Dissecting Drosophila embryonic brain development using photoactivated gene expression

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Abstract

The Drosophila brain is generated by a complex series of morphogenetic movements. To better understand brain development and to provide a guide for experimental manipulation of brain progenitors, we created a fate map using photoactivated gene expression to mark cells originating within specific mitotic domains and time-lapse microscopy to dynamically monitor their progeny. We show that mitotic domains 1, 5, and 9 give rise to discrete cell populations within specific regions of the brain. Two novel observations were that the antennal sensory system, composed of four disparate cell clusters, arose from mitotic domain 5 and that mitotic domain B produced glial cells, while neurons were produced from mitotic domains 1, 5, and 9. Time-lapse analysis of marked cells showed complex mitotic and migratory patterns for cells derived from these mitotic domains. Photoactivated gene expression was also used either to kill, to induce ectopic divisions, or to alter cell fate. This revealed that deficits were not repopulated, while ectopic cells were removed and extra glia were tolerated.

Keywords: Drosophila; Embryo; Brain; Mitotic domains; Glia; Pattern repair

Introduction

Fate maps serve as critical tools for developmental biologists to chart tissue morphogenesis and as guides for experimental manipulation. Detailed fate maps have been generated for many organisms. The most widely used fate map is that of Caenorhabditis elegans, which has been used to follow the mitotic and migratory behavior of all cells in this simple organism (Sulston et al., 1983). This fate map has allowed researchers to ask detailed questions about cell–cell interactions by providing a guide to ablate specific cells and determine the consequences. Combining fate mapping and perturbation studies with mutational and gene expression analysis has made C. elegans an excellent model system for studying development (McGhee, 1995). The application of similar methods to larger organisms that undergo extensive regulative development is desirable. The ideal fate map should contain information about cell movements, mitotic patterns, morphology, cell–cell contacts, and cell death as well as specific patterns of gene expression and the consequence of altered gene expression and cellular interactions.

Many fate maps of Drosophila melanogaster embryogenesis have been generated. However, for a variety of reasons, none have been used as broadly as the C. elegans fate map. The key differences between the C. elegans and Drosophila fate maps is the developmental stage onto which the fates are mapped and the coordinate systems. The C. elegans fate map starts with the fertilized egg and charts cell trajectory based on lineage and relative position in the embryo. This is possible because of the relatively small number of cells that need to be tracked. Upon fertilization, Drosophila embryos undergo 13 rounds of rapid syncytial nuclear division, where each nucleus is totipotent; there are no lineage-restricted fates at this stage, aside from the pole cells (St. Johnston, 1993). This initial period culminates in the formation of a cellular blastoderm composed of 5000-6000 cells (Foe et al., 1993). Embryonic fate maps generally start at this developmental stage. The only physical land-
marks at this stage are the anterior–posterior and dorsoventral axes. To fate map the embryo, a Cartesian coordinate system relative to percent position along these axes was used to mark the initial position of cells in the blastoderm (Hartenstein et al., 1985; Jürgens et al., 1986). Mapping was originally done by ablation (Underwood et al., 1980; Jürgens, 1986) and more recently by dye marking of cells (Technau and Campos-Ortega, 1985; Bossing and Technau, 1994). Ablation studies required the removal of rather large numbers of cells since embryos were able to compensate for small losses of cells (Underwood et al., 1980). The dye marking approaches have been very successful, but are limited in that they do not provide a means to alter the behavior of the marked cells.

Alternative fate mapping methods have been developed: these include gynandromorph analysis (Gehring et al., 1976) and the generation of mitotic clones (Janning, 1972). The latter has become the dominant method for producing marked clones of cells. This method provides a means to produce genetically perturbed clones of cells, but there is little control over the location of the clones.

The Cartesian coordinate method for subdividing the embryo is too coarse. A more reliable and precise coordinate system is required. The first sign of blastoderm specification is the appearance of mitotic domains, which are bilaterally symmetric groups of cells that divide in a stereotypic sequence (Foe, 1989). Mitotic domains were proposed to be indicators of cell fate (Foe, 1989; Minden et al., 1989). We have shown that mitotic domains are indeed fate domains. Cells within a mitotic domain are restricted to a limited set of fates that are distinct from the sets of cellular fates observed in neighboring mitotic domains (Cambridge et al., 1997; Namba and Minden, 1997). Thus, mitotic domains serve as a reliable coordinate system for precisely fate mapping the Drosophila embryo.

To enable the marking of cells in a spatially and temporally restricted manner, and alter their behavior, we developed a method for activating gene expression using a microbeam of light (Cambridge et al., 1997). This method, which is referred to as photoactivated gene expression, is based on the GAL4-expression method (Brand and Perrimon, 1993). Instead of supplying GAL4 genetically, chemically “caged” GAL4VP16 is injected into syncytial stage embryos that carry a UAS-transgene. Expression of the UAS-transgene is activated by briefly irradiating the cell, or cells, of choice with a long-wavelength UV microbeam, thus uncaging the GAL4VP16 protein. This method has been used to activate the expression of benign markers, such as LacZ and GFP, and to alter cell behavior with agents such as Cyclin E, Reaper (Rpr), and Head involution defective (Hid). Time-lapse microscopy and whole-mount embryo preparations are used to track the normal and altered behavior of marked cells.

This report focuses on the origin of the embryonic brain. There are very few reports on embryonic brain development because of its complex morphogenesis, which makes it a very difficult region of the embryo to study. Thus, a complete and accurate fate map of the embryonic brain relative to the blastoderm embryo still does not exist. We show that the brain is derived from five separate mitotic domains, each of which undergo distinct morphogenetic behaviors to generate discrete, nonoverlapping regions of the brain. Several different mechanisms are used to internalize blastoderm cells. Many of these fate assignments have not been previously documented, and in some cases, previously reported fates were incorrectly assigned.

In addition to benignly marking cells, cells were induced to undergo additional mitoses, change fate, or die. We report here that the brain has the capacity to efficiently eliminate ectopic cells, but the differentiation of affected regions is delayed. Ablated regions are not repaired by compensatory mitoses. Our results suggest that, because of its mitotic domain and regionalized development, the Drosophila brain will be a good model system in which to study brain development. Photoactivated gene expression of mitotic domains provides a precise, direct marking method to monitor and perturb cells in living embryos.

Materials and methods

Fly stocks

The following fly stocks were used (the chromosomal location of the UAS transgenes are shown in brackets): UAS-lacZ [1] (N. Perrimon), UAS-ricinA/CyO[2] (A. Brand), UAS-tauGFP [2] (A. Brand), UAS-rpr, UAS-hid/TM3 [3] (J. Nambu), UAS-nGFP = UGPnls14 [2], UAS-cycE [2], UAS-cycE [3], and UAS-gcm [2] were obtained from the Bloomington Stock Center. UAS-nGFP [2]; UAS-cycE [3], and UAS-lacZ [1]; UAS-cycE [2] were created from the above stocks.

Embryo preparation

Embryos were collected and prepared for photoactivation as previously described (Minden et al., 2000). The embryos were oriented with watchmaker’s forceps so that the mitotic domain to be photoactivated was flush with the coverslip. Embryos were staged by morphology according to Campos-Ortega and Hartenstein (1985).

Photoactivated gene expression

GAL4-VP16 was purified and caged with nitroveratryl chloroformate as previously described (Cambridge et al., 1997; Minden et al., 2000). The optimal concentration of caged GAL4-VP16 (115 μg/ml) was determined empirically by titration and injection into UAS-nGFP embryos followed by photoactivation. Photoactivation was performed on an inverted microscope (IX70, Olympus) through a 60X objective lens. When the desired mitotic
domain appeared (as a patch of enlarged cells in an appropriate location), they were irradiated with a beam of 365 nm light, using a DAPI bandpass filter. The beam diameter was determined by inserting a range of differently sized pinholes in the conjugate image plane on one side of a dual-beam, epi-fluorescence illuminator. Following photoactivation, the embryos were either imaged by time-lapse, fluorescence microscopy (see below) or incubated at 18°C until the desired stage and then fixed for immunohistochemistry. Irradiating a patch of 2-4 or 5-8 cells for 5 s was sufficient to produce a strong GFP signal limited to the irradiated cells in 50-60% of UAS-nGFP embryos. Single cell photoactivation required a 10- to 12-s irradiation pulse and resulted in a lower success rate.

**Antibodies**

The following primary antibodies were used in combination with fluorescent- or biotin-conjugated secondary antibodies: rat anti-ELAV (1:2000; Developmental Studies Hybridoma Bank, IA), mouse anti-GFP (1:500; Sigma), rabbit anti-phospho-Histone H3 (1:2500; Upstate biotechnology, NY), mouse anti-β-galactosidase (1:1500; Sigma), rabbit anti-Repo (1:500; G. Technau), mouse anti-FasII (1:20; C. Goodman). Secondary antibodies used were as follows: biotinylated rat anti-mouse pre-absorbed (1:1500; Vector Laboratories), biotinylated mouse anti-rat pre-absorbed (1:1500; Vector Laboratories), fluoresceinated rat anti-mouse preabsorbed (1:100, Vector Laboratories). Cy5-conjugated Streptavidin (1:600, Jackson ImmuNoResearch Laboratories) was used in conjunction with biotinylated labeled secondary antibodies for fluorescence visualization.

**Immunohistochemistry**

Fixation, devitellinization, and immunohistochemistry of photoactivated embryos were done as previously described (Namba and Minden, 1999; Minden et al., 2000). Histochemical detection of biotinylated secondary antibodies was performed with the Vectastain Elite ABC Kit (Vector Laboratories) as per instructions, and embryos were mounted in 1:2 Canada balsam:methyl salicylate. Fluorescent antibody-stained embryos were mounted in Vectashield (Vector Laboratories).

**Microscopy**

A Delta Vision microscope system with an Olympus IX 70 inverted microscope and softWoRx software (Applied Precision, WA) was used to examine fixed embryos and for three-dimensional, time-lapse, fluorescence microscopy. For time-lapse microscopy, 5-7 optical sections separated by 5 or 6 μm were imaged at 6- to 20-min intervals for up to 15 h. Similar amounts of cell death have been seen in fixed and stained embryos as in time-lapse recorded embryos, indicating that the recording process did not cause cell death (Pazdera, J.M. K.R., and J.S.M., unpublished results). The DeltaVision softWoRx software was used for

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Fig. 1. Fate mapping of 61, 65 and 89 cells. For all figures, the embryo anterior is to the left. Dorsally mounted embryos are indicated by a horizontal arrow pointing to the anterior, marked A. Laterally mounted embryos are indicated by a vertical arrow pointing dorsally, marked D. The stage of the embryo is indicated in the bottom left corner of each panel. (A) Schematic representation of the head mitotic domains at stage 7, which was used to guide photoactivation experiments. (B and C) Cartoon of the regions within the embryonic brain that are populated by each mitotic domain. Each line represents results from an individual embryo (61-red n = 31, 65-green n = 21, 89-blue n = 31). The remaining panels show micrographs of photoactivated embryos. The affected brain hemisphere is bounded by a solid line. (D–F) Photoactivation of 61. (D) Two to four cell photoactivation of a UAS-nGFP embryo stained with anti-GFP antibody (green) and anti-ELAV (red). The 61-derived cells are visible in both the brain (solid arrow) and the clypeolabrum (CL). (E and F) Two to four cell photoactivation of UAS-tauGFP embryos. The arrowheads indicate the pioneer axons of the embryonic peduncle. (G–K) Photoactivation of 65. All images are of five to eight cell photoactivations. (G) A photoactivated UAS-lacZ embryo showing the four different δ5 structures: the posterior group within the brain (solid arrow), the middle group just anterior to the brain (open arrow), the anterior group (open arrowhead), and the epithelial group (bracketed). The axon connecting the posterior and middle groups is indicated by the closed arrowhead. (H and I) A UAS-lacZ embryo stained with antibodies against β-galactosidase (green) and FasII (red). (H) Composite image of three adjacent optical sections showing FasII-positive: optic lobe (outlined with dashed line), Bolwig’s organ (asterisk), and Bolwig’s nerve (yellow arrowhead). GFP-expressing, δ5 brain cells are in a different focal plane (solid arrow) that is adjacent to optic lobe. The δ5 anterior group (open arrowhead) is adjacent to Bolwig’s organ. (I) An in-focus optical section of the GFP-positive δ5 cells within the brain (solid arrow). (J) A δ5 photoactivated UAS-nGFP embryo stained with anti-GFP (green) and anti-ELAV (red; using the same arrow scheme as G). (K) Composite of projected images of a δ5-photoactivated, UAS-tauGFP embryo (using the same arrow scheme as G). The bifurcated axon tract projecting to the maxillary complex is indicated by a notched arrow. (L–P) Photoactivation of 89. (L) Two to four cell photoactivation of a UAS-lacZ embryo stained with anti-β-galactosidase (green) and anti-ELAV (red). The closed arrowhead indicates an axon extending toward the ventral nerve cord. (M) Single cell photoactivation of a UAS-lacZ embryo histochemically stained with anti-β-galactosidase. The closed arrowhead indicates an axon extending to contralateral brain hemisphere. (N) Two to four cell photoactivation of a UAS-lacZ embryo stained with anti-β-galactosidase (green) and anti-Repo (red). The arrow indicates the patch of β-galactosidase-positive cells that were not expressing Repo. (O) Single cell photoactivation of 89 in a UAS-lacZ embryo histochemically stained with anti-β-galactosidase showing marked epidermal (closed arrowhead) and brain cells (closed arrow). (P) Five to eight cell photoactivation of 89 in a UAS-lacZ embryo histochemically stained with anti-β-galactosidase showing marked migratory cells (arrow) throughout the entire embryo. Yolk auto-fluorescence which appears in the green fluorescence channel is masked gray in (D, J, L, and N).

Fig. 2. Time-lapse images of an UAS-nGFP embryo following photoactivation. (Column A) A series of images from a time-lapse recording of a δ5 photoactivated embryo. The GFP fluorescence is shown in negative so that marked cells appear black overlaying transmitted light images. Lateral view of stages 9-16 as a projection of seven 5-μm optical sections. (Column B) Diagrammatic representation of the position of the progeny from mitotic domains 1, 5, and 9 from stage 9 to 16 shown as a lateral view. This series was constructed from multiple time-lapse experiments (61-red, 65-green, 89-blue, the brain is outlined in black).
image analysis and the projection of optical sections. Brain volume was determined by summing the brain area seen in sequential 5-μm optical sections of anti-ELAV-stained embryos. The volume was computed in μm³. The absolute brain volume was quite variable due to developmental stage differences and fixation effects. Therefore, we calculated the percent difference between the ablated hemisphere and the untreated hemisphere.

Results

Procephalic blastoderm fate map

The procephalic region of the embryo is made up of 13 mitotic domains (individual mitotic domains will be abbreviated as δN). To date, we have reported on the fate mapping of 7 procephalic mitotic domains (for δ2, δ8, δ10, δ15, see Cambridge et al., 1997; for δ3, δ18, δ20, see Namba and Minden, 1999). All of these mitotic domains produced non-overlapping sets of distinctly fated cells. These observations...
led to the conclusion that mitotic domains represent fate domains. Only one of these mitotic domains, δ20, gave rise to brain tissue, the optic lobe. Here, we report that mitotic domains 1, 5, 9, and B form the embryonic brain. We were interested in determining the morphogenetic movements of brain-forming cells. How are these cells internalized? Do they form discrete brain regions? Do they differentiate into neurons and glia? What other cell types are generated by these mitotic domains? To map the fates of cells within the brain-forming cells. How are these cells internalized? Do they differentiate into neurons and glia? What other cell types are generated by these mitotic domains? To map the fates of cells within the brain-forming cells. How are these cells internalized? Do they differentiate into neurons and glia? What other cell types are generated by these mitotic domains? To map the fates of cells within the brain-forming cells. How are these cells internalized? Do they differentiate into neurons and glia? What other cell types are generated by these mitotic domains?

Brain-forming mitotic domains populate distinct brain regions

Mitotic domains 1, 5, 9, and B occupy a large area that roughly corresponds to the procephalic neuroectoderm (Fig. 1A). The strategy for mapping how these mitotic domains contribute to the brain was to photoactivate patches of 2-4 or 5-8 cells within a chosen mitotic domain in UAS-lacZ or UAS-nGFP embryos. Photoactivated embryos were aged to stages 14-16 and immunostained for the expression of the UAS-transgene product. Mitotic domains 1, 5 and 9 generated cells that occupied discrete regions of the brain, suggesting that they may be neurons rather than glial cells which are scattered (Hartenstein et al., 1998). Mitotic domain B produced a dispersed population of cells that will be discussed later. A compendium of many mapping experiments was prepared (Fig. 1B and C; Fig. 2B). Each colored line in Fig. 1B and C (δ1 n = 31, δ5 n = 21, δ9 n = 31) outlines the region of marked cells observed in a single embryo mapped onto a dorsal or lateral view of the embryonic brain. These data show that all 3 mitotic domains give rise to 3 distinct, nonoverlapping regions of the embryonic brain, demonstrating their early regional specification. The slight overlap between cells derived from δ1 and δ5 that is seen in the cartoons is because the boundary between δ1- and δ5-derived brain cells is not orthogonal to the views being displayed, thus neither view is able to completely resolve the 2 domains. As will be discussed later, axons emanating from these mitotic domains follow very different paths, also indicating their distinct character. These results further support earlier findings that mitotic domain boundaries are cell fate boundaries (Cambridge et al., 1997; Namba and Minden, 1999).

Time-lapse recording of photoactivated UAS-nGFP embryos revealed the complex morphogenetic movements made by each of these mitotic domains to form part of the brain (Fig. 2; δ1 n = 4, δ5 n = 11, δ9 n = 25). The schematic shown in Fig. 2B starts at stage 9 when GFP fluorescence is clearly visible, 60-90 min following photoactivation. A significant amount of cell movement takes place in the head between stage 7, when cells were photoactivated, and stage 9, placing the cells from each mitotic domain some distance from the site of photoactivation (compare Fig. 1A and Fig. 2B, stage 9). The migration pattern is also distinct for each mitotic domain. Thus, the pattern and position of these mitotic domains bear little resemblance between stage 7 and stage 9 and beyond. The following sections will highlight unique features of these mitotic domains.

Mitotic domain 1 generates anterior protocerebrum neurons

Mitotic domain 1 is a large, two-lobed region. To determine whether there was any regional specification within δ1, patches of two to four cells were photoactivated in different locations. Photoactivation of δ1 generated clones of cells that were located in the clypeolabrum and the protocerebrum. Interestingly, there was a biased distribution of marked cells depending on the region photoactivated. Photoactivation of the anterior—ventral region of δ1 in UAS-nGFP embryos revealed that this region contributed mostly to the clypeolabrum (17/19 embryos; Fig. 1D, see the green fluorescence is clearly visible, 60-90 min following photoactivation. A significant amount of cell movement takes place in the head between stage 7, when cells were photoactivated, and stage 9, placing the cells from each mitotic domain some distance from the site of photoactivation (compare Fig. 1A and Fig. 2B, stage 9). The migration pattern is also distinct for each mitotic domain. Thus, the pattern and position of these mitotic domains bear little resemblance between stage 7 and stage 9 and beyond. The following sections will highlight unique features of these mitotic domains.

In the protocerebrum, marked δ1 cells populated two adjacent clusters of cells. δ1 photoactivation generated clones in one or both of these clusters. These cells colabeled with the pan-neuronal marker, ELAV (Sandhya et al., 1996) (19/19 embryos, 14-20 marked cells were generated per 2-4 cell photoactivation; Fig. 1D arrow). In contrast, very few δ1-derived cells expressed the glial cell marker, Repo (Hal-
The number of epidermal cells was not determined. In a later stage (Fig. 2A, frame 1, stage 9), the most anterior group of cells showed that these cells were internalized en masse rather than delaminating individually. The mass then moved posteriorly along the midline to their final position in the protocerebrum (Fig. 2B).

The location and double cluster appearance of δ1 neurons suggested that they may form the embryonic mushroom bodies. To further test this possibility, a 2- to 4-cell patch in the posterior–dorsal region of δ1 in UAS-tauGFP embryos was photoactivated. TauGFP binds to microtubules and highlights axons (Murray et al., 1998). TauGFP-marked neurons that had the typical morphology of the embryonic mushroom bodies were visible in 18/38 embryos (Tettamanti et al., 1997; Kurusu et al., 2002). They were large cells at the anterior tip of the protocerebrum arranged in two clusters that each projected pioneer axons toward the neuropil. These axons were fasciculated just inside the neuropil, where they made a turn toward the posterior of the neuropil, followed by a second, sharp medial turn (Fig. 1E and F, arrowhead) to form the embryonic peduncle. These observations support our hypothesis that δ1 gives rise to the embryonic mushroom bodies.

Mitotic domain 5 produces the embryonic antennal system

Mitotic domain 5, which is initially located just anterior to the cephalic furrow near the dorsal midline (Fig. 1A), produces 4 distinct cell populations that span the anterior third of the embryo (Figs. 1G, 2A, and 2B). Time-lapse analysis of photoactivated UAS-nGFP embryos, where a patch of 5-8 cells was irradiated, revealed the complex migration pattern of this mitotic domain (Fig. 2A). The photoactivated patch of cells first elongated along the edge of the cephalic furrow adjacent to the maxillary segment (Fig. 2A, frame 1, stage 9). The most anterior–ventral cells remained in the epidermis and moved to the anterior tip of the embryo. Head involution produced the most dramatic movements of δ5 cells. As head involution began, the non-epidermal δ5 progeny became internalized at the boundary between the mandibulary and maxillary segments and separated into two populations (Fig. 2A, frames 5-6, stages 13-14). The inward movement of these cells appeared to be via invagination. One population, the posterior group, which ultimately forms the antennal lobe of the brain, remained stationary at the midanterior region of the embryonic brain, while the second (middle) group migrated over the ventral surface of the developing brain (Fig. 2A, frames 6-7, stage 14-15). This was followed by a second separation of cells from the middle group, which migrated into the position of the antennal sensory organ (Fig. 2A, frame 7, stage 15). This culminated in populations of ~20 posterior group, ~5-6 middle group, and 2-4 anterior group cells; the number of epidermal cells was not determined.

Time-lapse recordings showed that the three internalized populations might be connected by axonal fibers. Immunohistochemical staining of δ5 photoactivated UAS-lacZ embryos confirmed the axonal nature of these connections (Fig. 1G, solid arrowhead). All four groups of cells arising from δ5 are shown in Fig. 1G. Not every photoactivated clone produced all four groups of marked cells. The anterior brain group was observed in all successfully photoactivated embryos (19/19 embryos); while the epidermal, middle, and anterior groups were seen with decreasing frequency, 90, 74, and 37%, respectively. These results argue that the four groups of cells were derived from the same progenitor population. The neuronal character of cells within these groups was revealed by counterstaining with anti-ELAV antibody; about half of the photoactivated cells within the anterior and posterior groups expressed ELAV (7 embryos examined, Fig. 1J, arrow, open arrowhead). Not all neurons express ELAV, such as the embryonic optic lobe (unpublished data) and possibly others (Robinow and White, 1991); however, the presence of an axon on these ELAV-negative brain cells confirm their neuronal phenotype (Fig. 1G and K, solid arrowhead). We further confirmed the neuronal nature of the δ5 derived brain cells, as well as those of the anterior group, by photoactivating a 5- to 8-cell patch in δ5 of UAS-tauGFP embryos. Tau-GFP highlighted the axons of the posterior group within the brain, the axon tracts between the groups and the structure of the most anterior group (Fig. 1K). The axons of the δ5-derived brain cells can also be seen extending into other parts of the brain in 34/37 embryos (Fig. 1K). Many of these processes appear to terminate in the region of the brain populated by δ1 cells, which we believe are mushroom body precursors (compare Fig. 1K and E, which correspond to δ5 and δ1, respectively). Only a small percent of δ5-derived cells stained with anti-Repo, thus δ5 primarily generates neurons, not glia.

This pattern of δ5 cell types was reminiscent of the cell types produced by δ20, which gives rise to the entire visual system (Namba and Minden, 1999). The morphogenetic movements of δ5 and δ20 were also similar; but not identical, δ20 cells form a more elongated pattern prior to internalization. Since there does not exist an early antennal marker, we confirmed that mitotic domains 5 and 20 yielded different structures by photoactivating a 5- to 8-cell patch of δ5 in UAS-tauGFP embryos. For all δ5-derived brain cells, as well as δ5-derived antennal cells (Holmes and Heilig, 1999). FasII was not expressed in any of the photoactivated δ5 cells (Fig. 1H and I), δ5-derived brain cells (Fig. 1H and L, closed arrow) were adjacent to the optic lobe (Fig. 1H and I, broken line); there was no overlap. Likewise, δ5 cells in the anterior group (Fig. 1H, open arrowhead) were adjacent to Bolwig’s organ, not overlapping (Fig. 1H, asterisk). Thus, δ5-derived cells do not contribute to any part of the visual system.

The morphology and position of the δ5-derived cells indicate that this mitotic domain gives rise to the antennal...
sensory system, where the anterior group corresponds to the antennal sensory organ and the posterior group, which is in the brain, corresponds to the antennal lobe. Of the 20 sensory organs and 7 sensory nerves in the embryonic Drosophila head, there are 3 predominant sensory organs located at the anterior tip of the embryo: the maxillary sense/terminal organ, the antennal sensory/dorsal organ, and Bolwig’s organ (Schmidt-Ott et al., 1994). The antennal nerve terminates in the brain, while the maxillary nerve terminates in the subesophageal ganglion, and Bolwig’s nerve terminates in the optic lobe (Schmidt-Ott et al., 1994). In addition, the antennal axon tract bifurcates, sending some of its axons ventrally to the maxillary sense organ; and some axons of the antennal lobe project toward the mushroom body via the inner antennocerebral tract (Python and Stocker, 2002). The axon tracts of cells derived from δ5 match the morphological characteristics of the antennal sensory system. The axon tract from the anterior group corresponds to the antennal axon tract that projects into the posterior group within the brain, which is the antennal lobe (Fig. 1G and K). Photoactivated δ5 cells in UAS-tauGFP embryos showed that the posterior group projected axons toward the mushroom body (Fig. 1K). Also, the UAS-tauGFP embryos displayed the characteristic branching of the δ5-derived axons, which extended ventrally toward the maxillary sense organ (Fig 1K, notched solid arrow). Together, these data indicate that δ5 gives rise to the larval antennal sensory system.

Mitotic domain 9 produces three apparently unrelated cell types

A unique feature of δ9 is that the entire cell population divides perpendicularly to the embryonic surface during the 14th mitosis, creating 2 populations of cells (Foe, 1989). It was predicted that δ9 would generate 2 populations, epider-
Table 1

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<th>Cell type(s)</th>
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*E indicates epithelial cells; B indicates brain cells; M indicates migratory cells. Different sized patches of 89 cells were photoactivated in UAS-lacz embryos just prior to, or during, the 14th mitosis. Three distinct cell types were derived from 89: posterior brain (Fig. 1L–N), dorsal midline epidermis (Fig. 1O, closed arrowhead), and an unidentified population of migratory cells (Fig. 1P).

Time-lapse analysis revealed that, initially, the brain and epidermal progenitors moved in unison anterior and dorsally, before separating, leaving the epidermal cells at the posterior brain (Foe, 1989). To ensure that both layers of progeny were marked, 89 cells were irradiated prior to, or during, the 14th mitosis. Three distinct cell types were derived from 89: posterior brain (Fig. 1L–N), dorsal midline epidermis (Fig. 1O, closed arrowhead), and an unidentified population of migratory cells (Fig. 1P).

To determine the lineage relationship of these three cell populations, different-sized patches of cells within 89 were photoactivated in UAS-lacz embryos (Table 1). Single cell photoactivation experiments yielded a large fraction (45%) of clones composed of brain and epidermal cells, which was expected given the oriented division. The next largest fraction was brain-only clones, indicating that some brain progenitors delaminated from the embryo surface without leaving an epidermal sister or that the epidermal sister progeny died. The remaining populations were composed of epidermal-only and migratory-only cells and clones of all three cell types.

Widening the beam to photoactivate two to four cells or five to eight cells increased the fraction of clones composed of all three cell types and decreased the fraction of epidermal-only and brain-only clones, but not migratory-only clones (Table 1). The fraction of migratory-only clones peaked at 21% for two to four photoactivation experiments. The fraction of clones composed of epithelial and brain cells decreased to a constant 13%; the fraction of brain and migratory cell clones plateaued at 28%.

Time-lapse recordings showed that all three cell types experienced significant amounts of cell death, making it extremely difficult to draw firm conclusions about lineage relationships. The origin of the migratory cells is not clear. None of the clones was composed of both epithelial and migratory cells, indicating that epithelial cells do not give rise to migratory cells directly. Thus, the migratory cells are either derived from brain progenitors or delaminated directly from the blastoderm. A significant fraction of embryos had marked migratory-only clones, particularly with two to four cell photoactivation, supporting the delamination hypothesis. The brain- and migratory-cell progenitors appear to be evenly distributed across 89. Closer examination of the origin and characterization of the 89 migratory cells will be reported elsewhere.

Fig. 5. Effect of ectopic cell division on the brain. Small and large regions of the prosophageal neurectoderm of UAS-nGFP; UAS-cycE embryos were photoactivated and later stained with anti-GFP (green) and anti-ELAV (red) antibodies. The experimental hemispheres are shown by the solid lines and the control hemisphere are indicated by the broken lines. (A) A stage 14 embryo after photoactivation of a 5-8 cell patch in 81. Notice the ectopic cells outside of the brain (solid arrow), as well as cells in the brain (solid arrowhead). (B) A stage 16 embryo photoactivated in 81 with a 5-8 cell patch showing double labeled cells (solid arrowhead) in the brain. Notice the reduction of GFP-positive cells outside of the brain. (C) A stage 14 embryo after photoactivation of a 5-8 cell patch in 89. Notice the cap of ectopic cells anterior to the affected hemisphere (solid arrow); the tip of control hemisphere is indicated by the open arrow. (D) A stage 16 embryo photoactivated in 89 (5-8 cell patch) showing marked cells in the correct brain location (solid arrow). (E and F) Photoactivation of large, 8-16 cell patches in mitotic domains 1, 5, and 9. (E) Shows an overlay of 2 optical sections highlighting the control and experimental hemispheres from a stage 16 embryo. Notice that very few of the GFP-positive cells in the photoactivated hemisphere also express ELAV; while most of the cells in the control hemisphere express ELAV. (F) A stage 17+ embryo showing coexpression of GFP and ELAV.
dorsal midline (Fig. 2B, stage 12, blue hatching), while the brain progenitors continue to move posteriorly to their final location in the brain (Fig. 2B). All photoactivated 89 brain cells expressed ELAV (24/24 embryos; Fig. 1L); none expressed Repo (18/18 embryos, Fig. 1N), indicating 89-derived brain cells are neurons, not glia. These neurons occupied the deutero-, proto-, and tritocerebrum (Fig. 1C and L); thus, the formation of the three cerebral structures does not appear to be specified by separate mitotic domains. In many embryos (16/56), an axon could be seen to project either through the tritocerebrum toward the ventral nerve cord (Fig. 1L, closed arrowhead) or toward the contralateral hemisphere through the tritocerebral commissure (Fig. 1M, closed arrowhead). These structures are similar to those described by Therianos et al. (1994).

Mitotic domain B generates brain glia

Progeny of mitotic domains 1, 5, and 9 populated almost all of the brain volume (Fig. 1B and C). None of these mitotic domains generated significant numbers of glial cells. Photoactivation of the remaining mitotic domain, 8B, revealed a major source of brain glia. Photoactivation of two to four cells in three locations along the length of this elongated mitotic domain (Fig. 1A) in UAS-nGFP embryos revealed that their progeny formed small clusters of cells in the presumptive protocerebrum at stage 14 (Fig. 3A, solid arrow). The distribution of these clusters in the stage 14 embryonic brain is diagrammed in Fig. 3E and F (n = 41). These clusters were located deep within the brain and were variable in size. Each of the clusters of marked 8B cells was surrounded by dispersed cells (Fig. 3A and cartooned as dots in Fig. 3E and F). Three-quarters (38/52) of 8B photoactivated embryos had marked, dispersed brain cells that also expressed Repo, indicating that they were glial cells (Fig. 3A–C). None of the embryos had any photoactivated cells, either within or outside of the cluster, that expressed ELAV (8/8; Fig. 3D), indicating that they are unlikely to be neurons. There are two classes of embryonic brain glia: the subperineurial glia that are mostly located in the brain periphery and the neuropil glia (Hartenstein et al., 1998). Glial cells arising from 8B were identified as subperineurial glia by their position. Neurupil glia were never observed, suggesting that this subtype of glial cells may arise from a different source.

Light-dependent ablation of small patches of brain progenitor cells

In addition to providing a means for direct fate mapping of selected cells, photoactivated gene expression allows one to specifically perturb cellular behavior in selected cells. Classically, cell ablation was used to determine cell fate by documenting the ensuing anatomical defects. An important issue raised by early ablation studies in Drosophila was the embryo’s ability to repair cellular defects. We wished to examine this in brain progenitors. Two schemes were used to kill cells: by inducing apoptosis by photoactivation of UAS-rpr, UAS-hid simultaneously (Lee and Baehrecke, 2000) or by apoptosis-independent cell killing by photoactivation of UAS-rin A (Hidalgo et al., 1995).

Patches of five to eight cells in δ1 or 89 of UAS-rin A or UAS-rpr, UAS-hid embryos were photoactivated and the volume and morphology of the embryonic brain was examined by ELAV expression at stages 14-16. To measure the extent of ablation, the volume of the photoactivated brain hemisphere was compared with the volume of the untreated brain hemisphere. The percent volume difference between brain hemispheres was calculated. In untreated embryos, there was a 2.7 ± 1.9% (n = 20) variation between the two brain hemispheres within the same embryo. Ablating five-to eight-cell patches within δ1 or 89 of UAS-rin A or UAS-rpr, UAS-hid embryos yielded very similar results in all four cases. The average reduction in brain volume was 20.4 ± 7.5% (n = 47). The reduction in brain volume was similar to the volume occupied by benignly marked cells in similarly photoactivated UAS-nGFP embryos. The location of the lost brain tissue did not correlate precisely with expectations based on our mitotic domain fate map (compare Fig. 1B and C with Fig. 4A–C). We suspect that the brain deformed to accommodate for the lost tissue. Therefore, one cannot use the location of gross morphological defects as a guide for fate mapping of the brain. More importantly, since there was a measurable decrease in brain volume, these data indicate that the embryos do not compensate for loss of tissue either by increased proliferation or by recruiting cells from other mitotic domains.

Ectopic brain cells are efficiently removed

We have previously shown that Drosophila embryos have the capacity to remove extra cells by apoptosis (Namba et al., 1997; Li et al., 1999). However, these studies dealt with rather large regions of extra cells. To examine whether small clones that experience an extra round of division are also repaired, we induced the expression of Cyclin E (Knoblich et al., 1994) in five to eight cell patches in δ1 or 89 of UAS-cycE embryos by photoactivation. These embryos also carried a UAS-nGFP transgene to mark the location of the photoactivated cells. Photoactivated embryos were fixed at stages 14-16 and immunostained for GFP and ELAV expression.

Mitotic domains 1 and 9 were able to remove ectopic cells, but appeared to have different capacities for finishing the repair process. In addition to marked cells in the expected brain location for both mitotic domains, numerous GFP-positive cells were seen outside of the brain (20/28 δ1-photoactivated embryos and 7/7 89-photoactivated embryos) at stage 14 (Fig. 5A and C, solid arrow). It is likely that the additional cells observed outside of the brain are brain progenitor cells that were never able to incorporate into the brain proper. None of these non-brain cells ex-
pressed ELAV; these cells were never observed in control UAS-nGFP-only embryos. By stage 16, most of the non-brain cells were absent and presumably dead. For δ1 photoactivated UAS-cycE embryos at stage 16, the number of marked brain cells decreased to usual numbers and most expressed ELAV (n = 15), indicating an almost complete repair (Fig. 5B). By stage 16, δ9-photoactivated, UAS-cycE embryos had largely removed the non-brain, ectopic cells (8/13 embryos, Fig. 5D) and the number of marked brain cells had been reduced to the expected number. But, there were often mislocalized marked cells (data not shown) and few expressed ELAV (Fig. 5D), indicating failed or delayed differentiation.

Large numbers of ectopic brain cells are removed, but survivors differentiate poorly

To determine the effect of inducing a large number of ectopic brain cells, 8-16 cell-sized patches in mitotic domains 1, 5, and 9 were photoactivated together in UAS-nGFP; UAS-cycE embryos, which were then analyzed at stages 16 and 17 for excess cells and neuronal differentiation. Surprisingly, at stage 16, the embryonic brain hemisphere on the affected side did not appear to be significantly larger than the control hemisphere; however, no ELAV expression was detected (18 embryos examined; Fig. 5E solid line). Staining similarly photoactivated UAS-nGFP; UAS-cycE embryos with anti-phosphohistone H3 (a marker for mitotic nuclei; Wei et al., 1999) revealed that the cycE transgene was no longer active and that the normal cell cycle had resumed (18/18 embryos, data not shown). In 2/7 stage 17 embryos, about one-third of the marked cells expressed ELAV (Fig. 5F, solid line); the remaining embryos failed to express ELAV in the ectopic cells. These results indicate that embryos are capable of removing large numbers of ectopic brain progenitors, but the surviving cells are delayed in differentiation. We previously showed that an extra round of mitosis caused by global overexpression of Cyc E resulted in a severe developmental delay (Li et al., 1999).

Induction of ectopic glial cells is tolerated in the embryonic brain

Ectopic brain progenitors are efficiently removed. Is the brain capable of regulating aberrant fate decisions? To address this question, normally neurogenic δ1 cells were induced to become glia by ectopic gcm expression (Hosoya et al., 1995; Jones et al., 1995; Giesen et al., 1997, Akiyama-Oda et al., 1998). UAS-gcm embryos were photoactivated in a 5-8 cell-sized patch, aged to stages 9-16 then stained with anti-Repo antibody. Fifty-three percent (20/38) of embryos analyzed at stages 9-11 showed premature, Repo-expression in the photoactivated cells (Fig. 6A), which was in keeping with the average photoactivation success rate. At stages 13-16, 47% (10/21) of the photoactivated embryos had a greater than 20% increase in the number of glial cells in the photoactivated hemisphere relative to the untreated hemisphere within the same embryo. An increase in glial cells of >20% was considered a successful photoactivation. Of the successfully photoactivated embryos, the average increase in the number of glia was 43%. Glial cells were counted over 6 × 4-μm optical sections in both the photoactivated (Fig. 6B) and the untreated hemispheres (Fig. 6C). The ectopic glial cells did not accumulate in the anterior protocerebrum, which is the normal destination of δ1 cells, but rather they were dispersed throughout the embryonic brain in a pattern similar to that of normal glial cell distribution (Fig. 6B). This indicates that the brain accommodates ectopic glia and does not repair aberrant neuronal-to-glia fate decisions up to stage 16. We are not certain of the postembryonic fate of the ectopic glia.

Discussion

The ideal fate map should contain information about the behavior of cells during normal development, the specific genes they express, and how they and their neighbors respond to altered behavior. The photoactivated gene expression method allows one to both mark cells to observe their normal behavior and to express fate-altering genes in real time. This approach to fate mapping and manipulation will provide new opportunities for dissecting embryonic development at the molecular/cellular level.

Five mitotic domains constitute the embryonic brain

The most difficult aspect of fate mapping the head region of the Drosophila embryo is its complex morphogenesis. We have fate mapped the majority of mitotic domains within the Drosophila procephalic blastoderm by using the photoactivated gene expression system and determined that the embryonic brain develops from five mitotic domains: the posterior–dorsal part of δ1 and δ5, δ9, δB (were shown...
in this study), and δ20 (Namba and Minden, 1999). The final position of the mitotic domain progeny within the brain does not reflect their relative blastoderm positions. Thus, the mitotic domains follow specific morphogenetic trajectories. Several different mechanisms are employed to internalize brain progenitors: the posterior–dorsal part of δ1 and δB invaginate en mass, δ5 and δ20 likely invaginate together, and δ9 uses oriented mitosis and possibly delamination. Together, these mitotic domains constitute nonoverlapping regions of the brain. This fate map will provide an avenue for performing region-specific experiments. The discrete behavior of the brain-forming mitotic domains raises several interesting questions about the ancestral origin of the brain. One such question is, did the various brain compartments evolve from a common group of cells and later specialize or did the compartments evolve independently and later coalesce to form the brain?

How does the mitotic domain-based fate map compare to previous studies? Younossi-Hartenstein et al. (1996) reported that expression of the neurogenic gene; lethal of scute (lsc) correlates with the location of mitotic domains, δ5, δ9, and the posterior-dorsal part of δ1. They also described a region of the blastoderm that invaginated en mass and gave rise to neurons and glia; this corresponds to the posterior–dorsal part of δ1 and δB. Noveen et al. (2000) suggested that the mushroom body neuroblasts arise from δB. However, lsc is only transiently expressed in δB (Younossi-Hartenstein et al., 1996), and our photoactivation-based fate mapping clearly shows that δB produces glia, not neurons. δ1 produces neurons that populate the region of the brain that forms the mushroom body (Tettemanti et al., 1997; Noveen et al., 2000; Kurusu et al., 2002). Finally, the anatomical classification of the deuterot-, proto-, and tritocerbrum does not correlate with separate mitotic domains; δ9 spans these three regions. The mitotic domain fate map correlates well with previous studies and provides a new level of precision and experimental options.

**Morphogenetic movements of mitotic domains 1, 5, 9, and B**

There is a tremendous amount of cell and tissue movement in the head region of the developing embryo. These movements are very difficult to describe without a context. Mitotic domains provide such a context by dividing the embryo into discrete regions with distinct boundaries. The following is a synopsis of the morphogenesis of mitotic domains 1, 5, 9, and B. Mitotic domain 1 first appears as a two-lobed structure with the posterior–dorsal lobe invaginating to form the anterior–medial part of the protocerebrum. The anterior–ventral portion of δ1 remained on the embryos surface forming the epithelial surface of the clypeolabrum. Thus, there is a margin within δ1 that forms a furrow boundary between the anterior–ventral and posterior–dorsal regions. The opposite side of the furrow is derived from δ9. The zippering up of this furrow joins δ1 to δ9, which causes the internalization of the posterior–dorsal portion of δ1 and δB. This, coupled to the posterior elongation of δ3 and δ18, draws δ9 dorsally and pulls δ5 and δ20 ventrally and anteriorly, creating a vortex centered on δ9. The initial division of δ9 is perpendicular to the embryo surface. Later δ9 divisions may also be perpendicular to the surface or cells may delaminate to generate a population of migratory cells. As δ5 and δ20 move ventrally along the cephalic furrow, a large portion of each mitotic domain forms a placode that is involuted to form the antennal and visual systems, respectively.

**The antennal sensory and visual systems are derived from adjacent mitotic domains**

There are three main sensory organs at the anterior tip of the embryonic brain: the antennal sensory/dorsal organ, Bolwig’s organ, and the maxillary sense/terminal organ, each of which generates an axonal tract that terminate in the brain (Schmidt-Ott et al., 1994). We have previously shown that δ20 gives rise to Bolwig’s organ and the maxillary sense/terminal organ (Namba and Minden, 1999; unpublished data). The morphology and position of the anterior δ5-derived cells indicates that they are the antennal sensory organ. Moreover, its axonal tract follows the path of the antennal nerve. There are many parallels between mitotic domains 5 and 20; they follow similar morphogenetic pathways, they produce brain neurons, distally located sense organs, and specific epithelial contact sites. The middle group of cells are likely nonneuronal guidance cells associated with the antennal sensory system. It is yet to be determined whether the antennal system epithelia ultimately form imaginal disc tissue, as is the case for the visual system. The way in which these sensory systems develop ensures that the correct axonal connections are maintained from the very beginning of the structure’s development. These similarities suggest a common ancestral sense organ.

The development of the antennal sensory system and antennal lobe from δ5 largely correlates with the engrailed expression patterns of the antennal stripe, antennal spot, and head spot at all stages of development (Schmidt-Ott and Technau, 1992). The engrailed head and antennal spots correspond to the location of the antennal lobe and middle group from δ5, respectively. The antennal stripe correlates with migration patterns of δ5 throughout development, for example, at stage 9 the antennal stripe is ventral–lateral as are δ5-derived cells (Fig. 2, frame 1). Time-lapse analysis of photoactivated cells provided evidence for the dynamic connection between the antennal sense organ, the antennal lobe, as well as associated cells, which could not be inferred from fixed preparations.

**Glial cells are derived from mitotic domain B**

Neurons and glia in the embryonic brain did not arise from the same stem cells, but rather glial cells were found
to be almost exclusively associated with δB. This surprising result was in contrast to previous findings in the ventral nerve cord (Bossing and Technau, 1994; Bossing et al., 1996; Schmid et al., 1999; Freeman and Doe, 2001). However, this neuron/glia specification is more reminiscent of vertebrate brain development (Price and Thurlow, 1988; Walsh and Cepko, 1992; Reid et al., 1995). Our data taken together with recent fate maps in the chicken (Cobos et al., 2002) and data which shows remarkable similarities between the olfactory systems of Drosophila and mice (Marin et al., 2002; Wong et al., 2002) suggest that the development of the Drosophila embryonic brain may be more similar to the embryonic vertebrate brain than previously thought.

δB contributes subperineural glial cells to the embryonic brain, not neuropil glia. We also observed clusters of GFPPositive/Repo-negative cells. The ultimate fate of these marked cells was not determined. They may represent as yet, undifferentiated cells; Repo labels postmitotic cells (Hartenstein et al., 1998). The origin of all brain glia was not determined. We did not photoactivate all of δB and procephalic mitotic domains δ23 and δ24, have yet to be mapped. A cluster of neuropil glia progenitors found at the deutero–tritocerebral boundary was previously mapped to a ventral–lateral region by Repo expression at stage 11 (Hartenstein et al., 1998). It is not clear how these cells arrived at this position. Further photofacilitation studies are needed to completely map the origin of brain glia.

Pattern repair in the embryonic brain

Embryogenesis is dependent on the establishment and maintenance of morphology, which requires balancing cell proliferation and death. Embryos of several species have been shown to have a remarkable ability to repair patterning defects as a consequence of cell ablation (French et al., 1976; Bryant et al., 1981; King and Bryant, 1982), increased cell density (Li et al., 1999), or alterations of the anterior–posterior fate map (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988; Namba et al., 1997). However, all of these studies affected large areas of the embryo. We tested the ability of the embryo to repair defects that were induced during the development of specific regions of the brain. The most commonly used method for function mapping has been cell ablation. We wished to examine the correlation between ablation mapping and photoactivated gene expression fate mapping, and to determine whether ablated regions were repaired by repopulating cell divisions. Cell ablation of brain progenitors led to a reduction in brain size, but the precise location of the defect was obscured by the tissue deforming to accommodate the missing volume. It was clear that the deficit was not repaired at the cellular level. These results agree with our previous studies of mispatterned embryos where compressed regions of the fate map were not repaired by new cell growth (Namba et al., 1997). The functional consequences of localized ablation will require further investigation.

Ectopic cells were efficiently removed. It was interesting that many ectopic cells were found outside of the brain, something never observed in control embryos. Time-lapse analysis showed that these extra cells failed to be incorporated into the brain, indicating that the selection, or counting process occurred during cell migration, before incorporation into the final brain structure. This selection process was not dependent on differentiation, since differentiation at the level of ELAV expression was often delayed. This observation is contrary to a common belief that cell death is a finishing step and occurs at the end of a developmental process.

Another common notion is that inappropriately differentiated cells are removed by apoptosis. To test this point, we induced glial cell differentiation in neurogenic δ1. The embryo appeared to accommodate this fate switch and incorporate these cells into the brain, in spite of their being in excess. Perhaps the excess glia were not removed because they are normally associated with neurons. Why weren’t glia counted when neuronal precursors were? It will be worthwhile to see the effect of switching neurons to a more unusual cell type, such as muscle.

Embryos need to continuously monitor cell density and eliminate excess or malfunctioning cells. This is the purpose of pattern repair. By further studying pattern repair in different tissues, we will learn more about the specific limits of pattern repair and the types of defects that lead to malformation and disease.

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