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Development of the *Drosophila* olfactory sense organs utilizes cell-cell interactions as well as lineage

G. Venugopala Reddy, Bhagwati Gupta, Krishanu Ray† and Veronica Rodrigues*

Molecular Biology Unit, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Bombay, 400 005, India

*Author for correspondence (e-mail: Veronica@tifrvax.tifr.res.in)
†Present address: Department of Cell and Molecular Medicine, University of California at San Diego, 9500 Gilman Drive, San Diego, CA 92093, USA

SUMMARY

We have examined the mechanisms underlying the development of the olfactory sense organs on the third segment of the antenna of *Drosophila*. Our studies suggest that a novel developmental strategy is employed. Specification of the founder or precursor cell is not governed by the genes of the *achaete-scute* complex. Another basic helix-loop-helix encoding gene, *atonal*, is essential for determination of only a subset of the sensilla types – the sensilla coeloconica. Therefore, we predict the existence of additional proneural genes for the selection of sensilla trichoidea and sensilla basiconica. The choice of a founder cell from the presumed proneural domain is regulated by *Notch* activity. Soon after delamination of the founder cell, two to three additional neighboring cells also take on a sensory fate and these cells together form a presensillum cluster. The selection of neighbors does not occur when endocytosis is blocked using a temperature sensitive allele of *shibire*, thus suggesting that cell-cell communication is required for this step. The cells of the cluster divide once before terminal differentiation which is influenced by *Notch* activity. The final cell number within each sensillum is controlled by programmed cell death.

Key words: sense organ development, cell specification, programmed cell death, *Drosophila*, basic helix-loop-helix protein

INTRODUCTION

Two main strategies have been described for the development of the sense organs in the *Drosophila* embryo and adult. The external sense organs utilize a mechanism that is largely lineage restricted (reviewed by Ghysen and Dambly-Chaudiere, 1992; Jan and Jan, 1993), in which the sensory precursor undergoes a series of stereotyped divisions to give rise to all the cells within the sensillum. In contrast, the photoreceptors of the compound eye are not related to each other by lineage, but depend on cellular interactions for their development (reviewed by Cagan and Zipursky, 1992; Dickson and Hafen, 1993). In this paper we present a third strategy which is utilized during development of the olfactory sense organs on the adult antenna. Our studies show that both lineage and cellular interactions are important mechanisms during the development of this sensory system.

There are three morphologically distinct types of sense organs on the surface of third segment of the antenna: the sensilla basiconica, sensilla trichoidea and the sensilla coeloconica (Venkatesh and Singh, 1983). Unlike the mechanosensory bristles or the ommatidia of the compound eye, these sensilla are not arranged in a precise pattern but are organized in broad domains on the antennal surface. The structure of each sensillum is composed of the trichogen cell which forms the shaft, the tormogen or socket cell and the thecogen or glial cell that surrounds the neurons. The neuronal composition of each sensillum is highly variant and depends on the position of the sensillum on the epidermis. Thus, the topography of sensilla on the antenna would suggest different mechanisms underlying determination of sense organ type, their arrangement on the surface of the appendage and how the cells of a sensillum acquire functionally distinct fates.

In all sensory systems thus far studied, the first step in development is the expression of proneural genes, which confer on a group of cells, the competence to take on a neural fate. The genes of the *achaete-scute* complex (*AS-C*) which specify most of the monoinnervated and polyinnervated external sense organs have been fairly intensively studied and shown to encode basic helix-loop-helix (bHLH) transcription factors (Jan and Jan, 1993). Their function is modulated by the positive regulator *daughterless* (*da*) and the negative regulators *hairy* (*h*) and *extramacrochaete* (*emc*) (Moscoso del Prado and Garcia-Bellido, 1984; Caudy et al, 1988). The gene *atonal* (*ato*) also encodes a bHLH protein and specifies the chordotonal organs of the embryo and adult and the photoreceptors of the compound eye (Jarman et al., 1993, 1994, 1995). The choice of the precursor cell from among a group of equivalent cells is made by a process of lateral inhibition mediated by the action of the 'neurogenic' class of genes (reviewed by Campos-Ortega, 1993). This process has been studied in some detail in the microchaete and macrochaete of the wing and notum and has been shown to be controlled by a complex regulatory loop involving the *Notch* (*N*) receptor, its ligand *Delta* (*Dl*) and products of the *Enhancer of split* [*E(spl)*] complex (Simpson, 1990; Heitzler and Simpson, 1996). Selection of the founder

photoreceptor cell (the R8 cell) also involves the action of *N* and *scabrous* (*sca*) genes (Baker et al., 1990).

The events involved in the so called 'lineage restricted' and 'lineage non-restricted' developmental systems diverge after selection of the founder or precursor cell. The sensory organ precursors (SOP) of the embryonic and adult peripheral nervous systems undergo divisions to give rise to shaft, socket, glia and neuron. In the multinnervated external sense organs the division pattern is somewhat more complex, but the first division of the SOP still segregates a lineage for the socket and shaft cell from that for the neurons and glia (Ray et al., 1993). The products of the *numb* and *prospero* (*pros*) genes are asymmetrically distributed during division of the SOP thus conferring different fates to the daughters. Numb appears to antagonize *N-Dl* signaling thus serving as a link between the intrinsic and extrinsic signals (reviewed by Jan and Jan, 1995).

In the case of the compound eye, careful mosaic analysis coupled with morphological data provide convincing evidence that the photoreceptor cells are not related by lineage but arise by interaction between cells (reviewed by Cagan and Zipursky, 1992). The contact between R8 and its neighbors results in the selection of the other photoreceptors and later the cone and pigment cells. While several of the genes involved in this process have been identified, the molecular mechanism underlying R7 selection is perhaps the most intensely studied. The interaction of the Sevenless (Sev) receptor tyrosine kinase on the presumptive R7 cell with its ligand, Bride of sevenless (Boss), on R8 initiates a Ras mediated signaling cascade resulting in the specification of the R7 cell. This pathway shares extensive similarity with other Ras signaling systems in different animals (Duffy and Perrimon, 1996).

In contrast to other sensory systems discussed above, very little information is available on the development of the olfactory sense organs on the antenna. Using antibody and cellular markers, we earlier showed, that the first step in development of the olfactory sensilla is the delamination of the founder cell (FC) (Ray and Rodrigues, 1995). A few hours later, two to three cells located adjacent to the FC also begin to express sensory markers and these cells together define a presensillum cluster (PSC). Analysis of mitotic patterns using bromodeoxyuridine (BrdU) incorporation showed that the cells of the PSC are not related to the FC by lineage. The PSC divides as a group to give rise to the cells of the olfactory sensillum. In this study we have analyzed the processes involved in this developmental program in some detail. We show that the olfactory system is unusual in that it uses features as well as molecules implicated in photoreceptor development as well as others from the external sense organs.

MATERIALS AND METHODS

Fly strains and antibodies

The P-(lacZ-ry^+) strain, A101.IF3, contains an 'enhancer-trap' insert in the *neuralized* gene and has been used previously to mark developing olfactory precursors (Ray and Rodrigues, 1995). Details of the *AS-C* rearrangements can be found in Lindsley and Zimm (1992) and Campuzano et al., (1985). Df(1)260-1, Df(1)sc^{19}; Df(1)$sc^{10\text{-}1}$ and Hw^1 were kindly provided by Jose Campos-Ortega, Univ. of Koln and the *hs-sc* strain (HSSC:3) by Juan Modolell, University of Madrid. The null allele of *ato* (*ato*^{1}) and Df(3R)p^{13} *red e*/TM3-*Ser* were obtained from Andrew Jarman, University of Edinburgh. shi^{ts2} was obtained from K. S. Krishnan, TIFR, Bombay, *cn* sca^{BP2} *bw* and the hs-FLP stocks from the Rubin lab Berkeley, and the Act5C>Draf1>lacZ strain from Gary Struhl, Columbia University. The hsp 70-*N*-intra and Act5C>y^+>*N*-intra were kindly provided by the Artavanis-Tsakonas lab, Yale University. The neuron-specific antibodies mAb22C10 and mAb44C11 were a gift from Seymour Benzer, CalTech.

For staging, white prepupae (0 hours after puparium formation; APF) were collected and allowed to develop further on a moist filter paper at 25°C. This stage lasts for 1 hour, hence the error in staging is ±30 minutes. When cultures were reared at other temperatures, ages were normalized with respect to growth at 25°C (Ashburner, 1989).

Cuticle preparation

Adult antennae were removed and placed in a drop of Faure's Solution (34% v/v chloral hydrate, 13% v/v glycerol, 20 mg/ml gum arabic, and 0.3% cocaine chlorohydrate) and allowed to clear overnight at 70°C. Sensilla were counted after projecting Nomarski images from the microscope onto a Videomonitor.

Production of mosaic animals

Gynandromorphs were generated by making use of the spontaneous loss of the Ring-X chromosome; R(1)2, In(1)w^{vco}/In(1)dl49, *y*, *w* lz^3 females were mated with males bearing a deficiency in the *AS-C* region and a duplication for the region on the Y chromosome to allow for survival. Adult progeny were scored for *yellow* (*y*) cuticular patches which serve as markers for mutant tissue.

For generating mitotic clones recombination was induced using the FLP/FRT system. In the *N* experiments the *y* N^{xk11} FRT101 and Act5C>*y*+>N_{intra} strains were used and clones were visualized by the *y* marker. In the lineage studies, we used a strain containing *Act5C>Draf1>lacZ* where clonal tissue is marked by the expression of β-galactosidase. In the Act5C>*y*+>N_{intra} and *Act5C>Draf1>lacZ* constructs, recombinase activity results in 'flipping-out' of the *Draf* and y^+ genes thus placing the gene of interest under control of the *Act5C* promoter. FLP recombinase activity was induced by subjecting larvae 36 hours after egg laying to a temperature pulse at 37°C for 30 minutes.

Immunohistochemistry

Discs were dissected, fixed as described previously (Ray and Rodrigues, 1995), and incubated in primary antibody appropriately diluted in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (PTX) for 1 hour at room temperature. Samples were washed in PTX, treated with biotinylated secondary antibodies followed by either streptavidin/biotin-horseradish peroxidase (HRP) or streptavidin coupled with FITC or Texas Red at dilutions recommended by the manufacturer. Stained discs were observed using Nomarski optics and in the case of fluorescently labeled preparations, using a BioRad MRC600 confocal microscope.

Staining of nuclear DNA with DAPI

Tissues were incubated in 1 μg/ml of DAPI in PBS for 2-4 minutes, rinsed in PBS and mounted in Vectashield (Vector Labs). Samples were observed using a fluorescent microscope (excitation, 359 nm; emission, 441 nm).

Examination of programmed cell death profiles

The TUNEL protocol was carried out as described by White et al. (1994) with minor modifications. Staged pupae were dissected, rinsed several times in PTX and once in terminal transferase buffer (Boehringer-Mannheim). The preparations were incubated for 3 hours at 37°C in reaction mixture and washed in PTX and incubated with avidin coupled RITC (Molecular Probes). In most experiments samples were double labeled with mAb22C10 to visualize the neurons within each sensillum cluster.

RESULTS

There are three morphologically distinct types of olfactory sense organs located in broad regions on the surface of the third segment of the antenna (Fig. 1A). The sensilla basiconica (Fig. 1C) and sensilla trichoidea (Fig. 1D) are located in defined domains on the antennal surface while the sensilla coeloconica (Fig. 1E) occupy a mixed region together with the other two (Fig. 1B). The majority of sensilla basiconica are innervated by either two or four neurons while most of the sensilla coeloconica have three sensory neurons. The sensilla trichoidea have between one and three neurons depending on their location on the antennal surface (Shubha Shanbhag, personal communication). Thus, there is a great variability in the neuronal composition of the sense organs depending on the sensillum type and its location on the antennal surface.

The b-HLH encoding genes of the *AS-C* are not essential for olfactory sense organ development, but *atonal* specifies the sensilla coeloconica

In an effort to identify the mechanisms involved in the specification of antennal sense organs we examined the roles, if any, of the previously identified proneural genes. All proneural genes so far identified encode b-HLH proteins which are expressed in a domain of cells and confer them with the competence to become neural (Campos-Ortega, 1993).

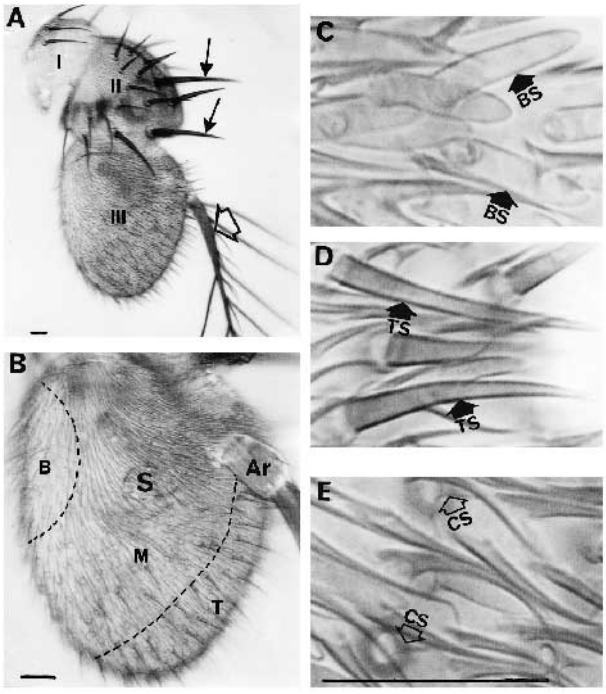

Fig. 1. The pattern of sensilla on the antennal surface. (A) Cuticular mount of an adult antenna showing segments I, II, and III (marked by roman numerals), open arrowhead indicates the arista; long arrows indicate the mechanosensory bristles on the second antennal segment. (B) The regions on the antennal surface occupied by sensilla basiconica (B), sensilla trichoidea (T) and the mixed region (M) are indicated. The sacculus (S) and the arista (Ar) are also seen. (C-E) Magnified regions of the antennal surface showing (C) basiconic sensilla (BS), (D) trichoid sensilla (TS). (E) sensilla coeloconica (CS). Scale bars, 25 μm.

We examined the effect of deletions of the *AS-C* on the development of the antennal sensilla. Since rearrangements that affect the *lethal of scute* (*l'sc*) locus result in embryonic lethality, their effects on adult development were studied by generating mosaic animals with mutant patches covering the third antennal segment. Clones of cells bearing a complete deletion of the *AS-C* [Df(1)260-1] still have normal numbers of all three kinds of antennal sensilla. A few sensilla appear smaller in size than their wild-type counterparts and a few show some morphological abnormalities. The deficiency Df(1)sc^{19}, which deletes *ac*, *sc* and *l'sc*, but not *asense* (*ase*) (Dambly-Chaudiere and Ghysen, 1987; Alonso et al., 1988; Gonzalez et al., 1989), produced completely normal numbers as well as morphology of olfactory sensilla (Fig. 2B). Results from both deficiencies taken together demonstrate that the *AS-C* genes are not essential for the development of the olfactory sensilla. ase^+ function could play a role in the differentiation of these sense organs and animals null for *ase* showed some morphological defects in the sensilla but the numbers and types of these sensilla were unaffected (Maria Dominguez, personal communication; Dominguez and Campuzano, 1993).

The lack of ac^+ and sc^+ requirement in antennal development was further verified by examination of the antenna from $sc^{10\text{-}1}$ animals. The lesion in this strain completely abolishes

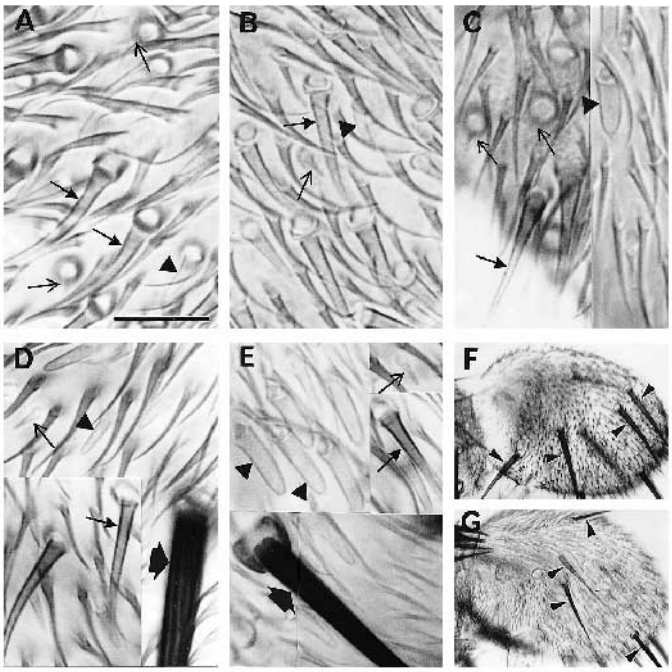

Fig. 2. Effects of the *achaete-scute* complex genes on the development of the olfactory sense organs. In all panels sensilla trichoidea are indicated with arrows, sensilla basiconica with arrowheads and sensilla coeloconica with fine arrows. (A) Wild-type antenna. (B) Comparable region from a mosaic antenna hemizygous for *Df(1)*sc^{19}. (C) Surface of the third antennal segment from a $sc^{10\text{-}1}$ animal. (D,F) Antenna from the *Hw* strain. Short thick arrow in D and arrowhead in F indicate the large 'macrochaete'. (E,G) *hs-sc* pupae were pulsed for three half-hour periods at 37°C begining at the onset of pupation. Pupae were returned to permissive temperature for 2 hours between each pulse. Short thick arrow in E and arrowhead in G indicate the ectopic macrochaete. A-E are at the same scale (bar, 10 μm).

Table 1. Numbers of sensilla on the surface of the third antennal segment in different mutants

Genotype	Sensilla basiconica	Sensilla trichoidea	Sensilla coeloconica
Canton-S	192±7	145±3	73±4
$sc^{10\text{-}1}$	178±8	133±9	74±5
Hw^{+}	171±11	130±7	70±5
hs-*sc*	171±8	133±9	74±5
ato/Df(3R)p^{13}	207±5	116±12	1±1

Each value represents the mean and standard deviation of at least 5 preparations.

both *ac* and *sc* function; surviving flies lack mechanosensory as well as gustatory sensilla on the body but the olfactory sensilla on the antenna develop normally (Campuzano et al., 1985; Fig. 2C; Table 1). Further, we were unable to detect transcripts of these genes in the third antennal segment using standard whole-mount hybridization protocols, while the expression was readily detected in the anlage of the second antennal segment (data not shown).

The gain-of-function allele of *ac*, *Hairywing* (*Hw*^{1}, Garcia-Bellido and Santamaria, 1978; Campuzano et al., 1986), or overexpression of the *sc* transcript using the *hsp-70* promoter transgene does not alter the numbers of olfactory sensilla on the antenna (Table 1; Fig. 2D-G). In both cases, however, we observed large bristles which resembled the macrochaetes elsewhere on the body (short broad arrows in Fig. 2D,E; arrowheads in Fig. 2F,G). Hence we conclude that the *AS-C* genes are neither essential nor sufficient to promote olfactory sense organ development on the surface of the antenna. It is interesting that misexpression of these genes can produce ectopic 'mechanosensory bristles' in an epidermal field normally dedicated to olfactory receptors.

The gene *atonal* has been identified as the proneural gene for the chordotonal organs of the embryo and the adult compound eye (Jarman, 1993, 1994, 1995) We examined the effect of complete removal of *ato* function on the development of the antenna. *ato*^{1} homozygotes are lethal, because of an unrelated lethal mutation on the same chromosome. The antenna of *ato*^{1}/Df(3R)*p*^{13} animals completely lack sensilla coeloconica while the basiconica and trichoidea are normal in number and morphology (Table 1). In addition, all such animals totally lack the sacculus, a region of the third antennal segment that contains thermo and hygroreceptors. The sacculus is a three chambered pit, each pit harboring a different type of sensilla: the outer chamber is lined with sensilla basiconica, the middle chamber contains a few blunt ended sensilla and the large inner chamber contains a large number of sensilla coeloconica (Itoh et al., 1991).

sc$^{10\text{-}1}$; *ato*^{1}/Df(3R)*p*^{13} males do not show any decrease in the numbers of sensilla basiconica or trichoidea (data not shown) arguing for the presence of an as yet unidentified proneural gene(s) which participates in antennal sensillar development.

Notch function is required for the choice of single founder cells

In most sensory systems, the selection of the precursor cell from the proneural domain is mediated by lateral inhibition involving N^{+} function (Wiggelsworth, 1942; Simpson, 1990).

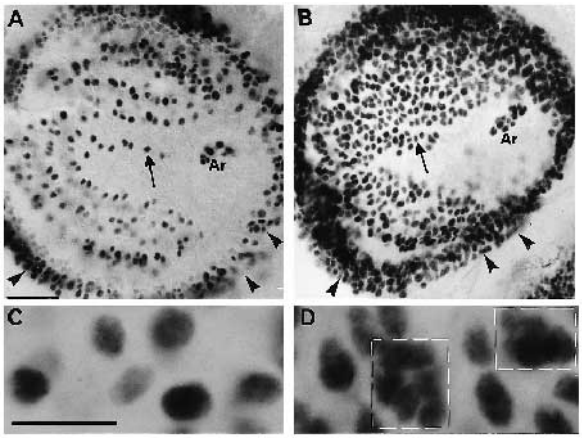

Fig. 3. Effect of *N* on the specification of olfactory organ precursors. Antennal discs from N^{ts1}; *neu*^{A101}/+ pupae pulsed between 0 and 6.5 hours at 32°C were stained to visualize sensory precursors. (A) N^{ts1}/+ ; *neu*^{A101}/+ disc. (B) N^{ts1}; *neu*^{A101}/+ disc. Increased formation of sensory precursors was also observed in the arista (Ar) and in the second antennal segment (arrowheads). Scale bar, 15 μm. C and D are enlarged regions of the discs shown in A and B respectively. The boxed area in D shows a group of FCs which could have arisen from a single proneural domain. Scale bar, 5 μm.

The first FCs in the antennal disc are detected in the *neu*^{A101} strain at the onset of pupation and between 20-30 new FCs are formed within each hour of development until 24 hours after puparium formation (APF). We tested the effect of removal of N^{+} activity during defined periods during sensory development using the temperature sensitive allele N^{ts1}.

White prepupae from a cross between N^{ts1} females and *neu*^{A101}/TM6-*Tb* males reared at 22°C, were transferred to 32°C for a further period of 6.5 hours. The antennal discs were dissected out just after the pulse and stained using an antibody against bacterial β-galactosidase. There was a large increase in the numbers of *lacZ*-expressing cells in N^{ts1} discs (Fig. 3B) as compared to N^{ts1}/+ controls (Fig. 3A). Most of the 'ectopic' cells were restricted to domains of four to six cells (Fig. 3D, boxed area) suggesting a failure of lateral inhibition thus allowing all the competent cells to take on a sensory fate. These groups of cells can be identified as ectopic FCs, because of their large nuclei which are placed apically in the disc membrane. In some experiments, pupae treated as described above were returned to permissive temperature until 16 hours APF before being dissected and stained. We observed that the 'extra' FCs generated by *N* loss-of-function were unable to form PSCs as seen in the *N*/+ controls (data not shown). Consistent with this, the adults from such flies did not show any ectopic olfactory sense organs unlike that seen on the wing and notum (Hartenstein and Posakony, 1989). The antenna was somewhat reduced in size and showed a reduction in the numbers of sensilla trichoidea and sensilla basiconica (Fig. 4B,G). The number of sensilla coeloconica was marginally increased after the pulse.

Absence of N^{+} function throughout development in N^{X81K1} clones resulted in a complete absence of bristles and a reduced antennal size (Fig. 4C). Methylene blue staining of sections through the mutant antenna showed that the cells that comprise the sensilla failed to develop. This is in contrast to clones

recovered elsewhere on the body where missing bristles were accompanied by a proliferation of sensory neurons at the expense of external cuticular structures. Clones where an activated *N* construct was expressed in the antenna were generated by flipase-induced recombination in the Act5c>y^+>N_{intra} construct. The clones closely resembled the *N* null clones and showed a failure of sensillar development (data not shown).

Pulses of high temperature given to N^{ts1} animals throughout antennal development (0-25 hours APF) resulted in a decrease in the number of sensilla (Fig. 4G). All the three types of sensilla were affected, but interestingly the sensilla coeloconica are only affected when temperature pulses are given between 7 and 17 hours APF. The interpretation of the N effects are complicated by the fact that new FCs continue to be specified up until 24 hours APF, making it difficult to distinguish the effect on lateral inhibition from that on PSC formation or differentiation (Ray and Rodrigues, 1995). Staining of pupae with mAb22C10 allowed us to infer that the missing sensilla reflected the complete absence of the sense organ, rather than the conversion of the cells forming the external structures to neurons. The activated *N* receptor when expressed at these times in development using the hsp70 promoter also resulted in an absence of olfactory sense organs.

Shifts to non-permissive temperatures of 6 hour duration between 7 and 25 hours APF resulted in ectopic formation of sacculus (Fig. 4E,F). The absence of *N* after the time of cell division of the PSC (16 hours APF) resulted in some defects in differentiation of the sense organs which will be discussed in subsequent sections.

shibire (*shi*) mutations affect the formation of the presensillum cluster

An FC is easily recognizable as an isolated cell with a large nucleus located apically in the disc membrane. Shortly after it is specified, each FC loses its distinctive appearance and becomes associated with 2-3 cells which begin to express β-galactosidase in the neu^{A101} line. The nuclei of these cells are located at different levels within the plane of the disc epithelium giving the appearance of an apical to basal location. Most of the FCs that were identified by 10 hours APF become part of the PSCs by 16 hours APF. We had previously shown that the cells of the PSC do not arise by division of the FC (Ray and Rodrigues, 1995). Data from BrdU incorporation studies as well as observations on discs stained with the DNA dye, DAPI, provide convincing evidence that the FC does not divide to form the cells of the PSC.

Since lineage does not determine the PSC, we asked whether cell-cell interactions in the disc could lead to the recruitment of neighboring cells to the sensory fate. Ablation of the FC to demonstrate its causal role in development of the PSC has not, so far, been possible for technical reasons. In several instances where cell-cell interaction has been demonstrated, endocytosis between the participating cells appears to occur. Perhaps the most dramatic example is the interaction between the R8 photoreceptor and the presumptive R7 cell. Here the Boss protein which is apically located on R8 is internalized after its inter-

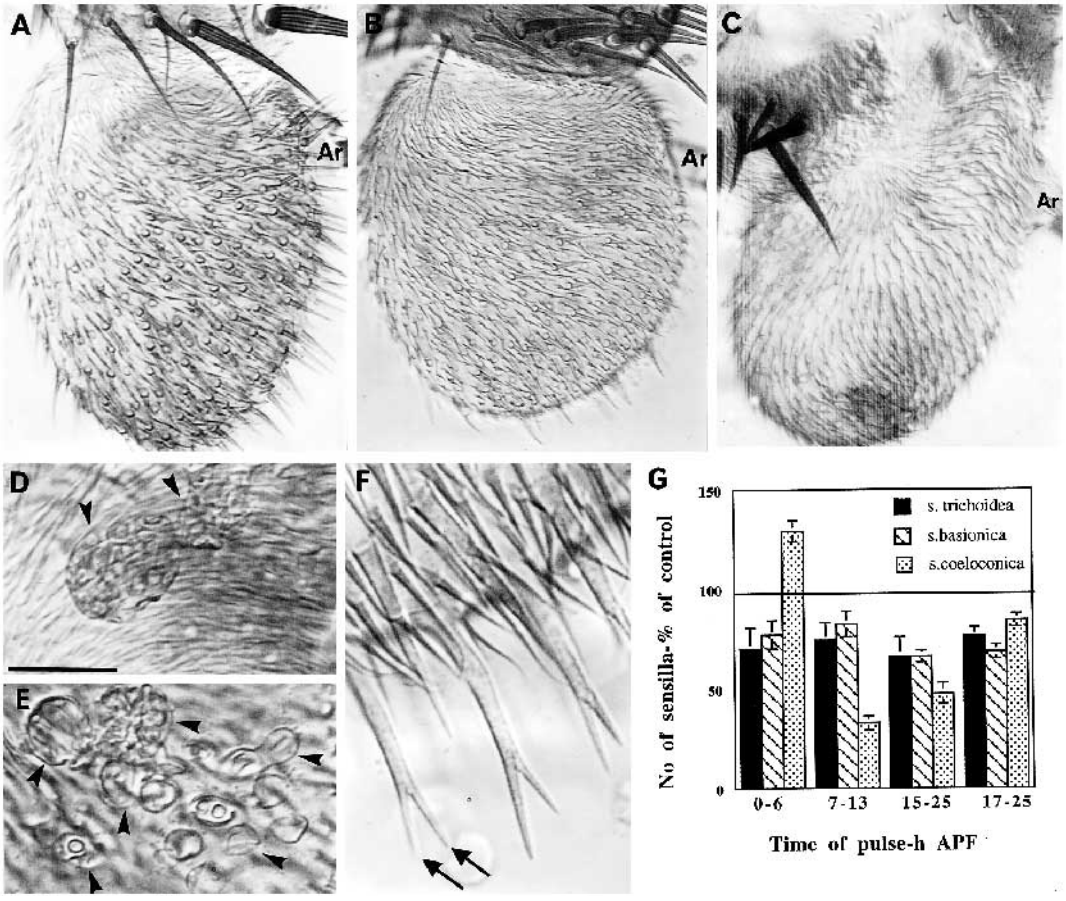

Fig. 4. Effect of *N* loss-of-function at different stages during antennal development. (A) Unpulsed N^{ts1} control. (B) N^{ts1} pulsed between 0 and 6.5 hours APF. (C). *N* null antenna generated as a clone of N^{tX81K1}. (D) Sacculus from N^{ts1} control animal grown at the permissive temperature. (E) Sacculus from N^{ts1} animal pulsed between 10 and 16 hours APF. (F) Bifurcated sensilla are seen in N^{ts1} animals when pulsed between 16 and 25 hours APF. (G) The numbers of sensilla trichoidea, sensilla basiconica and sensilla coleoconica are shown after different temperature pulses as a percentage of the numbers in the unpulsed controls. Scale bar for D-F, 10 μm.

action with the receptor, Sev, located on R7 (reviewed by Kramer, 1993). The availability of temperature-sensitive alleles of *shi* allowed us to block receptor-mediated endocytosis and study its effect on antennal development. White prepupae from a cross between shi^{ts2} females and neu^{A101}/TM6-Tb males were selected and reared at 25°C until 10 hours APF. They were then shifted to 31°C for a further 6 hours; this corresponds to a time period when maximal PSC formation occurs. Discs were dissected 1 hour after the pulse and stained using an antibody against β-galactosidase to visualize sensory cells. Since shi^{ts2} animals grow significantly slower than the wild type, we took care to compare experimental animals to controls which were at a comparable stage of development using several morphological criteria rather than by chronological age alone. The patterns of staining in pulsed shi^{ts2}; neu^{A101}/+ male discs were compared to those from pulsed shi^{ts2}/+; neu^{A101}/+ female controls, as well as shi^{ts2}; neu^{A101}/+ males which were reared to a comparable age continuously at the permissive temperature (Fig. 5). Control discs showed a large number of PSCs composed of 3 cells (Fig. 5A,B; graph in lower panel). When shi^{ts2} pupae were exposed to 31°C from 10-16 hours APF, the majority of sensory units were composed of single cells or two cell clusters (Fig. 5C,D; graph in lower panel). Sensory development in the anlage of the second antennal segment as well as the wing discs were comparable to those of control animals (not shown). Furthermore, morphological changes within the third segment of the antenna were unaffected by the temperature pulse in shi^{ts2} animals. Some of the pulsed pupae were allowed to develop to adulthood and showed a significant reduction in all three types of sensilla. Staining of late pupae with mAb22C10 showed that the entire sense organ and not just the external structures were missing (data not shown).

Interestingly we observed very different consequences of Shi absence in the olfactory sensory system as compared to the other external sense organs in the adult. Temperature pulses to shi^{ts2} animals between 0 and 8 hours APF had no effect on the FC selection process in the antennal discs. In the wing and notum, however, extra SOPs were observed resulting in ectopic bristles on the fly's body. shi^{+} removal later in development (14-24 hours APF) resulted in a conversion of trichogen and tormogen cells of the macrochaete and microchaete to neurons. The only effect seen in the antenna was the loss of complete sense organs presumably due to a failure in PSC formation.

The cells of the presensillum cluster divide once before terminal differentiation

Ray and Rodrigues (1995) showed using BrdU incorporation analysis that the cells of the PSC divide at least once before terminal differentiation. When BrdU was injected into pupae at 17-20 hours APF it could be detected in all the cells of several sensilla at 36 hours APF. In order to study the mitotic patterns in the disc in greater detail, we double-labeled antennal discs from neu^{A101} animals with an antibody against β-galactosidase and with DAPI. Dividing cells were observed within a short time window between 16 and 22 hours APF. Since the cell divisions within each sensillum are not synchronous, it is difficult to ascertain whether all the cells of a PSC, in fact, undergo division. Mitosis in the disc in this time period is restricted mainly to the sensory cells (data not shown). In a small number of cases we observed single epidermal cells located immediately adjacent to the PSC also undergoing division. It is possible that these cells are similar to the so-called 'mystery cells' observed during eye development which are associated with sensory structures and whose terminal fate remains unknown (Wolff and Ready, 1993).

The Pros molecule was detected in a subset of the PSC cells and was found to be asymmetrically partitioned in the daughter cells after cell division (G. V. Reddy unpublished). N^{+} as well as sca^{+} play important roles in the differentiation

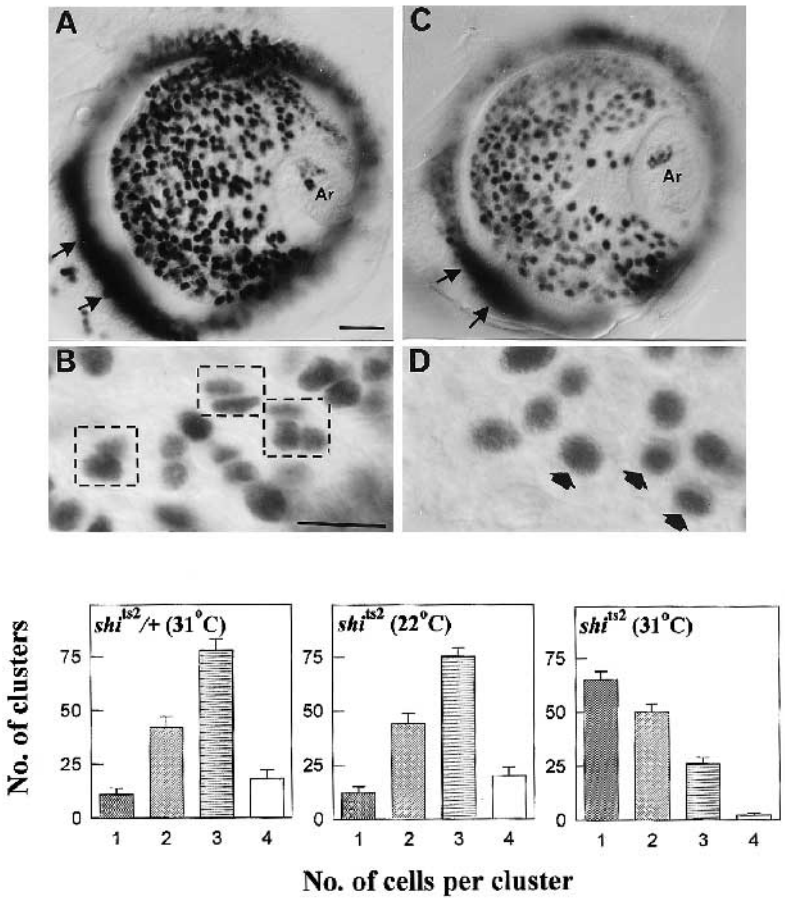

Fig. 5. Requirement for shi^{+} function in the formation of PSC. All animals are heterozygous for neu^{A101} to allow visualization of the sensory cells. shi^{ts2}/+ female (A,B) and shi^{ts2} male (C,D) pupae were pulsed at 31°C for a period corresponding to 10-17.5 hours APF at 25°C. Discs from the control animals (shi^{ts2}/+ animals pulsed at 31°C and shi^{ts2} unpulsed) of corresponding age revealed maximum clusters of 3 cells (Boxed; enlarged view in B and histograms in lower panel). When shi^{ts2} males were pulsed at high temperature, most of the clusters were found to be made up of only single or two cells (arrows in enlarged view in D and histograms in lower panel). The appearance of the arista (Ar) and second segment precursors (arrows in A and C) suggest that development in general was not stalled by *shi* loss-of-function. Scale bars for A and C, 15 μm and for B and D 10 μm. The data presented in the histograms is derived from 6 discs in the case of shi^{ts2}/+ (31°C) and shi^{ts2} (22°C) and from 10 discs in the case of shi^{ts2} (31°C).

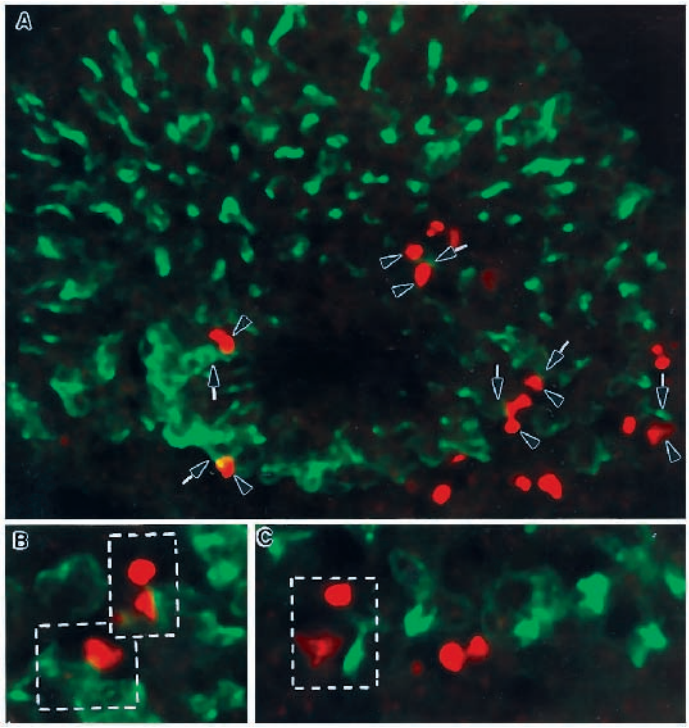

Fig. 6. Programmed cell death during development of the pupal antenna. 24-hour pupal antennae were labeled by the TUNEL protocol. Biotin incorporated into the apoptotic nuclei was visualized using streptavidin-coupled Texas Red (arrowheads in A). Neurons were stained using mAb22C10 and the reaction was visualized using FITC-coupled secondary antibodies (arrows). The preparations were observed using a laser confocal microscope. B and C are at higher magnification. Unstained cells of the sensillum could be seen by switching to Nomarski optics. Boxed regions correspond to single sensillum.

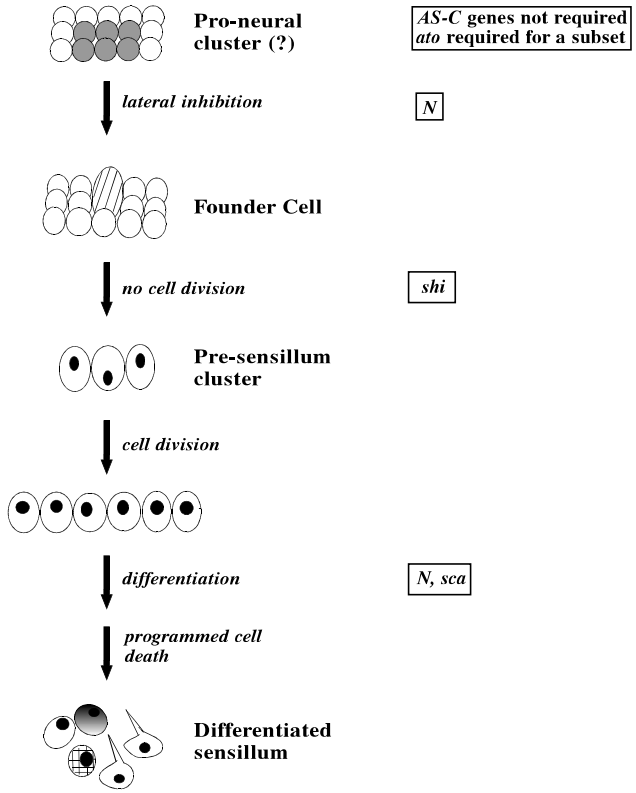

Fig. 7. Model summarizing the current knowledge on the events occurring during development of a sensillum on the antennal surface. Our conclusions are based mainly on our observations on a set of 150 sensilla which are specified during the first 10 hours of pupal development. It is possible that these conclusions are relevant to a subset of sensilla and not to all the sense organs on the antennal surface. Determination of whether this is the case awaits the availability to markers specific for different cell types.

of the olfactory sense organ. When N^{ts1} pupae were pulsed at non-permissive temperature later during development (16-25 hours APF), we observed several sensillar shafts which appeared bifurcated (Fig. 4F). Similar phenotypes were also seen in the complete loss-of-function allele sca^{BP2}. Serial sectioning and ultrastructural analysis of a bifurcated sensilla trichoidea revealed the presence of an extra trichogen cell. Hence the 'bifurcation' appears to result from two trichogen cells contributing to a single shaft. These sensilla were also found to be innervated by a single neuron while sensilla in corresponding positions on wild-type antennae are innervated by three neurons. These observations suggest that *sca* loss-of-function results in the conversion of a neuron to a support cell.

The first fully differentiated sense organs were detected by 22 hours APF. The monoclonal antibody mAb44C11 which recognizes differentiated neurons first stained cells within the sensillum 22 hours APF (data not shown).

Cell number in the sensillum is controlled by mechanisms that include programmed cell death

Each sensillum is composed of three non-neuronal support cells and a variable number of neurons. The number of neurons innervating each sensillum, appears to depend on the sensillum type and its location on the antennal surface (S. Shanbhag, unpublished). This means that there must exist a mechanism that controls the final cell number within each sensillum. We examined the developing antennal disc for the presence of apoptotic nuclei during different stages of development. Terminal transferase catalyzed dUTP end labeling (TUNEL) allows the visualization of apoptotic nuclei in wholemount antennal preparations, double labeled with mAb22C10 to reveal sensory cells. At 10 hours APF most of the apoptotic nuclei are restricted to the second antennal segment; only a few stained cells become apparent in the third antennal segment by 15 hours APF (not shown). The appearance of these cells was dynamic and progresses from the tip of the appendage to the base. The peak of apoptosis in the third antennal segment was observed between 22 and 30 hours APF. A very large number of dying cells were seen in the arista at this stage (not shown). A few TUNEL positive nuclei could still be detected on the surface of the antenna up to 50 hours APF. Double staining with mAb22C10 (Fig. 6, green) showed that the TUNEL-positive cells (Fig. 6, red) were associated with the sensory clusters. We analyzed at least 300 different sensory structures at different time points and found that >75% of them had at least one apoptotic nucleus per sensillum. The close association of the apoptotic nuclei with the sensory clusters was ascertained by double

staining with mAb22C10 (Fig. 6B,C) as well as by observation using Nomarski optics.

Hence programmed cell death is likely to be involved in removing undifferentiated cells from the sensillum cluster. Additional mechanisms must exist to control the number of cells that differentiate as neurons in each sense organ.

Clonal analysis reveals that olfactory sense organs are of mixed lineage

Our current view of olfactory sense organ development is that subsequent to FC selection additional neighboring cells are recruited to form a PSC. This cluster of cells divides to finally form the differentiated sensillum (Fig. 7). In such a model the cells constituting a single sensillum would be derived from different lineages. We used the FRT/FLP system designed by Struhl and Basler (1993), to generate mitotic clones in the third segment of the antenna. FLP-recombinase activity was induced in larvae of the *Act5C>Draf1>lacZ* strain 36 hours after egg-laying by providing a temperature pulse at 37°C for 30 minutes. Recombination at the FRT sites results in clones of cells which express *lacZ* under control of the *Act5C* promoter in all cell types. The clones can therefore be identified using an antibody against β-galactosidase (green in Fig. 8), while neurons were recognized using the antibody mAb22C10, (red staining in Fig. 8A,B). Clonal boundaries that segregate the cells of a single sensillum are strong evidence of the cells possessing a mixed lineage. We examined 29 randomly selected sensillar clusters in pupae 48 hours APF for the presence of clonal patches. At this stage the shaft of the sensillum is not fully developed, making it difficult for us to ascertain the sense organ type. In all cases, only some of the cells stained positive for β-galactosidase suggesting that the sensillum was of a mixed lineage. In 8 sensilla, all the neuronal cells but none of the support cells expressed *lacZ* (Fig. 8); while in 5 the support cells but not the neurons were stained. In 15 of the sensilla examined, a subset of the neurons and support cells expressed reporter enzyme activity. While our analysis is not large enough to speculate about the relationship between cells within a sensillum, we can conclude that the cells constituting a single sensory unit are not all clonally derived.

DISCUSSION

Our current view of the development of the olfactory sense organs is based largely on observations on the first set of 150 FCs which appear within the first 10 hours of pupation. The lack of appropriate markers prevents us from assigning these FCs to specific sensilla types and we assume that the mechanisms occurring in these first set of precursors are generally applicable to all the sensilla on the antennal surface (Fig. 7). The process occurs through several sequential steps involving specification of the precursor cell, interactions involving endocytosis with neighboring cells leading to formation of a cluster, cell division followed by terminal differentiation and programmed cell death. These mechanisms show several important variations from those employed in the development of the external sense organs of the embryo and the adult as well as the photoreceptors of the adult compound eye. The significance of this complex pattern of cellular events in relation to the function of regulatory circuits at the molecular level needs further investigation. In this paper we have highlighted some of the novel mechanisms used in the development of the olfactory system and provide the basic groundwork on which these events can be further analyzed.

Specification of the founder cell

In most sensory fields so far studied, choice of the precursor cell involves expression of a proneural gene in a domain of ectodermal cells, creating an 'equivalence group' in which each cell is competent to assume a neural fate. The proneural genes so far identified are bHLH genes belonging to the *AS-C* complex which specifies the majority of the external sense organs in the embryo and adult, and the gene *ato* which is required for the chordotonal organs of the embryo and the adult. We find that the *AS-C* genes are neither essential nor sufficient for directing proneural function in the antenna. *ato* is required for the normal development of the sensilla coeloconica and the sacculus on the antennal surface. The nature of the proneural gene(s) required for the sensilla trichoidea and sensilla basiconica remains obscure. Overexpression of the negative regulator of bHLH proteins, *emc*, results in a reduction of all the sensilla on the antennal surface (B. Gupta and V. Rodrigues, unpublished data). This predicts that the, as yet unidentified, gene(s) that serves a proneural function in the antenna could belong to the bHLH group of transcription factors.

The choice of the FC from the proneural domain involves the activity of the N^+ gene. Loss of N function at this time leads to a larger numbers of FCs being specified, however these 'extra' founders are unable to develop into mature sensilla. Temperature shifts at different time points lead to a reduction in numbers of sensilla on the antenna. The fact that the olfactory sense organs develop continuously rather than synchronously makes it difficult to demarcate the effects of N function at different stages in the development of a sensillum. The wide range of effects of *N* loss-of-function and gain-of-function lead us to conclude that *N* plays a permissive role in antennal development, allowing cells to receive and act on different signals rather than itself deciding a particular cell fate.

Endocytosis is required for the formation of the presensillum cluster

In the developing antennal disc, following selection of the FC, a group of cells neighboring it express the neu^{A101} marker and are therefore believed to have adopted a sensillogenic fate. There are two possible scenarios to explain the formation of the PSC. One possibility is that the FC actively interacts with neighboring cells leading to their recruitment to form a cluster of cells. In this case ablation of the FC should lead to an absence of PSC formation. Such ablation studies have so far been elusive because of the small size of the disc cells and the lack of specific transformants (for example Gal4 strains) that could express toxins specifically in the precursor cells (Smith et al., 1996). An alternative hypothesis for the presence of PSCs is that in a densely populated epidermal field like the antennal disc, several precursor cells could come together to form a sensilla. This would lead to sensilla of mixed lineages. Mixing of cells from different sensilla have been reported in the sensilla of the wing blade which also represents a highly populated sensory region (Hartenstein and Posakony, 1989). The fact that the cells of the PSC appear distinct from the FCs

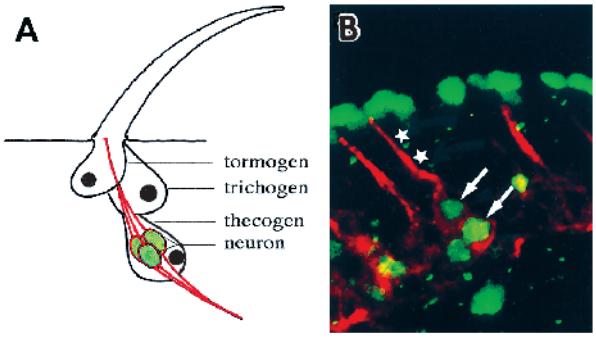

Fig. 8. Clonal analysis of the olfactory sensillum. Pupal antennal were stained with an antibody against β-galactosidase and a secondary antibody linked to FITC (green staining). Neurons were stained with mAb22C10 and the secondary antibody reaction was visualized using Texas Red. (A) Diagrammatic representation of the clone shown in B. The neuronal nuclei show the presence of β-galactosidase (arrows in B) while the nuclei of the support cells shown in A do not. In B stars indicate the positions of the tormogen and trichogen cell nuclei. The cells are not seen in fluorescence but can be identified by shifting to Nomarski optics on the same preparation.

in morphology and position of their nuclei within the epidermis argues against the idea that the PSC is merely a collection of FCs.

We were able to show that a block in endocytosis during development of the antennal disc does not effect the specification of the FC, but affects the clustering of cells to form PSC. Hence a different mechanisms must exist for their formation than that involved in FC selection. We therefore favor the working hypothesis that the PSC is formed by a recruitment of neighboring cells to enter the sensory fate. The occurrence of endocytosis has precedents in other developmental systems where cellular interactions are involved. The best studied example is the internalization of the entire Boss ligand from the surface of the R8 cell by the presumptive R7 which expresses the Sev receptor tyrosine kinase on its surface (reviewed by Kramer, 1993). The physiological significance of this internalization is as yet unclear. In the case of the olfactory system where a block in formation of cell clusters is observed, endocytosis would appear to act in downregulating molecule(s) which play a role in inhibiting cluster formation. The observation that high levels of the *Drosophila* EGF receptor inhibits cluster formation during eye development provide an important clue to this process (Baker and Rubin, 1992).

Terminal differentiation of the cells of the sensillum relies on cellular interactions as well as lineage

Our studies show that the development of the olfactory sensillum is neither strictly lineage related nor does it depend on a mechanism that entirely uses cell-cell interactions. The cellular events occurring early in the pathway viz. selection of the FC and formation of PSCs resemble mechanisms operating during eye development where the R8 cell recruits its neighbors through cell-cell contact. The proneural gene *ato* which specifies R8 is also involved in the development of one of the three types of sensilla; the sensilla coeloconica. Moreover the formation of the PSC requires endocytosis suggesting a mechanism paralleling the R8-R7 interaction which also requires *shi*-mediated endocytosis. After the formation of the PSC the mechanisms involved become more akin to those involved in the 'lineage-restricted' sensilla in the rest of the peripheral nervous system. The cells of the PSC divide at least once before the terminal differentiation. Our preliminary observations suggest that fate acquisition in the olfactory sensilla is likely to rely on both cellular interactions and lineage (Posakony, 1994). We have seen that Pros becomes asymmetrically segregated among the cells of the sensillum after division (V. Reddy, unpublished). In addition the function of N and also sca play important roles in the specification of cell fate.

The selective advantage of this mode of development in the olfactory sense organs remains speculative. The basic mechanisms used here, like those in other sensory systems, involve precursor cell selection, and cell fate acquisition to give rise to cells of different functional types. The variation of this theme used during olfactory development needs to be examined in an evolutionary context.

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