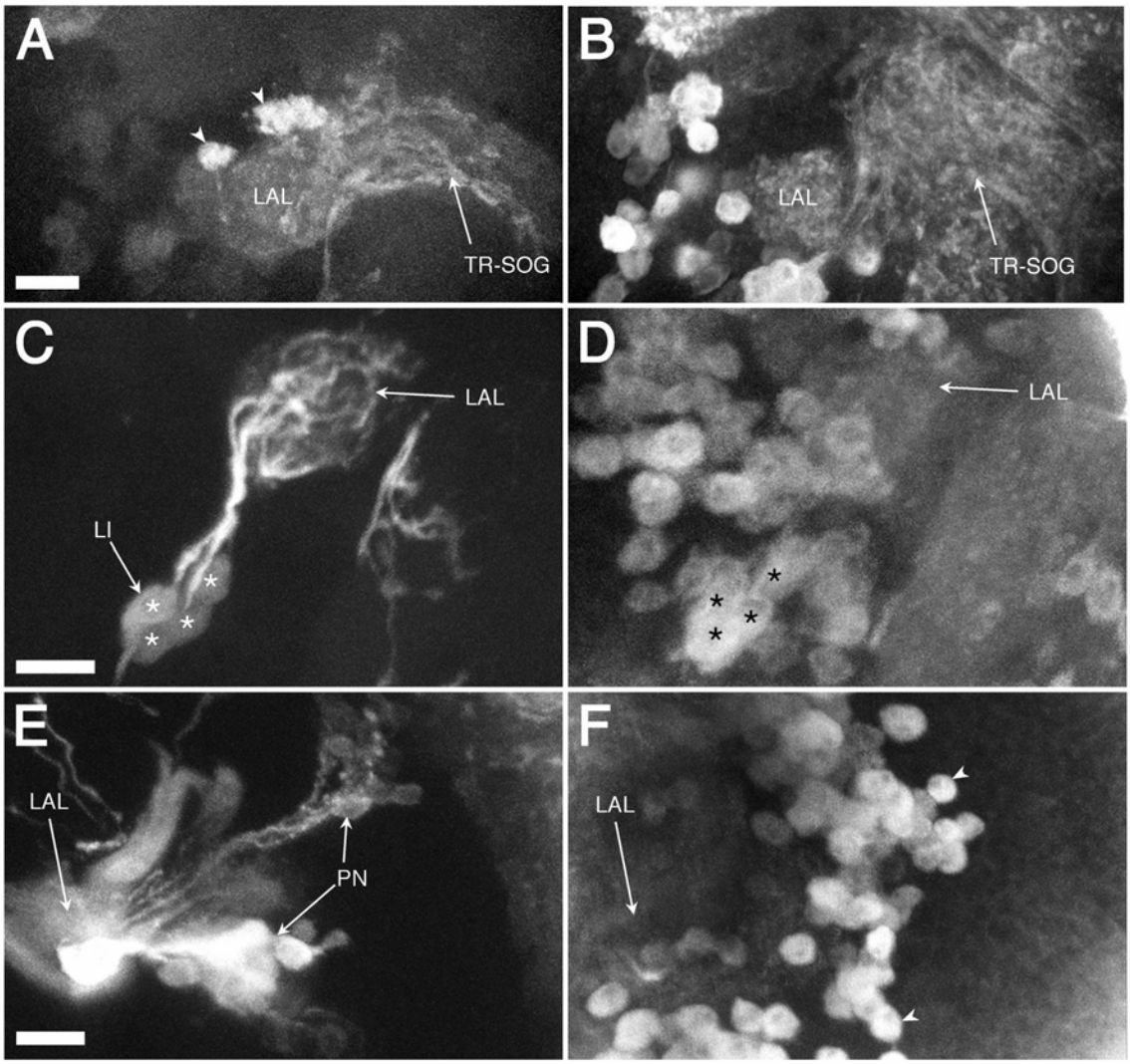

Fig. 2

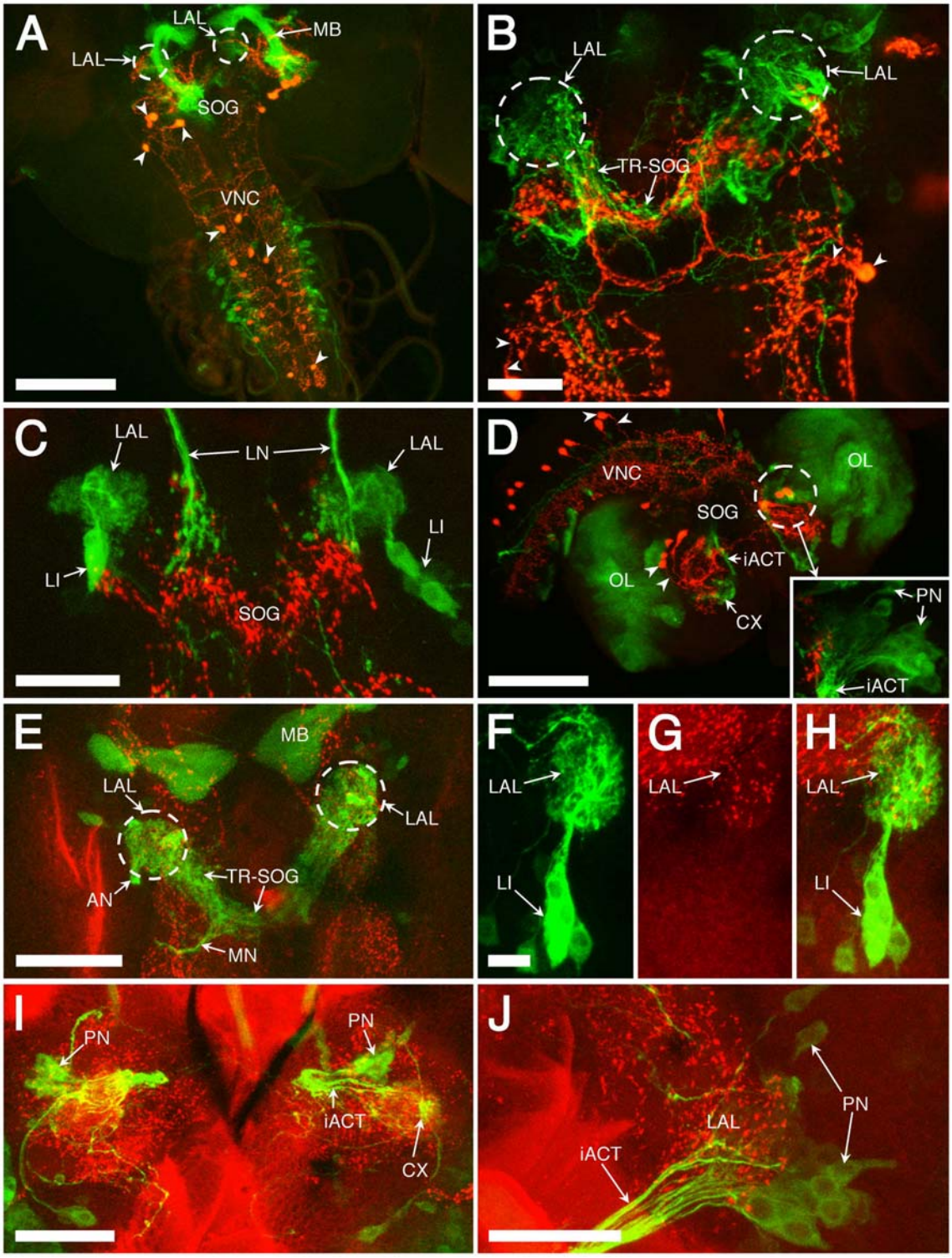
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Fig. 3

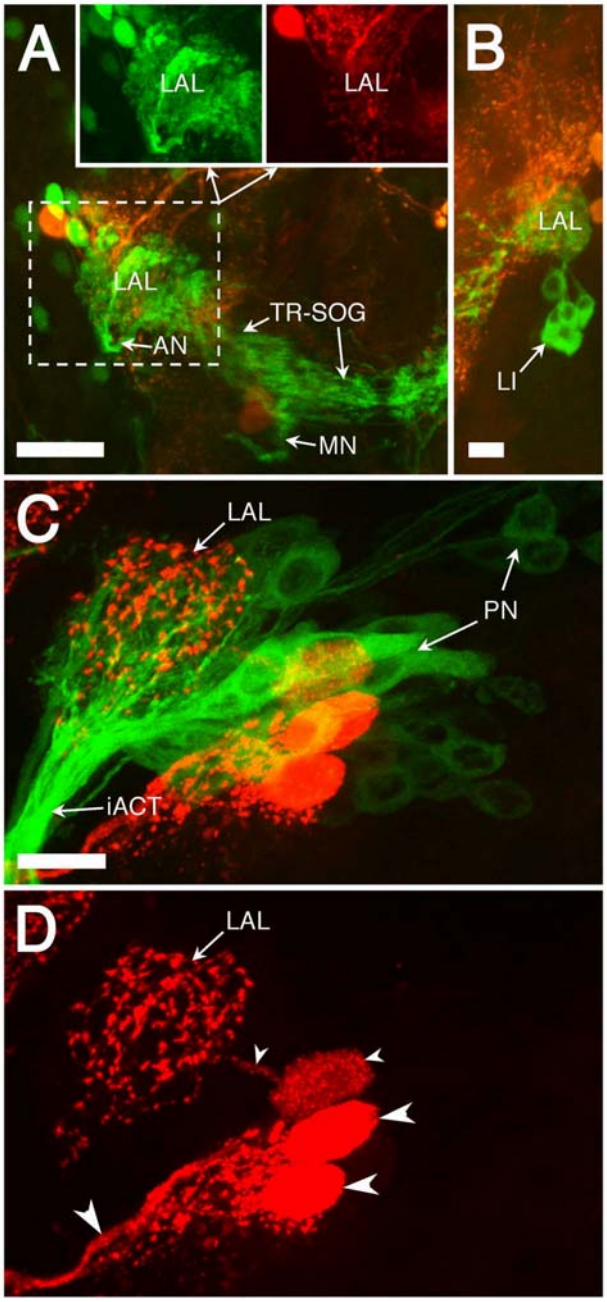

Fig. 4

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