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

Nanaë Gendre¹, Karin Lüer², Sandrine Friche¹, Nicola Grillenzoni¹, Ariane Ramaekers¹, Gerhard M. Technau² and Reinhard F. Stocker¹,*

¹Department of Biology and Program in Neuroscience, University of Fribourg, CH-1700 Fribourg, Switzerland
²Institute of Genetics, University of Mainz, D-55099 Mainz, Germany

*Author for correspondence (e-mail: reinhard.stocker@unifr.ch)

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 sensilla develop on adult-specific appendages. Yet, this design might also relate to the fact that larval and adult sensilla 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 holometabolan 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 FLPout 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 prosvi (Voia1)/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 prosvi was studied in the prosvi-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 FLPout 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 fragile pupae, spiracles and the posterior end of the pupal case were removed. An incision was made along the dorsal midline from Pupal age is indicated as hours after puparium formation. Adults were studied 3-5 days old.

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 FLPout. 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. After 12-18 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/~dshwww) 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 HRP/lacZ and BrdU/lacZ 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 (τ-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 1300×1030 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 prosvi (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 suggest that, during this interval, cell divisions were still going on. The frequency of FLPouts in late embryos was relatively low (Fig. 2H-I, Table 2). The presence of labeled neurons was observed in sensillum 7 of the lso (Fig. 2K,L). Neurons arranged in pairs were observed five times in sensilla 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 low.

### 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>Function</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>6 (n=4)</td>
<td>6 MJ94</td>
</tr>
<tr>
<td></td>
<td>Anterior</td>
<td>3</td>
<td>Gustatory</td>
<td>6 (n=15)</td>
<td>6 MJ94</td>
</tr>
<tr>
<td>Accessory cells</td>
<td>Proximal*</td>
<td>2*</td>
<td>Gustatory*?</td>
<td>8 (n=11)</td>
<td>8 (n=9)</td>
</tr>
<tr>
<td>vcso</td>
<td>Middle</td>
<td>2</td>
<td>Gustatory</td>
<td>8 MJ94</td>
<td>8 MJ94</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>4</td>
<td>Gustatory</td>
<td>18 (n=11)</td>
<td>18 (n=7)</td>
</tr>
<tr>
<td>Accessory cells</td>
<td>Proximal*</td>
<td>2*</td>
<td>Gustatory*?</td>
<td>18 (n=11)</td>
<td>18 (n=7)</td>
</tr>
<tr>
<td>Large/small accessory cells</td>
<td>1-6</td>
<td>6x1</td>
<td>Mechanosensory</td>
<td>18 (n=11)</td>
<td>18 (n=7)</td>
</tr>
<tr>
<td>Fishtrap bristles (two rows)</td>
<td>18-23</td>
<td>One each</td>
<td>Mechanosensory</td>
<td>One each</td>
<td>One each</td>
</tr>
<tr>
<td>Accessory cells</td>
<td>0</td>
<td>One each</td>
<td>Mechanosensory</td>
<td>One each</td>
<td>0</td>
</tr>
<tr>
<td>Fishtrap bristles (two rows)</td>
<td>23 sensilla</td>
<td>One each</td>
<td>Mechanosensory</td>
<td>One each</td>
<td>0</td>
</tr>
<tr>
<td>Accessory cells</td>
<td>0</td>
<td>One each</td>
<td>Mechanosensory</td>
<td>One each</td>
<td>0</td>
</tr>
</tbody>
</table>

Data in columns 2-4 are from Nayak and Singh (1985), except * (this paper).

**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 sensilla 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 sensilla 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 low.
larval sense organs in adult Drosophila

high, as shown by several observations of two putative independent events in left and right sense organs (Fig. 2F) or in different sensilla of the lso (Fig. 2G). A similar explanation might apply to an exceptional case of two labeled neurons in the multineuronal sensillum 7 (A: arrow) and of the vcsO (B: arrow), as well as in dendrites, cell bodies and axons of dcsO neurons (consecutive sections C, C’: arrows). D) Fishtrap bristles labeled by MJ94/UAS-lacZ (arrows) lack HRP staining, in contrast to the vcsO (arrowhead). E-L) When FLPouts are induced in the MJ94 line at 12-24 hours after egg laying (AEL), single neurons are labeled in the lso, in both sensillum 7 (composed of eight neurons) (E-G: 7) and the mononeuronal sensilla 1-6 (G: 3). By contrast, the bineuronal sensilla 8 and 9 always exhibit paired labeled neurons (H-I: arrows). Simultaneous labeling of two sensilla suggests the occurrence of two independent FLPout events (F,G). I) FLPouts induced earlier (6-18 hours AEL) lead to simultaneous label in many neurons (e.g. three in sensillum 7). (K,L) FLPout induced at 12-24 hours AEL invariably leads to single labeled neurons in the multineuronal sensilla of the vcsO (K) and dcsO (L). The two large neurons in K (arrows) are outside the vcsO. ftb, fishtrap bristles. Distal is to the bottom except in (A) in which distal is to the left. F shows both left and right lso. Scale bars, 20 μm (A shows scale for A-D; E shows scale for 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>FLP-out induced 12-24 hours AEL</th>
<th>Number of labeled neurons observed</th>
<th>FLP-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>1x: 3 neurons</td>
</tr>
<tr>
<td></td>
<td>Distal (4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lso</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>3x*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 (2)</td>
<td>1x†‡</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.

Extra neurons are added during metamorphosis

Interestingly, late embryonic FLPouts always led to pairs of labeled neurons in sensilla 8 and 9 of the lso (Fig. 2H,I, Table 2), suggesting that cell divisions occurred after the FLPout. To discover whether the terminal cell divisions of these neurons had taken place during metamorphosis, we used the mitotic marker BrdU (Truman and Bate, 1988; Stocker et al., 1995) to MJ94 and wild-type CS animals, in experiments ranging from 0 hours to 18 hours APF. Indeed, in adult flies that had been treated in this way as pupae, subsets of neuronal and non-neuronal cells of the pharyngeal sense organs were labeled. In
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 *prosvI* (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 dpo (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
Larval sense organs in adult Drosophila

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
observations showing: (i) an almost identical organization of the larval pps and the adult dcso; (ii) the presence of the pps and dps sensilla continuously through metamorphosis; (iii) an uninterrupted expression of the P[GAL4] lines used in these two organs; and (iv) the persistence of dendrites and axons in all surviving neurons.

HRP injected at the syncytial blastoderm stage becomes incorporated into every cell upon cellularization. During subsequent development, the marker remains at high levels in cells that divide only a few times but becomes diluted in cells that undergo repeated divisions (Technau, 1986). Consequently, labeling in the adult is expected in many neurons of the central nervous system known to be persisting larval neurons [e.g. optic lobe pioneers (Tix et al., 1989b)] but should be absent from tissues derived from imaginal discs (Levine et al., 1995; Tissot and Stocker, 2000; Truman, 1996). This corresponds to what we observe and allows us to postulate an embryonic origin for the elements containing high HRP levels in adult pharyngeal sense organs.

This interpretation is supported by the FLPout experiments (cf. Wong et al., 2002) performed at late embryonic stages with the neuron-specific MJ94 line. In adults deriving from this treatment, we detected exclusively single labeled neurons in sensillum 7 of the lso (containing eight neurons) and in the five multiply innervated sensilla of the vcsso and dcso (Table 2). Although we did not study the cell lineage of these sensilla, they are probably homologous to other multineuronal terminal-pore gustatory sensilla, which derive from a common sensory mother cell (Ray et al., 1993). Indeed, apart from its eight neurons, sensillum 7 of the lso corresponds to a typical insect gustatory sensillum in terms of fine structural and cellular organization, containing no more than three accessory cells (Nayak and Singh, 1983). Hence, the single labeled neurons in this sensillum and in all sensilla of the vcsso and dcso must have been postmitotic during FLPout. This agrees with the observation that formation of head nerves is complete by embryonic stage 15 (Campos-Ortega and Hartenstein, 1997).

Could these neurons have remained immature during larval life, differentiating only during metamorphosis, similar to subsets of postmitotic cells in the larval central nervous system CNS (Booker and Truman, 1987; Truman, 1990)? We believe this rather unlikely because it would require either the entire 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 lines mCD8-GFP. Thus, we suggest that all the neurons of the dcso and vcsso, and sensillum 7 of the lso derive from the persistence of neurons of the central nervous system known to be persisting larval sensillum.

**Cell proliferation and cell death in pharyngeal sense organs through metamorphosis**

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 sensilla 8 and 9 of the lso. Neuronal identity of the labeled cells was also established for the lso sensillum 7 as well as for the fishtrap bristles. Consequently, these neurons must have arisen by a terminal division after the FLPout and after BrdU application (i.e. during metamorphosis) (Fig. 5). Hence, of the total of 32 sensory neurons of the three main adult sense organs, only the birth dates of four monoinnervated lso sensilla remain unclear. Yet, their 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 vcsso 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 dps (Fig. 5). By contrast, the vps undergoes programmed cell death, and the fishtrap bristles are entirely adult specific, lacking any larval counterpart. In conclusion, metamorphosis of the internal gustatory sensory system is an intricate process involving neuronal persistence, generation of additional neurons and neuronal death.

Fig. 5. Fate of pharyngeal sensory neurons through metamorphosis. Persisting neurons are shown in color, neurons undergoing apoptosis in black, and newly born neurons with a dot. Individual sensilla are shown as arrowhead symbols. (A) Third larval instar: the dorsal pharyngeal sense organ (dps) consists of two groups of neurons, a and pl. Precise numbers of sensilla in the dps, dpo (dorsal pharyngeal organ) and vps (ventral pharyngeal sense organ) are unclear. lbn, labial nerve; lrn, labral nerve; pps, posterior pharyngeal sense organ. (B) Adult: the dorsal cibarial sense organ (dcso) corresponds to the conserved pps. The labral sense organ (lso) and the ventral cibarial sense organ (vcsso) derive essentially from the dps. However, the vcsso also includes two neurons of the dpo. Seven small sensilla of the lso as well as the fishtrap bristles (ftb; numbers in the diagram reduced) are born during metamorphosis. Remaining neurons of the dps and dpo are located along the accessory pharyngeal nerve (apn). pn, pharyngeal nerve.
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 vcs0, 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; Tix et al., 1989a; Williams and Shepheard, 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 affrents to navigate from the imaginal discs to their central targets (Usui-Ishihara et al., 2000; Williams and Shepheard, 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 Kρ-driven reporter line suggested the incorporation of four receptor neurons of the larval eye into the so-called adult eyeflet (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 theIso, a pathway function for the newly developing affrents 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.

This work was supported by Swiss National Funds (grants 31-52639.97 and 31-063447.00), the Roche Foundation and the DFG (grant TE 1307-4). We thank P. Gehr and B. Rothen-Rutishauser (Institute of Anatomy, University of Bern) for providing confocal facilities, and B. Gerber (Würzburg), F. Python (Fribourg) and K. Stürtkühl (Bochum) for valuable comments on the manuscript.

References


Gehring, W. and Seippel, S. (1967). Die Imaginalzellen des Clypeo-Labrums (Institute of Anatomy, University of Bern) for providing confocal facilities, and B. Gerber (Würzburg), F. Python (Fribourg) and K. Stürtkühl (Bochum) for valuable comments on the manuscript.

This work was supported by Swiss National Funds (grants 31-52639.97 and 31-063447.00), the Roche Foundation and the DFG (grant TE 1307-4). We thank P. Gehr and B. Rothen-Rutishauser (Institute of Anatomy, University of Bern) for providing confocal facilities, and B. Gerber (Würzburg), F. Python (Fribourg) and K. Stürtkühl (Bochum) for valuable comments on the manuscript.

References


Nayak, S. V. and Singh, R. N. (1983). Sensilla on the tarsal segments and


