Integration of complex larval chemosensory organs into the adult nervous system of Drosophila

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Summary

The sense organs of adult Drosophila, and holometabolous insects in general, derive essentially from imaginal discs and hence are adult specific. Experimental evidence presented here, however, suggests a different developmental design for the three largely gustatory sense organs located along the pharynx. In a comprehensive cellular analysis, we show that the postemergent of the three organs derives directly from a similar larval organ and that the two other organs arise by splitting of a second larval organ. Interestingly, these two larval organs persist despite extensive reorganization of the pharynx. Thus, most of the neurons of the three adult organs are surviving larval neurons. However, the anterior organ includes some sensilla that are generated during pupal stages. Also, we observe apoptosis in a third larval pharyngeal organ. Hence, our experimental data show for the first time the integration of complex, fully differentiated larval sense organs into the nervous system of the adult fly and demonstrate the embryonic origin of their neurons. Moreover, they identify metamorphosis of this sensory system as a complex process involving neuronal persistence, generation of additional neurons and neuronal death. Our conclusions are based on combined analysis of reporter expression from P⁴GAL4 driver lines, horseradish peroxidase injections into blastoderm stage embryos, cell labeling via heat-shock-induced flip-out in the embryo, bromodeoxyuridine birth dating and staining for programmed cell death. They challenge the general view that sense organs are replaced during metamorphosis.

Key words: Pharyngeal sense organs, Sensory neurons, Metamorphosis, FLput labeling, Embryonic labeling, Mitotic labeling

Introduction

Holometabolous insects have a unique way of generating their adult sensory apparatus. The vast majority of their larval sensilla is lost during metamorphosis and becomes replaced by adult sensilla that originate from imaginal discs (reviewed by Levine et al., 1995; Tissot and Stocker, 2000; Truman, 1996). This is certainly because many adult sense organs develop on adult-specific appendages. Yet, this design might also relate to the fact that larval and adult sense organs are often very different in terms of cell numbers, organization and function, reflecting the different life styles of the two stages. For example, in the creeping Drosophila larva, odor sensation is accomplished by no more than 21 sensory neurons. They are assembled in a single sensillum, which is part of a mixed olfactory/gustatory sense organ (Heimbeck et al., 1999; Python and Stocker, 2002; Singh and Singh, 1984). By contrast, in the flying adult, olfaction relies on 1300 sensory neurons allocated to 550-600 sensilla (Stocker, 2001) that are sited on two exclusively olfactory appendages. Hence, during ontogeny, it might be more economical to replace the simple larval olfactory apparatus than to reorganize it.

As a seeming exception to the rule of sensory replacement, small subsets of neurons associated with leg imaginal discs or abdominal segments have been shown to persist during metamorphosis (Jan et al., 1985; Shepherd and Smith, 1996; Tix et al., 1989a; Williams and Shepherd, 1999). However, experimental evidence suggests that these neurons might have a specialized function, serving as pioneers for growing adult afferents (Usui-Ishihara et al., 2000; Williams and Shepherd, 2002). Whether they become truly integrated in the adult nervous system is not known.

The external gustatory sensilla of the Drosophila larva appear to follow the general holometabolon fate: they degenerate during metamorphosis and are replaced by adult-specific sensilla that derive from the labial imaginal disc (Ray et al., 1993; Ray and Rodrigues, 1994; Wildermuth and Hadorn, 1965). Here, we study whether this rule also applies to the internal gustatory system that is located along the pharyngeal tube. Interestingly, the adult pharynx derives essentially from small, densely packed imaginal cells – the clypeolabral bud – that are closely associated with the larval pharyngeal skeleton (Bryant, 1978; Gehring and Seippel, 1967; Struhl, 1981). Does this imply that adult pharyngeal sensilla are born during metamorphosis, like their external counterparts, or do the anatomical similarities of certain larval (Python and Stocker, 2002; Singh, 1997; Singh and Singh, 1984) and adult pharyngeal organs (Nayak and Singh, 1983; Stocker, 1994; Stocker and Schorderet, 1981) rather suggest persistence of sensilla through metamorphosis?
To study possible links between the two sets of sense organs, we undertook a combined approach involving reporter expression from P[GAL4] driver lines, horseradish peroxidase (HRP) injections into syncytial blastoderm stage embryos, cell labeling through heat shock induced FL.Pout in the late embryo, bromodeoxyuridine (BrdU) birth dating and staining for programmed cell death. Our data include, neuron by neuron, the entire pharyngeal sensory system. They demonstrate that each of the three adult pharyngeal organs is of embryonic origin and derives from mature larval sense organs. Only a few sensilla are added through metamorphosis and few larval sensilla degenerate. This design is in marked contrast to nearly all other sense organs, which originate entirely from imaginal discs. The overall persistence of the larval sensory system is particularly striking because the pharynx undergoes extensive reorganization during this period.

Materials and methods

Fly strains and staging

Only female flies were used in this study. Flies were kept at 25°C in a 12:12 hours light:dark cycle under non-crowded conditions. Apart from the wild-type Canton S (CS) strain, the P[GAL4] strains MJ94/FM7 (Joiner and Griffith, 1999) and prosV (Voilà) /TM3 (Balakireva et al., 1998) were studied. Male MJ94 flies were crossed with female UAS-lacZ (Brand and Perrimon, 1993), UAS-GFP (Yeh et al., 1995) (kindly provided by A. H. Brand, Wellcome/CRC, Cambridge, UK) or UAS-mCD8-GFP (Bloomington Stock Center). Expression of prosV was studied in the prosV-UAS-GFP/TM3 strain. The y w hs-flp; Sp/Cyo; UAS>CD2 y+ >mCD8-GFP/Cyo stock (supplied by R. Axel, Columbia University) was used for the FL.Pout experiments (see below). Larval patterns were analyzed in feeding third instars. Pupal age is indicated as hours after puparium formation (APF). Adults were studied 3-5 days old.

Dissection

Larvae were cut at about half of the body length in PBS containing 0.2% Triton X-100 (PBST). Anterior halves were turned inside out. Gut, fat body and salivary glands were removed. Tissues were then fixed on ice for 1 hour in 4% paraformaldehyde (PA) in PBST. In the fragile pupae, spiracles and the posterior end of the pupal case were removed. An incision was made along the dorsal midline from head to cerci. The proboscis was opened distally with a razor blade and cut into distal and proximal parts that were fixed in PA for 2-3 hours on ice. At all stages, tissues were prepared for whole mounts. In adults, cryosections (10-16 μm intervals) were also made.

Tracing embryonic lineage

HRP was used as a cellular tracer of embryonic lineage, as described previously (Technau, 1986; Tix et al., 1989a). Briefly, HRP 10% in 0.2 M KCl was injected into stage 4 (syncytial blastoderm) embryos (wild-type CS or MJ94/UAS-lacZ). The injected embryos were allowed to develop to adulthood. 1-2-day-old adults were dissected, fixed, cryosectioned and processed for HRP and/or lacZ histochemistry (see below). A second method for tracing embryonic origin was to generate mCD8-GFP-labeled cells by heat-shock-induced FL.Pout. For this purpose, the progeny of the cross MJ94 × y w hs-flp; Sp/Cyo; UAS>CD2 y+ >mCD8-GFP (Wong et al., 2002) was exposed to 35°C for 1 hour. Owing to egg-laying periods of 6 hours, the age of these animals varied between 18-24 hours and 12-18 hours after egg laying (AEL).

BrdU labeling

BrdU labeling (Stocker et al., 1995; Truman and Bate, 1988) was performed in the progeny of the cross MJ94 × UAS-lacZ or MJ94 × UAS-mCD8-GFP at 0 hours, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 12 hours and 18 hours APF. BrdU was applied topically (Winberg et al., 1992): 2-3 μg of a BrdU solution (100 mg ml⁻¹) in a mixture of dimethylsulfoxide and acetone (1:1) was applied onto the animals’ intact anterior surface. In pupae at more than 12 hours APF, the puparium was partially removed. Animals were then allowed to undergo metamorphosis and were sacrificed as 2-3-day-old adults.

Immunocytochemistry and histochemistry

Green fluorescent protein (GFP) and anti-CD8 antibody (Caltag) coupled with green fluorescence label Alexa 488 (Molecular Probes) were used as fluorescent markers for the confocal microscope. This was done often in combination with the neuronal nuclear marker anti-Elav 9F8A9 (Developmental Studies Hybridoma Bank; http://www.uiowa.edu/~dshww) coupled with red fluorescence Cy3 (Jackson ImmunoResearch) or Alexa 568 (Molecular Probes). These double labelings were performed in both whole mounts and cryosections (Python and Stocker, 2002). The embryonic tracer HRP was identified in cryosections using the diaminobenzidine method. For lacZ labeling or HRPlacZ and BrdUlacZ double labeling, β-galactosidase was visualized histochemically using X-Gal (Gibco) (Brand and Perrimon, 1993). BrdU immunocytochemistry with an anti-BrdU antibody (G3G4; Developmental Studies Hybridoma Bank) was performed as described by Stocker et al. (Stocker et al., 1995).

Programmed cell death

Apoptosis was studied in flies MJ94/UAS-mCD8-GFP by applying an antibody against Drosophila caspase (α-active Drice), generously supplied by B. Hay (California Institute of Technology, Pasadena, CA). Double labeling was accomplished by applying two secondary antibodies, tagged with green fluorescence Alexa 488 (Molecular Probes) for CD8 and with red fluorescence Alexa 568 for caspase, respectively.

Confocal and light microscopy

Multiple series of confocal images were taken at 0.5-1 μm intervals with a BioRad MRC 1024 microscope (equipped with a Kr/Ar laser). Image analysis was performed on a Macintosh computer using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/). Color selection of images was done using Adobe PhotoShop. Light microscopic images were taken with a Zeiss AxioCam digital camera at 1300x1030 resolution and stored and processed with the AxioVision program.

Results

Larval and adult pharyngeal sense organs

Two P[GAL4] enhancer trap lines were used to investigate the pharyngeal sensory system. MJ94 (Joiner and Griffith, 1999) expresses GAL4 in the entire set of pharyngeal sensory neurons, and prosV (Balakireva et al., 1998) in subsets of accessory cells. mCD8-GFP or lacZ were used as reporters and anti-Elav served as a pan-neuronal marker (Robinow and White, 1988). The essentials of this study are shown in Fig. 5.

Three paired sense organs consisting of several sensilla each are located along the larval pharynx (Fig. 1A) (Python and Stocker, 2002). Sensilla contain between one and nine sensory neurons, collected below a common terminal pore or bristle (Singh and Singh, 1984). The dorsal and ventral pharyngeal sense organs (dps, vps) sit behind the mouth-hooks; a smaller posterior organ (pps) is located considerably further back. For
possible homologies with embryonic sense organs (Campos-Ortega and Hartenstein, 1997; Schmidt-Ott et al., 1994), see Python and Stocker (Python and Stocker, 2002). The nerves associated with the three organs are shown in Fig. 5A. The pps consists of two sensilla with three neurons each (MJ94, n=14; prosV1, n=18) (Fig. 1B,G, Fig. 5A). In MJ94, we established 17-18 anti-Elav-antibody-stained neurons for the dps (n=18) and 16 for the vps (n=15) (Fig. 1I,J, Fig. 5A). An additional five neurons which probably correspond to the dorsal pharyngeal organ (dpo) (Campos-Ortega and Hartenstein, 1997; Schmidt-Ott et al., 1994) are located about 100 μm behind the dps (Fig. 1A,I).

In the adult fly, dorsal and ventral cibarial sense organs (dcso, vcso) occupy the upper and middle part of the pharynx, respectively, whereas the distalmost labral sense organ (Iso) extends along the hypopharynx (Fig. 1C-F) (Nayak and Singh, 1983; Stocker, 1994; Stocker and Schorderet, 1981). The larval ridge-like cuticular pattern of the pharynx (Fig. 1A,B) is replaced by a rugged pattern (Fig. 1D), indicating shedding and regeneration of the larval cuticle. The dcso and vcso were reported to include two sensilla each, containing three and three gustatory neurons in the dcso and two and four in the vcso (Nayak and Singh, 1983). We confirm this for the dcso but find an additional proximal sensillum with two neurons in the vcso (Fig. 1D,E,K,M; Fig. 5B; Table 1).

Two rows of singly innervated mechanosensory ‘fishtrap'
bristles accompany the vcso distally and proximally (Nayak and Singh, 1983) (Fig. 1E,M). The lso consists of a heterogeneous group of nine sensilla (Fig. 1F, Fig. 5B). According to fine structural criteria, the distalmost sensilla (8 and 9) each possess a gustatory and a mechanoreceptive neuron, sensillum 7 comprises eight gustatory neurons, and the remaining sensilla are singly innervated mechanoreceptors (Nayak and Singh, 1983). The total of 18 neurons is confirmed by our Elav-binding data (Fig. 1N, Table 1). The nerves carrying the afferents from the adult organs are shown in Fig. 5B.

Interestingly, the larval pps appears to be anatomically equivalent to the adult dcso. Similarity refers to the position on the pharynx (cf. Fig. 1A,C), the presence of two sensilla with three neurons each (cf. Fig. 1G,K) and the labeling of two large accessory cells in prosV1 (cf. Fig. 1H,L). Hence, the dcso might derive directly from the pps (Fig. 5), suggesting embryonic birth dates for its neurons. For the other sense organs, the anatomy was found to be different at the two stages.

Most of the adult pharyngeal neurons are persisting larval neurons born in the embryo

To study whether some of the adult sensory neurons were indeed born during embryonic development, we followed two different approaches. First, we injected the lineage tracer HRP at the syncytial blastoderm stage (Technau, 1986). Using this technique, adult cells born during embryogenesis remain labeled, whereas adult cells born during postembryonic stages undergo an almost complete dilution of the marker owing to massive proliferation and growth (Tix et al., 1989a). Second, we generated mCD8-GFP-labeled pharyngeal neurons by heat-shock-induced recombination in the progeny of a cross between MJ94 and y w hs-flp; Sp/CyO; UAS>CD2 y+ >mCD8-GFP (cf. Wong et al., 2002). When the heat shock is applied late during embryogenesis, the presence of labeled single neurons in adult multineuronal sensilla should prove their embryonic origin. This is because multineuronal sensilla derive from a common sensory mother cell, the neurons being born last in the cell lineage (Ray et al., 1993).

In the adult, the embryonically injected HRP was observed in many cells, except in tissues that are known to derive from imaginal discs, such as the antennae. Remarkably, many cells in all three pharyngeal sense organs showed the HRP product, mostly in their cytoplasm. In the lso, strong HRP staining was present in sensillum 7 (Fig. 2A), but label was weak or absent in the other sensilla. Whether HRP resided in neurons or in sheath cells was not clear. Intense HRP staining was also present in the vcso and dcso. In the vcso, the label was strongest in what seemed to be dendritic extensions (Fig. 2B). In the dcso, HRP product was clearly present in neurons, as judged by the labeling of dendritic and axonal processes. All six neurons were probably HRP positive (Fig. 2C,C’). No label was found in fishtrap bristle sensilla (Fig. 2D).

FLPout clones performed in the MJ94 line were fully compatible with these results. In all three pharyngeal organs, we observed single mCD8-GFP-labeled cells when the 1 hour heat treatment was done between 12 hours and 24 hours AEL. These cells were clearly neurons, as judged by the neuron-specific expression of the MJ94 line, the presence of dendrites and axons, and simultaneous labeling by anti-Elav antibody. More precisely, of the 64 GFP-labeled neurons observed, 52 were single cells (Table 2). Exclusively single labeled neurons were found in sensillum 1-7 of the lso (Fig. 2E-G) (with one exception; see below) and in all five sensilla of the vcso and dcso (Fig. 2K,L). Neurons arranged in pairs were observed five times in sensillum 8 and 9 of the lso (Fig. 2H,I, Table 2). The fact that in none of the multiply innervated sensilla, except sensilla 8 and 9 of the lso, were more than one labeled neuron found indicates that these neurons had undergone their terminal mitosis during embryogenesis. By contrast, when the FLPout was performed earlier (6-18 hours AEL), a clone of three labeled neurons was observed in sensillum 7 of the lso (Fig. 2J) and a clone of two neurons in the vcso (not shown). This suggests that, during this interval, cell divisions were still going on. The frequency of FLPouts in late embryos was relatively

Table 1. Adult pharyngeal sense organs: neuronal composition, function and expression pattern of P[GAL4] lines used

<table>
<thead>
<tr>
<th>Sense organ</th>
<th>Sensillum type</th>
<th>Number of neurons</th>
<th>Total number of Elav-positive cells</th>
<th>Number of GAL4-expressing cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>dcso</td>
<td>Posterior</td>
<td>3</td>
<td>Gustatory</td>
<td>CS 6 (n=4) MJ94 6 (n=15)</td>
</tr>
<tr>
<td></td>
<td>Anterior</td>
<td>3</td>
<td>Gustatory</td>
<td>MJ94 6 (n=15) prosV1 0</td>
</tr>
<tr>
<td>Accessory cells</td>
<td>Proximal* 2*</td>
<td>Gustatory*?</td>
<td>8 (n=11) 8 (n=9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>2</td>
<td>Gustatory</td>
<td>8 (n=9) 8 (n=11)</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>4</td>
<td>Gustatory and mechanosensory</td>
<td>18 (n=11) 18 (n=7)</td>
</tr>
<tr>
<td>lcso</td>
<td>1-6</td>
<td>6x1</td>
<td>Mechanosensory</td>
<td>18 (n=11) 18 (n=7)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>8</td>
<td>Gustatory</td>
<td>18 (n=11) 18 (n=7)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2</td>
<td>Gustatory and mechanosensory</td>
<td>18 (n=11) 18 (n=7)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2</td>
<td>Gustatory and mechanosensory</td>
<td>18 (n=11) 18 (n=7)</td>
</tr>
<tr>
<td>Large/small accessory cells</td>
<td>Eighteen to 23 sensilla</td>
<td>One each</td>
<td>Mechanosensory</td>
<td>One each</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>One each</td>
</tr>
<tr>
<td>Fishtrap bristles (two rows)</td>
<td></td>
<td></td>
<td></td>
<td>0 3/8</td>
</tr>
<tr>
<td>Accessory cells</td>
<td>0</td>
<td>One each</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Data in columns 2-4 are from Nayak and Singh (1985), except * (this paper).
Fig. 2. Embryonic origin of subsets of neurons and non-neuronal cells in the pharyngeal sense organs, as shown by HRP injection at the syncytial blastoderm stage (A-D) and by FLPout (Wong et al., 2002) induced in F1 embryos of the cross MJ94 × y w hs-FLP; Spi/CyO; UAS>CD2 y+;mcCD8-GFP (E-L).

Table 2. Clones induced in pharyngeal sensilla by hs flip-out in the MJ94 line

<table>
<thead>
<tr>
<th>Sense organ</th>
<th>Sensillum type (neuron number)</th>
<th>Flip-out induced 12-24 hours AEL</th>
<th>Number of labeled neurons observed</th>
<th>Flip-out induced early (6-18 hours AEL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dcso</td>
<td>Posterior (3)</td>
<td>5x</td>
<td>0x</td>
<td>1x: 2 neurons</td>
</tr>
<tr>
<td></td>
<td>Anterior (3)</td>
<td>4x</td>
<td>0x</td>
<td></td>
</tr>
<tr>
<td>vcso</td>
<td>Proximal (2)</td>
<td>9x</td>
<td>0x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Middle (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distal (4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iso</td>
<td>1 (1)</td>
<td>1x</td>
<td>0x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 (1)</td>
<td>1x</td>
<td>0x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (1)</td>
<td>5x</td>
<td>0x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 (1)</td>
<td>3x</td>
<td>0x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 (1)</td>
<td>3x</td>
<td>0x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 (1)</td>
<td>6x</td>
<td>0x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7 (8)</td>
<td>14x</td>
<td>1x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 (2)</td>
<td>0x</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 (2)</td>
<td>11x</td>
<td>3x*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2x†</td>
<td></td>
</tr>
<tr>
<td>Fishtrap bristles</td>
<td>Proximal (1)</td>
<td>0x</td>
<td>0x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distal (1)</td>
<td>0x</td>
<td>0x</td>
<td></td>
</tr>
</tbody>
</table>

The three sensilla of the VCSO were not identified in the flip-outs.

*Two of the cases were induced at 12-18 hours after egg laying (AEL), one case at 18-24 hours AEL.
†Induced at 12-18 hours AEL.
‡Perhaps caused by abnormal loss of second neuron.

Total number of left/right sides observed is 86.
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the lso, we observed BrdU-positive neuronal and non-neuronal cells at the distal tip, belonging to sensilla 8 and 9 (Fig. 3A-C). Additional take-up had occurred in cells situated more proximally in the lso (Fig. 3A-E), for example in the neuron associated with sensillum 6 (Fig. 3D). In the vcso and dcso, none of the preparations revealed any labeled neurons. However, two accessory cells were clearly BrdU positive in both of these organs (Fig. 3F-I). Also, we observed labeling in the neurons of fishtrap bristles (Fig. 3H) and massive take-up in cells of the pharyngeal wall (Fig. 3G,H). Thus, the BrdU studies suggest that subsets of cells in the three sense organs undergo terminal division during metamorphosis. In summary, the adult pharyngeal sense organs appear to consist of both embryonically and postembryonically derived neurons and accessory cells.

Fate of pharyngeal sense organs through metamorphosis

Having established the embryonic origin of most of the adult pharyngeal sensory neurons, we wanted to study the relations between larval and adult pharyngeal organs directly. To this end, we investigated the expression pattern of the MJ94 line in progressively older pupal stages (Fig. 4). Until 20 hours APF, development was studied at hourly intervals; thereafter, it was studied at 24 hours, 48 hours, 64 hours and 68 hours APF. As expected, the most stable in terms of anatomy was the pps/dcso, which always exhibited six GAL4-positive and Elav-positive neurons, characterized by dendritic and axonal processes (Fig. 4A-C), and two GAL4-positive accessory cells in prosvl (not shown). This conserved organization strongly supports developmental continuity between the two organs (Fig. 5).

The fate of the vps was very different. A few hours APF, its neurons began to lose coherence and Elav- and mCD8-positive cellular debris appeared (Fig. 4D). The antibody α-active Drice, which displays a pattern in the pupal retina similar to the known pattern of apoptosis (Brachmann and Cagan, 2003) (not shown), labeled the neurons of the vps at early pupal stages (Fig. 4E,E' but not those of the dps (Fig. 4F,F') or the pps (not shown). These data suggest that the vps is subject to programmed cell death (Fig. 5).

In contrast to the pps and vps, the dps underwent complex transformation. Initially, its dendrites appeared to be collected in two clusters, anterior and posterior (a, p1) (Fig. 4G). Two neurons of the dps (Fig. 1A,I, Fig. 4G) then joined the dps, leading to an additional cluster p2 (Fig. 4H,I). From 14 hours APF onward, this entire complex began to split into two distinct organs (vcso and lso) in parallel with the distal elongation of the pharynx (cf. Fig. 4J,K,M). The posterior organ, vcso, was derived from p1 and p2; p1 gave rise to the distal and middle sensilla of the vcso, p2 to the proximal sensillum (Fig. 4K,L). Five to six additional neurons, probably remnants of the dps and dpo, were found scattered on the associated nerve, outside the vcso proper (Fig. 4J-L). They persisted up to the adult stage (Fig. 1M, Fig. 2K). At 24 hours APF, Elav staining revealed for the first time the two rows of fishtrap bristles (Fig. 4L).

The anterior organ, lso, derived mostly from cluster a. It gave rise to two sensilla: a complex one with eight neurons (sensillum 7) and a mononeuronal sensillum (Fig. 4M), perhaps sensillum 3, judged by its distinct size. Around 20 hours APF, an additional four cells in the distalmost part of the lso, encompassing bineuronal sensilla 8 and 9, began to express Elav and GAL4, and another five neurons appeared more proximally (Fig. 4M-O). The latter formed mononeuronal sensilla 1-6 together with the persisting putative sensillum 3 (see above).

These observations are fully compatible with the experimental data regarding the persistence of most of the larval neurons and the birth of a new, smaller set during
metamorphosis. In addition they demonstrate the conservation through metamorphosis of entire sense organs, pps and dps, the existence of complex morphogenetic movements including the split of a larval sense organ in two, and the degeneration of the vps.

Discussion
Most of the larval pharyngeal neurons persist through metamorphosis
Our data prove that most of the neurons of the three major adult pharyngeal sense organs are persisting larval neurons that were born in the embryo (Fig. 5). This is unlike other adult sensory neurons, nearly all of which derive from imaginal discs. Our interpretation relies on two independent experimental approaches for demonstrating embryonic birth dates (the use of the embryonic lineage tracer HRP and cell labeling by FLPout at late embryonic stages, a novel use of this technique). The experimental data are supported by anatomical
The persistence of mature larval neurons does not exclude the addition of cells during larva-adult transition. Indeed, BrdU applied during metamorphosis labeled subsets of cells in all three sense organs and in the fishtrap bristles. In agreement with these data, late embryonic FLPouts displayed pairs of labeled neurons in the sensillum 8 and 9 of the lso. Neuronal identity of the labeled cells was also established for the lso sensillum 6 as well as for the fishtrap bristles. Consequently, these neurons must have arisen by a terminal division after the metamorphic origin is suggested by BrdU uptake in sensillum 6 and the gradual appearance of Elav staining at 24 hours APF in all four sensilla.

Taken together, these data suggest that the lso is composed of both larval and adult-specific sensilla, whereas the vcs o and dcso consist exclusively of larval sensilla. Seen from a larval perspective, both the pps and the dps are conserved through metamorphosis, although with considerable modification of the sensillum or subsets of neurons in multineuronal sensilla to remain immature. Moreover, there is no indication for immature neurons from tracing their development with the marker line mCD8-GFP. Thus, we suggest that all the neurons of the dcso and vcs o, and sensillum 7 of the lso derive from mature, functional larval neurons. Also, continuous reporter expression through metamorphosis suggests that one of the mononeural lso sensilla (perhaps sensillum 3) might be another persisting larval sensillum.

Cell proliferation and cell death in pharyngeal sense organs through metamorphosis

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Larval sense organs persist despite extensive reorganization of the pharynx

The fact that the pps and dps persist through metamorphosis is remarkable given the origin of the adult labrum and cibarium from imaginal cells of the clypeolabral bud (Bryant, 1978; Gehring and Seippel, 1967; Struhl, 1981). In agreement with these reports, we observe massive labeling of pharyngeal epithelial cells after early pupal BrDU application. Moreover, the pharyngeal cuticle is shed and regenerates, a process that includes the cuticular part of the sensilla in question. Perhaps the birth of additional accessory cells during metamorphosis (e.g. in the dcso or vcsv, containing exclusively persisting neurons) is related to this modification. Formation of new cuticular structures is also known from persisting external sensilla during larval molts, but the survival of pharyngeal sensilla during the extensive remodeling of the pharynx remains stunning. The morphogenetic movements we observe in the sensory system certainly reflect these dramatic changes.

Why is the larval pharyngeal sensory apparatus largely conserved through metamorphosis?

Small subsets of neurons associated with leg imaginal discs or with abdominal segments have previously been shown to persist through metamorphosis (Jan et al., 1985; Shepherd and Smith, 1996; Tim et al., 1989a; Williams and Shephered, 1999). In the fly Phormia, such leg-disc-associated neurons remain immature (Lakes-Harlan et al., 1991a; Lakes-Harlan et al., 1991b), implying that they are non-functional. Laser ablation studies suggest that persisting neurons might help adult afferents to navigate from the imaginal discs to their central targets (Usui-Ishihara et al., 2000; Williams and Shephered, 2002). Whether they become truly integrated in the adult nervous system or die after reaching adulthood (having completed their pathway role) remains to be shown. Recently, tracing the expression pattern of a K driven reporter line suggested the incorporation of four receptor neurons of the larval eye into the so-called adult eycket (Helfrich-Förster et al., 2002; Hofbauer and Buchner, 1989), but this was not tested experimentally.

Our data demonstrate for the first time experimentally the integration of larval sensory neurons into the adult nervous system of Drosophila. Particularly striking and novel is the fact that entire, fully differentiated larval sense organs become incorporated. Also, this is the first observation of metamorphic survival in the chemosensory system.

Concerning the persisting neurons of the lso, a pathway function for the newly developing afferents towards and inside the central nervous system is certainly possible. However, the integration of the surviving pharyngeal neurons into the adult sensory system invites other interpretations. For example, these neurons and/or their central projections might be particularly precious, allowing, for example, the persistence of specific feeding-associated gustatory tasks through metamorphosis. As an alternative explanation, survival might be due to reasons of economy, a principle that governs the metamorphosis of the nervous system (Tissot and Stocker, 2000). Although neuronal reorganization is indispensable owing to the changing demands of larval and adult life, it is kept at a minimum, as shown by the survival of most larval interneurons and motor neurons (Truman, 1996). Sophisticated adult sense organs, however, might be easier to build de novo than by the transformation of simple larval organs, explaining the almost complete replacement of the larval sensory system. Why pharyngeal sense organs do not follow this general rule might relate to their largely conserved function at the two stages of life (analyzing the quality of ingested food of similar composition). The presence of larva-specific and adult-specific sensilla, however, suggests the existence of stage-specific gustatory tasks.

Using the genetic potential of the fly, it will be intriguing to dissect the functions of the different types of sensilla. Moreover, our analysis invites us to study at single cell level the genetic basis of many essential developmental processes, including cell determination, differentiation and apoptosis.

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