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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; Skeath 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 (LNs) (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 proneural 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

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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. *amos*^{1}, *amos*^{2}, *amos*^{3} 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 *UAS-LacZ,UAS-mCD8::GFP/CyO; FRT82B ems*^{3}*/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 ems*^{3}*/TM6B* were crossed to males of the following genotypes:

wt^{1118}*,OR59b-* Gal4*/FM7a;;FRT82B Tub-Gal80/MKRS*,
wt^{1118}*,OR85f-*Gal4*/FM7a;;FRT82B Tub-Gal80/MKRS*,
w^{1118}*;OR10a-Gal4/CyO; FRT82B Tub-Gal80/TM6B*,
wt^{1118}*,OR67d-* Gal4*/FM7a;;FRT82B Tub-Gal80/MKRS*,
w^{1118}*;OR43a-Gal4/CyO-GFP; FRT82B Tub Gal80/MKRS*,
w^{1118}*; OR88a-*Gal4*/CyO-GFP; FRT82B Tub Gal80/MKRS*,
w^{1118}*; OR83c-*Gal4*/CyO-GFP; FRT82B Tub Gal80/MKRS*,
w^{1118}*; OR47b-*Gal4*/CyO-GFP; FRT82B Tub-Gal80/MKRS*,
w^{1118}*; OR23a-*Gal4*/CyO; FRT82B Tub Gal80/MKRS* or
w^{1118}*;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 ems*^{3}*/TM6B* females were crossed to *w*^{1118}*; FRT82B, Ubi::GFPnls, 3R P{A92}RpS3[Plac92]/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; DSHB, Iowa, USA); rabbit anti-Ems (1:500) and rat anti-Ems (1:100; U. Walldorf, University of Saarland, Hamburg, Germany); mouse anti-Lz (1:10; DSHB Iowa, USA); sheep anti-Atonal (1:5000) and rabbit anti-Amos (1:500; A. Jarman, University of Edinburgh, UK); and guinea-pig anti-Senseless (1:1000; H. Bellen, Baylor College of Medicine, USA). The secondary antibodies used were 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/).

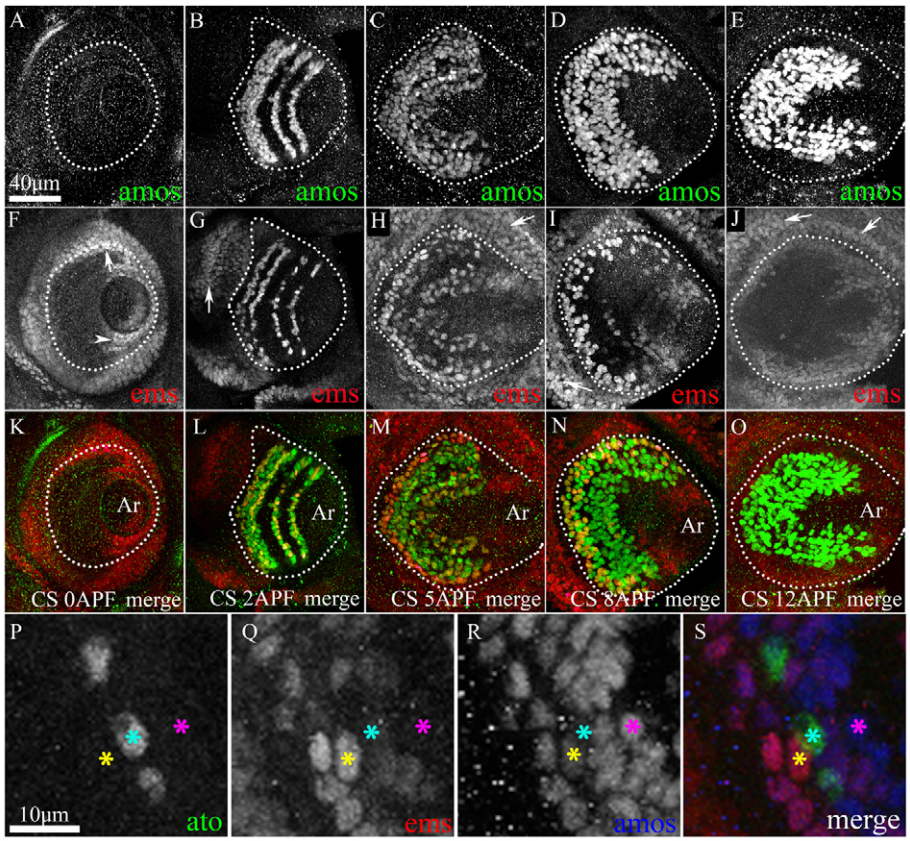

Fig. 1. *ems* is co-expressed in a subset of *amos*-expressing cells in the developing antennal disc. (**A-O**) The third antennal segment in the antennal disc is highlighted within broken lines. Ar, arista. (A,F,K) 0 hours APF; (B,G,L) 2 hours APF; (C,H,M) 5 hours APF; (D,I,N) 8 hours APF; (E,J,O) 12 hours APF. Merged images of discs immunolabeled with anti-Amos (A-E) and anti-Ems (F-J) shown in K-O. Both Amos (A) and Ems (F) are absent from sensory cells of the disc at 0 hours APF. At 2 hours APF, expression of both Amos and Ems is seen (B,G); at 12 hours APF, Ems expression has decayed (J), while Amos expression is still strong (E). In addition to sensory cells, Ems can be detected in the superficial layer of the disc (arrows in F-J) and in the epidermal cells at the periphery of the presumptive arista (arrowhead in F). (**P-S**) Magnified images of the third antennal segment stained with anti-Ato (P), anti-Ems (Q) and anti-Amos (R). The merged image (S) shows that Ems is always co-expressed with Amos (yellow star), but never with Atonal (cyan star). However, not all Amos-positive cells express Ems (pink star). Scale bars: 40 μm for A-O, 10 μm for P-S.

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 *lz*, 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.

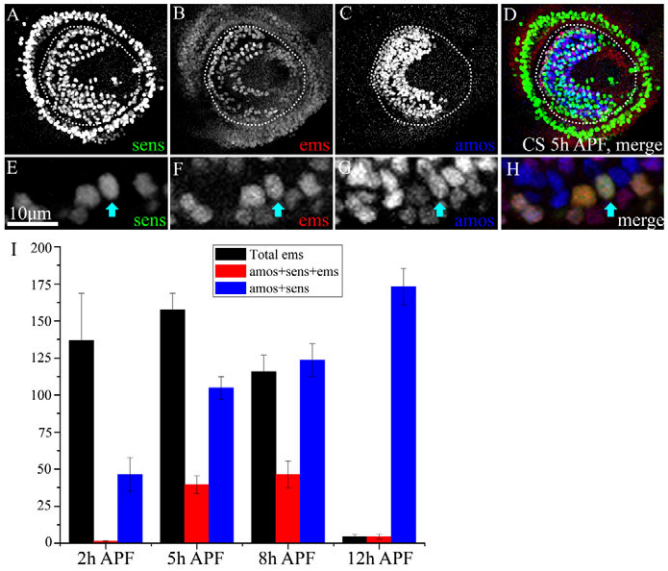

Fig. 2. *ems* is expressed in a subset of olfactory sense organ progenitors. (**A-D**) 5 hours APF antennal disc co-stained with anti-Senseless (A), anti-Ems (B) and anti-Amos (C). (D) Merge. (**E-H**) Magnified images from a 5 hour APF antennal disc. Scale bar: 10 μm. The cell marked with arrows expresses all three markers and are identified as an Ems-positive, Amos-positive SOP. Ems-positive cells that express Amos but not Sens are likely to be cells of the proneural domains. (**I**) Quantification of Ems-positive and Amos-positive cells, which also express Sens. Histograms represent the mean ± s.d.; *n*=4.

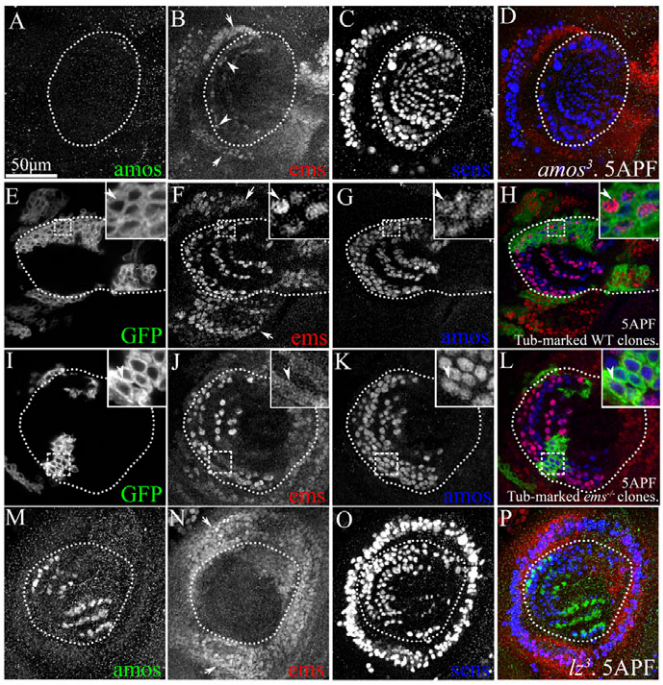

Fig. 3. Lz and Amos regulate *ems* expression in the developing third antennal segment. (**A-D**) Antennal discs from 5 hour APF animals. Anlage of the third antennal segment (and arista) is highlighted with broken lines. (A) *amos*^{3} completely lacks Amos immunoreactivity. (B) Ems expression is present in the non-sensory cells in the superficial layers of the disc (arrows) and in a few cells within the third segment (arrowheads). (C) Sens-positive cells are reduced in number when compared with wild type (Fig. 2A). (D) Merge of A-C. (**E-L**) MARCM clones labeled with *tub*-Gal4>UAS-GFP. In each case, a region of the clone (boxed) is magnified in the inset. (E-H) Control clones show normal expression of Ems (F, arrow indicates the non-sensory staining) and Amos (G). (H) Merge of GFP, Ems and Amos. Arrowheads in E-H indicate cells that co-expresses Amos and Ems. (I-L) *ems*-null clones. Anti-Ems recognizes the non-functional truncated Ems in the mutant (arrowhead in J). In these clones, cells mutant for Ems express Amos (arrowheads in K,L) and the overall domains of *amos* expression remain normal (K). (**M-P**) Antennal discs of 5 hours APF *lz*^{3} animals. Expression of Amos is reduced, leaving a small domain of cells (M). Ems staining within sensory progenitors is absent, leaving only the staining in superficial cells (N, arrows). The number of Sens-positive (SOPs) (O) is greatly reduced. (P) Merge. Scale bar: 50 μm.

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 (110.33±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* 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, lz^{3} and lz^{34}. 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 lz^{3} 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; lz^{3} 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 $ems^{-/-}$ 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*/+) leads to a decrease in the number of basiconic and trichoid sensilla (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* (lz^{ts1}). 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 (lz^{ts1}/Y; ems^{3}/+), 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, lz^{ts1} 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

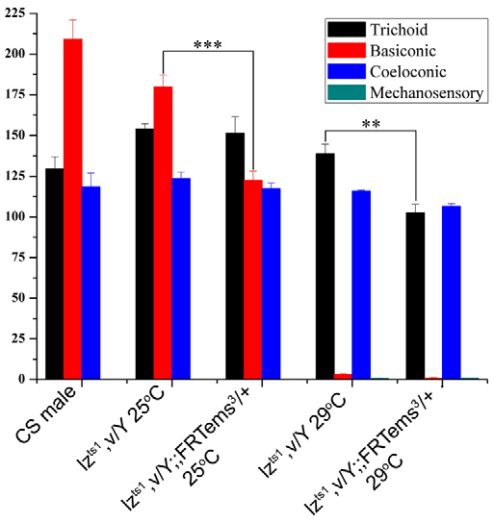

Fig. 4. *ems* interacts genetically with *lz* mutants to produce phenotypes that affect the antennal sense organs. Numbers of trichoid, basiconic and coeloconic sensilla, as well as ectopic mechanosensory bristles are indicated. Bars represent mean ± s.e.m. [n=3 for wild type (Canton special, CS); n=5 in all other genotypes]. Numbers of sensilla are marginally affected in lz^{ts1}/Y reared at 25°C. When one mutant copy of the *ems* mutant is introduced (lz^{ts1}/Y; ems^{3}/+), the number of basiconica is reduced (P<0.001). lz^{ts1}/Y animals reared at 29°C show almost no basiconica, leaving trichoidea unaffected. In lz^{ts1} /Y; ems^{3}/+ animals, the numbers of trichoidea are also reduced (P<0.001).

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 lz^{ts1} mutants at 29°C and in lz^{ts1}/Y; ems^{3}/+ at 29°C; these were never observed in controls (data not shown).

In view of the observation that *ems*-null 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 neurons of basiconic sense organs (OR10a, OR59b, OR85f) and seven Gal4 drivers specific for ORs expressed in neurons of trichoid sense 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 $ems^{-/-}$ neuroblast clones in the antennal lobe, which are rarely generated 48 hours after larval

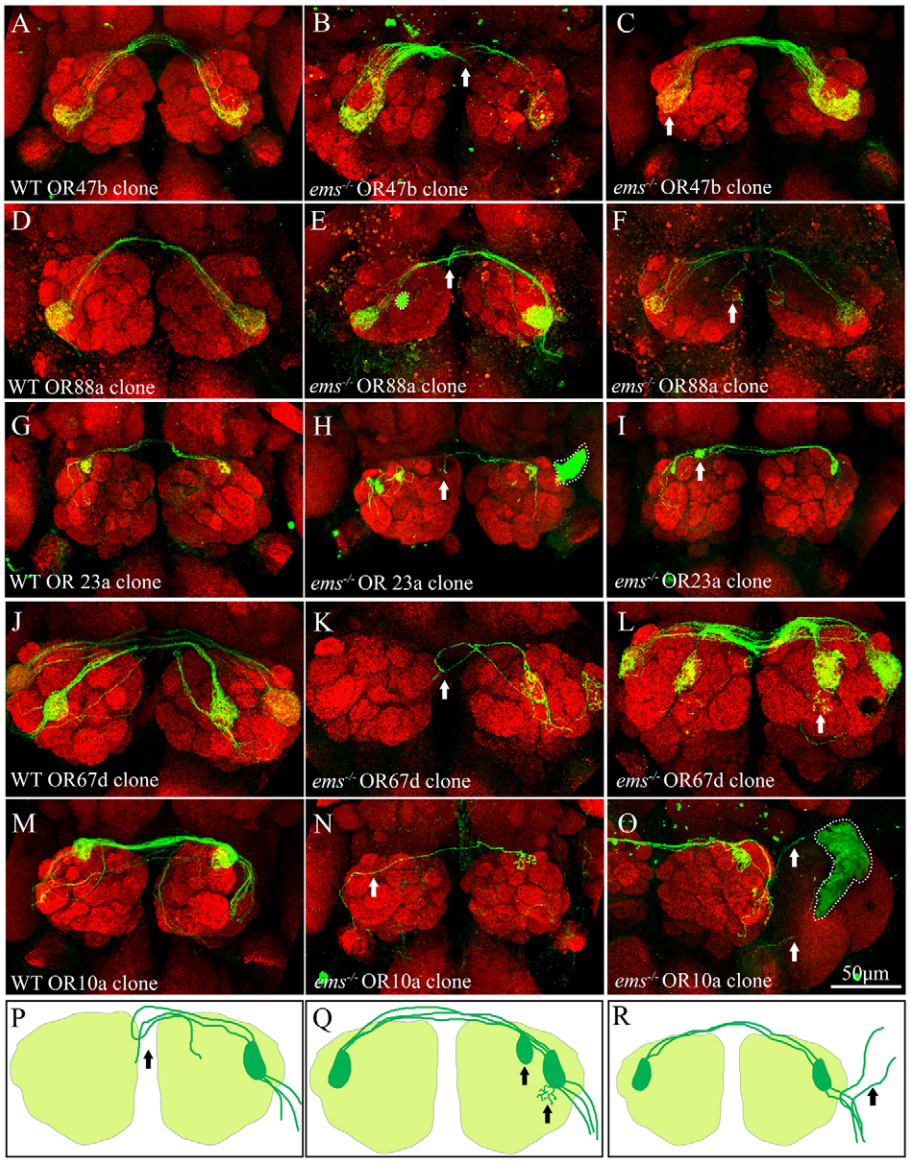

Fig. 5. Ems is necessary for wiring of OSNs to the antennal glomeruli. Adult antennal lobes immunostained with mAbnc82 to visualize the glomeruli. Clonal OSNs are marked by expression of GFP induced by MARCM. (**A-O**) OSNs expressing OR-47b (A-C), OR-88a (D-F), OR-23a (G-I), OR-67d (J-L) and OR-10a (M-O) are labeled. Wild-type OSNs (A,D,G,J,M) target to their cognate glomeruli and send collaterals to the contralateral lobe via the antennal commissure. (**P-R**) The connectivity defects seen in *ems*$^{-/-}$ OSNs that lack *ems* function are summarized. (1) Midline crossover defects, marked by arrows in B,E,H,K, where projection across the commissure is compromised. In C, OSNs innervate the contralateral glomerulus less than the ipsilateral glomerulus, indicating a possible crossover defect (arrow). (2) Target recognition defects where OSNs target ectopic glomeruli (F,I, arrows; compare with controls in D,G,Q). In some cases, OSNs fail to innervate the ipsilateral glomerulus (N, arrow). (3) OSNs spill-over defects, where terminals extend beyond their target glomeruli 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 µm.

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 glomerulus 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 these cases, labeled mutant axons often failed to enter the contralateral lobe and either stalled or mis-projected back to the ipsilateral lobe (schematic in Fig. 5P). Moreover, when multiple receptor axons were labeled a marked reduction in the intensity of contralateral versus ipsilateral glomerular innervations was seen (arrow in Fig. 5C). We also observed targeting defects in which OSNs innervated ectopic glomeruli, in addition to the cognate glomerulus in the antennal lobe (e.g. OR23a; arrow in Fig. 5I). In some cases, OSN terminals 'spilled out' of the boundaries of the cognate glomerulus into neighboring regions in antennal lobe (OR67d; arrow in Fig. 5L; schematic in Fig. 5Q). Only a subset of the clones obtained (~25%) showed the defects described above (see Table S1 in the supplementary material for details). This could imply that the penetrance of the phenotype is low. However, because only a subset of the basiconic/trichoid SOPs express *ems*, it is possible that low frequencies of phenotypes are to be expected even if penetrance is high.

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

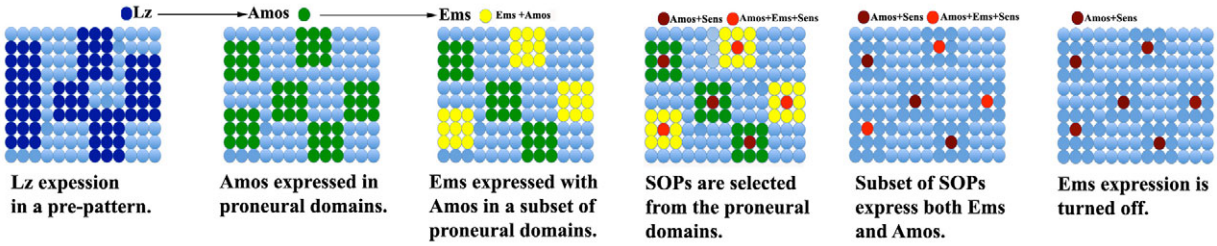

Fig. 6. Summary diagram of the development of SOPs in the antennal disc. Lz is expressed in a zone of epidermal cells defining a prepattern in the third antennal disc. *amos* is regulated by *lz* and is turned on within a set of cells called the proneural domain. Although *amos* and *ems* are expressed 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 Ems and Sens. Expression of *ems* is turned off prior to *amos*. The gene(s) that negatively regulate *ems* have not been identified.

number of labeled clones in the antennal lobe than in their wild-type controls (see Table S1 in the supplementary material). This observation could have two possible explanations. (1) *ems* could regulate the expression of some ORs; hence the promoter would be unable to drive Gal4 expression in mutant clones. In the mouse, Emx2 is known to initiate transcription of several OR genes (McIntyre et al., 2008). (2) Some OSNs lacking *ems* function are unable to target to the antennal glomeruli. These possibilities require further investigation.

Taken together, these findings show that *ems* plays an important role in axonal pathfinding and targeting of OSNs, which is seen most prominently among neurons of the trichoid sensilla, but is also observed in basiconic OSNs. We conclude that *ems* is not only involved in the formation of external sensillar structures but also plays an important, non-redundant role in OSN axonal wiring in the antennal lobe.

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 coeloconic 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-patterning 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 sensillar 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) (Jhaveri et al., 2004), semaphorins (Komiyama 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 (Komiyama 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 neuromere (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; Jürgens 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 neuromere, 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 (Jefferis and Hummel, 2006; Rodrigues and Hummel, 2008). In the anterodorsal 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; Komiyama 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*, has 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 (Mallamaci 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

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Table S1. Frequencies of clones obtained among the trichoid and basiconic sensilla

OR	Sensillum type	Glomerulus	Wild-type clonal frequency (%)	*ems* clonal frequency (%)	Phenotype frequencies
Trichoid-specific ORs					
23a	at2	DA3	70	65	5/17 targeting defects (including ectopic glomerulus targeting)
47b	at4	VA1v	100	100	8/15 targeting defects
67d	at1	DA1 and VA6	62.5	65	3/13 targeting defects
88a	at4	VA1d	100	100	3/17 targeting defects
43a	at3	DA4l	61	40	0/11 targeting defects
83c	at2	DC3	100	87.5	0/14 targeting defects
65a	at4	DL3	82	81.8	0/16 targeting defects
Basiconic-specific ORs					
10a	ab1	DL1	52	47	4/10 targeting defects (including one non-olfactory neuropile target)
59b	ab2	DM4	83.3	42.9	0/15 targeting defects
85f	ab10	DL4	100	59	0/13 targeting defects