Expression and function of the empty spiracles gene in olfactory sense organ development of Drosophila melanogaster

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SUMMARY
In Drosophila, the cephalic gap gene empty spiracles plays key roles in embryonic patterning of the peripheral and central nervous system. During postembryonic development, it is involved in the development of central olfactory circuitry in the antennal lobe of the adult. However, its possible role in the postembryonic development of peripheral olfactory sense organs has not been investigated. Here, we show that empty spiracles acts in a subset of precursors that generate the olfactory sense organs of the adult antenna. All empty spiracles-expressing precursor cells co-express the proneural gene amos and the early patterning gene lozenge. Moreover, the expression of empty spiracles in these precursor cells is dependent on both amos and lozenge. Functional analysis reveals two distinct roles of empty spiracles in the development of olfactory sense organs. Genetic interaction studies in a lozenge-sensitized background uncover a requirement of empty spiracles in the formation of trichoid and basiconic olfactory sensilla. MARCM-based clonal mutant analysis reveals an additional role during axonal targeting of olfactory sensory neurons to glomeruli within the antennal lobe. Our findings on empty spiracles action in olfactory sense organ development complement previous studies that demonstrate its requirement in olfactory interneurons and, taken together with studies on the murine homologs of empty spiracles, suggest that conserved molecular genetic programs might be responsible for the formation of both peripheral and central olfactory circuitry in insects and mammals.

KEY WORDS: Olfactory sense organs, empty spiracles, amos, lozenge, Axonal targeting

INTRODUCTION
The emergence of function in sensory circuits requires accurate synaptic connectivity between peripheral sensory neurons and their central targets. A large body of literature has shown that this depends on a complex interplay of molecular mechanisms for proliferation control, neuronal fate specification, neuronal pathfinding and target recognition. An important contribution to the generation of neuronal circuits between sensory neurons in the periphery and their target neurons in the CNS is made by ensembles of transcription factors that operate as cell-intrinsic determinants in the regulation of temporal and spatial gene expression in the developing neurons (Chen et al., 2003; Komiyama and Luo, 2006; Shirasaki and Pfaff, 2002; Sheath and Thor, 2003).

An excellent model for the analysis of the molecular mechanisms that control sensory neuron connectivity is the developing olfactory system. In both insects and mammals, precise neuronal circuitry is established by the ordered axonal projection of olfactory sensory neurons (OSNs) to specific target neurons in the olfactory glomeruli (Axel, 1995; Jefferis and Hummel, 2006; Mombaerts et al., 1996; Rodrigues and Hummel, 2008). In Drosophila, OSNs are housed in ~500 hair-like sense organs called sensilla, located on two peripheral structures: the third segment of the antenna and the maxillary palps. Morphologically, three major types of olfactory sensilla can be identified on the third antennal segment, namely the sensilla basiconica, trichoidea, and coeloconica; each of these antennal sense organs contains between one and four sensory neurons (Shanbhag et al., 2000). Each OSN expresses a single odorant receptor (OR) molecule and sends its axon to a specific glomerulus in the antennal lobe where it forms connections with postsynaptic target neurons, the projection neurons (PNs) and local interneurons (LN)s (Fishilevich and Vosshall, 2005).

Sense organs on the antenna derive from progenitor cells (sense organ precursors; SOPs) in the eye-antennal disc. Initial disc development begins during embryogenesis when a set of cells acquire antennal identity through the combinatorial action of the homeodomain transcription factors Homothorax, extradenticle and Distalless, as well as the basic helix-loop-helix (bHLH) protein Spineless (Haynie and Bryant, 1986). The interaction of these transcription factors with the EGF signaling pathway generates a prepattern upon which neurogenic and proneural genes act to generate SOPs. The molecular mechanisms that control the selection of a single progenitor from the undifferentiated field of epidermal cells in the antennal disc are thought to be similar to those involved in the development of other sense organs in the fly PNS, where neurogenic domains are refined to single SOPs through Notch signaling (Rhyu et al., 1994). Two proneural genes are involved in the specification of SOPs for the different sensillar types on the antennal surface (Goulding et al., 2000; Gupta et al., 1998; zur Lage et al., 2003). atonal (ato), a bHLH transcription factor-encoding gene, specifies the progenitors that give rise to coeloconic sensilla, while amos, a second bHLH transcription factor-encoding gene specifies the basiconic and trichoid SOPs. The choice between basiconic and trichoid sensillar development appears to be controlled by the dosage of the Runx family
transcription factor Lozenge (Lz), which regulates amos expression; high levels of Lz produce basiconic sensilla, while lower levels produce trichoidea (Gupta et al., 1998).

After their generation, OSNs initiate axogenesis and target specific glomeruli in the antennal lobe. Considerable progress has been made in understanding the mechanisms that control this wiring specificity, and a number of molecules involved in signaling, cell adhesion and axonal guidance have been identified (Rodrigues and Hummel, 2008). However, the role of transcription factors in the control of OSN pathfinding and connectivity remains far less well understood; so far, only two POU domain transcription factors, Acj6 and Pdm3 (Bai et al., 2009; Komiyama et al., 2004; Tichy et al., 2008), and mastermind (mam), a nuclear factor required for Notch signaling, have been implicated (Sakurai et al., 2009). This paucity of information on transcription factors involved in OSN axonal wiring specificity contrasts with the large number of transcription factors known to control the wiring of the PNs into the antennal glomeruli (Komiyama and Luo, 2006).

The cephalic gap gene empty spiracles (ems) encodes a homeodomain-containing transcription factor that is required during embryogenesis for the development of the antennal head segment from which the larval olfactory sense organs derive. ems loss-of-function mutations result in a gap-like phenotype in the embryonic head and brain, and an absence of peripheral sensory structures in the antennal cephalic segment (Cohen and Jurgens, 1990; Dalton et al., 1989; Schmidt-Ott et al., 1994; Walldorf and Gehring, 1992). During postembryonic brain development, ems is expressed in two of the deutocerebral neuroblast lineages that give rise to the antennal lobe PNs and LNs; ems function is necessary for the specification of these olfactory interneurons, as well as for targeting of their neurites in the antennal lobe (Das et al., 2008; Lichtneckert et al., 2008). By contrast, virtually nothing is known about the function of ems in antennal olfactory sense organ development in Drosophila.

This lack of information on ems action in the development of the adult olfactory sense organs contrasts with the large amount of information on the role of the ems orthologs Emx1 and Emx2 in the formation of the mammalian olfactory system. In the mouse, both genes are expressed in the developing olfactory epithelium, as well as in the developing olfactory bulb, and mutant analysis indicates that they play important roles in proliferation and axonal wiring (Bishop et al., 2003; Mallamaci et al., 1998; Matsuo et al., 1997; Nedelec et al., 2004; Shinozaki et al., 2004; Simeone et al., 1992a; Simeone et al., 1992b). Given the importance of Emx1/2 in the development of axonal projections of murine olfactory receptor neurons, we set out to investigate whether the fly ems gene might also be required for targeting of OSNs in Drosophila.

Here, we show that ems is expressed post-embryonically in a subset of precursors of olfactory sense organs, and acts in their development as well as in the correct targeting of the OSN axons that derive from them. ems is expressed transiently during early pupal life in a subset of progenitors that co-express amos and Lz, and genetic analysis using loss-of-function mutations suggests that ems expression in the developing olfactory sense organs is dependent on both Lz and amos. Although ems-null clones did not reveal any obvious defects in sense organ development, analysis of heterozygotes in a Lz mutant background uncovers a functional requirement in the formation of trichoid and basiconic sensilla. MARCM-based mutant analysis using OR-specific Gal4 drivers reveals a further functional requirement of ems in OSN axonal pathfinding. Our findings on the role of ems in the development of olfactory sense organs in the PNS complement previous reports of a requirement for ems in the development of olfactory interneurons in the CNS. Taken together with studies on the murine homologs of ems, these results suggest that conserved molecular genetic programs might be responsible for the formation of peripheral and central olfactory circuit elements in insects and mammals.

MATERIALS AND METHODS

Fly strains and genetics

Fly stocks were obtained from the Bloomington Stock Centre (IN, USA) and, unless otherwise stated, were grown on cornmeal media, at 25°C. amos1, amos2, and amos3 and the amos-Gal4 stock were kindly provided by Andrew Jarman (University of Edinburgh, UK). For staging, white prepupae (0 hours after puparium formation; APF) were collected on a moist filter paper and aged under humid conditions at 25°C.

MARCM experiments

For tubulin marked MARCM clones (Lee and Luo, 2001), females of genotype yhsFLP; Tubulin-Gal4, UAS-mCD8::GFP; UAS-LacZ/CyO-GFP; FRT82B Gal80/TM6B were crossed to males of either UAS-LacZ,UAS-mCD8::GFP/CyO; FRT82B/TM6B or yhsFLP; UAS-LacZ,UAS-mCD8::GFP/CyO; FRT82B ems1/TM6B.

In MARCM experiments where OR-Gal4 lines were used to mark specific OSNs, females of genotype yhsFLP; UAS-LacZ,UAS-mCD8::GFP/CyO; FRT82B/TM6B or yhsFLP; UAS-LacZ,UAS-mCD8::GFP/CyO; FRT82B ems1/TM6B were crossed to males of the following genotypes: 

- w\textsuperscript{1182}, OR59b- Gal4/FM7a.; FRT82B Tub-Gal80/MKRS,
- w\textsuperscript{1182}, OR85f-Gal4/FM7a.; FRT82B Tub-Gal80/MKRS,
- w\textsuperscript{1182}, OR10a-4 Gal4/CyO; FRT82B Tub-Gal80/TM6B,
- w\textsuperscript{1182}, OR67d- Gal4/FM7a.; FRT82B Tub-Gal80/MKRS,
- w\textsuperscript{1182}, OR43a-Gal4/CyO-GFP; FRT82B Tub Gal80/MKRS,
- w\textsuperscript{1182}, OR88a-Gal4/CyO-GFP; FRT82B Tub Gal80/MKRS,
- w\textsuperscript{1182}, OR83c-Gal4/CyO-GFP; FRT82B Tub Gal80/MKRS,
- w\textsuperscript{1182}, OR477-Gal4/CyO-GFP; FRT82B Tub Gal80/MKRS,
- w\textsuperscript{1182}, OR25a-Gal4/CyO; FRT82B Tub Gal80/MKRS or
- w\textsuperscript{1182}, OR65a-Gal4/CyO-GFP; FRT82B Tub-Gal80/MKRS.

For generating large clones in the antenna, the Minute technique was used. Here, yhsFLP; UAS-LacZ,UAS-mCD8::GFP/CyO; FRT82B/TM6B or yhsFLP; UAS-LacZ,UAS-mCD8::GFP/CyO; FRT82B ems1/TM6B females were crossed to w\textsuperscript{1182}, FRT82B, Ubiv::GFPnicc, 3R P{y[492]Rps3}[Pлаe92]/TM6B males. Progeny of all the clonal crosses were heat shocked for 1 hour at 37°C at the late second instar stage. Results were observed in the adult, except for experiments shown in Fig. 3E-L, where 5 hour APF animals were dissected.

Immunolabeling

Pupal antennal discs and adult brains were dissected and stained as described previously (Jhaveri and Rodrigues, 2002; Wu and Luo, 2006). Primary antibodies used were: rabbit anti-GFP (1:10,000; Molecular Probes, Invitrogen, Delhi, India); mouse anti-Bruchpilot (mAbnc82, 1:20; Walldorf, University of Saarland, Hamburg, Germany); mouse anti-Lz (1:500; H. Bellen, Baylor College of Medicine, USA). The secondary antibodies were used: Alexa Fluor-488-, Alexa Fluor-568- and Alexa Fluor-647-coupled antibodies generated in goat (1:400; Molecular Probes).

Cuticle preparation and sensillar counting

Adult antennae were dissected in PBS, placed on a glass slide with Faure’s solution, covered with a coverslip and allowed to clear at 75°C overnight (Gupta et al., 1998). Cuticle mounts were imaged using Nomarski optics in a Nikon E-1000 microscope. Static images of the antenna taken at different focal planes were imported into Image J and sensilla were counted using the ‘cell counter’ plug-in (http://rsbweb.nih.gov/ij/).
Microscopy and image processing
Fluorescent preparations were imaged on an Olympus Fluoview (FV1000) or Leica TCS SP5 scanning confocal microscope. Optical sections were taken at 1 μm intervals with a picture size of 512×512 pixels and digitally processed using Image J and Adobe Photoshop CS3 (Adobe Systems, San Jose, CA, USA).

RESULTS
ems is co-expressed in a subset of amos-expressing cells in the developing antennal disc
The progenitors of the olfactory sense organs are generated in the third segment of the antennal disc and are specified by two bHLH transcription factor-encoding genes, amos and ato. For a precise characterization of the spatial and temporal pattern of ems expression during this process, we first studied ems in the context of amos and ato using immunocytochemistry.

Fig. 1A-E shows the spatiotemporal expression of amos in the third antennal segment from 0-12 hours APF. At 0 hours APF, amos is not yet expressed (Fig. 1A) and first appears at 1 hour APF (not shown). At 2 hours APF, Amos appears in three to four semi-circular domains in the third antennal segment (Fig. 1B), which broaden by 8 hours APF and 12 hours APF to merge into a single large ‘C-shaped’ band of cells (Fig. 1C-E). The time course of ems expression is comparable with that of amos, although it is sparser and terminates earlier (Fig. 1F-J). At 0 hours APF, ems is detected in superficial cells located along the periphery of the disc and in the second antennal segment (arrow in Fig. 1F) and at the base of the presumptive arista (arrowhead in Fig. 1F). This staining is probably within the epidermal cells of the disc, including the peripodial membrane, and was not analyzed further in this study. No ems-expressing cells are seen within the third antennal segment proper at this time. At 1 hour APF, initial expression is observed in the third antennal segment (not shown), and by 2 hours APF this increases to include cells in four semi-circular domains (Fig. 1G). Expression in these domains broadens by 5 hours APF (Fig. 1H). By 8 hours APF, ems levels begin to diminish (Fig. 1I) and are undetectable by 12 hours APF (Fig. 1J).

A comparison of merged spatiotemporal expression patterns of amos and ems shows that the onset of ems expression is temporally coincident with that of amos, but is downregulated earlier (Fig. 1K-O). ems is present in a subset of amos-expressing but in none of the ato-expressing cells (Fig. 1P-S). This was confirmed at all pupal stages in the third antennal segment (data not shown). We conclude that although all ems-expressing cells co-express amos (and none co-express ato), not all amos-expressing cells co-express ems.

As lz is known to activate amos (zur Lage et al., 2003), both genes should be expressed together in precursors of the trichoid and basiconic (but not coeloconic) sensilla. As expected, we find that all ems-expressing cells also co-express ato, and that ems expression is restricted to a subset of lz-expressing cells (see Fig. S1 in the supplementary material).

The ems gene is expressed in a subset of olfactory sense organ precursors
Although amos is expressed in the proneural cell clusters from which the trichoid and basiconic SOPs derive, the senseless (sens) gene is highly expressed in the SOPs themselves and is, thus, a marker for these progenitor cells (Nolo et al., 2000). Fig. 2A-E shows SOPs marked by Sens in the antennal disc 5 hours APF. Only a small proportion of the ems and amos-expressing cells co-express sens (Fig. 2A-H). We conclude from this that ems is expressed (with amos) in a subset of the proneural domains from which SOPs are selected.
Initial observations indicate that the number of ems-expressing SOPs increases transiently during early pupal development. In order to quantify this, we counted the number of cells co-expressing ems, sens and amos within the anlage of the third antennal segment at different time points during early pupal development (Fig. 2I). At 5 hours APF, ~25% of ems- and amos-expressing cells also co-express sens; this number increases to ~40% at 8 hours APF. At 12 hours APF, ems expression declines markedly and is found in only a few cells.

Taken together, these findings indicate that ems is expressed, together with amos, in a subset of proneural clusters within the third segment of the antennal disc. Moreover, its expression is transient within SOPs, which are selected from these clusters. This suggests that ems could be involved in the development of a subset of the trichoid and/or basiconic olfactory sense organs.

amos regulates ems expression in the developing third antennal segment

Given the central role of amos in the specification of basiconic and trichoid SOPs, we reasoned that expression of ems in the third antennal segment might require amos. We characterized two mutant alleles of amos, amos$^{1}$ and amos$^{3}$, which show a strong reduction in basiconic and trichoid antennal sensilla (zur Lage et al., 2003). In both mutant alleles, ems expression was almost totally absent in the third antennal segment except for a few cells seen in the periphery of the third antennal segment (white arrowhead in Fig. 3B). Fig. 3A-D demonstrates that ems expression is absent in proneural clusters and SOPs (ems is detected only in non-sensory/non-progenitor cells; compare with control discs, Fig. 1H). As expected, ato expression is unaffected (not shown), whereas the number of sens-expressing SOPs was reduced as compared with controls (Fig. 3C; Fig. 2A). The ~250 (252.3±38.8; n=3) Sens-positive cells in wild-type antennal discs at 5 hours APF are reduced to ~110 (110.3±6.8; n=3) in amos$^{3}$ mutants.

To determine whether ems might in turn regulate amos expression, we generated marked clones of a loss-of-function allele of ems (which is embryonic lethal) using MARCM (mosaic analysis with a repressible cell marker) (Lee and Luo, 2001). In these experiments, ubiquitously expressed tub-Gal4>UAS-GFP was used to drive UAS-mCD8::GFP in clones induced at the second larval instar stage and recovered at 5 hours APF. In control clones, as expected, all ems-expressing cells co-expressed amos, and the overall expression domains of the two genes were comparable with wild type (Fig. 3E-H). In ems mutant clones, the overall expression domains of amos also remained normal and all cells that expressed the mutant ems gene invariably co-expressed amos (Fig. 3I-L). [The ems mutant allele used encodes a truncated non-
functional protein that is still detected in the cytoplasm by the anti-Ems antibody (see Lichtneckert et al., 2007).] We conclude that ems does not regulate amos in the developing third antennal segment.

As lz is known to regulate amos (zur Lage et al., 2003), loss-of-function lz alleles might be expected to affect ems expression in the third antennal segment. To investigate this, we characterized the developing antennal disc of two viable strong hypomorphic lz alleles, lzt1 and lzt4. In both alleles, the number of amos-expressing cells was strongly reduced (Fig. 3M) and ems expression was completely absent in the anlage of the third antennal segment, leaving only the epidermal staining (Fig. 3N). This is shown for lzt3 in Fig. 3M-P, which also documents the fact that the overall number of SOPs as visualized by sens-expression was reduced as expected (wild-type 252.3±38.8, n=3; lzt3 92.7±0.6, n=3). These results are in accordance with the notion that lz, by acting through amos, is also involved in regulation of ems in the anlage of the developing third antennal segment.

**ems is involved in the development of olfactory sensilla**

As the two genes known to specify trichoid and basiconic sensilla, lz and amos, also regulate ems expression, we reasoned that ems might itself play a role in olfactory sensillar development. In order to test this, we generated large emsnull mutant clones using the Minute method (see Materials and methods) in the antenna and examined sensillar types in cuticular whole mounts. This analysis failed to reveal any change in the number of any of the three sensillar types in mutant versus wild-type antennae (see Fig. S2 in the supplementary material).

This negative result could mean that ems is not necessary for olfactory sensillar development. Alternatively, it could reflect a functional redundancy of ems with other genes that control the development of the olfactory sensilla. Ectopic expression of ems in the sense organ precursors using lz-Gal4 (lz-Gal4/+;UAS-ems/+;P>0.001; see Fig. S3 in the supplementary material). When ems is expressed using a later driver amos-Gal4, only the trichoid sensilla are affected (P<0.01; see Fig. S3 in the supplementary material). These results suggest that the dynamic expression of Ems in the sensory precursors is important for determination of sensillar types.

Because lz (acting through amos) is a key player in sense organ development, a possible functional redundancy of ems might be uncovered by testing for genetic interactions with lz alleles. To investigate this, we examined the sensillar numbers of flies heterozygous for ems in the background of a temperature-sensitive allele of lz (lznull). When reared at a permissive temperature (25°C), expression patterns of amos and ems were only mildly affected (see Fig. S4A-C in the supplementary material) and the number of different sensillar types was comparable with that of the wild type (Fig. 4). However, when these hemizygous mutants also carried a heterozygous ems-null allele (lznull/Y; emsnull/+), they showed a marked reduction in the number of basiconic sensilla (Fig. 4; P<0.001). The trichoid and coeloconic sensilla were unaffected (P>0.3). As expected, lznull animals reared at non-permissive temperature (29°C) showed a severe reduction in basiconic sensilla (Fig. 4). Staining of 5 hours APF antennal discs from these animals revealed a strong decrease in ems as well as in amos expression (see Fig. S4D-F in the supplementary material). Significantly, however, in the background of an ems heterozygote at 29°C, these animals showed an additional reduction in the number of trichoid sensillae (Fig. 4; P<0.001). The coeloconic sensilla were not affected in any of these experiments. A small number of mechanosensory bristles appeared on the antennae of the lznull mutants at 29°C and in lznull/Y; emsnull/+ at 29°C; these were never observed in controls (data not shown).

In view of the observation that emsnull mutations by themselves do not affect sensillar development, we hypothesize that ems is involved in a functionally redundant manner in the development of a subset of trichoid and basiconic (but not coeloconic) sense organs. It therefore seems likely that ems acts in a redundant pathway together with other gene(s) regulated by lz.

**ems is required for olfactory receptor neuron wiring**

The experiments described above were assayed at the level of external sensillar morphology and uncovered a redundant role of ems in sense organ development. However, in these experiments, the development of the internal cells comprising the sense organs – neurons and support cells – was not analyzed. Could ems have an additional non-redundant role in the development of the OSNs that are generated from the SOPs? To investigate this, we carried out a MARCM-based mutant analysis using OR-specific Gal4 drivers and assayed for axonal wiring defects in the adult antennal lobe. Three Gal4 drivers specific for ORs expressed in basiconic sense organs (OR10a, OR59b, OR85f) and seven Gal4 drivers specific for ORs expressed in neurons of trichoid sens organs (OR23a, OR47b, OR67d, OR88a, OR43a, OR83c, OR65a) were used. Clones were induced at the late second larval instar stage and recovered in the adult (see Materials and methods). This protocol allowed us to avoid large emsnull neuroblast clones in the antennal lobe, which are rarely generated 48 hours after larval
hatching; nevertheless, we excluded brains that showed anatomical defects visible upon immunostaining with mAbnc82. Previous investigators have shown that in most cases clones including only one or two interneurons are likely to be generated at these time points (Das et al., 2008).

OSNs deficient for ems showed a variety of connectivity defects exemplified in Fig. 5 and summarized in Table S1 in the supplementary material. In control clones, labeled receptor neuron axons enter the antennal lobe, converge onto the appropriate ipsilateral glomerulus, and also innervate the corresponding contralateral lobe via a commissural projection (Fig. 5A,D,G,J,M). By contrast, marked defects in projections across the midline were observed in mutant clones induced with four out of seven trichoid-specific driver lines (OR47b, OR88a, OR23a and OR67d; arrow in Fig. 5B,E,H,K). In some cases, OSNs fail to innervate the ipsilateral glomerulus (N, arrow). (3) OSNs spill-over defects, where terminals extend beyond their target glomerulus to a neighboring glomerulus (arrow, L). (4) Misrouting defects, where OSNs project outside the antennal lobe to non-olfactory neuropile (arrows in O,R). The green staining demarcated with broken lines in E,H,O is a staining artifact. Scale bar: 50 \( \mu \text{m} \).

Targeting defects were also observed in one of the three basiconic specific OR lines (OR10a). Mutant axons that projected across the midline often failed to target to the appropriate contralateral glomerulus and meandered across the lobe, whereas in other cases, they failed to form ipsilateral projections and innervated only the contralateral glomerulus (Fig. 5N). In one preparation, we observed mutant axons projecting beyond the antennal lobe into adjacent ‘non-olfactory’ neuropile (arrowheads in Fig. 5O; schematic in Fig. 5R). The remaining basiconic-specific OR lines (OR59b and OR85f) yielded a significantly smaller
Fig. 6. Summary diagram of the development of SOPs in the antennal disc. Lz is expressed in a zone of epidermal cells defining a prepatterning in the third antennal disc. amos is regulated by Lz and is turned on within a set of cells called the proneural domain. Although amos and ems are expressed simultaneously within the resolution of our experiments, genetic experiments have shown that amos turns on ems in a subset of proneural domains. Lateral inhibition acts upon these cells, leading to the selection of a single SOP that expresses higher levels of Amos and/or Em's and Sens. Expression of ems is turned off prior to amos. The gene(s) that negatively regulate ems have not been identified.

DISCUSSION

ems is expressed in a subset of proneural clusters and SOPs specified by amos

In this report, we studied the expression and function of ems in the developing third antennal segment of Drosophila. Our analysis of ems expression combined with data on co-expression of other key genes involved in antennal sense organ development allowed the identification of the ems-positive cell types. Moreover, it demonstrated that ems is expressed in a restricted spatial and temporal pattern in these developing sense organs.

In antennal development, SOPs derived from proneural domains specified by amos form trichoid and basiconic sensilla and those specified by ato form coelocoonic sensilla (Gupta et al., 1998; zur Lage et al., 2003). We find that ems-expressing cells comprise a subset of the cells in the amos-expressing proneural domains and SOPs, and argue that ems confers specific properties on this subset of SOPs. Although the precise identity of sensilla that are influenced by ems expression has not been determined, it is unlikely that these are confined to a specific region of the antennal surface. Furthermore, it is unlikely that ems-expressing SOPs develop into a distinct sensillar type because the sensillar phenotypes we observed upon interaction with Lz alleles affect both trichoid and basiconic lineages.

Previous work has established that the pre-patternning gene Lz first appears early in the antennal disc (at late third instar larva) and regulates the expression of amos in proneural clusters (Goulding et al., 2000; Gupta et al., 1998). Our immunocytochemical studies show that ems expression is coincident with the appearance of amos, arguing that Lz could regulate both genes in proneural clusters.

Genetic analysis demonstrates that ems expression is lost in amos mutants, favoring the idea of a hierarchy of gene function with Lz controlling amos, which in turn regulates ems (model in Fig. 6).

What is the mechanism that selects ems expression in a spatially defined pattern of cells in the antennal disc? It seems unlikely that expression could be determined solely through the action of Lz and amos, as ems is expressed in a small subset of these lineages. We propose that additional genes interact in a genetic cascade, together with Lz and amos to select specific ems-expressing proneural clusters and SOPs. This idea is supported by our observation of a haplo-insufficient interaction of ems with Lz mutations. The identity of these unknown genes needs to be deciphered by further genetic studies.

Function of ems in adult olfactory sense organ development

Two mutant phenotypes in olfactory sense organ development were observed in ems loss-of-function experiments: deficits in the number of external olfactory sensilla and axonal path finding/targeting defects of mutant OSNs in the antennal lobe.

Although ems loss-of-function mutations did not provide evidence for a requirement of ems in sensilla development, we did observe a clear haplo-insufficient interaction between ems mutations and Lz alleles that affected the formation of trichoid and basiconic sense organs. This leads us to propose that ems plays a redundant role, acting with gene(s) regulated by Lz, in the development of some of the basiconic and trichoid sensilla. Genes that act together with Lz to regulate formation of trichoid or basiconic sensilla have not yet been identified. Ha and his colleagues (Ha and Smith, 2006) identified two genes, tod1 and tot1, that affect trichoid sensilla alone without affecting basiconica. However, the nature of these genes has not been characterized, and it will be interesting to study whether they impinge on ems expression.

A marked non-redundant role of ems was observed in OSN axon pathfinding and targeting in MARCM-based analyses. Several different types of axonal projection defects were seen in the ems mutant OSNs of trichoid sensilla (schematized in Fig. 5P-R). These include defects in commissural projections, mistargeting to inappropriate glomeruli, spillover of axon terminals and unequal innervation of ipsilateral versus contralateral glomeruli. Comparable defects were seen in the ems mutant OSNs of basiconic sensilla, albeit at lower frequency.

As ems is expressed transiently in the SOPs and their proneural domains during early pupal development, it is likely that ems acts as an early intrinsic determinant in sense organ progenitors to influence cell fate decisions, which indirectly result in appropriate axonal projections of OSNs later in postembryonic development.
Thus, OSN axonal targeting is likely to be mediated by other factors that are themselves regulated by ems and subsequently affect components of the wiring machinery.

Targeting of the OSNs to cognate glomeruli has been studied in several laboratories and the roles of several cell adhesion and signaling molecules have been identified. Roundabout proteins (Robo1, Robo2 and Robo3) (Jhaiveri et al., 2004), semaphorins (Komiyma et al., 2007; Sweeney et al., 2007), N-cadherin (Hummel and Zipursky, 2004), DSCAM (Hummel et al., 2003), Wnt5 (Sakurai et al., 2009; Yao et al., 2007) and the small GTPases Pak and Dock (Ang et al., 2003) have been implicated. However, to date, the only transcription factors other than Ems that have been shown to affect OSN targeting are the POU domain molecules Acj6 and Pdm3 (Komiyma et al., 2004; Tichy et al., 2008), and the Notch signaling pathway acting through Mastermind (Sakurai et al., 2009). Although Ems, along with other transcription factors, has also been shown to be required for the precise targeting of PNs and LNs, it remains to be determined how these transcription factors can regulate cell surface and signaling molecules in developing OSN axons.

**ems is required for the development of both larval and adult olfactory sense organs and olfactory interneurons**

During embryonic development, ems is first expressed at the early cellular blastoderm stage in a single circumferential stripe at the anterior end of the embryo that subsequently becomes regionalized to discrete ectodermal patches of the labral, antennal and intercalary segment of the anterior head (Dalton et al., 1989; Walldorf and Gehring, 1992). The large ems domain in the ectoderm/neuroectoderm of the antennal segment gives rise to a set of peripheral cephalic sense organs and to the anlage of the antennal brain neurome (Hartmann et al., 2000; Lichtneckert and Reichert, 2008; Urbach and Technau, 2004).

Mutation of ems leads to a gap-like phenotype in the embryonic head, which includes deletions of cephalic sense organs in the antennal (and intercalary) segments (Cohen and Jurgens, 1990; Dalton et al., 1989; Jürges et al., 1984; Walldorf and Gehring, 1992). The major olfactory sense organ of the larva – the dorsal organ – is also lacking in ems mutants (Schmidt-Ott et al., 1994; Stocker, 2008). In addition, lack of ems function also results in defects in the embryonic brain, including a deletion of the deutocerebral brain neurome, which contains the larval olfactory lobe (Hirth et al., 1995). This phenotype is due to defective specification of the neuroectoderm and correlates with the absence of the proneural gene lethal of scute, which is thought to be required for neuroectodermal cells to adopt the competence to become neuroblasts (Younossi-Hartenstein et al., 1997).

Remarkably, ems plays important roles in both peripheral and central olfactory system development of both the larval and adult. This is despite the fact that the olfactory sense organs of the adult antenna (and maxillary palps) have a distinct origin from those of the larvae. Most of the olfactory interneurons of the adult are generated postembryonically from a set of deutocerebral brain neuroblasts, and previous reports showed that ems function is necessary in at least two of these neuroblast lineages (Jeffers and Hummel, 2006; Rodrigues and Hummel, 2008). In the antennodorsal neuroblast lineage, ems plays a role in the appropriate dendritic targeting of PNs to the olfactory glomeruli, whereas in the lateral neuroblast lineage it determines the correct number of PNs and LNs. It is interesting that ems acts in the development of both the peripheral and central olfactory system; the possibility that this transcription factor plays a role in matching these classes of neurons is intriguing.

**Evolutionary conservation of ems/Emx roles in olfactory system development?**

The organization of the olfactory system is strikingly similar in insects and mammals (Hildebrand and Shepherd, 1997; Komiyma and Luo, 2006). OSNs expressing a given OR project to the same glomerulus in the antennal lobe of insects and the olfactory bulb of mammals. In the glomeruli of both animal groups, OSNs make specific synaptic connections with olfactory interneurons: PNs and LNs in insects; and the mitral, tufted cells, the periglomerular cells and granule cells in mammals. In Drosophila, ems plays important roles in the development of both peripheral sense organs and central olfactory interneurons at both larval and adult stages. Remarkably, the vertebrate homologs of ems, Emx1/2, have comparable functions in the development of the olfactory system.

In the mouse, genes of the ems/Emx family have been shown to be important both for the development of the OSNs and the olfactory interneurons. The two murine ems gene homologs, Emx1 and Emx2, are expressed peripherally in the developing olfactory epithelium and centrally in cells in the developing olfactory bulb, notably the mitral cells, which are the vertebrate counterpart of the insect olfactory PNs (Mallamaa et al., 1998; Simeone et al., 1992a; Simeone et al., 1992b). Mutational loss of the Emx genes leads to marked defects in both nasal epithelium and olfactory bulb (Bishop et al., 2003; Cecchi and Boncinelli, 2000; Shinozaki et al., 2004). In Emx2 mutants, the olfactory nerve is present; however, no connection is formed between the nerve and the bulb, implying that most of the olfactory sensory neurons fail to project to the brain. In Emx1/2 double mutants the olfactory bulbs are reduced and severely disorganized, and the olfactory tract is deficient.

The comparable expression and function of ems/Emx genes in the development of olfactory sensory neurons and olfactory interneurons in insects and mammals, argue for evolutionarily conserved roles of the ems/Emx genes in olfactory system development. Thus, although the similarity in anatomical organization of the peripheral and central olfactory system in insects and mammals may be due to functional convergence, it might also reflect a remarkable conservation in the molecular mechanisms for central and peripheral olfactory system development in both animal groups.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at [http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.052407/-/DC1](http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.052407/-/DC1)

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in the peripheral olfactory system

DEVELOPMENT

Graded expression of semaphorin-1a cell-autonomously directs dendritic targeting: intrinsic transcriptional control and hierarchical interactions.


Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster.


As a result, the empty spiracles gene is necessary and sufficient for sensory organ development in Drosophila. Cell 102, 349-362.


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