Cell lineage-specific expression and function of the empty spiracles gene in adult brain development of Drosophila melanogaster

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The empty spiracles (ems) gene, encoding a homeodomain transcription factor, is a member of the cephalic gap gene family that acts in early specification of the anterior neuroectoderm in the embryonic brain of Drosophila. Here we show that ems is also expressed in the mature adult brain in the lineage-restricted clonal progeny of a single neuroblast in each brain hemisphere. These ems-expressing neuronal cells are located ventral to the antennal lobes and project a fascicle to the superior medial protocerebrum. All adult-specific secondary neurons in this lineage consistently express ems during postembryonic larval development and continue to do so throughout metamorphosis and into the adult. Mosaic-based MARCM mutant analysis and genetic rescue experiments demonstrate that ems function is autonomously required for the correct number of cells in the persistently expressing adult-specific lineage. Moreover, they indicate that ems is also required cell autonomously for the formation of the correct projections in this specific lineage. This analysis of ems expression and function reveals novel and unexpected roles of a cephalic gap gene in translating lineage information into cell number control and projection specificity in an individual clonal unit of the adult brain.

KEY WORDS: empty spiracles, ems, Brain development, Neuroblast lineage, MARCM, Drosophila, Neurogenesis

INTRODUCTION

The insect brain is generated by stem-cell-like neuroblasts that derive from the cephalic neuroectoderm. Neuroblasts divide repeatedly in an asymmetric mode that is self-renewing and generates smaller ganglion mother cells, which usually divide once to produce two postmitotic progeny (Pearson and Doe, 2004; Skeath and Thor, 2003). In insects such as Drosophila, which has complete metamorphosis, neuroblasts generate the primary neurons of the larval brain during embryonic development. Following a period of quiescence, most neuroblasts resume their asymmetric mode of proliferation during postembryonic larval development and generate the adult-specific secondary neurons that make up the bulk of the adult central nervous system (Prokop and Technau, 1991; Truman and Bate, 1988). The adult-specific neurons that are generated during larval life from each persistent neuroblast form a lineage-related cluster of immature neurons that extend fasciated primary neurites into the neuropile but wait until metamorphosis to complete their extension to synaptic targets (Dumstrei et al., 2003; Pereanu and Hartenstein, 2006; Truman et al., 2004; Zheng et al., 2006). During metamorphosis, development of the adult brain is completed through neuronal remodelling of larval functional neurons and final morphogenesis of adult-specific neurons (Lee et al., 2000; Marin et al., 2005; Zheng et al., 2006).

Recent analyses of Drosophila neurogenesis have identified developmental control genes that are involved in generating the larval brain. Expression analysis for over 30 of these genes has shown that specific combinations of gene expression characterize each of the approximately 100 embryonic brain neuroblasts (Urbach and Technau, 2003). For a number of these genes, loss-of-function analyses have revealed severe defects in neurogenesis, patterning and circuit formation during 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). By contrast, only a few of these developmental control genes have been studied in postembryonic development of the adult brain (Callaerts et al., 2001; Hassan et al., 2000; Hitier et al., 2001; Kurusu et al., 2000; Pereanu et al., 2000; Pereanu and Hartenstein, 2006).

The empty spiracles (ems) gene plays a central role in embryonic development of the brain (Lichtneckert and Reichert, 2005). The ems gene encodes a homeodomain transcription factor that acts as a cephalic gap gene during early embryogenesis (Cohen and Jurgens, 1990; Dalton et al., 1989; Walldorf and Gehring, 1992). During embryonic neurogenesis, ems is expressed in 11 bilaterally symmetrical neuroblasts and later is found in the deutocerebral and tritocerebral embryonic brain neuromeres (Hirth et al., 1995; Urbach and Technau, 2003). Mutation of the ems gene results in the absence of cells in the deutocerebral and tritocerebral anlagen; this is due to the failure of neuroblasts to form in the mutant domain (Younossi-Hartenstein et al., 1997). By contrast to the insight into the role of ems in embryonic brain development, virtually nothing is known about expression or function of ems during postembryonic development of the adult brain.

This lack of information on ems action in postembryonic brain development in Drosophila contrasts with the wealth of information on the role of the ems orthologues, Emx1 and Emx2, in mammalian brain development. Both of these mammalian genes are expressed in the early neuroectoderm and the embryonic progenitor cells that give rise to telencephalic brain regions (Simeone et al., 1992a; Simeone et al., 1992b). Mutant analysis indicates that these genes play important roles in early patterning and proliferation of anterior brain regions (Bishop et al., 2003; Cecchi and Boncinelli, 2000; Shinozaki et al., 2002). Mammalian Emx genes also act in later phases of brain development and are expressed in cells of the adult brain (Briata et al., 1996; Cecchi, 2002; Gulisano et al., 1996). Moreover, mutant analysis suggests a role of Emx genes in differentiation and maintenance of cortical neurons and in pathfinding of cortical efferents (Bishop et al., 2003; Shinozaki et al., 2002).
The finding that mammalian Emx genes play multiple roles in different stages of brain development underscores an emerging theme in vertebrate neuronal development. Thus, many developmental control genes implicated in early neurogenesis and patterning, are re-expressed and have different roles in later embryogenesis and postembryonic brain development (Salie et al., 2005; Zapala et al., 2005). Given the conservation of expression and function of fly ems and mammalian Emx genes in embryonic brain development, we set out to determine if the Drosophila ems gene might also play important roles in postembryonic development of the adult brain.

Here we show that ems is expressed in the adult brain in the clonal progeny of a single neuroblast in each brain hemisphere. All adult-specific secondary neurons in this lineage already express ems during larval development and continue to do so throughout metamorphosis and into the adult. To investigate the role of the ems gene in the persistently expressing, adult-specific brain lineage, we used mosaic analysis with a repressible cell marker (MARCM). Our findings demonstrate that ems function is cell-autonomously required for the correct number of cells in this lineage. Moreover, they indicate that ems is also required cell autonomously for the formation of the correct neuritic projections in this specific lineage. This analysis of ems function reveals novel and unexpected roles of a cephalic gap gene in determining the anatomical features of an individual lineage-based unit in the adult brain of Drosophila.

MATERIALS AND METHODS

Fly strains and genetics

Unless otherwise stated, fly stocks were obtained from the Bloomington Stock Center. Wild type was Oregon R. Two recombinant chromosomes were constructed: FRT82B, ems93G (Jurgens et al., 1984) on chromosomal arm 3R and tub-P-GAL4, UAS-mCD8::GFP on chromosome 2. For MARCM analysis (Lee and Luo, 1999) +: UAS-mCD8::GFP; UAS-nlslacZ20b, FRT82B, ems93G or UAS-mCD8::GFP; UAS-nlslacZ20b, FRT82B males were crossed to hs-FLP; tub-P-GAL4; FRT82B tub-P-GAL80 lethals (Bello et al., 2003), resulting in ems mutant or wild-type clones. Ems or P35 rescue experiments were performed by combining UAS-ems (H.R., unpublished) or UAS-P35BH1 on chromosome 2 with FRT82B, ems93G and crossing them to the stock hs-FLP; tub-P-GAL4, UAS-mCD8::GFP; FRT82B tub-P-GAL80 lethals respectively to generate MARCM clones.

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 to 25 hours before heat-shock treatment (37°C for 60 minutes).

Immunolabelling

Larval and adult brains were fixed and immunostained as previously described (Bello et al., 2003). The following antibodies were used: rat anti-Em (1:200; gift of U. Walldorf, University of Saarland, Homburg, Germany), rabbit anti-Em (1:500; gift of U. Walldorf), rabbit anti-Grh (1:200), rabbit anti-H3P (1:400; Upstate Biotechnology), rabbit anti-cleaved caspase 3 (1:75; Cell Signalling Technologies) rat anti-Elav Mab7E8A10 (1:30; DSHB), mouse anti-cyclin (1:50; gift of H. Richardson, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia), mouse anti-Pros MaMR1A (1:10; DSHB), mouse anti-βGAL (1:20; DSHB), mouse anti-Nrt BP106 (1:10; DSHB), mouse anti-BrU (1:100; DSHB), mouse monoclonal nc82 (1:20; gift of A. Hofbauer, University of Regensburg, Regensburg, Germany), rabbit anti-Cas (Kambadur et al., 1998). Secondary antibodies were Alexa 488, Alexa 568 and Alexa 647 antibodies generated in goat (1:300; Molecular Probes).

To estimate the number of dividing cells in wild-type or ems mutant clones induced at early first instar stage, larvae were transferred to BrdU-containing standard medium (final concentration: 1 mg/ml) at 60 hours after hatching and raised for 12 hours before dissection (Truman and Bate, 1988). Brains were fixed immediately in 4% paraformaldehyde for 15 minutes at RT and incubated with the anti-Em and anti-βGAL primary antibodies. Subsequently, an additional fixation step in 2% paraformaldehyde was applied and the brains were incubated in 2N HCl for 30 minutes to denature BrdU-labelled DNA before incubation with the anti-BrdU antibody.

Microscopy and image processing

Fluorescent images were recorded using a Leica TCS SP scanning 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). For visualizing particular MARCM clones, image stacks with few non-interfering clones were selected and stained processes and cell bodies from other clones were removed using the lasso tool in every single optical section. Digital 3D-models were generated using the AMIRA software by manually labelling structures of interest such as cell bodies, processes, whole clones or neurite and subsequent automated 3D surface rendering.

RESULTS

The ems gene is expressed in the adult brain of Drosophila

To investigate if the ems gene is expressed in cells of the adult brain, we carried out an immunocytochemical analysis of whole-mount brains 1-10 days after eclosion using an anti-Em antibody. In all cases, ems expression was detected in two bilaterally symmetrical cell clusters of the central brain (Fig. 1A). To locate these ems-expressing cell clusters relative to the neuropile, double labelling experiments were carried out using the neuropile marker anti-nc82 in combination with the anti-Em antibody. These studies showed that the two symmetrically arranged ems-expressing cell clusters are located ventrally to the antennal lobes and dorsally to the suboesophageal ganglion near the anterior midline of the brain (Fig. 1B,C). No other cells in the central brain or optic lobes expressed ems in the adult brain.

The compact aspect of the ems-expressing cell clusters suggests that they might represent clonally restricted neuroblast lineages. To investigate this, we carried out a MARCM-based analysis (Lee and Luo, 1999; Lee and Luo, 2001). In these experiments, GFP-labelled wild-type clones were induced at random in early first instar larvae (21-25 hours after egg-laying) to specifically label the secondary, adult-specific lineage of individual larval neuroblasts. The brains of adult flies that contained GFP-labelled MARCM clones were then co-labelled with anti-Em and anti-Nc82. Brains, in which GFP-labelled clones were anti-Em-immunoreactive, were analysed further by confocal microscopy.

Co-labelling of cell bodies with GFP and anti-Em was restricted to one clone per brain hemisphere (Fig. 1D). All the GFP-labelled cells of this particular clone coexpressed ems. In addition, a few ems-expressing cells that were not GFP-labelled were closely associated with the cell cluster that coexpressed GFP and ems (Fig. 1F). These findings indicate that the majority of the cells in the ems-expressing clusters of the adult brain are secondary adult-specific neurons that derive in a clonal manner from a single larval neuroblast. (The non-GFP-labelled cells in the ems-expressing clusters may represent primary neurons generated by the same persistent neuroblast during embryogenesis.)

The ems-expressing cells in the GFP-labelled neuroblast clones were associated with a GFP-labelled fascicle that extended to more dorsal brain regions. To facilitate the analysis of this fascicle, a digital 3D model of the projection and the major neuropile compartments along which the fascicle projected was generated (Fig. 1E). This showed that the fascicle projected from the GFP-labelled cell bodies medi ally along the antennal lobe to the ipsilateral superior medial protocerebrum. Hereafter this will be referred to as
The ems gene is expressed in brain neuroblast clones during larval development

The restricted expression of ems in the adult brain suggested that it might be required for the development of the neuroblast lineage. This prompted us to examine the expression of ems at earlier stages. Anti-Em immunolabelling was found in several distinct cell clusters in each brain hemisphere of late third instar larvae, including a prominent cluster located near the medial edge of each hemisphere (Fig. 2A). (Additionally, scattered cells in the suboesophageal ganglion also expressed ems; these cells were not considered further in this study.) No ems expression was seen in the developing optic lobes. The architecture of these ems-expressing clusters was further examined in double immunolabelling experiments using anti-Em in combination with anti-Neurotactin (Fig. 2B). Neurotactin is highly expressed on fasciculated neurites of immature neurons, and anti-Neurotactin-labelling can therefore be used to reconstruct secondary lineages in the larval brain (de la Escalera et al., 1990; Pereanu and Hartenstein, 2006).

A total of eight cell clusters with ems expression were found in each brain hemisphere. These cell clusters could be unambiguously identified based on their relative positions and on the projection pattern of their primary neurite bundles (Pereanu and Hartenstein, 2006; Truman et al., 2004). Each cell cluster contained a large ems-expressing cell near the cortex surface associated with a columnar-like aggregate of smaller cells, which co-expressed ems and Neurotactin (Fig. 2C,D). In each cell cluster, a Neurotactin-expressing fascicle emerged from the smaller ems-expressing cells (Fig. 2D). This fascicle extended towards the brain neuropile (Fig. 2E). A digital 3D model of all eight ems-expressing cell clusters and their primary neurites is shown in Fig. 2F. The medial cluster (asterisk) with its dorsally projecting fascicle (arrow) is clearly identifiable in this model.

These findings suggest that each ems-expressing cell cluster in the late third instar brain is composed of a persistent neuroblast and its progeny, which project fasciculated primary neurites into the neuropile. To determine if ems expression in the late third instar brain is restricted to adult-specific lineages, a MARCM-based clonal analysis was carried out. GFP-labelled wild-type MARCM clones, induced in early first instar larvae, were recovered in the late third instar brain for all eight Em-positive cell clusters. This indicates that each cell cluster represents a neuroblast clone. A clear difference in ems expression was observed between the medial ems-expressing lineage (hereafter referred to as the EM lineage) and the remaining seven ems-expressing lineages. In these seven lineages, ems expression was present in the neuroblast and in a small subset of the adult-specific neurons located adjacent to the neuroblast; these represent the late born cells in the neuroblast clone (Fig. 2G). By contrast, early born neurons located further away from the neuroblast did not express ems (Fig. 2H). This spatially restricted ems-expression pattern within neuroblast clones is illustrated in a digital 3D model of one representative of the seven lineages (Fig. 2I; arrowhead in 2F).

A markedly different ems-expression pattern was observed in the EM lineage. In this lineage, ems-expression was present throughout the secondary lineage, including the neuroblast and the adult-
specific neurons, irrespective of their position within the clone (Fig. 2J,K). A digital 3D model of the EM lineage (Fig. 2L) illustrates the fact that \textit{ems} is expressed throughout the GFP-labelled clone. In addition, GFP-negative cells located close to the early born, adult-specific neurons of the GFP-labelled clone also expressed \textit{ems}, albeit at a lower level (open arrowheads in Fig. 2K; lightly coloured cells in Fig. 2L). These could be progeny generated by the EM neuroblast during embryonic development before MARCM clone induction.

\textbf{\textit{ems}-expression in the EM lineage persists through metamorphosis of the brain}

Among the eight neuroblast lineages that express \textit{ems} in the larval brain, only one, the EM lineage, expresses the gene in all its secondary, adult-specific cells. This expression pattern also characterizes the single \textit{ems}-expressing lineage in the adult brain and, together with their similar location and neurite projection pattern, suggests that larval EM lineage might correspond to the adult lineage.

To investigate this, GFP-labelled wild-type MARCM clones induced in early first instar larvae were examined at late third instar stage and at 24, 48 and 72 hours after puparium formation (APF). In all cases, only one GFP-labelled clone, which coexpressed \textit{ems} in all labelled cells, was observed per brain hemisphere (Fig. 3). These clones were comparable in size and location in the brain cortex and had similar fascicle projections. Moreover, their overall morphology at 72 hours APF was very similar to that of the single \textit{ems}-expressing clone in the adult brain (compare Fig. 3D with Fig. 1D). By contrast, \textit{ems} expression in the other lineages was strongly reduced in the early pupa and completely disappeared at later pupal stages. This indicates that the larval EM lineage persists through metamorphosis, maintaining both its \textit{ems} expression features and its neurite fascicle projection pattern. One morphological change that did occur during metamorphosis in the cells of the EM lineage was the emergence of dense dendrite-like arborizations (Fig. 3 insets). In the early pupa, these short arborizations extended ventrally into the neuropile of the suboesophageal ganglia. They were retained through metamorphosis and remained present in the adult brain (Fig. 1G).

Based on its overall morphology, we tentatively assign the EM lineage to the BAmas2 secondary lineage defined by Pereanu and Hartenstein (Pereanu and Hartenstein, 2006). Accordingly, this lineage is a member the basoanterior group of the ventral deutocerebrum, which surrounds the antennal compartment and projects its secondary lineage axon tract (SAT) upwards along the medial edge of the brain along the median bundle.

\textbf{Neuronal precursors and postmitotic neurons are present in \textit{ems} mutant EM lineages}

To determine the role of the \textit{ems} gene in the development of the EM lineage, \textit{ems} mutant and wild-type MARCM clones were induced randomly in early first instar larvae and analysed in late third instar brains. Mutant lineages were homozygous for \textit{ems}^9Q64, an embryonic lethal loss-of-function allele of \textit{ems}. This allele encodes a truncated non-functional protein that is detected by the anti-Ems antibody in the cytoplasm.

All labelled wild-type and \textit{ems} mutant EM lineages contained one large cell, the neuroblast, which consistently expressed the transcription factor Grainyhead (Grh) (Fig. 4A,B). Smaller Grh-expressing ganglion mother cells (GMCs) were found directly
adjacent to the neuroblast in both wild-type and mutant EM lineages. Moreover, expression of the mitotic markers Cyclin E (CycE) and anti-phosphorylated histone-H3 (H3p) was seen in neuroblasts and GMCs of both wild-type and mutant clones (Fig. 4C-F). These findings indicate that neuroblasts and GMCs are present and mitotically active in wild-type and *ems* mutant EM lineages at late third instar stage (Almeida and Bray, 2005; Bello et al., 2003; Cenci and Gould, 2005).

In addition to precursors, the EM clones contained a number of smaller cells representing adult-specific neural progeny of the lineage that expressed the neuron-specific label Elav in wild-type and mutant clones (Fig. 4G,H). In addition, two differentiation markers, Prospero (Pros) and Castor (Cas) (Almeida and Bray, 2005), which were expressed in postmitotic neurons of the wild-type EM lineage, were also seen in the neural progeny of the *ems* mutant EM lineage (Fig. 4I-L). Taken together, these results indicate that mitotically active progenitor cells and differentiating neuronal progeny are present in the wild-type and *ems* mutant EM lineage in the late third instar brain.

### The *ems* gene is required for correct neuronal cell number in the EM lineage

Although *ems* mutant EM lineages did contain postmitotic neurons, the number of neurons per clone seemed to be reduced (Fig. 4). Reduction in clone size was clearly manifest in mutant clones of late third instar brains (Fig. 5B-G). Quantification of GFP-labelled cells revealed that the wild-type EM lineage contains on average 79 adult-specific cells (s.d.=3.4; n=7), whereas *ems* mutant clones had an average of only 36 labelled cells (s.d.=12.8; n=20) (Fig. 5A). To confirm that this reduction was due to *ems* loss of function, we carried out a clonal rescue experiment. For this, *ems* expression was targeted in *ems* homozygous mutant clones using a UAS-ems transgene under the control of the MARCM tub-GAL4 driver. When examined in late third instar, the size of these rescued clones was restored to an average of 77 cells (s.d.=4.5; n=10), which was almost wild type (Fig. 5A, also compare Fig. 5H-J with Fig. 5B-D). These findings indicate that the *ems* gene is required cell autonomously for the correct number of adult-specific neurons in the EM lineage.

In order to obtain insight into the mechanisms of clone size reduction in *ems* mutants, a more detailed characterization of the EM lineage during postembryonic development was carried out for both wild-type and mutant clones. First, the number of cells in EM clones (induced at early first instar) was determined at different larval and pupal stages (Fig. 6). At 48 hours after larval hatching (ALH), mutant and wild-type clones contained a similar number of cells, suggesting that initially postembryonic proliferative activity in the EM clones was not affected by *ems* loss of function. Marked differences between mutant and wild-type clones became apparent at 72 hours ALH in that the *ems* mutant clones contained fewer cells than the wild-type clones. This difference had increased at 96 hours ALH and remained large through pupal development and in the adult.

To determine if this difference in clonal cell number might be due to reduced proliferative activity in the *ems* mutants, we next studied the incorporation of BrdU into wild-type and mutant clones at the mid-third instar stage (see Materials and methods). In wild-type clones, an average of 16.2 (s.d.=2.2; n=13) and in *ems* mutant clones an average of 15.9 (s.d.=1.9; n=13) labelled cells were observed, indicating that mitotic activity was similar in the two cases. Furthermore, the percentage of EM neuroblasts expressing the mitotic marker H3p at mid-third instar stages was comparable in both cases; 43% of wild-type neuroblasts (n=74) and 50% of *ems* mutant neuroblasts (n=14) expressed the marker. (Comparable findings were obtained for late third instar larva; data not shown.) Taken together, these findings imply that the proliferative activity was not significantly reduced in *ems* mutant EM clones.

To investigate if the reduction in clonal cell number might involve the death of postmitotic cells, we initially stained *ems* mutant clones in late third instar brains with the apoptosis marker cleaved Caspase 3. All the *ems* mutant EM clones studied contained one to four cleaved Caspase 3-positive cells (average=2.4, s.d.=1.0, n=10), indicating the presence of apoptosis in the mutant lineages. To determine if apoptosis can account for the reduction in clonal cell number observed in the *ems* mutant lineage, we next blocked cell death in *ems* mutant clones through misexpression of the pancaspase inhibitor P35. For this, clones were induced in early first instar larva and cell numbers determined at the late third larval stage. Blocking cell death resulted in mutant clones containing an average of 70 (s.d.=15; n=9) cells. This is comparable to an average of 79 cells in wild-type EM clones and an average of 77 cells in *ems* mutant clones misexpressing an *ems* transgene, and it is significantly higher than the average of 36 cells in *ems* mutant clones (see above). These findings imply that the reduction in clonal cell number in *ems* mutant EM lineages is due to apoptosis.

### The *ems* gene is required for correct projections in the EM lineage

When examined in the adult brain, *ems* mutant MARCM clones in the EM lineage showed a second marked *ems* mutant phenotype. In many cases, mutant clones lacked the prominent protocerebral fascicle that projected to the superior medial protocerebrum in the wild-type control (Fig. 7A-D). In other mutant clones, a somewhat
reduced protocerebral fascicle was formed (Fig. 7E,F). Moreover, in all ems mutant clones examined (n=8), aberrant projections extended without obvious pattern towards adjacent neuropiles (Fig. 7C-F, arrowheads). Misdirected projections of this type were never observed in the wild-type control. These projection defects were fully restored in rescue experiments in which the ems transgene was misexpressed in the ems mutant EM clone (Fig. 7G,H). Rescued clones had a normal protocerebral fascicle and never showed short aberrant or misdirected process extensions. These findings indicate that the ems gene is required cell autonomously for the correct projection pattern of adult-specific neurons in the EM lineage.

As the primary fascicle of the EM lineage is formed in larval stages, it is possible that the projection phenotype observed in the adult ems mutant EM lineage first manifests itself in the larval brain. Alternatively, the larval fascicle may develop normally in the mutant lineage and then become disrupted during metamorphosis. To investigate this, we characterized the primary fascicle of the EM lineage in wild-type and ems mutant MARCM clones at the late third instar. By contrast to the wild-type clones, approximately half the ems mutant EM lineages (11/20) showed a complete lack of the primary fascicle, and all the mutant lineages (20/20) had ectopic misdirected process extensions (Fig. 7I-N). These larval projection defects were fully restored in rescue experiments in which the ems transgene was misexpressed in the ems mutant EM clone (Fig. 7O,P). This indicates that ems function is already required during larval stages for the formation of correct projections by adult-specific EM neurons.

To determine if the cell-autonomous requirement of the ems gene for correct projection of the EM lineage occurs at the level of postmitotic cells, we analysed single cell MARCM clones in third instar larval brains. Single labelled wild-type cells had neuronal morphologies that were expected for the EM lineage (Fig. 8A,B). Thus, labelled cells had a cell body located in the appropriate region of the brain cortex as well as a projection pattern in the brain neuropile consisting of a single process that extended towards the midline, turned rostrally, projected to the anterior protocerebrum, and there formed arborizations. By contrast, most of the single labelled ems mutant cells showed dramatic projection defects. In some cases, labelled cells extended processes posteriorly that arborized in the suboesophageal ganglion (Fig. 8C,D). In other
cases, labelled cells failed to extend any process (Fig. 8E,F). These observations indicate that individual postmitotic EM cells manifest a cell-autonomous requirement for the ems gene in order to establish their appropriate neuronal morphology.

Taken together, our MARCM-based mutant analysis indicates that ems loss of function leads to two cell-autonomous phenotypes in the adult-specific EM lineage of the brain. First, the number of adult-specific cells in the mutant EM lineage is dramatically reduced. Second, marked projection defects occur in the protocerebral fascicle of the adult-specific neurons in the mutant EM lineage.

**DISCUSSION**

**Expression of ems in postembryonic neuroblast lineages**

During postembryonic development of the Drosophila brain, expression of the ems gene is observed in eight neuroblast lineages per hemisphere. In seven of these, ems expression is transient and disappears during pupal development. This cessation of expression during metamorphosis could be related to the dynamic pattern of ems expression within each lineage. Thus, during larval development of these lineages, ems expression appears limited to the neuroblast and its recently generated progeny, suggesting that expression in the progeny may be transient. This type of dynamic expression could explain the fading out of the Ems signal in the seven lineages once their neuroblasts stop proliferation at the early pupal stage.

By contrast, in the eighth neuroblast lineage, ems expression is persistent. During larval development the neuroblast and all its adult-specific progeny express ems; this expression continues throughout metamorphosis and into the adult in all postmitotic cells of the EM lineage. The mechanisms responsible for the maintenance of ems expression in the adult-specific cells of the EM lineage are currently unknown. However, there is some evidence that ems is also expressed and maintained in the primary neurons of the EM lineage generated during embryogenesis. In all postembryonic stages and in the adult, approximately 30 ems-expressing neurons are closely associated with the early born, adult-specific neurons of the EM clone. These neurons are not generated postembryonically, and their number does not change significantly during postembryonic development. This suggests that the mechanisms responsible for the persistence of ems expression in the EM lineage may operate in all cells of the lineage, embryonic and postembryonic.

**Functional roles of ems in the EM lineage**

For mutant analysis of ems function we focused on the EM lineage and used clonal techniques to ensure that the secondary adult-specific neurons are mutant from the time of their birth onwards. Two lineage-specific mutant phenotypes are apparent in these loss-of-function experiments. The number of adult-specific neurons is reduced and projection defects occur in mutant clones. Both phenotypes are cell autonomous, and both can be fully restored in genetic rescue experiments. Moreover, both mutant phenotypes are seen in larval stages and persist in the adult brain. These findings implicate the ems transcription factor in translating lineage information into neuronal cell number control and neurite projection specificity.

During early embryogenesis, ems is expressed in a total of eleven neuroblasts per embryonic brain hemisphere (Urbach and Technau, 2003). An unambiguous link between these embryonic brain neuroblasts and the eight postembryonic ems-expressing neuroblasts has not yet been established. If the persistent expression of ems is a unique feature of the EM lineage, it should be possible to trace this lineage back into embryonic stages and identify its embryonic neuroblast of origin. For the remaining seven postembryonic ems-expressing neuroblasts, this may be more difficult and require a combination of molecular markers and neuroanatomical lineage mapping (Pereanu and Hartenstein, 2006; Younossi-Hartenstein et al., 2006).

The postembryonic expression of ems in the fly brain has interesting parallels to the expression of the Emx1 and Emx2 genes in the mammalian brain. In addition to early expression in the neural plate, the Emx1 gene is expressed in many differentiating and mature neurons of the murine cortex (Briata et al., 1996; Gulisano et al., 1996). Brain-specific expression of Emx2 appears to be more transient in later stages and in the adult brain seems to be restricted to neural stem cells (Gangemi et al., 2001; Mallamaci et al., 1998; Mallamaci et al., 2000). Thus, spatially restricted persistent and transient expression patterns are observed for the ems/Emx genes in neural progenitors and in neurons during brain development and maturation in flies and mice.

**Fig. 6. Wild-type and ems mutant EM clone size at different developmental stages.**

MARCM clone induction occurred at 0 hours ALH. Average number of cells is plotted against the time of analysis. Numbers of clones analysed indicated in brackets.
of ganglion mother cells might be suppressed in favour of a direct differentiation of each neuroblast progeny into a single neuron, resulting in a total clone size reduction of 50%. This also appears unlikely, as GMCs expressing a cell proliferation marker can be identified repeatedly in mutant clones, indicating that they divide normally to produce two daughter cells. Third, the time window of proliferative activity or the proliferation rate of the persistent neuroblast is shortened in *ems* mutants. While we cannot rule out this possibility, it also appears unlikely for the following three reasons: first, mutant and wild-type clones contain a similar number of cells at 48 hours ALH, suggesting that the proliferation rate is not affected at this stage; second, BrdU-incorporation studies reveal no difference in mitotic activity at late larval stage brains of wild-type versus *ems* mutant clones; third, the percentage of neuroblasts expressing the mitotic marker H3p at late larval stages was comparable for wild-type and *ems* mutant clones. The final explanation for the marked reduction in cell number seen in mutant clones is that postmitotic cells die due to apoptosis. This possibility is supported by two observations: (1) late larval *ems* mutant EM clones contain apoptotic cells, as assayed by the apoptosis marker cleaved Caspase 3; (2) blockage of cell death in the *ems* mutant lineage through a pancaspase inhibitor results in significant restoration of the clonal cell number to a value comparable to that observed in the wild type. Based on these findings, we posit that *ems* is required in the adult-specific EM lineage for survival of clonal postmitotic progeny.

Two types of neurite projection defects are observed in *ems* mutant EM lineages. First, in the adult brain of all *ems* mutants, short aberrant projections extend from the cell bodies in a misdirected manner into adjacent neuropile. Misdirected projections of this type are also present in the larval *ems* mutant EM lineages. This suggests that *ems* is already required during larval stages to prevent the formation of these misprojections. Whether the aberrant projections formed in the larva persist into the adult, or whether misprojections of this type are continuously formed (and retracted) during metamorphosis and in the adult, is currently not known. However, the fact that neurite projections, albeit short and ectopic, are formed in all mutant EM clones implies that the *ems* gene is not required for process outgrowth per se. Rather, the *ems* gene appears to be required to prevent the formation of misdirected processes, suggesting a role of the gene in neuronal pathfinding.

A second projection defect is observed in the adult brain in approximately half the *ems* mutant EM lineages. It consists in the complete absence of the fascicle projecting to the superior medial protocerebrum. This projection phenotype in the adult has a corresponding projection phenotype in the larva, in that the primary neurite bundle is missing in approximately half the mutant lineages. These observations suggest that the formation of the primary neurite bundle during larval development might be a prerequisite for the process extension to adult-specific targets during metamorphosis; this would indicate a larval requirement of *ems* for neurite fascicle formation.

Both projection phenotypes seen in mutant neuroblast clones, short ectopic neurite projections and the absence of the fascicle to the protocerebrum, are also apparent in *ems* mutant single cell clones of the larval brain. Given that all other cells in the lineage, including the EM neuroblast, are wild-type-like in these experiments, this finding indicates that individual postmitotic neurons of the EM lineage have a cell-autonomous requirement for the *ems* gene in order to form correct projections in larval brain development.
Are features of ems function in brain development general?

Our analysis of ems function in the EM lineage demonstrates that homeobox transcription factors can influence adult brain architecture in a cell-autonomous and lineage-specific way. A lineage-specific, cell-autonomous requirement of other transcription factors in brain development has been shown for the olfactory projection neurons and for mushroom body neurons in Drosophila (Callaerts et al., 2001; Komiyama et al., 2003; Kurusu et al., 2000; Martini and Davis, 2005; Martini et al., 2000; Noveen et al., 2000). Thus, increasing evidence indicates that key developmental control genes, which operate early in embryogenesis, also act later in a lineage-specific manner in controlling anatomical features of the adult Drosophila brain. It may be a general feature of brain development that developmental control genes implicated in early neurogenesis and patterning are re-expressed and have different roles in later embryogenesis and postembryonic brain development (Salie et al., 2005; Zapala et al., 2005).

A comparison of the role of ems in Drosophila brain development with that of Emx1 and Emx2 in mammalian brain development is interesting, especially when the cortical phenotypes of Emx1/Emx2 double mutants are considered (Bishop et al., 2003; Shinozaki et al., 2002). The cortical surface area of Emx1/Emx2 double mutants is about half that of wild type, and the thickness of the preplate and cortical plate is reduced, suggesting that Emx genes regulate the numbers of cortical neurons. Moreover, Emx1/Emx2 double mutants have major defects in the pathfinding of most cortical axons, implying an important role for Emx genes in axonal pathfinding in the brain. Thus, mutant analyses in Drosophila and mouse suggest that loss of function of ems/Emx genes may result in comparable brain phenotypes, namely in reduction of neuronal cell number and in neurite projection defects. This in turn suggests that the morphological differentiation of brain architecture in both flies and mammals may involve conserved functions of orthologous ems/Emx homeobox genes, not only in the early embryo but also during later stages of brain development.

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References
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