Development 135, 2415-2424 (2008) doi:10.1242/dev.022210

empty spiracles is required for the development of olfactory projection neuron circuitry in *Drosophila*

Robert Lichtneckert*, Lionel Nobs and Heinrich Reichert

In both insects and mammals, second-order olfactory neurons receive input from olfactory receptor neurons and relay olfactory input to higher brain centers. In *Drosophila*, the wiring specificity of these olfactory projection neurons (PNs) is predetermined by their lineage identity and birth order. However, the genetic programs that control this wiring specificity are not well understood. The cephalic gap gene *empty spiracles* (*ems*) encodes a homeodomain transcription factor required for embryonic development of the antennal brain neuromere. Here we show that *ems* is expressed postembryonically in the progenitors of the two major olfactory PN lineages. Moreover, we show that *ems* has cell lineage-specific functions in postembryonic PN development. Thus, in the lateral PN lineage, transient *ems* expression is essential for development of the correct number of PNs; in *ems* mutants, the number of PNs in the lineage is dramatically reduced by apoptosis. By contrast, in the anterodorsal PN lineage, transient *ems* expression is necessary for precise targeting of PN dendrites to appropriate glomeruli; in *ems* mutants, these PNs fail to innervate correct glomeruli, innervate inappropriate glomeruli, or mistarget dendrites to other brain regions. Furthermore, in the anterodorsal PN lineage, *ems* controls the expression of the POU-domain transcription factor Acj6 in approximately half of the cells and, in at least one glomerulus, *ems* function in dendritic targeting is mediated through Acj6. The finding that *Drosophila ems*, like its murine homologs *Emx1/2*, is required for the formation of olfactory circuitry implies that conserved genetic programs control olfactory system development in insects and mammals.

KEY WORDS: *ems*, Brain, Olfactory system, Projection neurons, Neuroblast lineage, Dendritic targeting

INTRODUCTION

The formation of appropriate interconnections between specific neurons during development is an important prerequisite for correct functioning of the adult nervous system. This interconnectivity process requires molecular mechanisms for spatiotemporal control of proliferation, specification of neuronal fate, selection of process outgrowth pathways, and identification of synaptic partner neurons. A significant contribution to the formation of neuronal circuitry is made by transcription factors that act as cell-intrinsic determinants to mediate temporal and spatial regulation of gene expression in the developing nervous system (Chen et al., 2003; Shirasaki and Pfaff, 2002; Skeath and Thor, 2003).

An excellent model system for the analysis of neuronal connectivity is the developing olfactory system. In both mammals and flies, precise neuronal circuitry is established by the ordered axonal projection of olfactory receptor neurons, which manifest the same olfactory receptor molecules to specific target glomeruli in the brain (Axel, 1995; Mombaerts et al., 1996; Vosshall et al., 2000). Comparably precise circuitry is established by the second-order olfactory neurons. These projection neurons (PNs) in the insect antennal lobe and mitral/tufted cells in the olfactory bulb of vertebrates receive input from olfactory receptor axons in specific glomeruli and relay information to target neurons in higher olfactory centers of the brain (Komiyama and Luo, 2006; Vosshall and Stocker, 2007). The developmental origin of the highly stereotyped PN network has been intensively studied in *Drosophila*, in which ~150 PNs from three deutocerebral neuroblast lineages relay olfactory information from the antennal lobe to the mushroom body and lateral horn (Jefferis et al., 2005; Jefferis et al., 2001; Lai and Lee, 2006; Marin et al., 2002; Stocker et al., 1997; Wong et al., 2002). In the antennal lobe, most PNs manifest stereotyped uniglomerular targeting, with a similar degree of specificity as olfactory receptor neuron axons. This targeting specificity of PNs is prespecified by lineage and birth order, and initial dendritic targeting in the antennal lobe occurs prior to ingrowth of receptor axons (Jefferis et al., 2005; Jefferis et al., 2001; Lai and Lee, 2006; Marin et al., 2002; Stocker et al., 1997; Wong et al., 2002).

Initial insight into the mechanisms mediating targeting specificity of PNs comes from the analysis of transcription factors expressed in subsets of these neurons during the dendritic targeting process in late postembryonic development. Among these are the POU-domain transcription factors Acj6 and Drifter. Acj6 is expressed in the anterodorsal PNs and is required for correct dendritic targeting to anterodorsal PN-specific glomeruli. Drifter (Ventral veins lacking – FlyBase) is specifically expressed in the lateral PNs and is required for correct dendritic targeting to lateral PN-specific glomeruli (Komiyama et al., 2003). Further transcription factors involved in mediating PN-intrinsic targeting are the LIM-homeodomain proteins Islet (Tailup – FlyBase) and Lim1, the homeodomain protein Cut, the zinc-finger protein Squeeze, the LIM co-factor Chip and the BTB zinc-finger protein Lola, all of which act during development of antennal lobe connectivity (Komiyama and Luo, 2007; Spletter et al., 2007). Although PN connectivity is at least partially defined by combinatorial expression of these transcription factors, it is likely that they represent only a subset of those involved in PN targeting specificity, given the hundreds of predicted transcription factors in the *Drosophila* genome (Adams et al., 2000).

Recent analyses of *Drosophila* neurogenesis have identified a number of transcription factor-encoding genes that are expressed in specific combinations in the embryonic neuroblasts of the central brain (Urbach and Technau, 2003). For a number of these genes,

Biozentrum, University of Basel, CH-4056 Basel, Switzerland.

*Author for correspondence (e-mail: robert.lichtneckert@unibas.ch)

Accepted 16 May 2008

loss-of-function analyses have revealed severe defects in embryonic brain development (Hirth et al., 1998; Hirth et al., 2003; Hirth et al., 1995; Kammermeier et al., 2001; Noveen et al., 2000; Urbach and Technau, 2003). One of these genes is *empty spiracles* (*ems*), which is required for embryonic head and brain development (Lichtneckert and Reichert, 2005). *ems* encodes a homeodomain transcription factor that is required for the development of the antennal segment from which the antennal sense organs derive (Cohen and Jurgens, 1990; Dalton et al., 1989; Walldorf and Gehring, 1992). *ems* is also expressed in the antennal (deutocerebral) brain neuromere, and mutation of *ems* results in a gap-like brain phenotype (Younossi-Hartenstein et al., 1997). In contrast to our understanding of the role of *ems* in embryonic development of the larval antennal sense organs and antennal brain neuromere, virtually nothing is known about the expression or function of *ems* during postembryonic development of the corresponding adult structures (antenna, deutocerebrum), which contain key elements of the olfactory system.

This lack of information on *ems* action in postembryonic development of the olfactory system in *Drosophila* contrasts with the large amount of information on the role of the *ems* orthologs *Emx1* and *Emx2* in the formation of the murine olfactory system. In the mouse, *Emx2* is expressed in the developing olfactory epithelium, and both mammalian Emx genes are regionally expressed in the developing olfactory bulb, notably in 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). Mutant analysis indicates that these genes play important roles in proliferation and tract formation of the olfactory system (Bishop et al., 2003; Cecchi and Boncinelli, 2000; Shinozaki et al., 2002). Thus, the olfactory bulbs in *Emx1/2* double mutants are reduced and severely disorganized, the mitral cell layer, external plexiform layer and glomerular layer are thin and poorly organized, and the olfactory tract is deficient. These prominent roles of Emx genes in vertebrate olfactory system development prompted us to investigate whether *ems* might also be important in olfactory system development in *Drosophila*.

Here we show that *ems* is expressed postembryonically in the progenitors of the two major PN lineages and that *ems* expression is essential for correct PN development. Loss-of-function studies demonstrate that the role of *ems* in PN development is lineage-specific. In the lateral PN (lPN) lineage, *ems* is essential for development of the correct number of PNs; in *ems* mutants, the number of neurons in this lineage is dramatically reduced. By contrast, in the anterodorsal PN (adPN) lineage, *ems* is necessary for precise targeting of PN dendrites to appropriate glomeruli; in *ems* mutants, PNs fail to innervate correct glomeruli, innervate inappropriate glomeruli, or mistarget dendrites to other brain regions. Furthermore, we show that Acj6 expression is lost in approximately half of the *ems* mutant adPNs, and that the reduced innervation of the VA1lm glomerulus by mutant adPNs is significantly rescued by the misexpression of *acj6*. Our findings on *ems* expression and function in *Drosophila*, together with studies on the murine homologs, suggest that conserved molecular genetic programs might be responsible for formation of circuitry that relays olfactory information to higher brain centers in insects and mammals.

MATERIALS AND METHODS

Fly strains and genetics

Unless otherwise stated, fly stocks were obtained from the Bloomington Stock Center. For wild-type, *ems* mutant or *ems* mutant P35 rescue MARCM clones in larvae, *UAS-mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b}; *FRT82B* or +; *UAS-mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b}; *FRT82B*, *ems*^{9Q64} or +; *UAS-P35*^{BH1}, *UAS-mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b}; *FRT82B*, *ems*^{9Q64} males, respectively, were crossed to *hs-FLP*; *tubP-GAL4*; *FRT82B tubP-GAL80*^{LL3} females (Lichtneckert et al., 2007). For wild-type or mutant MARCM clones in the adult projection neurons expressing GH146-GAL4 (Stocker et al., 1997) +; *UAS-mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b}; *FRT82B*, *ems*^{9Q64} or *UAS-mCD8::GFP*^{LL5}, *UAS-nlslacZ*^{20b}; *FRT82B* males were crossed to *hs-FLP; GH146-GAL4*; *FRT82B tubP-GAL80*^{LL3} females. For *acj6* misexpression in *ems* mutant MARCM clones and for *ems* misexpression in adult projection neurons +; *UAS-acj6*; *FRT82B*, *ems*^{9Q64} or +; *UAS*-ems; *FRT82B* males, respectively, were crossed to *hs-FLP*; *GH146-GAL4*, *UAS-mCD8::GFP*^{LL5}; *FRT82B tubP-GAL80*^{LL3}. The following genotype was used to generate the dual-expression-control MARCM *tubP-lexA::GAD/+*; *FRTG13*, *GAL4-GH146*, *UAS-mCD8/FRTG13*, *hs-FLP*, *tubP-GAL80*; *lexAop-rCD2::GFP/+* (Lai and Lee, 2006).

For MARCM experiments, embryos of appropriate genotype were collected on standard medium over a 4-hour time window and raised at 25°C for 21-25 hours before a 1-hour heat shock (except for dual-expression-control clones, for which a 1-hour heat shock was provided 3-6 hours after egg laying).

Immunolabeling

Brains were fixed and immunostained as previously described (Lichtneckert et al., 2007). Antibodies used were: rabbit anti-Ems (1:500; gift of U. Walldorf, University of Saarland, Homburg, Germany), rabbit anti-Grh (1:200; Bello et al., 2006), rat anti-Elav Mab7E8A10 (1:30; DSHB), mouse anti-Nrt BP106 (1:10; DSHB), mouse anti-Acj6 (1:5; DSHB), mouse monoclonal anti-Nc82 (Nc82 is also known as Bruchpilot – FlyBase) (1:20; gift of A. Hofbauer, University of Regensburg, Regensburg, Germany), rat anti-mouse (m) CD8 (Caltech Laboratories, Burlingame, CA) and rab anti-GFP (Torrey Pines Biolabs, Houston, TX). Secondary antibodies were Alexa488-, Alexa568- and Alexa647-conjugated antibodies generated in goat (1:300; Molecular Probes).

Microscopy and image processing

Fluorescent images were recorded using a Leica TCS SP confocal microscope. Optical sections were taken at 1 μm intervals in line average mode with a picture size of 512×512 pixels. Digital image stacks were processed using ImageJ (http://rsb.info.nih.gov/ij/). Digital 3D models were generated using AMIRA software (Mercury Computer Systems, SAS, Merignac, France) by manually labeling structures of interest, such as cell bodies, and subsequent automated 3D surface rendering.

RESULTS

Expression of the *ems* gene in PN lineages during postembryonic development

To determine whether *ems* is expressed in olfactory PNs during postembryonic development, we first focused on the larval PNs that express the enhancer trap line Gal4-GH146, which labels subsets of PNs once they begin to extend neuronal processes (Jefferis et al., 2001; Stocker et al., 1997). Gal4-GH146-positive cells were identified via UAS-mCD8::GFP (Fig. 1A); expression of *ems* was assayed by anti-Ems immunohistochemistry (Fig. 1B). As expected, mCD8::GFP-labeled cells corresponded to a mixture of larval- and adult-specific adPNs and lPNs; labeling included cell bodies, dendritic domains and axons projecting in the inner antenno-cerebral tract to the mushroom body calyx and lateral horn. Anti-Ems immunoreactivity labeled clusters of cells located between antennal lobes and optic lobes. Two groups of *ems*-expressing cell bodies were found in close proximity to the two clusters of mCD8::GFP-expressing PN cell bodies (Fig. 1C, arrowheads). However, colabeling of cell bodies with anti-Ems and mCD8::GFP was never observed at any larval or pupal stages (Fig. 1D,E; data not shown). Thus, during postembryonic development, PNs that express Gal4-GH146 do not (simultaneously) coexpress *ems*.

Given that Gal4-GH146 is only expressed in a subset of PNs, it is possible that the two groups of Ems-positive, Gal4-GH146-negative cell bodies correspond to the complementary subset of PNs that do

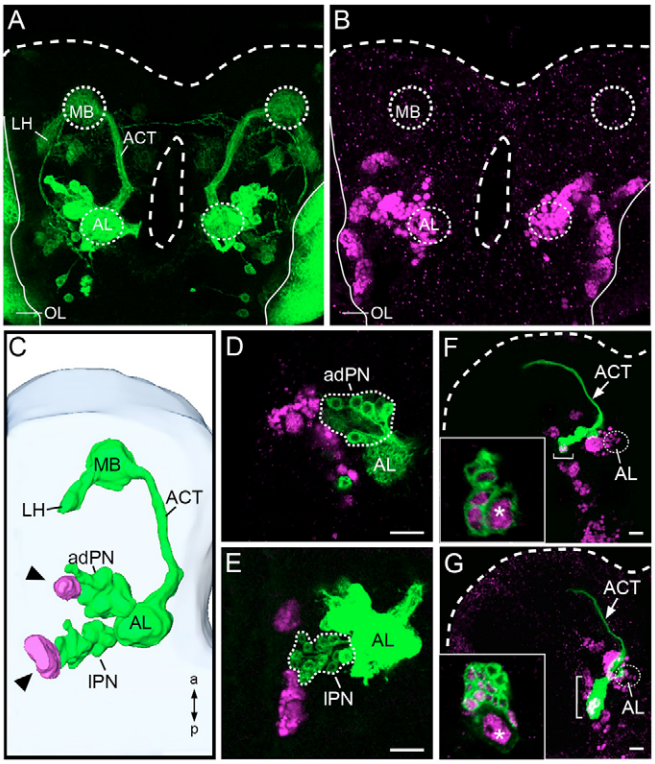

Fig. 1. *ems* expression and olfactory projection neurons in the *Drosophila* late larval brain. (**A**,**B**) *z*-projection of central brain expressing GAL4-GH146 UAS-CD8::GFP (green) or labeled for Ems (magenta). *ems*-positive cells and GAL4-GH146-positive cells are both located close to antennal lobes. (**C**) Digital model of 3D reconstructed optical sections providing an overview of GAL4-GH146-positive adPNs and lPNs (green) and adjacent *ems*-expressing cell body clusters (magenta, arrowheads). (**D**,**E**) *ems*-expressing cell bodies (magenta) are in close proximity to the GAL4-GH146-positive adPNs and lPNs (green); *ems*-expressing cells are never GAL4-GH146-positve. Cell bodies of PNs are delimited by dotted lines. Single optical sections. (**F**,**G**) Two GFP-labeled MARCM clones (green) that extend axons in the antenno-cerebral tract, coexpress *ems* (magenta) in a subset of their cell bodies. *z*-projections of optical sections. Insets show that *ems* is expressed in the neuroblast (asterisk) and in cells located close to the neuroblast. Brackets indicate position of cells shown in insets. Single optical sections. Ventral views, anterior to the top. adPN, anterodorsal projection neuron; lPN, lateral projection neuron; ACT, antenno-cerebral tract; AL, antennal lobe; LH, lateral horn; MB, mushroom body calyx; OL, optic lobe. Dashed lines outline the brain; dotted lines outline the labeled anatomical structures within the brain. Scale bars: 10 μm.

not express Gal4-GH146. To investigate this, we examined these two groups of *ems*-expressing cell bodies using anti-Ems in a MARCM-based clonal analysis with a ubiquitous tub-Gal4 driving UAS-mCD8::GFP. Clones were induced at larval hatching and therefore only adult-specific cells were labeled. If the two groups of *ems*-expressing cells in the larval brain are indeed PNs, then they should have cell bodies near the antennal lobe and axon fascicles projecting through the antenno-cerebral tract. As expected, the two groups of labeled cells had their cell bodies adjacent to the larval antennal lobe and extended their axon fascicles through the larval antenno-cerebral tract, indicating that they were olfactory PNs (Fig. 1F,G). Since the adult-specific cells in the MARCM clone are not yet fully differentiated at the third instar larval stage, dendritic and axonal terminals have not extended into their respective target areas. Importantly, in each of these two MARCM-labeled clonal lineages, *ems* expression was present in the neuroblast and in a subset of the cells located adjacent to the neuroblast, but not in the other cells located further away from the neuroblast (Fig. 1F,G, insets).

For a more detailed characterization of the *ems*-expressing cells in these two lineages, MARCM-labeling was combined with cell-type-specific markers in late third instar larval stage. Both PN clones contained one large cell, the neuroblast, which expressed the transcription factor *grainy head* (*grh*); these neuroblasts always coexpressed *ems* (Fig. 2A,B). Several smaller *grh*-expressing ganglion mother cells (GMCs) were found directly adjacent to the neuroblast in all PN lineages; these GMCs also consistently coexpressed *ems*. Ems-positive cells that coexpressed the neuron-specific marker Elav were also found in the lineages (Fig. 2C,D). Approximately a fifth of the Elav-positive neurons in each clone were also Ems-positive, and these neurons were generally located near the neuroblast in the superficial cortical layers. The remaining four-fifths of the Elav-positive neurons were Ems-negative and were generally located in deeper cortical layers closer to the antennal lobe neuropile. Thus, in PN neuroblast clones, the progenitor cells, neuroblasts and GMCs consistently expressed *ems*, and *ems* expression was also observed in a subset of neurons located near these progenitors, but not in the remaining neurons. Based on their location relative to the neuroblast, the Ems-positive neurons are likely to represent younger, more recently born neurons, whereas the remaining Ems-negative neurons are likely to represent older, earlier-born neurons.

Might some of these older, earlier-born neurons in the two neuroblast clones correspond to those Ems-negative PNs that express Gal4-GH146? To address this, we carried out dual-expression-control MARCM experiments with Gal4-GH146 and ubiquitous tubP-LexA::GAD as drivers (Lai and Lee, 2006). These experiments allow simultaneous differential labeling of the GH146-expressing PNs (via Gal4-GH146-driven UAS-mCD8) and of all cells in a neuroblast clone (via tubP-LexA::GAD-driven lexAop-rCD2::GFP). Clones were induced during embryogenesis, recovered 48 and 96 hours after larval hatching (ALH), and co-immunostained with anti-Ems. In the dual-labeled clones, tubP-lexA-driven marker expression labeled all cells in the adPN and lPN lineages, including the neuroblast, as expected. In both of these lineages, the neuroblast and a subset of cells located near the neuroblast in the outermost cortical cell layers always expressed *ems* (Fig. 3A-D, arrowheads indicate neuroblasts). By contrast, Gal4-GH146-driven marker expression labeled a different subset of the cells in the two lineages that was generally located in deeper cortical layers closer to the antennal lobe neuropile and that was always Ems-negative (Fig. 3A′-D′). Thus, although the adPNs and lPNs that express Gal4-GH146 do not concomitantly coexpress *ems*, they belong to postembryonic neuroblast lineages in which the neuroblast does express *ems* (Fig. 3E). We conclude that the neurons of the adPN and lPN lineages derive from progenitor cells that persistently express *ems* and posit that *ems* is expressed transiently in young, recently born PNs and disappears from older PNs during subsequent development.

ems is required for correct neuronal cell number in the lPN lineage

To determine the role of *ems* in postembryonic development of adPN and lPN lineages, wild-type and *ems* mutant MARCM clones were generated. For this, ubiquitously expressed tub-Gal4 was used to drive a UAS-mCD8::GFP reporter; clones were induced in the

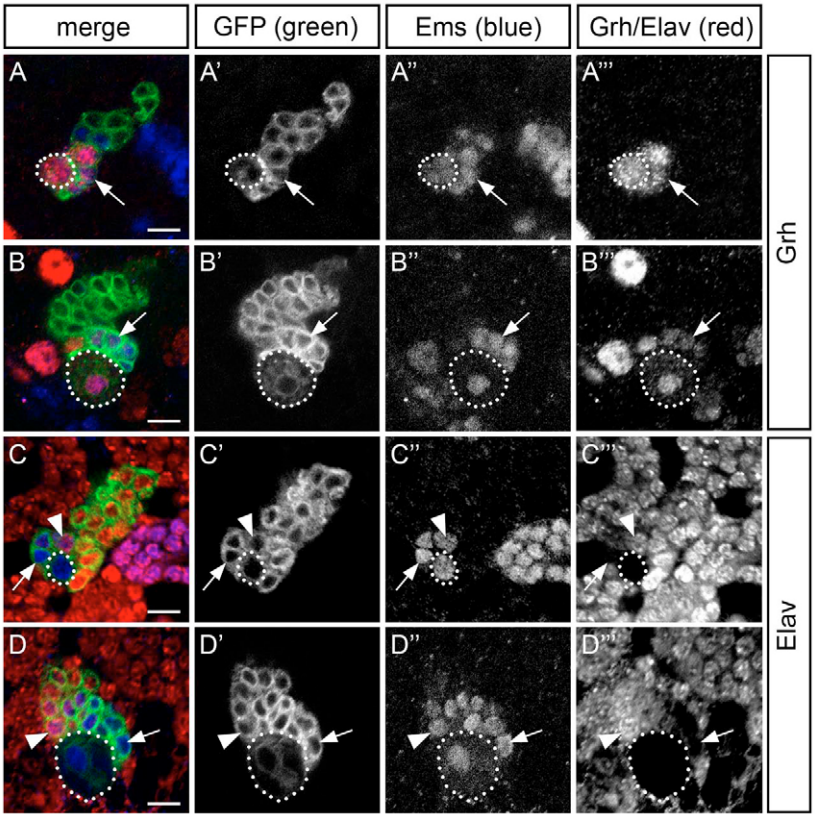

Fig. 2. *ems*-expressing cell types in the adPN and lPN lineages at the late third instar larval stage. (**A-D'''**) MARCM clones induced in early first instar *Drosophila* larvae. (A,C) Lineage as shown in Fig. 1F. (B,D) Lineage as shown in Fig. 1G. MARCM clones were co-immunostained with (A,B) anti-Ems (blue) and anti-Grh (red) or (C,D) anti-Ems (blue) and anti-Elav (red). Single channels are shown separately. Dotted outline, *ems*-expressing neuroblasts; arrow, ganglion mother cells (GMCs); arrowhead, postmitotic neurons. Scale bars: 5 μm.

early first instar larva and analyzed in the late third instar larva. A comparison of wild-type and *ems* mutant clone size revealed a marked difference in *ems* requirement for the two different PN lineages (Table 1).

For the adPN lineage, the average cell number in wild-type and *ems* mutant clones was virtually identical (64 and 62 cells, respectively). Moreover, wild-type and *ems* mutant adPN clones were recovered with equal frequency; clones were observed in ~25% of the brains examined. By contrast, for the lPN lineage, the average clone size in wild-type and *ems* mutant MARCM clones was strikingly different (201 and 28 cells, respectively). Whereas wild-type lPN clones were also recovered in ~25% of the brains examined, less than 2% contained *ems* mutant lPN clones. Thus, *ems* loss-of-function results in lPN clones that have less than 15% of the number of cells seen in wild-type clones. Moreover, given the low frequency of recovery of *ems* mutant lPN clones, it is likely that *ems* mutation often leads to the complete absence of lPN cells. The dramatic reduction in cell number observed in *ems* mutant lPN clones implies that mutant cells are either not generated in appropriate numbers or that they die during postembryonic development.

To determine whether apoptosis could account for this marked reduction in cell number, we blocked cell death in *ems* mutant clones through targeted misexpression of the pancaspase inhibitor P35. Clones were induced in early first instar larvae and cell numbers determined at the late third instar stage. Mutant *ems* lPN clones involving P35 misexpression were recovered in ~25% of the brains examined, comparable to the wild-type recovery rate. Blocking cell death in *ems* mutant clones resulted in clones containing an average of 143 cells. This represents a fivefold increase in cell number as compared with *ems* mutant clones, and corresponds to 71% of the cell number observed in wild-type clones (Table 1). This marked rescue effect implies that the cell number reduction in *ems* mutant lPN lineages is largely owing to apoptosis during larval development.

ems is required for correct dendritic targeting in the adPN lineage

Although *ems* loss-of-function did not affect clonal cell number in adPNs, *ems* might play a role in proper innervation of adPN targets in the brain. To investigate this, wild-type and *ems* mutant MARCM clones were generated in adPNs in the early first instar larva using Gal4-GH146 to drive UAS-mCD8::GFP, and were analyzed in the adult brain. All wild-type clones showed the typical adPN morphology: the position of cell bodies, projection of axons from the antennal lobe to the mushroom body calyx and lateral horn, and dendritic innervation of the subset of glomeruli in the antennal lobe specific for adPNs, were all normal (Fig. 4A,C,E,G,I). In *ems* mutant adPN neuroblast clones, cell body position and axonal projection trajectory were also normal; however, marked defects in dendritic targeting were apparent.

Three types of dendritic targeting phenotype were observed. In the first, mutant adPNs failed to innervate specific glomeruli that were always innervated by wild-type adPNs. For example, mutant adPNs innervated the VA1lm glomerulus in only 63% of the clones examined, versus 100% innervation observed for wild-type clones, and similar targeting defects were found in the VA3 and VM2 glomeruli (Fig. 4B,D; Table 2). In the second phenotype, mutant adPNs ectopically innervated inappropriate glomeruli including those normally targeted by lPNs. For example, mutant adPNs ectopically innervated the DL2 glomerulus in 71% of the clones examined, as compared with 0% innervation by wild-type adPN clones, and comparable ectopic targeting defects were observed in the DA2, VA6, DL5 and VL2 (Fig. 4F,H), as well as VM1, DA1, DA4 and DC1 glomeruli (Table 2). In the third phenotype, observed in approximately a third of the clones examined, mutant adPNs formed inappropriate misprojections into the subesophageal ganglion (Fig. 4J). Misprojections of this type were never seen in

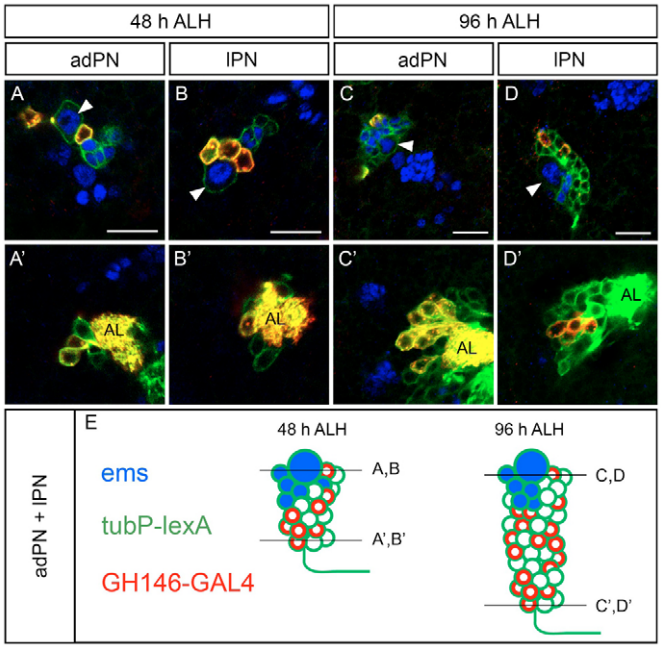

Fig. 3. *ems* expression in adPN and lPN lineages. (**A-D'**) Dual-expression MARCM. tubP-lexA-positive cells are green, GAL4-GH146-positive cells are red, coexpressing cells appear yellow; Ems-expressing cells are blue. Clones were induced during embryogenesis and analyzed 48 and 96 hours after larval hatching (ALH). Confocal images show single optical sections from four preparations. (A-D) Optical sections taken at superficial cortex layers include the *ems*-positive neuroblast (arrowhead). (A'-D') Optical sections taken at deeper cortical layers close to the antennal neuropile. (**E**) Summary of *ems* expression (blue) in larval PNs (see text for details). Scale bars: 10 μm.

Table 1. Cell number in adPN and lPN clones

	Wild type	*ems* mutant	*ems* mutant UAS-P35
adPN	64±2.3 (*n*=18)	62±7.3 (*n*=19)	NT
lPN	201±4.9 (*n*=8)	28±14.9 (*n*=4)	143±34.4 (*n*=8)

Average clonal cell number (±s.d.) in late third instar larvae, comparing wild-type clones, *ems* mutant clones and *ems* mutant clones misexpressing the apoptosis blocker P35.
n, the number of clones examined for each lineage and genotype.
NT, no *ems* mutant UAS-P35-misexpressing clones were examined for the adPN lineage.

wild-type adPN clones. Taken together, these findings show that *ems* is required during postembryonic development for correct dendritic targeting in the adPN lineage.

Expression of *ems* is absent in the Gal4-GH146-labeled adPNs during the developmental period in which glomerular innervation occurs (Jefferis et al., 2005; Stocker et al., 1997). Indeed, in both PN lineages, no *ems* expression was seen later than 24 hours APF. Might the absence of *ems* in these cells during this later differentiation phase be necessary for the formation of correct innervation by the adPNs? To investigate this, we misexpressed *ems* in adPNs during this later differentiation period using the MARCM system with Gal4-GH146 driving UAS-ems. Clones were induced at the early first instar larval stage and examined in the adult.

In adPN *ems* misexpression clones, cell number, cell body position and axonal projections were the same as in the wild type. However, compared with wild-type clones (Fig. 5A,C,E), marked defects in adPN dendritic targeting became apparent in these *ems* misexpression experiments. In all mutant clones examined, ectopic innervation of one or more inappropriate glomeruli was observed. For a given glomerulus, the frequency of inappropriate innervation by mutant adPNs varied. For example, the DA2 glomerulus was ectopically innervated in 78%, whereas DC1 was ectopically innervated in 44% of the clones examined (Fig. 5B,D; the frequency of inappropriate innervation for seven other glomeruli is given in Table 2). The absence of appropriate glomerular innervation was also observed. However, this phenotype was limited to the DL1 glomerulus: clones of adPNs misexpressing *ems* never innervated this glomerulus (Fig. 5F; Table 2). Thus, misexpression of *ems* in GH146-positive adPNs beyond the time of endogenous wild-type *ems* expression leads to severe dendritic mistargeting effects.

Axon terminal arborization defects caused by *ems* misexpression

Next, we investigated whether *ems* loss-of-function or misexpression might influence the stereotyped axon arborizations of adPNs in the lateral horn, one of the two central targets for PN axons. These experiments, which involved the generation of single-cell clones induced by early larval heat shock, concentrated on DL1-innervating adPNs because these are the only class that can be unequivocally identified based on time of clone induction (Jefferis et al., 2001; Komiyama et al., 2003; Marin et al., 2002).

The axons of wild-type aPN neuroblast clones project as a fascicle to the lateral horn and there form a main arborization area that appears as a continuation of the axon fascicle and a secondary, more dorsal arborization area that branches out more or less perpendicularly from the main fascicle (Marin et al., 2002). Single-cell clones of DL1 PNs, which had the expected dendritic domain in the antennal lobe (Fig. 6B), also projected their axons to the lateral horn and bifurcated into a main lateral terminal process and a secondary dorsal terminal process (Fig. 6C).

The axons of *ems* loss-of-function adPN neuroblast clones also projected a fascicle to the lateral horn and there formed two arborization areas comparable to those of the wild type (Fig. 6D). Single-cell clones of *ems* loss-of-function DL1 PNs, which correctly innervated the DL1 glomerulus (Fig. 6E), projected their axons to the lateral horn, bifurcated and formed two wild-type-like terminal processes (Fig. 6F). This suggests that *ems* is not required for correct axon terminal arborizations in DL1 PNs.

Table 2. Quantification of innervation of antennal glomeruli in wild-type adPN clones, *ems* loss-of-function mutant adPN clones and *ems*-misexpressing adPN clones

	VA1lm	VA3	VM2	DL1	VA2	VA6	VL2	VM1	VM4	DA1	DA2	DA4	DC1	DL2	DL5
WT	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0
LOF	**63**	**71**	**83**	100	0	**21**	**54**	**17**	0	**37**	**29**	**54**	**42**	**71**	**21**
GOF	100	100	100	**0**	**100**	**78**	**78**	**11**	**44**	0	**78**	0	**44**	**89**	**22**

The observed frequency (%) of adPN innervation for 15 glomeruli in 9 wild-type clones (WT), 24 *ems* loss-of-function mutant clones (LOF), and 9 *ems* gain-of-function clones misexpressing *ems* (GOF). Percentages in bold differ from those of the wild type.

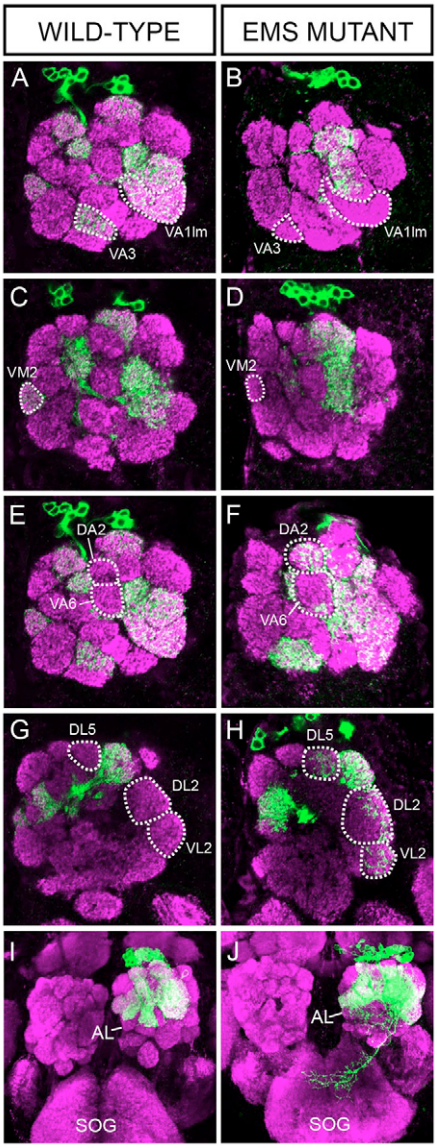

Fig. 4. *ems* is required for the dendritic targeting of adPN-specific glomeruli. Anti-GFP-labeled GAL4-GH146 UAS-mCD8::GFP MARCM clones (green); neuropile marker anti-Nc82 (magenta). Clones were induced in early first instar *Drosophila* larvae and analyzed in adult. Dotted lines mark the outline of single glomeruli. (**A-H**) Single confocal sections of antennal lobes with dorsal to top and medial to left showing dendritic innervation by wild-type (A,C,E,G) or *ems* mutant (B,D,F,H) clones. Loss of innervation of VA3, VA1lm (B) and VM2 (D) glomeruli by *ems* mutant clones as compared with wild-type clones (A,C). Ectopic innervation of inappropriate glomeruli DA2, VA6 (F) and DL2, DL5, VL2 (H) by *ems* mutant clones as compared with wild-type clones (E,G). (**I**,**J**) *z*-projections of frontal optical sections showing antennal lobes and subesophageal gangion (SOG). No innervation of SOG is observed in the wild type (I), whereas *ems* mutant adPN clones extend ectopic projection into SOG (J).

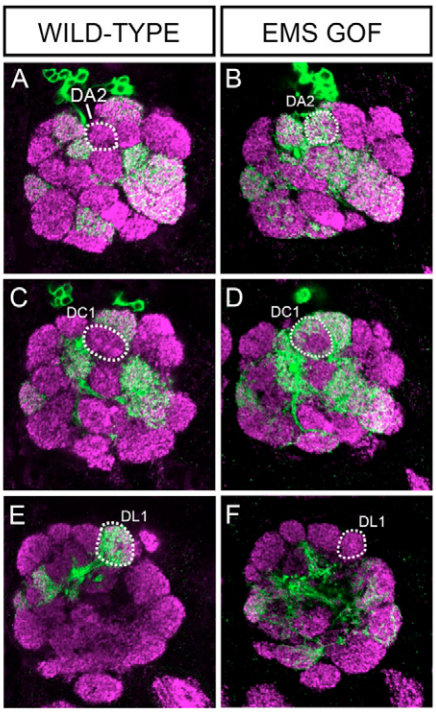

Fig. 5. Misexpression of *ems* in mature adPNs causes dendritic targeting defects. Anti-GFP-labeled GAL4-GH146 UAS-mCD8::GFP MARCM clones (green); anti-Nc82 (magenta). Clones were induced in early first instar *Drosophila* larvae and analyzed in adult. Single confocal sections of single antennal lobes; dorsal to top, medial to left showing dendritic innervation by wild-type clones (**A**,**C**,**E**) or *ems* gain-of-function (GOF) clones misexpressing *ems* (**B**,**D**,**F**). Ectopic innervation of inappropriate glomeruli DA2 (B) and DC1 (D) by *ems*-misexpressing clones as compared with wild-type clones (A,C). Loss of innervation of the DL1 glomerulus (F) by *ems*-misexpressing clones as compared with wild-type clones (E). Dotted lines mark the outline of single glomeruli.

By contrast, in *ems* misexpression experiments, the axons of adPN neuroblast clones had ectopic terminal arbors between the main arborization area and the secondary arborization area in the lateral horn (Fig. 6G). Single-cell cones of DL1 PNs, which failed to innervate the DL1 glomerulus and ectopically innervated the DA2 and DM6 glomeruli (Fig. 6H), consistently formed ectopic terminal branches between the main lateral terminal process and the secondary dorsal terminal process in the lateral horn (Fig. 6I). Thus, at least for the DL1 PNs, misexpression of *ems* beyond the time of normal endogenous expression leads to distinct axon terminal arborization defects.

acj6 expression in the adPN lineage is *ems* dependent

The POU-domain transcription factor Acj6 has been shown to be specifically expressed in all postmitotic GAL4-GH146-labeled adPNs. Indeed, Acj6 function is required for the correct innervation of adPN-specific glomeruli (Komiyama et al., 2003). In order to investigate the expression patterns of *ems* and *acj6* in the adPN lineage, we induced wild-type MARCM clones in the early first instar larva using a ubiquitous tub-Gal4-driven UAS-mCD8::GFP and colabeled the clones with anti-Ems and anti-Acj6 in the late third instar larva (Fig. 7A). As expected, *ems* expression was found in the neuroblast and in a small number of adjacent cells, whereas Acj6 staining was found in a nearly complementary pattern that excluded the neuroblast and most of the immediately adjacent cells but labeled all other cells of the lineage. Interestingly, a small number of cells at the interface between the *ems* expression domain and the *acj6* expression domain consistently showed colabeling for the two transcription factors (Fig. 7A,B). These findings are in

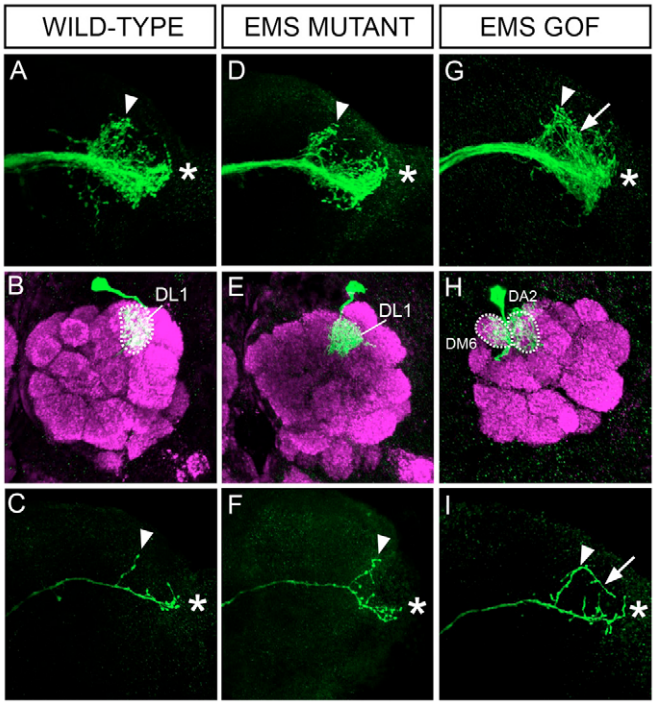

Fig. 6. *ems*-misexpressing adPN clones but not *ems* mutant clones show axon terminal defects. Anti-GFP-labeled GAL4-GH146 UAS-mCD8::GFP MARCM clones (green); anti-Nc82 (magenta). Wild-type clones (**A-C**), *ems* mutant clones (**D-F**), and *ems* gain-of-function (GOF) clones misexpressing *ems* (**G-I**) were induced in early first instar *Drosophila* larvae. At this time, only neuroblast clones and DL1 class single-cell clones can be induced in the adPN lineage. Analysis was in adult. (A,D,G) *z*-projection of confocal sections of lateral horn with anterior to top and medial to left. Neuroblast clones of all three genotypes show two main arborization areas in lateral (asterisk) and dorsal (arrowhead) lateral horn. In the *ems* GOF clones, ectopic innervation is seen between two main arborization areas (arrow in G). Wild-type and *ems* mutant DL1 class single-cell clones innervate the DL1 glomerulus of antennal lobe (B,E, respectively) and bifurcate into a dorsal (arrowhead) and lateral (asterisk) process in lateral horn (C,F). By contrast, *ems*-misexpressing clones ectopically innervate DA2 and DM6 glomeruli (H) and show ectopic terminal branches (arrow) between dorsal terminal process (arrowhead) and main lateral terminal process (asterisk) in the lateral horn (I). Dotted lines mark the outline of single glomeruli.

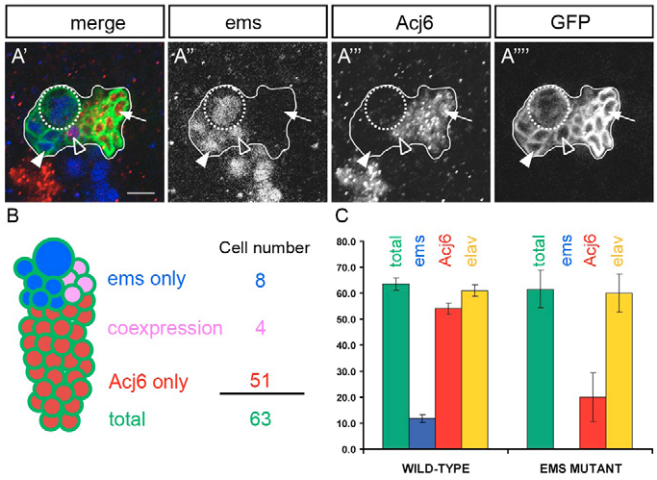

Fig. 7. *ems* and *acj6* expression in the adPN lineage at the late third instar larval stage. MARCM clones induced in early first instar *Drosophila* larvae. (**A'-A'''**) MARCM clone (continous outline) co-immunostained with anti-Ems (blue) and anti-Acj6 (red). Single optical section. Single channels shown separately. The neuroblast (dotted outline) and adjacent cells (white arrowhead) express *ems* alone. Whereas only a few cells coexpress *ems* and *acj6* (open arrowhead), most cells express exclusively *acj6* (arrow). (**B**) Summary of *ems* and *acj6* expression in larval adPNs with average cell numbers indicated for each expression class (see text for details). (**C**) Comparison of the number of cells expressing *ems*, *acj6* and *elav* in wild-type versus *ems* mutant MARCM clones at the late third instar larval stage. In the absence of *ems*, the number of *acj6*-expressing cells is reduced. Scale bar: 5 μm.

accordance with the notion that, in contrast to *acj6*, *ems* is expressed in the progenitors and young postmitotic neurons and therefore precedes *acj6* expression during differentiation of adPNs.

To test whether *ems* expression in progenitors and young postmitotic cells is required for *acj6* expression, wild-type clones were compared with *ems* mutant clones and the number of cells expressing *ems*, *acj6* and *elav* determined. Neither the total clone size nor the number of cells expressing Elav was altered in the *ems* mutant as compared with the wild type. However, the number of *acj6*-expressing cells was reduced by more than half, from 54 (s.d.=2.2) in the wild type to an average of 20 (s.d.=9.4) in the mutant (Fig. 7C). These findings indicate that the expression of *ems* in adPN progenitors and/or in young postmitotic cells is required for proper expression of Acj6 in the more mature and differentiated adPNs.

We also analyzed *acj6* expression in wild-type and *ems* mutant adPN MARCM clones 48 hours after puparium formation (APF) and in the adult. For this we used the GH146-GAL4 driver, which is expressed in postmitotic adPNs. As expected, all wild-type adPNs that expressed GH146-GAL4 also expressed *acj6* (Komiyama et al., 2003). By contrast, the number of *acj6*-positive adPNs in *ems* mutant clones was found to be reduced during metamorphosis and at the adult stage: 53% (n=9) and 56% (n=7) of the GH146-GAL4-positive adPNs expressed *acj6* at 48 hours APF and in the adult, respectively. Thus, loss of *acj6* expression in approximately half of the *ems* mutant adPNs persists through metamorphosis, when innervation of glomeruli takes place, and is also observed in the adult.

Since approximately half of adPNs fail to express *acj6* at the absence of *ems*, and because a similar loss of innervation phenotype occurs in *acj6* mutant adPN clones (Komiyama et al., 2003), we wanted to test whether at least some of the glomerular phenotypes in *ems* mutant clones might be due to the control of *acj6* by *ems*. For this, we misexpressed *acj6* in *ems* mutant adPN clones using the GAL4-GH146 driver and compared the innervation of the VA1lm, VM2 and VA3 glomeruli in this misexpression experiment with the innervation observed in the *ems* mutant PNs alone. We found a significant rescue of the innervation phenotype (P<0.02, χ^2 test) for the VA1lm glomerulus (Fig. 8): correct innervation was restored from 63% in *ems* mutant adPN clones (n=24) to 87% in *acj6*-misexpressing *ems* mutant clones (n=23). In the VA3 and VM2 glomeruli, where the loss of innervation phenotype was less penetrant in the *ems* mutant adPN (71% and 83% correct innervation, respectively; see also Table 2), no significant rescue of innervation was observed in *acj6*-misexpressing *ems* mutant clones (83% and 87% correct innervation, respectively). Thus, at least in the VA1lm class of PNs, the loss of appropriate innervation seen in the *ems* mutant appears to be due, at least in part, to the downregulation of *acj6*, and misexpression of *acj6* in these cells is able to rescue the phenotype.

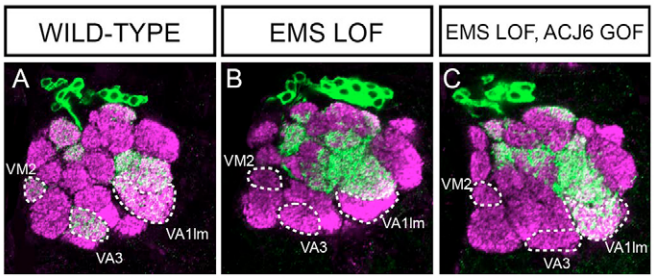

Fig. 8. Misexpression of *acj6* in the *ems* mutant adPN lineage rescues the loss of innervation phenotype in the VA1lm glomerulus. Anti-GFP-labeled GAL4-GH146 UAS-mCD8::GFP MARCM clones (green); anti-Nc82 (magenta). Clones were induced in early first instar *Drosophila* larvae and analyzed in adult. Single confocal sections of single antennal lobes (dorsal to top, medial to left) showing dendritic innervation by wild-type clones (**A**), *ems* loss-of-function (LOF) clones (**B**) and *ems* mutant clones (GOF) misexpressing *acj6* (**C**). Loss of innervation of VA1lm glomeruli by *ems* mutant clones is significantly rescued by the misexpression of *acj6*, whereas no significant rescue could be found for the VA3 and VM2 glomeruli. Dotted lines mark the outline of single glomeruli.

DISCUSSION

Expression of *ems* in postembryonic olfactory PN lineages

The two major groups of olfactory projection neurons, adPNs and lPNs, derive from two brain neuroblasts that generate most of their progeny postembryonically (Jefferis et al., 2001; Lai and Lee, 2006). Here we show that *ems* is expressed in these neuroblasts as well as in their GMCs and a subset of their postmitotic neuronal progeny during postembryonic development. Our expression data support a model in which *ems* is persistently expressed in the postembryonic progenitors (neuroblasts and GMCs) of adPN and lPN lineages, remains transiently expressed in their neuronal progeny, and subsequently disappears in these neurons during their differentiation phase and in the adult. This is corroborated by the observation that *ems* is never coexpressed with the Gal-GH146 driver, which only begins to be expressed in PNs once they differentiate and initiate process outgrowth (Jefferis et al., 2005; Stocker et al., 1997). The experimental findings that support this model have implications for our understanding of *ems* action in olfactory system development.

During embryogenesis, *ems* is expressed in a subset of the neuroblasts that give rise to the deutocerebral neuromere (Urbach and Technau, 2003; Younossi-Hartenstein et al., 1997). This raises the possibility that *ems* might also be involved in the development of the small group of olfactory PNs generated in the embryo (Marin et al., 2005; Python and Stocker, 2002). Although an unambiguous link between embryonic *ems*-expressing neuroblasts and the two postembryonic *ems*-expressing neuroblasts that generate the adPN and lPN lineages is lacking (Urbach and Technau, 2003), it is conceivable that *ems* is required for the development of the entire complement of adPNs and lPNs, embryonic and postembryonic. Indeed, as *ems* is not only expressed in the embryonic neuroectoderm from which the deutocerebrum derives, but also in the embryonic antennal segment from which the antennal sense organs derive (Cohen and Jurgens, 1990; Dalton et al., 1989; Walldorf and Gehring, 1992), *ems* might play a key role in the development of both the peripheral and central olfactory systems.

Lineage-specific functional roles of *ems* in postembryonic olfactory PN development

MARCM-based clonal loss-of-function experiments reveal two lineage-specific mutant phenotypes in adPN versus lPN lineages. In the lPN lineage, *ems* loss-of-function results in a dramatic cell-autonomous reduction in cell number. A significant contribution to this phenotype is made by cell death. Blocking cell death in mutant clones restores cell number to ~70% of the wild-type number. Although these findings imply that cells in *ems* mutant lPN lineages die during postembryonic development, we do not know whether cell death occurs at the level of the progenitors or postmitotic neurons. *ems* is persistently expressed in the neuroblast and GMCs in this lineage and might be required for the survival of these progenitors. Alternatively, as *ems* is transiently expressed in postmitotic neurons in the PN lineages, this transient neuronal expression might be required for PN survival. Finally, because blocking apoptosis does not always result in a complete rescue of cell number, unknown lineage-specific proliferation defects might also occur.

In the postembryonic adPN lineage, *ems* loss-of-function does not affect cell number, implying that *ems* is not required for proliferation or survival of adPNs. Furthermore, adPNs in *ems* mutant clones have the overall dendritic and axonal features of wild-type PNs, suggesting that *ems* is not required for general process outgrowth in this lineage. However, *ems* mutant adPNs do show marked cell-autonomous defects in dendritic targeting: they fail to innervate appropriate glomeruli, ectopically innervate inappropriate glomeruli, or mistarget dendrites. These targeting defects are not random in nature but are limited to a subset of glomeruli. This relative specificity of the mistargeting phenotypes indicates that *ems* loss-of-function does not simply result in non-specific spillover of adPN dendrites. Moreover, it argues for the existence of other cell-intrinsic determinants that participate in translating adPN lineage information into dendritic targeting specificity.

Previous studies have identified an ensemble of transcription factors that act as intrinsic regulators of dendritic targeting in PNs (Komiyama et al., 2003; Komiyama and Luo, 2007). For example, the two POU-domain transcription factors Acj6 and Drifter are differentially expressed in adPNs and lPNs, are required for the specific connectivity of these PNs in their lineage, and cause mistargeting when misexpressed in PNs of the alternate lineage. Acj6 and Drifter are expressed in postmitotic Gal4-GH146-positive PNs during their dendritic targeting phase. Hence, the developmental time period in which these transcription factors are expressed coincides with that in which their mutant phenotypes appear. By contrast, the transcription factor-encoding *ems* gene is expressed in the precursors of PN lineages, but not in Gal4-GH146-positive PNs during their dendritic targeting phase. Thus, *ems* expression and appearance of the *ems* mutant dendritic targeting phenotype occur sequentially and do not overlap in developmental time. This suggests that *ems* acts as an early intrinsic determinant in the adPN lineage to influence cell fate decisions that indirectly result in dendritic targeting later in postembryonic development. Therefore, the mechanism of *ems* action on dendritic targeting might be mediated by other factors that are themselves regulated by *ems* and that subsequently affect components of the wiring machinery.

Our findings indicate that Acj6 is one of these factors. We have identified Acj6 as downstream mediator of *ems* action, at least in one class of adPNs. Acj6 expression is lost in approximately half of the *ems* mutant adPNs. Moreover, the reduced innervation of the VA1lm

glomerulus in the *ems* mutant is significantly rescued by the misexpression of *acj6*. It is noteworthy that the innervation of the VA1lm glomerulus has been reported to be lost in 63% of *acj6* mutant adPN clones and that misexpression of *acj6* in these clones rescued innervation to a level similar to that observed in our Acj6 rescue experiments (Komiyama et al., 2003). Given that Acj6 expression is lost in only about half of the *ems* mutant adPNs, misexpression of Acj6 should not affect the other half of the *ems* mutant adPNs. Indeed, for the innervation of the VA3 and VM2 glomeruli, we observe that misexpression of Acj6 in *ems* mutant adPNs does not result in significant rescue of the projection phenotype, implying that Acj6 expression is *ems*-independent in the PNs that innervate VA3 and VM2.

The fact that the other phenotypes observed in this investigation for *ems* mutant adPN and lPN clones have not been reported in *acj6* mutant clones does, however, imply that there are other downstream mediators of *ems* action in PN development. Similarly, because Acj6 is lost in only about half of the *ems* mutant adPNs, other upstream regulators of *acj6* expression in adPNs are also likely to be present.

Although transient early *ems* expression is important for appropriate development of the adPN lineage, more prolonged, later expression of *ems* in the differentiating adPNs can have detrimental effects. Ectopic misexpression of *ems* in adPNs in differentiating PNs via the Gal4-GH146 driver results in dendritic targeting defects comparable to those caused by *ems* loss-of-function. Interestingly, ectopic *ems* misexpression also causes axonal targeting defects in at least one of the adPNs, the DL1 neurons. Since misexpression of *ems* beyond the time of normal endogenous expression can lead to dual targeting defects (axonal and dendritic), precise temporal regulation of *ems* expression is likely to be crucial for the correct development of adPNs.

Evolutionary conservation of *ems*/Emx gene functions in olfactory projection neuron development?

The organization of the insect and mammalian olfactory system is remarkably similar (Hildebrand and Shepherd, 1997; Komiyama and Luo, 2006). Olfactory receptor neurons expressing the same receptor project their axons to the same glomeruli in the insect antennal lobe as in the mammalian olfactory bulb. In these glomeruli, the olfactory receptor neurons make specific synaptic connections with the dendrites of second-order olfactory neurons, the PNs in insects and the mitral cells in mammals. Finally, PNs and mitral cells send processed olfactory information to specific regions of higher olfactory centers in the brain.

In both insects and mammals, genes of the *ems/*Emx family are important for the development of these second-order neurons. In *Drosophila*, *ems* is expressed in the two main PN lineages and is required for correct cell number and precise dendritic targeting of these neurons. In the mouse, the two *ems* gene homologs, *Emx1* and *Emx2*, are expressed in two complementary groups of mitral cells (Mallamaci et al., 1998; Simeone et al., 1992a; Simeone et al., 1992b), and the loss of both genes leads to marked defects in the mitral cell layer (Bishop et al., 2003).

The remarkably similar expression and function of the *ems/*Emx genes in the development of second-order olfactory neurons in insects and mammals, together with the similarities in expression of these genes in developing olfactory sensory structures in both groups, argue for evolutionarily conserved roles of the *ems/*Emx genes in olfactory system development. Thus, while the astonishing similarity in anatomical organization of the olfactory system in insects and mammals may be the result of functional convergence, it might also reflect, at least in part, a hitherto unexpected conservation of the molecular genetic mechanisms for olfactory system development in these animals.

We thank B. Bello for MARCM lines, S. Flister for assistance, U. Walldorf for the Ems antibody, R. Stocker for the Gal4-GH146 line, L. Luo for the UAS-Acj6 line and S. L. Lai and T. Lee for dual-expression-control MARCM lines. This work was supported by the Swiss NSF.

References

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (2000). The genome sequence of Drosophila melanogaster. *Science* **287**, 2185-2195.

Axel, R. (1995). The molecular logic of smell. *Sci. Am.* **273**, 154-159.

Bello, B., Reichert, H. and Hirth, F. (2006). The *brain tumor* gene negatively regulates neural progenitor cell proliferation in the larval central brain of *Drosophila*. *Development* **133**, 2639-2648.

Bishop, K. M., Garel, S., Nakagawa, Y., Rubenstein, J. L. and O'Leary, D. D. (2003). Emx1 and Emx2 cooperate to regulate cortical size, lamination, neuronal differentiation, development of cortical efferents, and thalamocortical pathfinding. *J. Comp. Neurol.* **457**, 345-360.

Cecchi, C. and Boncinelli, E. (2000). Emx homeogenes and mouse brain development. *Trends Neurosci.* **23**, 347-352.

Chen, H. H., Hippenmeyer, S., Arber, S. and Frank, E. (2003). Development of the monosynaptic stretch reflex circuit. *Curr. Opin. Neurobiol.* **13**, 96-102.

Cohen, S. M. and Jurgens, G. (1990). Mediation of Drosophila head development by gap-like segmentation genes. *Nature* **346**, 482-485.

Dalton, D., Chadwick, R. and McGinnis, W. (1989). Expression and embryonic function of empty spiracles: a Drosophila homeo box gene with two patterning functions on the anterior-posterior axis of the embryo. *Genes Dev.* **3**, 1940-1956.

Hildebrand, J. G. and Shepherd, G. M. (1997). Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Annu. Rev. Neurosci.* **20**, 595-631.

Hirth, F., Therianos, S., Loop, T., Gehring, W. J., Reichert, H. and Furukubo-Tokunaga, K. (1995). Developmental defects in brain segmentation caused by mutations of the homeobox genes orthodenticle and empty spiracles in Drosophila. *Neuron* **15**, 769-778.

Hirth, F., Hartmann, B. and Reichert, H. (1998). Homeotic gene action in embryonic brain development of Drosophila. *Development* **125**, 1579-1589.

Hirth, F., Kammermeier, L., Frei, E., Walldorf, U., Noll, M. and Reichert, H. (2003). An urbilaterian origin of the tripartite brain: developmental genetic insights from Drosophila. *Development* **130**, 2365-2373.

Jefferis, G. S., Marin, E. C., Stocker, R. F. and Luo, L. (2001). Target neuron prespecification in the olfactory map of Drosophila. *Nature* **414**, 204-208.

Jefferis, G. S., Marin, E. C., Komiyama, T., Zhu, H., Chihara, T., Berdnik, D. and Luo, L. (2005). Development of wiring specificity of the Drosophila olfactory system. *Chem. Senses* **30 Suppl. 1**, i94.

Kammermeier, L., Leemans, R., Hirth, F., Flister, S., Wenger, U., Walldorf, U., Gehring, W. J. and Reichert, H. (2001). Differential expression and function of the Drosophila Pax6 genes eyeless and twin of eyeless in embryonic central nervous system development. *Mech. Dev.* **103**, 71-78.

Komiyama, T. and Luo, L. (2006). Development of wiring specificity in the olfactory system. *Curr. Opin. Neurobiol.***16**, 67-73.

Komiyama, T. and Luo, L. (2007). Intrinsic control of precise dendritic targeting by an ensemble of transcription factors. *Curr. Biol.* **17**, 278-285.

Komiyama, T., Johnson, W. A., Luo, L. and Jefferis, G. S. (2003). From lineage to wiring specificity. POU domain transcription factors control precise connections of Drosophila olfactory projection neurons. *Cell* **112**, 157-167.

Lai, S. L. and Lee, T. (2006). Genetic mosaic with dual binary transcriptional systems in Drosophila. *Nat. Neurosci.* **9**, 703-709.

Lichtneckert, R. and Reichert, H. (2005). Insights into the urbilaterian brain: conserved genetic patterning mechanisms in insect and vertebrate brain development. *Heredity* **94**, 465-477.

Lichtneckert, R., Bello, B. and Reichert, H. (2007). Cell lineage-specific expression and function of the empty spiracles gene in adult brain development of Drosophila melanogaster. *Development* **134**, 1291-1300.

Mallamaci, A., Iannone, R., Briata, P., Pintonello, L., Mercurio, S., Boncinelli, E. and Corte, G. (1998). EMX2 protein in the developing mouse brain and olfactory area. *Mech. Dev.* **77**, 165-172.

Marin, E. C., Jefferis, G. S., Komiyama, T., Zhu, H. and Luo, L. (2002). Representation of the glomerular olfactory map in the Drosophila brain. *Cell* **109**, 243-255.

Marin, E. C., Watts, R. J., Tanaka, N. K., Ito, K. and Luo, L. (2005). Developmentally programmed remodeling of the Drosophila olfactory circuit. *Development* **132**, 725-737.

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., Edmondson, J. and Axel, R. (1996). Visualizing an olfactory sensory map. *Cell* **87**, 675-686.
Noveen, A., Daniel, A. and Hartenstein, V. (2000). Early development of the Drosophila mushroom body: the roles of eyeless and dachshund. *Development* **127**, 3475-3488.
Python, F. and Stocker, R. F. (2002). Adult-like complexity of the larval antennal lobe of D. melanogaster despite markedly low numbers of odorant receptor neurons. *J. Comp. Neurol.* **445**, 374-387.
Shinozaki, K., Miyagi, T., Yoshida, M., Miyata, T., Ogawa, M., Aizawa, S. and Suda, Y. (2002). Absence of Cajal-Retzius cells and subplate neurons associated with defects of tangential cell migration from ganglionic eminence in Emx1/2 double mutant cerebral cortex. *Development* **129**, 3479-3492.
Shirasaki, R. and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. *Annu. Rev. Neurosci.* **25**, 251-281.
Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A. and Boncinelli, E. (1992a). Nested expression domains of four homeobox genes in developing rostral brain. *Nature* **358**, 687-690.
Simeone, A., Gulisano, M., Acampora, D., Stornaiuolo, A., Rambaldi, M. and Boncinelli, E. (1992b). Two vertebrate homeobox genes related to the Drosophila empty spiracles gene are expressed in the embryonic cerebral cortex. *EMBO J.* **11**, 2541-2550.
Skeath, J. B. and Thor, S. (2003). Genetic control of Drosophila nerve cord development. *Curr. Opin. Neurobiol.* **13**, 8-15.
Spletter, M. L., Liu, J., Liu, J., Su, H., Giniger, E., Komiyama, T., Quake, S. and Luo, L. (2007). Lola regulates Drosophila olfactory projection neuron identity and targeting specificity. *Neural Develop.* **2**, 14.
Stocker, R. F., Heimbeck, G., Gendre, N. and de Belle, J. S. (1997). Neuroblast ablation in Drosophila P[GAL4] lines reveals origins of olfactory interneurons. *J. Neurobiol.* **32**, 443-456.
Urbach, R. and Technau, G. M. (2003). Molecular markers for identified neuroblasts in the developing brain of Drosophila. *Development* **130**, 3621-3637.
Vosshall, L. B. and Stocker, R. F. (2007). Molecular architecture of smell and taste in Drosophila. *Annu. Rev. Neurosci.* **30**, 505-533.
Vosshall, L. B., Wong, A. M. and Axel, R. (2000). An olfactory sensory map in the fly brain. *Cell* **102**, 147-159.
Walldorf, U. and Gehring, W. J. (1992). Empty spiracles, a gap gene containing a homeobox involved in Drosophila head development. *EMBO J.* **11**, 2247-2259.
Wong, A. M., Wang, J. W. and Axel, R. (2002). Spatial representation of the glomerular map in the Drosophila protocerebrum. *Cell* **109**, 229-241.
Younossi-Hartenstein, A., Green, P., Liaw, G. J., Rudolph, K., Lengyel, J. and Hartenstein, V. (1997). Control of early neurogenesis of the Drosophila brain by the head gap genes tll, otd, ems, and btd. *Dev. Biol.* **182**, 270-283.