Chapter 3

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

*Introduction*

Sensory systems govern behaviour in animals and for the emergence of any function in sensory circuits, accurate synaptic connectivity must occur between peripheral sensory neurons and their central targets. Wide varieties of cellular and developmental processes such as cell proliferation control, fate specification, neuronal outgrowth and path finding have been shown to be required in this process. An excellent model for the analysis of molecular mechanisms that control these processes has been the developing olfactory system of *Drosophila melanogaster*. The extremely precise connectivity seen between olfactory sensory neurons (OSNs) and projection neurons (PNs), and the detailed architecture of the antennal lobe lend themselves well to the analysis of any deviations from the norm.

As described earlier, in both insects and vertebrates precise neuronal circuitry is established by the axonal projection of olfactory sensory neurons (OSNs) to specific target projection neurons in the olfactory glomeruli (Axel, 1995; Mombaerts et al., 1996; Jefferis and Hummel, 2006; Rodrigues and Hummel, 2008). 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). In *Drosophila*, OSNs are housed in approximately 500 hair-like sense organs called sensilla, and, based on morphology, three major types of sensilla exist - sensilla basiconica, trichoidea, and coeloconica. Each of these contains between one and four sensory neurons and develops from sense organ precursors (SOPs) that are specified on the antennal disc during pupal life (Shanbhag et al., 2000). The antennal disc cells are set aside during embryogenesis through the combinatorial action of the homeodomain transcription factors *Homothorax*, *Extradenticle* and *Distalless*, and the basic helix-loop-helix (bHLH) protein *Spineless* (Haynie and Bryant, 1986). The interaction of these transcription factors with the EGF signalling pathway generates a prepattern upon which neurogenic and proneural genes act to generate SOPs. Selection of a single progenitor cell from the field of undifferentiated epidermal cells of the antennal disc occurs by generation of proneural domains that are then refined to single SOPs through Notch signalling (Rhyu et al., 1994). Two proneural genes are involved in the specification of SOPs of the different sensillar types on the...
antenna (Goulding et al., 2000; Gupta et al., 1998; zur Lage et al., 2003). The coeloconic sensilla are specified by *atonal* (*ato*), a bHLH transcription factor and *amos*, another similar transcription factor specifies the basiconic and trichoid SOPs. The choice between basiconic and trichoid sensillar development is governed by the dosage of the Runx family transcription factor Lozenge (Lz) which regulates *amos* expression. At high levels, Lz promotes the formation of basiconic sensilla while lower levels promote trichoidea formation (Gupta et al., 1998). After the SOP has been selected, a series of stereotyped divisions results in the generation of cells that will form the neurons of the sensilla (OSNs), the external structure of the sensilla (shaft) and the associated support cells (sheath cells). Once they have been generated, OSNs initiate axogenesis and target specific glomeruli in the antennal lobe. We have learnt a considerable amount about the mechanisms that control this wiring specificity, and a number of signalling molecules, cell adhesion molecules and axonal guidance molecules have been identified (Rodrigues and Hummel, 2008).

The homeodomain-containing transcription factor *empty spiracles* (*ems*) is required during embryogenesis for the development of the antennal head segment. Upon the loss *ems*, a gap-like phenotype is seen in the embryonic head and brain, and peripheral sensory structures that derive from this segment, such as the larval dorsal organ, fail to develop (Cohen and Jurgens, 1990; Dalton et al., 1989; Schmidt-Ott et al., 1994; Walldorf and Gehring, 1992). Postembryonically, *ems* continues to be expressed in eight neuroblast lineages of the larval brain (Lichtneckert et al., 2007). Two of these neuroblast lineages in the deutocerebral brain region give rise to the antennal lobe PNs and LNs. This expression of *ems* in the antennal lobe lineages has been shown to be essential 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).

In vertebrates however, the *ems* orthologues *Emx1* and *Emx2* have been shown to be required for diverse aspects of brain development, including for the development of olfactory sensory neurons. During development, the *Emx* genes are expressed in various regions of the brain associated with olfaction, such as the olfactory placodes, which give rise to the nasal epithelium, and the developing olfactory bulb. In the absence of these genes severe defects have been reported in various olfactory structures in the brain. The nasal epithelium, where the cell bodies of the OSNs reside is disrupted. The axons of the OSNs fasciculate into a normal olfactory nerve, however, this nerve does not form connections with the olfactory bulb, implying that the OSNs are unable to target to the bulb. The olfactory bulb of these animals is itself extremely reduced in size and the mitral cell layer of the olfactory bulb is disorganised. These phenotypes can be attributed to the requirement of these genes in neuronal proliferation and axonal targeting (Simeone et al., 1992a, 1992b; Matsuo et al., 1997; Mallamaci et al., 1998; Bishop et al., 2003; Nédélec et al., 2004; Shinozaki et al., 2004). In addition to these morphological defects, *Emx2* has also been shown to regulate the expression of various

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odorant receptor genes – either increasing their domain of expression, or restricting it (McIntyre et al 2008).

Given the requirement of Emx1/2 in the development of murine olfactory sensory neurons, and the additional similarity in the requirement of these genes in the development of the olfactory interneurons of both the fly and mouse, we decided to investigate whether the fly ems gene might also be required for targeting of OSNs in Drosophila.

In this chapter I show that ems is expressed in a small subset of sensillar precursors, and is required in their development. Additionally, it is also required for the correct targeting of the OSN axons that derive from these SOPs. The expression of ems is seen in SOPs that co-express Amos and Lz and this expression is very transient. Loss-of-function analysis of Lz and Amos suggest that ems expression in the developing olfactory sense organs requires the action of both lz and amos. While mutational inactivation of ems from the antennal disc did not reveal any obvious defects in sense organ development, analysis of heterozygotes in a genetically sensitized background of lz mutation revealed a functional requirement of ems in the formation of trichoid and basiconic sensilla. Although the loss-of-function of ems did not obviously affect the formation of the antennal sense organs, it did have effects on the development of the OSNs of these sense organs. OSNs that lack functional ems have severe defects in targeting their axons to their cognate glomeruli. These findings on the role of ems in the development of the olfactory PNS in the fly complement the requirement for ems in the development of olfactory interneurons in the CNS. Furthermore, seen in the light of the vertebrate data, they suggest that conserved molecular mechanisms might be responsible for the formation of peripheral and central olfactory circuit elements in insects and mammals.
Results

Ems is co-expressed with Lz and Amos, and not with Ato in the developing antennal disc

To be able to characterize the spatiotemporal expression pattern of ems in the developing antennal disc, the expression of Ems was first studied in the context of Amos and Atonal expression using immunohistochemistry. Amos and Ato are proneural proteins that specify the SOPs of the basiconic/trichoid sensilla and the coeloconic sensilla respectively. Figure 1 documents the spatiotemporal expression analysis of these three proteins from puparium formation (0 APF) till 12 APF. Figure 1 A-E documents the expression profile of Amos, fig 1 F-J of Ems and fig 1 K-N of Ato. At puparium formation only Ato expression can be seen in the anlage of the third antennal segment and Amos expression is not evident at all (figure 1O). At 0 h APF Amos expression is not yet evident in the disc (figure 1A). The first evidence of Amos expression is seen in the disc at 1h APF (not shown), and at 2h APF it can be detected in three to four semi-circular stripes (figure 1B). These stripes broaden over time and by 12h APF they merge into a single large, ‘C-shaped’ band of cells (figure 1C-E). In contrast, Ato expression does not increase during this time (figure 1L-N). The expression of Ems in the disc is similar to that of Amos, however, it is expressed in far fewer cells and its expression in the disc terminates earlier than Amos (figure 1F-J). Therefore, Ems is not detectable in the antennal disc at 0h APF except in cells located superficially along the periphery of the disc and at the base of the presumptive arista (arrows in figure 1F). These cells are likely to be epidermal cells of the disc and the peripodial membrane and were therefore not analyzed further in this study. The first expression of Ems in the third antennal segment proper is seen at 1h APF (not shown); by 2h APF Ems expression is seen in four semi-circular domains (figure 1G). By 5h APF this expression broadens (figure 1H) and by 8h APF Ems expression already begins to diminish (figure 1I). By 12h APF Ems is undetectable in the third antennal disc proper (figure 1J). In the merged images of the expression patterns of Ato, Amos and Ems it is evident that the onset of Ems expression coincides with that of Amos and it is down regulated well before Amos expression diminishes (figure 1O-S). Additionally, in the merged images it becomes clear that Ems is expressed only in a subset of the Amos-expressing cells and is excluded from the cells that express Ato (magnified images in figure 1T-W). This exclusion of Ems expression from the Atonal expressing cells was observed at all pupal stages in the third antennal segment. In summary therefore, all Ems-expressing cells of the third antennal segment co-express Amos and none co-express Ato. However, Ems is expressed by only a subset of the Amos expressing cells, and therefore, not all of the Amos expressing cells co-express Ems.
Figure 1. *ems* is co-expressed in a subset of *amos*-expressing cells in the developing antennal disc. The anlage of the third antennal segment in the antennal disc is highlighted within dotted lines (A-S). Ar-region gives rise to the Arista. Scale bar for figures A-S = 40µm, for T-W = 10µm. (A,F,K,O)-0h APF; (B,G,L,P)-2h APF; (C,H,M,Q)-5h APF; (D,I,N,R)-8h APF; (E,J,S)-12h APF. Merged images of discs immunolabelled with anti- *Amos* (A-E), anti- *Ems* (F-J) and anti- *Ato* (K-N) are shown in (O-S). Both *Amos* (A) and *Ems* (F) are absent from the antennal disc at 0h APF. At 2h APF, expression of both *Amos* and *Ems* is seen (B and G); at 12APF *Ems* expression in the third segment has decayed (J) while *Amos* expression is still strong (E). In addition to the sensory cells *Ems* can be detected in the superficial layer of the disc and in the epidermal cells at the periphery of the presumptive arista (arrows in F). (T-W) show magnified images of the third antennal segment (of a different antennal disc) stained with antibodies against *Amos* (T), *Ems* (U) and *Ato* (V). The merged image (W) 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).
The expression of *amos* has been shown to be regulated by one of the Runt family of transcription factors, *lozenge* (*lz*) (zur Lage et al., 2003). The co-expression of Ems with Amos suggested that Ems might also be co-expressed with Lz. Co-expression studies were carried out with these proteins on the antennal disc of third instar larvae (figure 2A-C) and 4APF pupae (figure 2D-F). The expression of Lz can be detected in three to four semi-circular rings in the antennal disc of the third larval instar, much earlier than the first detectable Amos expression (figure 2A). Like Amos expression, these rings broaden into two broad bands by 4APF (figure 2D). As expected, Ems expression is not seen in the third larval instar antennal disc, but by 4APF is visible in broad bands (figure 2B,E). In the merge and the higher magnification images it is evident that all of the Ems expressing cells also express Lz, while the converse of this is not true (figure 2. D-I). Lz and Amos are together are known to be expressed in the precursors of the trichoid and basiconic (but not coeloconic) sensilla.

**Figure 2. Ems is co-expressed with Lz in the pupal antennal disc.** Antennal discs from L3 (A-C) and 4h APF (D-F) wild type animals immunostained with antibodies against Lz (A,D) and Ems (B,E). Note that Lz, and not ems, is expressed in the disc at L3 stage (A,B). By 4APF, Ems expression can be seen within the domain of Lz expression (D,E). Scale bar = 50µm. The box in F has been...
magnified in G-I. All Ems-positive cells co-express Lz (cell marked by cyan asterisk in G-I). However, not all Lz-positive cells co-express Ems (cell marked by yellow asterisk in A-C).

**The ems gene is expressed in a subset of olfactory sense organ precursors**

Given its co-expression with the proneural gene *amos*, it is possible but not necessary that the Ems expressing cells are SOPs. To test this, another bHLH containing transcription factor *senseless (sens)*, which is highly upregulated in the SOPs themselves, was used as a marker for sense organ precursors (Nolo et al., 2000). In figure 3A and E SOPs can be seen marked by Sens in a 5h APF antennal disc. Some of these cells also co-express Ems, however, not all of the Ems and Amos-expressing cells co-express Sens (figure 3A-H). The Ems (and Amos) expressing cells that co-express Sens are likely to be SOPs, whereas, the Ems (and Amos) expressing cells that do not express Sens are likely to be proneural clusters. In summary, co-expression studies with Amos and Sens reveal that Ems is expressed in a subset of proneural clusters from which SOPs are selected (Figure 3I).

**Figure 3.** *ems* is expressed in a subset of olfactory sense organ progenitors. In A-D, a 5h APF antennal disc is shown co-stained with antibodies against Senseless (A), Ems (B) and Amos (C). D is the merged image. E-H are the magnified images from a 5h APF antennal disc. Scale bar=10µm. In the magnified image, the cell marked with the arrow is an example of a cell that expresses all three markers and is therefore identified as the 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 the
total number of Ems-positive and Amos-positive cells that also express Sens. Histograms represent the mean and standard deviation from four preparations.

An apparent increase in the number of Ems-expressing SOPs was observed in the initial phase of antennal disc development. This was quantified by counting the number of cells that were positive for Ems, Sens and Amos proteins (i.e. basiconic and trichoid specific SOPs that co-expressed Ems) at different times during pupal development (counts were averaged from 4 preparations; figure 3I). At 2h APF, a very small proportion of the Ems and Amos expressing cells also expressed Sens, therefore, at this stage Ems expression in trichoid/basiconic SOPs is negligible. At 5h APF there is a sharp increase in the number of Ems/Amos expressing cells that also express Sens. That is, approximately 25% of these Ems and Amos expressing cells also express Sens 5h APF. At 8h APF this number increases to approximately 40% and by 12h APF Ems expression diminishes and is only found in very few cells. Taken together, these findings suggest that Ems is expressed in a subset of proneural clusters from which trichoid/basiconic SOPs derive and its expression persists transiently in the SOPs that are selected from these clusters. These data suggest that ems could be involved in the development of a subset of the trichoid and/or basiconic olfactory sense organs.

**Lz and amos regulate the expression of ems in the developing third antennal segment**

The co-expression of Ems with Lz and Amos raises the possibility that ems might either be regulated by, or might itself regulate, the expression of these genes. To explore the possibility that ems might be downstream of the activities of Lz and amos, the expression of Ems was analysed in antennal discs derived from animals mutant for either of these genes (figure 4A-D and M-P). Two mutant alleles of amos, amos¹ and amos³, were analysed. These alleles have a drastic reduction in numbers of basiconic and trichoid antennal sensilla (zur Lage et al., 2003). In both the mutant alleles of amos, the expression of Ems was almost totally absent from the third antennal segment (figure 4B). The cells which retained Ems expression were those seen in the periphery of the third antennal segment (white arrowhead in figure 4B). A representative image of this is shown for the amos³ allele in figure 4A-D, which demonstrates that Ems expression is absent in proneural clusters and in SOPs. The remnant Ems expression is only seen in non-SOP and non-proneural cluster cells. As expected, the expression of Ato was unaltered in the amos mutant discs (not shown), while the total number of SOPs as marked by Sens staining was reduced as compared to wild type controls (compare figures 4C, 3A). In 5h APF wild type antennal discs have of an average of 252.33 SOPs (standard deviation = 38.76, N=3), in similarly aged amos¹ mutant discs, an average of only 110.33 SOPs were seen (standard deviation = 6.79, N=3). This reduction in the total number of progenitor cells is not unexpected and can be attributed to the loss of the Amos positive SOPs upon the loss of amos function. In conclusion therefore, amos regulates the expression of ems in the developing third antennal segment.
Figure 4. *lz* and *amos* regulate *ems* expression in the developing third antennal segment. Developing antennal discs from 5h APF animals. The anlage of the third antennal segment (and arista) is highlighted with dotted lines. Scale bar for A-P = 50µm. (A-D) *amos*\(^3\) completely lacks Amos immunoreactivity (A). (B) Ems expression is present only in the non-sensory cells in the superficial layers of the disc (arrow); staining is greatly reduced within the third segment. (C) The number of Sens-positive cells are reduced as compared to wildtype (compare with figure 3A). (D) Merge of A-C. (E-L) MARCM clones labelled with tub-Gal4\(\triangleright\)UAS::GFP. In each case, a part of the clone is magnified in the inset and the boundaries of this part are indicated by the box. (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 are shown. The arrowheads in E-H indicate a cell 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 the insets of K-L). In these antennae, the overall domains of Amos expression remain normal (K). (M-P) Antennal discs of 5h APF *lz*\(^3\) animals. Expression of Amos is reduced leaving a small domain of cells (M). Ems staining within the sensory progenitors is absent leaving only the staining in superficial cells (N arrows). The number of SOPs as indicated by Sens staining (O) is greatly reduced. The merge is shown in (P).
To address the possibility that *ems* might in turn regulate *amos* expression, GFP labelled clones of a loss-of-function allele of *ems*, which is embryonic lethal, were generated using mosaic analysis with a repressible cell marker (MARCM, Lee and Luo, 2001). The ubiquitously expressed *tub-Gal4* was used to drive *UAS-mCD8::GFP* in clones that were induced at the second larval instar stage. These clones were recovered at 5h APF (see methods) and probed for the presence of Amos and Ems protein. Similar clones were generated with a wild type chromosome for comparison. As expected, in such clonal controls, all Ems-expressing cells also expressed Amos, and the overall expression pattern of both the proteins was comparable to wild type (figure 4E-H). In the *ems* mutant clonal discs, the overall expression pattern of Amos remained unaffected and the specific expression of Amos was also unaffected within the *ems* null clones (identified by the cytoplasmic ems staining which occurs because 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) (figure 4I-L). These experiments establish that although *amos* regulates *ems* in the antennal disc, *ems* does not impinge on *amos* expression in the antennal disc.

Given that *lz* regulates the expression of *amos* (zur Lage et al., 2003), and *amos* in turn regulates the expression of *ems*, it is likely that *lz* also regulates the expression of *ems* in antennal disc. To investigate this, two viable, strong hypomorphic *lz* alleles, *lz*3 and *lz*34 were characterized for Ems expression in the developing antennal disc. As expected, in both these alleles, there was a strong reduction in the number of cells expressing Amos (figure 4M) and there was an overall reduction in the number of SOPs in the antennal disc (Figure 4O. Wild type average = 252.33, standard deviation = 38.76, N=3; *lz*3 average = 92.67, standard deviation = 0.58, N=3). In these antennal discs, Ems expression was completely absent from the third segment, leaving only the epidermal staining (Figure 4N). A representative image of this is shown for *lz*3 in figure 4M-P. The results from these experiments suggest that *lz*, through the action of *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**

The expression analysis and the genetic regulation of *ems* described above demonstrates that this homeodomain containing transcription factor is expressed in SOPs of the basiconic/trichoid sensilla and is regulated by genes that specify these SOPs. This poses the possibility that *ems* might also be involved in the specification of these SOPs/sensilla. To investigate this possibility, large *ems*<sup>−/−</sup> mutant clones were generated in the antennal disc using the Minute method and cuticular whole-mounts of the adult antenna were examined for the number of different sensillar types (see methods). Under these circumstances, no defects were seen in the numbers of any sensillar types on the antennal surface (Figure. 5). This result suggests that *ems* is not required for the development of olfactory
sensilla. However, it is also possible that this negative result reflects a functional redundancy of \textit{ems} with other genes that control the development of olfactory sensilla.

\textbf{Figure 5.} \textit{ems} null antennae do not have any defects in the number of sensilla on the antennal surface. The number of sensilla trichoidea, basiconica and coeloconica were counted from antennae of wildtype (Canton-Special; CS) and large \textit{ems} null clones covering most of the antennal surface. The clones were generated using the Minute strategy to increase clone size. Each bar represents the mean and SEM from three antennae. There was no significant difference in sensillar numbers between wildtype and \textit{ems} null animals.

This second possibility was supported by the observation that overexpression of \textit{ems} in the domain of Lz using the \textit{iz-Gal4} driver, lead to a phenotype in the eye and antenna that was similar to the \textit{iz} mutant phenotype (Figure 6). Whereas the heterozygous \textit{iz-Gal4} driver line alone at 29\textdegree C displayed no defects in either the eye (Figure 6A,D) or the antenna (not shown), when \textit{ems} was overexpressed using this driver glossy, almond shaped eyes were obtained at 25\textdegree C and 29\textdegree C (Figure 6 B,C,E,F). Furthermore, a loss of eye pigmentation was observed at 29\textdegree C, which is characteristic of \textit{iz} loss of function (arrows in Figure 6F). At 29\textdegree C, the antennae of these animals also showed a drastic reduction in the number of basiconic and trichoid sensilla, and the appearance of mechanosensory sensilla, which are also characteristic of \textit{iz} loss of function (Figure 6G). This was also observed when \textit{ems} was overexpressed using the \textit{amos-Gal4} driver line (Figure 6G). These observations from overexpression studies suggest that \textit{ems} exerts its influence on members of the genetic cascade that specifies antennal sense organs. It is therefore possible that \textit{ems} plays a redundant role in sense organ specification and therefore, the removal of \textit{ems} alone does not affect the specification of sensilla.
Figure 6. Overexpression of *ems* leads to defects in the eye and antenna.  

A shows *lz>GFP/+* flies at 29°C. Note that the eyes are not defective in these flies (magnified in D). B shows *lz>GFP/+* that overexpress Ems in the Lz domain, and have been grown at 25°C. Note that the eyes of these flies are glazed (magnified in E). The same flies, when grown at 29°C have a more severe defect in the eyes (C and F). Arrows in F indicate pigment loss in the eyes. G is a quantification of sensilla in these genotypes.

*lz*<sup>ts1</sup> flies, when reared at permissive temperatures (25°C), have only mildly affected expression patterns of Amos and Ems (figure 7B-D). The sensillar numbers under these conditions were also comparable to that of the wild type (figure 7A). However, when animals hemizygous for *lz*<sup>ts1</sup> were also heterozygous for a null allele of *ems* (*lz*<sup>ts1</sup>/Y; *ems*<sup>3/+</sup>), a marked reduction in the number
of basiconic sensilla was observed at permissive temperatures (figure 7A; p<0.001). The trichoid and coeloconic sensilla remained unaffected (p<0.3). At non-permissive temperatures of (29°C), the $lz^{nl}$ animals showed a strong reduction in the number of basiconic sensilla (figure 7A). Correspondingly, there was a strong reduction in the expression of both Ems and Amos in antennal discs of these animals at 5h APF (figure 7 E-G). Under these conditions, when the dosage of ems was reduced with one copy of the null allele of ems ($lz^{nl}/Y; ems^{3}/+$), these animals showed a further reduction in the number of trichoid sensilla (figure 7A; p< 0.001). The numbers of coeloconic sensilla were not affected in any of these experiments. Interestingly, small numbers of mechanosensory bristles were observed on the antennal surface of the $lz^{nl}$ mutants at 29°C and in the $lz^{nl}/Y; ems^{3}/+$ at 29°C, which were never seen in control animals (data not shown).

Taken together, these results suggest that although ems is not necessary for the development of antennal sensilla, it probably acts in a functionally redundant pathway with other genes that are also regulated by $lz$.  


Figure 7. *ems* interacts genetically with *lz* mutants to result in phenotypes affecting the antennal sense organs. (A) The numbers of trichoid, basiconic and coeloconic sensilla as well as the mechanosensory bristles are indicated. Each bar represents the mean and SEM from three (wild type-Canton-Special; CS) or five (all others) antennae. Numbers of sensilla are marginally affected in *lz*/*Y* reared at 25°C. When one mutant copy of the *ems* mutant is introduced (*lz*/*Y*; *ems*+/+); the basiconic sensillar numbers are reduced (p<0.001). *lz*/*Y* animals reared at 29°C show almost no basiconica leaving trichoidea unaffected. In *lz*/*Y*; *ems*+/+ animals reared at 29°C the numbers of trichoidea are reduced, over and above the loss of basiconic sensilla (p<0.001). (B-G) Antennal discs from 5h APF *lz*/*Y* pupae immunostained with antibodies against Ems (B,E) and Amos (C,F). When *lz*/*Y* animals were reared at 25°C (permissive temperature; B-D) the expression patterns of Ems (B) and Amos (C) were largely unaffected. When grown at 29°C the sensory cells labelled with Ems were not observed (E-G). Only a few cells labelled with Amos still remain under these conditions.
ems is required for olfactory receptor neuron wiring

The mutational analysis described above investigated the role of ems at the level of the shaft cells, which constitute the external parts of a sensillum. However, apart from the shaft cells, other internal cell types were not investigated in the experiments described above. These experiments therefore, do not address whether the neurons or the support cells, require ems for their development or not. Could ems have an additional non-redundant role in the development of the internal cells of a sensillum? When the differentiation of appropriate cell types was investigated using antibodies to Elav and Cut, which are cellular markers for neurons and support cells respectively, no differences were observed in ems null clones as compared to wild type clones (data not shown). This suggests that ems does not affect the cell division pattern of the SOP. However, it was still possible that ems function was required in the targeting of the receptor neurons to the antennal lobe.

To explore this possibility, specific odorant receptor Gal4 lines were used to label ems null OSNs and their termini in the antennal lobe were analysed for targeting defects. For this analysis, three Gal4 drivers expressed in basiconic OSNs (OR10a, OR59b, OR85f) and seven Gal4 drivers expressed in trichoid OSNs (OR23a, OR47b, OR67d, OR88a, OR43a, OR83c, OR65a) were used. Females of the 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:

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\begin{align*}
\text{wt}^{1118} & ; \text{OR59b-Gal4/FM7a} & \text{FRT82B Tub-Gal80/MKRS}, \\
\text{wt}^{1118} & ; \text{OR85f-Gal4/FM7a} & \text{FRT82B Tub-Gal80/MKRS}, \\
\text{w}^{1118} & ; \text{OR10a-Gal4/CyO} & \text{FRT82B Tub-Gal80/TM6B}, \\
\text{wt}^{1118} & ; \text{OR67d-Gal4/FM7a} & \text{FRT82B Tub-Gal80/MKRS}, \\
\text{w}^{1118} & ; \text{OR43a-Gal4/CyO-GFP} & \text{FRT82B Tub Gal80/MKRS}, \\
\text{w}^{1118} & ; \text{OR88a-Gal4/CyO-GFP} & \text{FRT82B Tub Gal80/MKRS}, \\
\text{w}^{1118} & ; \text{OR83c-Gal4/CyO-GFP} & \text{FRT82B Tub Gal80/MKRS}, \\
\text{w}^{1118} & ; \text{OR47b-Gal4/CyO-GFP} & \text{FRT82B Tub-Gal80/MKRS}, \\
\text{w}^{1118} & ; \text{OR23a-Gal4/CyO} & \text{FRT82B Tub Gal80/MKRS or} \\
\text{w}^{1118} & ; \text{OR65a-Gal4/CyO-GFP} & \text{FRT82B Tub-Gal80/MKRS}.
\end{align*}
\]

Clones were induced in the late second larval instar and were recovered in the adult. This protocol was employed to avoid large ems^- neuroblast clones in the antennal lobe lineages, as these events are rare after 48 hours after larval hatching. On the rare occasion of a neuroblast clone, the brains were identifiable due to the anatomical defects seen upon immunostaining with mAbnc82 and
these were excluded from the analysis. This does not exclude the possibility that one or two cell clones of interneurons are generated at these time points (Das et al., 2008).

**Figure 8.** *ems* is necessary for wiring of OSNs to the antennal glomeruli. Antennal lobes of adult animals immunostained with NC82 (red) to visualize the glomeruli. Clonal OSNs are marked by expression of GFP (green) induced by the MARCM method. Scale bar = 50µm for 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 labelled. Wild type OSNs (A, D, G, J, M); target to their cognate glomeruli and also send collaterals to the contralateral lobe via the antennal commissure. A range of connectivity defects seen in *ems*−/−.
OSNs lacking \textit{ems} function are summarized in Figure P-R. i) Midline crossover defects are 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 also indicating a possible crossover defect (arrow). ii) Target recognition defects where OSNs target ectopic glomeruli (F, I arrows; compare to controls in D, G; Q). In some cases OSNs fail to innervate the ipsilateral glomerulus (N arrow). iii) OSN spill-over defects where terminals extend beyond their target glomeruli to a neighbouring glomerulus (arrow L). iv) Misrouting defects where OSNs project outside the antennal lobe to non-olfactory neuropile (arrows in O, R). The green staining demarcated with dotted lines in E, H and O are staining artefacts.

When the axonal termini of \textit{ems} null OSNs were examined in the antennal lobe, a variety of connectivity defects were observed when compared to their corresponding control clones. Representative images of these are shown in figure 8 and the data has been summarized in Table 1. In the control clones, OSN axons enter the antennal lobe, innervate the appropriate ipsilateral glomerulus, and send a projection across the dorsal commissure onto the contralateral lobe where it innervates the identical contralateral glomerulus (figure 8A, D, G, J, M). The projections of the \textit{ems} null OSNs are in stark contrast to this. In four of the seven trichoid specific OSNs examined, \textit{ems} null OSNs have difficulty in sending contralateral projections (OR47b, OR88a, OR23a and OR67d; arrow in figure 8B, E, H, K). In some cases, the axonal termini were able to cross over, but either failed to enter the contralateral lobe, or crossed back towards the ipsilateral lobe (figure 8K and schematic in 8P). In some cases they entered the lobe, but sent aberrant projections in the contralateral lobe. When the clone size was large, a marked reduction in the intensity of contralateral innervations was observed when compared to the ipsilateral glomerular innervations (arrow in figure 8C). There were also cases where OSNs innervated ectopic glomeruli, in addition to the cognate glomerulus in the antennal lobe (e.g. OR23a; arrow in 8I). In some cases, the terminals of \textit{ems} null OSNs were unable to restrict their innervations to the confines of their cognate glomeruli and ‘spilled out’ into neighbouring regions in antennal lobe (OR67d; arrow in figure 8L; schematic in figure 8Q). Importantly, not all of the mutant OSNs were defective; only about 25% of the clones analysed displayed the phenotypes described above. This could mean that the penetrance of the phenotype is low. It is also likely that, since only a subset of the basiconic/trichoid SOPs express \textit{ems}, these frequencies of phenotypes are to be expected.

Among the three basiconic specific OSNs, only one showed targeting defects (OR10a). These mutant axons projected across the midline, but were unable to target the correct contralateral and meandered across the lobe. In some cases they were unable to form ipsilateral glomerular projections at all and innervated only the contralateral glomerulus (figure 8N). In one case, mutant axons projected beyond the antennal lobe neuropile and into adjacent ‘non-olfactory’ neuropile (arrowheads in figure 8O; schematic in figure 8R). In the other two basiconic-specific OSNs (OR59b and OR85f), significantly smaller number of clones were recovered when compared to their wild type
controls (see Table 1). This can be explained if *ems* has a role in regulating the expression of some odorant receptors themselves. In such a scenario, the OR promoter would be unable to drive Gal4 expression in mutant clones. There is precedent for this in the mouse where *Emx2* is known to initiate the transcription of several OR genes (McIntyre et al., 2008). Alternatively, it is also possible that some OSNs are unable to reach the vicinity of the antennal glomeruli in the absence of *ems*. These possibilities require further investigation.

<table>
<thead>
<tr>
<th>OR</th>
<th>Sensillum type</th>
<th>Glomerulus innervated</th>
<th>WT clonal frequency %</th>
<th><em>ems</em>−/− clonal frequency %</th>
<th>Phenotype frequencies in <em>ems</em>−/− OSNs</th>
</tr>
</thead>
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<tr>
<td></td>
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<tr>
<td>TRICHOID SPECIFIC ORs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23a</td>
<td>at2</td>
<td>DA3</td>
<td>70</td>
<td>65</td>
<td>5/17 targeting defects, including ectopic glomerulus targeting.</td>
</tr>
<tr>
<td>47b</td>
<td>at4</td>
<td>VA1v</td>
<td>100</td>
<td>100</td>
<td>8/15 targeting defects.</td>
</tr>
<tr>
<td>67d</td>
<td>at1</td>
<td>DA1 and VA6</td>
<td>62.5</td>
<td>65</td>
<td>3/13 targeting defects.</td>
</tr>
<tr>
<td>88a</td>
<td>at4</td>
<td>VA1d</td>
<td>100</td>
<td>100</td>
<td>3/17 targeting defects.</td>
</tr>
<tr>
<td>43a</td>
<td>at3</td>
<td>DA4l</td>
<td>61</td>
<td>40</td>
<td>0/11 targeting defects.</td>
</tr>
<tr>
<td>83c</td>
<td>at2</td>
<td>DC3</td>
<td>100</td>
<td>87.5</td>
<td>0/14 targeting defects.</td>
</tr>
<tr>
<td>65a</td>
<td>at4</td>
<td>DL3</td>
<td>82</td>
<td>81.8</td>
<td>0/16 targeting defects.</td>
</tr>
<tr>
<td></td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>10a</td>
<td>ab1</td>
<td>DL1</td>
<td>52</td>
<td>47</td>
<td>4/10 targeting defects, including one non-olfactory neuropile target.</td>
</tr>
<tr>
<td>59b</td>
<td>ab2</td>
<td>DM4</td>
<td>83.3</td>
<td>42.9</td>
<td>0/15 targeting defects.</td>
</tr>
<tr>
<td>85f</td>
<td>ab10</td>
<td>DL4</td>
<td>100</td>
<td>59</td>
<td>0/13 targeting defects.</td>
</tr>
</tbody>
</table>

Table 1. Frequencies of clone generation and phenotype frequencies seen in WT and *ems*−/− OSNs. In the sensillum type column, ‘at’ refers to antennal trichoid sensilla and ‘ab’ refers to antennal basiconic sensilla. The numbers alongside refer to functional subtypes of these sensillar types.

In summary, these data show that *ems* plays an important role in axonal path finding and targeting of OSNs. This requirement of *ems* is most prominently among OSNs of the trichoid sensilla, but is also observed in basiconic OSNs. Therefore, apart from its role in the development of the external sensillar structures, *ems* 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*

This series of experiments has described in detail the expression and function of *ems* in the developing third antennal segment of *Drosophila*. The analysis of Ems expression with other key proteins involved in sensillar development allowed the identification of the Ems-positive cell types and demonstrated that Ems expression is spatially and temporally restricted in these developing sense organs.

The three kinds of antennal sense organs – basiconica, trichoidea and coeloconica – arise from SOPs on the third antennal segment, which are specified by the action of two proneural genes. The proneural gene *atona*1 specifies the coeloconic SOPs and *amos* specifies the basiconic and trichoid SOPs (Gupta et al., 1998; zur Lage et al., 2003). The expression pattern analysis of Ems revealed that the Ems-expressing cells comprise a subset of the Amos-expressing proneural domains and SOPs. This suggests that Ems confers on this Amos expressing subset of SOPs specific properties. It was not possible to determine which sensilla on the adult antenna derived from SOPs that experienced Ems. However, it is unlikely that these sensilla are restricted to a specific region of the antennal surface. It is also unlikely that the Ems-expressing SOPs develop into either basiconic or trichoid alone, as interactions with *lz* alleles affect both these sensillar types.

What determines the expression of Ems in the antennal disc? The expression pattern of the pre-patterning gene *lz* appears early in the antennal disc, at the late third instar larva, and it regulates the expression of Amos in proneural clusters (Goulding et al., 2000; Gupta et al., 1998). The spatiotemporal expression analysis of Ems showed that it is coincident with the onset of Amos, but terminates before it. The expression of both these genes is lost upon the loss of *lz*, posing the possibility that *lz* could activate both genes in proneural clusters independently or that there exists a hierarchical activation of these three genes. As it was not possible to decipher which gene’s expression preceded the other, it was also possible that the expression of one required the other. Genetic analysis revealed that the loss of *amos* function abolished Ems expression, however, the loss of *ems* did not affect the expression of Amos. This favours the idea of a hierarchy of gene function in which *lz* activates the expression of Amos, which in turn regulates Ems expression (model in figure 9).

It remains to be understood what restricts Ems expression to a subset of the Lz and Amos expressing SOPs. Lz and Amos alone are not sufficient for this process as other SOPs that express these genes do not express Ems. It is likely therefore, that additional genes interact with *lz* and *amos* in a genetic cascade to finally select Ems-expressing proneural clusters and SOPs. This idea is supported by the observation that more severe sensillar phenotypes are recovered when, in an *ems*
heterozygote, the dosage of lozenge is reduced. Further studies are required to identify what these genes could be.

**Figure 9. Summary diagram of the development of SOPs in the antennal disc.** Lz is expressed in a zone of epidermal cells defining a prepatter 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.

*Function of ems in adult olfactory sense organ development*

While probing for the function of ems in the SOPs in which it is expressed, two kinds of mutant phenotypes were observed. Compromising ems function in the disc lead to the reduction in the number of some olfactory sensilla, and OSNs of these sensilla had axonal path finding/targeting defects when deficient for ems.

A complete loss-of-function of ems did not reveal any requirement for this gene in the development of olfactory sensilla. However, a clear haplo-insufficient interaction between ems and Lz alleles was seen in the formation of basiconic and trichoid sensilla. Specifically, when the function of Lz is compromised, a reduction in the dosage of ems results in a clear enhancement of the sensillar phenotype – reduction in the basiconic and trichoid sensilla. This suggests that although ems specifies basiconic and trichoid sensilla in the developing antennal disc, its function in SOP development is a redundant one. Its interaction with Lz suggests that the genes with which ems shares redundancy are also regulated by Lz. These experiments also demonstrate that ems function impinges on both the trichoid and basiconic sensilla and therefore cannot be involved in the development of one or the other specifically. It is possible however, that ems itself is regulated differently in the basiconic and trichoid subsets of SOPs. Till date only two genes have been shown to affect the formation of one or the other sensillum type. tod1 and tot1 affect the formation of trichoid sensilla without affecting basiconic sensilla (Ha and Smith, 2006). However, the molecular nature of these genes has not been characterized and it will be interesting to see if they impinge on ems expression or not.
A second mutant phenotype was seen in the targeting of OSNs of the basiconic and trichoid sensilla. Although the complete loss of function of *ems* did not affect the development of these sensilla it had a marked effect on the targeting of their OSNs and uncovered a further, non-redundant role of *ems* in this process. OSNs of the trichoid sensilla showed a variety of defects when deficient for *ems* function (schematized in figure 8P-R). Many OSNs had defects in the various stages of OSN targeting, such as commissure formation and contralateral innervation, glomerular targeting and restricting axonal terminals to the cognate glomerulus. The OSNs of the basiconic sensilla displayed similar defects, however, the frequencies of recovering these defects were lower.

Axon targeting of OSNs occurs long after the expression of Ems subsides in the SOPs. Therefore, it is unlikely that *ems* impinges directly on this process. The role of *ems* is likely to be that of an early intrinsic determinant that has an impact on cell fate decisions and therefore indirectly impacts axonal projections of OSNs later in postembryonic development. In other words, OSN axonal targeting is probably regulated by other factors that are themselves regulated by *ems*. Several studies have demonstrated the role of cell adhesion and signalling molecules in the process of OSN path finding. Roundabout (Robo 1, 2 and 3) (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), the small GTPases Pak and Dock (Ang et al., 2003) and the Notch signalling pathway acting through mastermind (Endo et al., 2007) have been implicated. However, apart from Ems, the only other transcription factors that have been shown to affect OSN targeting are the POU domain molecules Acj6 and Pdm3 (Komiyama et al., 2004; Tichy et al., 2008). In the targeting of PN dendrites *ems* has been shown to act via the activation of *acj6* (Lichtneckert et al 2008). However, how *ems*, or any other transcription factor, regulates signalling and cell surface molecules is not understood and needs to be determined.

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

In the embryo, the expression of Ems is initially seen in the cellular blastoderm in a circumferential stripe. Subsequently, its expression becomes restricted to ectodermal patches of the labral, antennal and intercalary segment of the head (Dalton et al., 1989; Walldorf and Gehring, 1992). These expression domains of Ems in the ectoderm/neuroectoderm of the antennal segment gives rise the deutocerebral brain neuromere (also called the antennal brain neuromere) and also to a set of peripheral sense organs in the cephalic region (Hartmann et al., 2000; Lichtneckert and Reichert, 2008; Urbach and Technau, 2004).

Corresponding to its early expression domain, mutations in *ems* lead to a gap-like phenotype in the embryonic head, this includes the loss of the antennal brain neuromere and also a deletion of peripheral sense organs of antennal and intercalary segments (Jürgens et al., 1984; Dalton et al., 1989;
Among the cephalic sense organs that are deleted upon the loss of ems is the dorsal organ - the major olfactory sense organ of the larva (Schmidt-Ott et al., 1994; Stocker, 2008). When clonal experiments were performed to remove ems function only from larval OSNs without affecting dorsal organ specification, defects were seen in targeting of these neurons to the larval antennal lobe (data not shown). The larval antennal lobe is a deutocerebral brain structure, which is deleted entirely in ems null embryos (Hirth et al., 1995). This deletion phenotype has been shown to be due to loss of the proneural gene lethal of scute leading to the loss of proneural cluster formation and, as a consequence, the complete loss of neuroblast formation/specification from this region of the neuroectoderm (Younossi-Hartenstein et al., 1997). Clonal experiments that bypass this early neuroblast specification phenotype reveal that the neuroblasts and neurons of the larval antennal lobe also require ems for the targeting of their dendrites (data not shown). Later, in postembryonic development, ems is also required in some of the neuroblasts and neurons of the adult antennal lobe for either their survival or their targeting (Lichtneckert et al 2008, Das et al 2008).

These observations highlight a requirement for ems in the development of the peripheral and central olfactory systems of both the larva and the adult. This is remarkable as the adult olfactory sense organ, the antenna, develops from the antennal disc, and is distinct from the larval olfactory sense organ. Most olfactory interneurons of the adult and the larva are generated from four deutocerebral brain neuroblasts that generate the larval interneurons during embryogenesis and the adult interneurons postembryonically (Jefferis and Hummel, 2006; Rodrigues and Hummel, 2008). Therefore, loss of ems function affect both the larval and the adult interneurons.

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

The connectivity of the olfactory circuit of insects and mammals is strikingly similar (Hildebrand and Shepherd, 1997; Komiyama and Luo, 2006). OSNs express a given OR exclusively and OSNs that express the same OR target the same glomerulus in the antennal lobe of insects and the olfactory bulb of mammals. Here they synapse with the dendrites of the olfactory interneurons. These are of two types – the interneurons that take the olfactory information out of the antennal lobe or the olfactory bulb (PNs or mitral, tufted cells) and those that are responsible for local information processing within the antennal lobe/olfactory bulb (LNs or periglomerular and granule cells). This similarity extends beyond the structural level. In mice, the homologues of ems, Emx1/2 have been shown to be required for the development of various aspects of olfactory system development. They are expressed in the developing peripheral and central structures and also function there (Mallamaci et al., 1998; Simeone et al., 1992a; Simeone et al., 1992b). In the periphery, the formation of the nasal epithelia, the expression of ORs in the OSNs and the targeting of the OSNs to the olfactory bulb are all disrupted in Emx loss of function animals. In the centre, loss of Emx function leads to a loss of
mitral cells, a reduction in the size of the bulb and a disruption of the organization of the bulb (Cecchi and Boncinelli, 2000; Bishop et al., 2003; Shinozaki et al., 2004; McIntyre et al., 2008). This similarity in the expression and function of the \textit{ems/Emx} genes in the development of peripheral and central olfactory systems of insects and mammals hint at evolutionarily conserved roles for these genes in olfactory system development.

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