Mosaic Analysis with a Repressible Cell Marker for Studies of Gene Function in Neuronal Morphogenesis

Neurotechnique

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Summary

We describe a genetic mosaic system in *Drosophila*, in which a dominant repressor of a cell marker is placed in *trans* to a mutant gene of interest. Mitotic recombination events between homologous chromosomes generate homozygous mutant cells, which are exclusively labeled due to loss of the repressor. Using this system, we are able to visualize axonal projections and dendritic elaboration in large neuroblast clones and single neuron clones with a membrane-targeted GFP marker. This new method allows for the study of gene functions in neuroblast proliferation, axon guidance, and dendritic elaboration in the complex central nervous system. As an example, we show that the *short stop* gene is required in mushroom body neurons for the extension and guidance of their axons.

Introduction

The functional organization of the nervous system depends on individual neurons sending out dendrites and axons and forming precise connections with other neurons. At times, dendrites can be highly elaborate and axons can take long and complex routes to reach their targets in the densely packed nervous tissue. The classical Golgi staining and more recent intracellular labeling methods (reviewed in Cowan, 1998) allow for the visualization of single isolated neurons in the nervous tissue. Thus, the dendritic morphology and axonal projection pattern can be clearly revealed. Indeed, it is mostly the Golgi method that allowed Ramon y Cajal to study the organization of nervous tissue systematically throughout the developing and adult stages of many different species (Cajal, 1911), thus laying much of the foundation for modern neuroscience.

Important extracellular cues and their receptors that guide axons to their targets have been recently identified (reviewed in Tessier-Lavigne and Goodman, 1996). Some of these were identified using genetic approaches in *C. elegans* and *Drosophila* (Hedgecock et al., 1990; Seeger et al., 1993). These genetic analyses rely on the premise that if a particular guidance molecule is removed from the organism by a mutation in the gene, an informative phenotype may arise. Relatively little is known about the intracellular signaling mechanisms through which many of these guidance cues exert their functions and lead to the reorganization of cytoskeleton in growth cones. One reason could be that the proteins involved in the intracellular signaling are also required for many other developmental processes (e.g., Luo et al., 1994). Thus, mutations in the genes encoding these proteins are likely to cause pleiotropic developmental defects that preclude their identification as being required for axon guidance.

Genetically mosaic organisms, in which clones of somatic tissues have different genotypes compared to the rest of the organism, have been used to analyze gene functions in many biological processes. Mosaic analysis is especially useful for studying genes with pleiotropic functions because at a desired time specific tissues mutant for the candidate gene can be made in an otherwise wild-type organism. Mosaic analysis has been widely used in *Drosophila* for studying the functions of known genes, while the more recent introduction of the high efficiency FLP/FRT system (Golic and Lindquist, 1989), together with means to effectively mark clones (Xu and Rubin, 1993), has allowed the use of mosaic systems to screen for new mutants.

In theory, one could use such mosaic analysis to create clones in the nervous system for the study of neuronal morphogenesis. However, existing systems for identifying mosaic clones in *Drosophila* negatively label mutant cells and are not suitable for revealing the morphology of mutant neurons. Here, we describe the "MARCM" system (for mosaic analysis with a repressible cell marker) whereby mutant cells are the only cells labeled. By engineering the marker to label the plasma membrane and therefore to be highly concentrated in neuronal processes, we can visualize single isolated neurons in the complex developing and adult *Drosophila* central nervous system with Golgi staining-like resolution. Moreover, these positively marked neurons can be made homozygous mutant for a particular gene of interest in an otherwise wild-type environment. Thus, this method allows the molecular and cellular analysis of gene function in the morphogenesis of CNS neurons. As an example, we show that the *short stop* gene is required in mushroom body neurons for the extension and guidance of their axons.

Results and Discussion

Strategy for Generating Mosaic Organisms in which Mutant Cells Are Exclusively Labeled

Figure 1A illustrates the scheme for marking mosaic clones using conventional FLP/FRT methods (Golic and Lindquist, 1989; Xu and Rubin, 1993). Cells that express the yeast FLP recombinase (FLP) may recombine their chromosomes between two FRT sequences present as transgenes at homologous chromosomal locations (FRT sites). If the parental cells are heterozygous for a recessive mutation x, located distal to the FRT site, 25% of the recombinant daughter cells, or somewhat more (Pimpinelli and Ripoll, 1986), will be homozygous for x (for simplicity of description, we refer to these cells as mutant cells hereafter, even if mutant x is hypothetical). The same number of cells will be homozygous wild type. To identify the mutant cells, a marker under the control

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A Parental cell | Mitotic recombination after DNA replication | Two distinct mosaic progeny
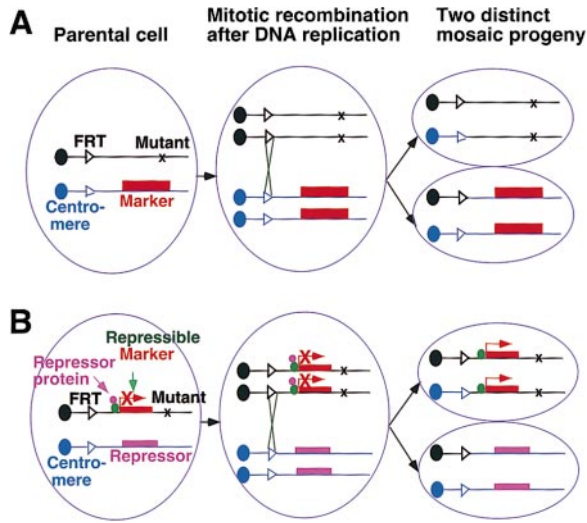

Figure 1. Different Designs for Marking Mutant Clones in a Mosaic Organism

(A) In conventional mosaic analysis, homozygous mutant cells are identified as unstained cells if the marker gene is placed distal to the FRT site on the homologous chromosome arm in *trans* to the mutant gene.

(B) In the MARCM system, a transgene encoding the repressor of marker gene expression is placed distal to the FRT site on the homologous chromosome arm from the mutant gene. Only in homozygous mutant cells can the marker gene be expressed because of the loss of the repressor transgene.

of the heat shock promoter is placed distal to the FRT site on the chromosome arm in *trans* to mutation *x*, such that mutant cells will be the only cells that are unstained with the marker.

Unfortunately, this marking systems is not suitable for the analysis of neuronal morphology because one needs to visualize the mutant cells. The ideal situation would be if the mutant cells were the only cells labeled by a histological marker. Our design to achieve this situation is to place the marker under the control of a repressible promoter and to introduce a transgene that allows the ubiquitous expression of the repressor. This repressor transgene is placed distal to the FRT site in *trans* to the mutant *x* chromosome arm. In heterozygous cells, marker expression is "off" due to the presence of the ubiquitously expressed repressor. If mitotic recombination between the two FRT sites creates daughter cells that are homozygous for mutant *x*, these same cells will also lack the repressor transgene and allow expression of the marker (Figure 1B).

To find a suitable repressible system, we have taken advantage of the well-established GAL4-UAS targeted expression system in flies (Brand and Perrimon, 1993). The GAL4 transcription factor activates transcription of reporter genes under the control of the GAL4 upstream activation sequence (UAS) in yeast (Giniger et al., 1985) and flies (Fischer et al., 1988; Brand and Perrimon, 1993). In yeast, the GAL80 protein antagonizes GAL4 activity by binding to the activation domain of GAL4, preventing interaction between GAL4 and the transcriptional machinery (Ma and Ptashne, 1987). We reasoned that if GAL80 can antagonize GAL4 activity in flies, it could be

used along with GAL4/UAS-marker to achieve repressible expression of the marker gene (Figure 1B).

GAL80 Potently Antagonizes GAL4 Activity in Developing and Adult Flies

Transgenic flies expressing GAL80 protein under the control of *Drosophila* tubulin 1α promoter (tubP), which allows ubiquitous expression (O'Donnell et al., 1994), are viable and fertile. No external morphological or behavioral abnormalities were detected, suggesting that high-level, ubiquitous GAL80 protein expression does not cause significant cell or organismal toxicity. We employed several assays to test whether GAL80 can inhibit GAL4 activity in flies. First, we tested whether GAL80 can rescue the eye phenotype caused by GAL4-driven expression of a dominant *Drac1* mutant (*Drac1^L89.6*; Luo et al., 1994) in eye pigment cells (Figure 2A). The presence of one copy of the *tubP-GAL80* transgene completely suppressed such GAL4-induced defects (Figure 2B).

Next, we directly tested the ability of GAL80 to antagonize GAL4-induced marker expression. Potent suppression (see below) was observed with a number of different markers, including *UAS-lacZ*, *UAS-GFP*, and two transgenic markers we generated, *UAS-tau-lacZ* (a microtubule-binding protein Tau fused with *lacZ* [Callahan and Thomas, 1994]) and *UAS-mCD8-GFP* (a fusion protein between mouse lymphocyte marker CD8 and the green fluorescence protein). For most experiments described in this study, we used mCD8-GFP as a marker because: (1) it labels the cell surface as a result of mCD8 being a transmembrane protein; (2) it is highly concentrated in neuronal processes including axons and dendrites (see below) due to the high surface/volume ratio in neuronal processes; (3) we have not detected any toxicity due to overexpression of this fusion protein throughout a cell's life; and (4) the fusion protein can be visualized directly by GFP fluorescence or by monoclonal antibody staining against mCD8.

Ubiquitous neuronal expression of the marker resulting from the *GAL4^C155* driver that allows reporter expression in all postmitotic neurons (Lin and Goodman, 1994; Robinow and White, 1991) is completely suppressed in the presence of one copy of the *tubP-GAL80* transgene. Such suppression is observed in third instar larval stage (Figure 2D, compared with Figure 2C), in adult brain (Figure 2F, compared with Figure 2E), and in all other embryonic, larval, and pupal stages tested (data not shown). Expression of markers in the adult sensory organ precursors of imaginal discs resulting from the *GAL4^109–68* driver (Guo et al., 1996) was also completely suppressed by the *tubP-GAL80* transgene (Figure 3C, compared with Figure 3A).

We also generated a *tubP-GAL4* transgene that should allow expression of the reporter in all cells in any developmental stage. Again, GAL80 potently inhibited GAL4-induced marker expression in embryos (Figures 2G and 2H), third instar larval brain (data not shown) and imaginal discs (see Figure 4), and adult tissues including the brain (data not shown), the wing (Figures 2I and 2J), and legs (data not shown). Thus, GAL80 can potently inhibit GAL4-induced expression of target genes in developing and adult *Drosophila*.
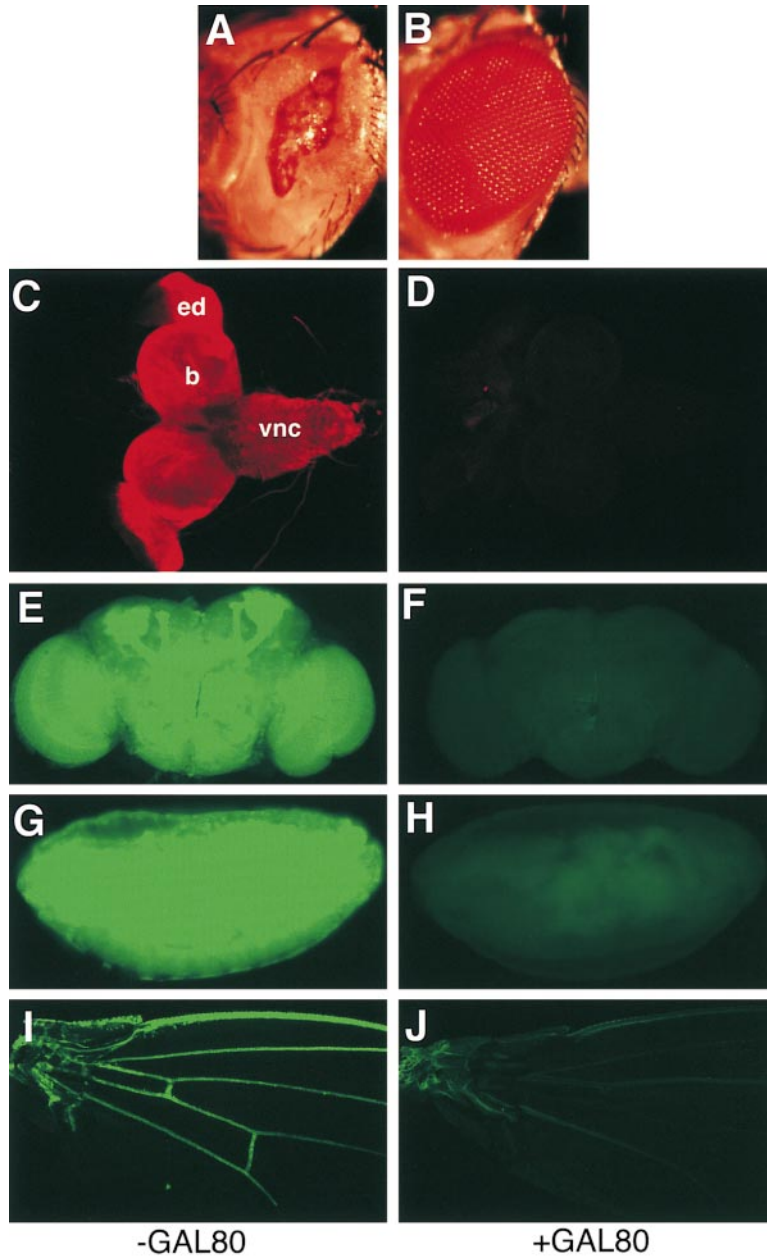
Figure 2. Suppression of GAL4 Activity by GAL80 in *Drosophila*

(A and B) The deformed eye phenotype (A) resulting from *GAL4⁵⁴*-dependent expression of a dominant *Drac1* mutant gene was suppressed by the presence of one copy of *tubP-GAL80* (B).

(C–F) The *GAL4^C155* driven panneuronal expression of the marker mCD8-GFP in third instar larval brain and eye disc (C) or adult head (E) was suppressed by the presence of one copy of the *tubP-GAL80* transgene (D and F). (C and D), anti-mCD8 immunofluorescence; (E and F), GFP fluorescence. b, brain; vnc, ventral nerve cord; ed, eye disc.

(G–J) The *tubP-GAL4*-driven expression of *mCD8-GFP* was suppressed by *tubP-GAL80* in embryos (G and H) and adult wings (I and J), based on the endogenous GFP fluorescence. Note that (D) was 20-fold overexposed compared to (C). (F), (H), and (J) were 5-fold overexposed compared to (E), (G), and (I), respectively.

Genotypes: (A), *GAL4⁵⁴,UAS-Drac1^L89/CyO;* (B), *GAL4⁵⁴,UAS-Drac1^L89/tubP-GAL80;* (C and E), *GAL4^C155/+; UAS-mCD8-GFP/+;* (D and F), *GAL4^C155/+; UAS-mCD8-GFP/tubP-GAL80;* (G and I), *UAS-mCD8-GFP/+; tubP-GAL4/+;* and (H and J), *UAS-mCD8-GFP/tubP-GAL80; tubP-GAL4/+.*

Removal of GAL80 in FLP/FRT-Mediated Mitotic Recombination Results in Expression of the Marker

Next, we investigated whether removal of GAL80 results in expression of the marker (Figure 1B). Figure 3 illustrates examples of wing disc and eye disc clones generated by inducing FLP expression using heat shock. In the absence of GAL80, the marker is expressed in a large subset of sensory organ precursors in the wing disc (Figure 3A) and R8 photoreceptor cells in the eye disc (Figure 3D) due to the expression pattern of *GAL4^109-68*. To remove the *tubP-GAL80* transgene during mitotic recombination, we placed it distal to the FRT site on the right arm of the second chromosome (*FRT^G13*) (Chou and Perrimon, 1996) and introduced into these flies another *FRT^G13* on the homologous chromosome, as well as other

transgenes including the FLP recombinase under the control of heat shock promoter (*hs-FLP*), *GAL4^109-68*, and the UAS-marker. We observed that in different animals the reporter is expressed in different small subsets of sensory organ precursors (Figure 3B, compared with Figure 3A) or R8 photoreceptor cells (Figures 3E and 3F, compared with Figure 3D) in response to heat shock induction of FLP. In the absence of heat shock, no marker expression was ever detected in wing discs (Figure 3C) or eye discs (data not shown), indicating that marker expression was dependent on FLP activity and, hence, was a result of mitotic recombination. In the case of the photoreceptor clones, one can clearly trace the projection of single labeled neurons from the cell body in the eye disc (Figure 3E, arrow) to the growth cone in the optic lobe (Figure 3F, arrow).
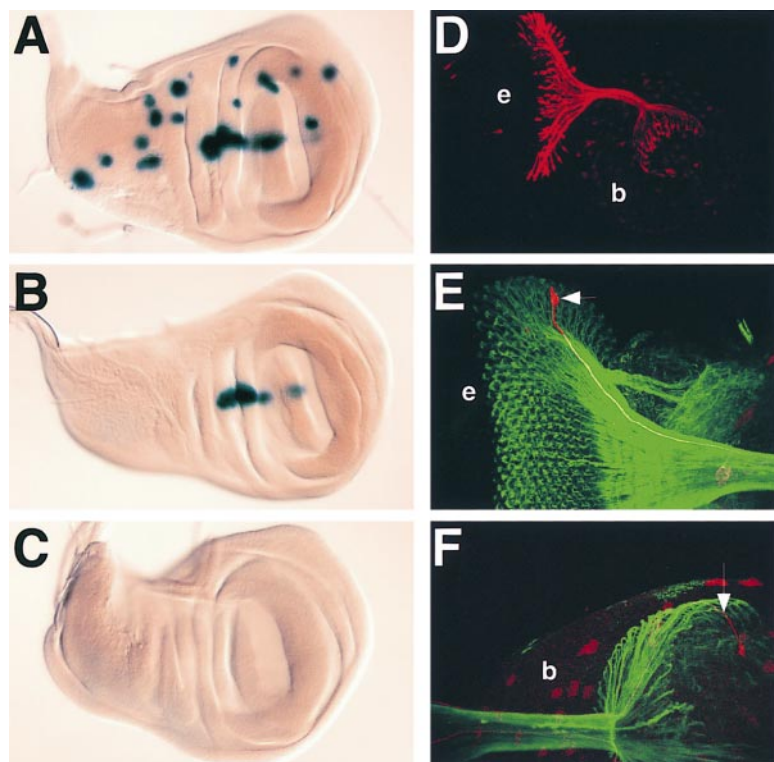
Figure 3. Expression of GAL4-Dependent Markers after Loss of GAL80 Due to Mitotic Recombination

(A–C) X-gal staining of third instar wing discs revealed the $GAL4^{109-68}$-driven expression of β-galactosidase in a large subset of sensory organ precursors (A), which was suppressed in the presence of $tubP$-$GAL80$ (C). However, heat shock-dependent mitotic recombination allows expression of β-galactosidase in small subsets of sensory organ precursors, presumably due to loss of the $tubP$-$GAL80$ transgene (B). Genotypes: (A), UAS-$lacZ/GAL4^{109-68}$, (B and C), hs-$FLP/+$; UAS-$lacZ$,-FRT^{G13},$GAL4^{109-68}/FRT^{G13}$,$tubP$-$GAL80$. (B) was from a larva that had been heat shocked for 30 min two days before the dissection. (C) was from a larva that was not heat shocked. (D–F) Composite confocal images of third instar larval photoreceptors revealed the $GAL4^{109-68}$-driven expression of UAS-tau-$lacZ$ in a large subset of R8 photoreceptors (D). In the MARCM system, heat shock-induced mitotic recombination allows the generation of small clones of labeled photoreceptor cells (red in [E] and [F]; green represents all developing photoreceptors labeled by mAb 22C10). A single photoreceptor can be traced from cell body in eye disc (arrow in [E]) to the end of the extending axon in the optic lobe (arrow in [F]). e, eye disc; b, brain. Genotypes: (D), UAS-tau-$lacZ/GAL4^{109-68}$; (E–F), hs-$FLP/+$; FRT^{G13},UAS-tau-$lacZ$,$GAL4^{109-68}/FRT^{G13}$,$tubP$-$GAL80$.

Specificity and Sensitivity of the MARCM System

In assessing the usefulness of the MARCM system for marking mutant clones, it is important to examine two properties: (1) specificity—whether all marked cells are homozygous mutant for gene x; and (2) sensitivity—whether all cells devoid of the $tubP$-$GAL80$ transgene are marked.

The fact that GAL80 can potently inhibit GAL4-induced marker expression in all cell types tested (Figure 2) argues that cells expressing the marker would have to be devoid of the $tubP$-$GAL80$ transgene and, hence, homozygous for x (Figure 1B). Therefore, the MARCM system is predicted to be highly specific. To confirm this, we constructed flies in which mutant cells are simultaneously marked positively by the MARCM system and negatively by the πMYC expressed from a transgene located on same chromosome arm as the $tubP$-$GAL80$ transgene distal to the FRT site (Xu and Rubin, 1993). To ensure that every cell that loses the GAL80 protein would be able to express the marker, we introduced the $tubP$-$GAL4$ driver into these flies (except in Figures 4I and 4J). This allowed us to compare the number and the size of the clones independently and simultaneously by two different methods (Figure 1; see illustrations in Figure 4A).

Using confocal microscopy, we examined mosaic clones in the third instar wing disc epithelium double-labeled with positive marker (mAb against mCD8) and negative marker (mAb against MYC). The green staining (Figure 4C) is for the reporter mCD8-GFP and, hence, represents the cells that have lost $tubP$-$GAL80$ and are homozygous mutant for x. The red staining (Figure 4B) is for MYC, and therefore cells that are devoid of red

staining are homozygous mutant for x. When the red and green staining are superimposed, we observe that all green cells fall into the holes lacking red staining (Figure 4D). At high magnification, we also observe that all the single cells labeled for mCD8 are devoid of MYC staining (Figures 4G and 4H). These experiments confirm that the MARCM system is highly specific.

Sensitivity of the MARCM system can also be measured in the same experiment. If all mutant cells are labeled, one would expect that green staining would fill all the blank holes in the red staining. In theory, the mutant cells would only express the marker after the concentration of the GAL80 protein inherited from parental cells is significantly reduced. Thus, one would expect poor sensitivity shortly after mitotic recombination. When wing discs were examined 24 hr after heat shock, very few holes in red staining were filled with green staining, and the existing green staining was weak in intensity (data not shown). At 36 hr after heat shock, about half of the holes were filled (data not shown). At 48 hr, almost all the holes were filled when examined under low magnification (Figures 4B–4D). When small clones were examined under high magnification (Figures 4G and 4H), although most holes in red staining are filled, there are also weakly stained or unstained regions for red that are not stained for green (arrowhead in Figure 4G).

This small number of cells that were not stained by either red (MYC) or green (mCD8-GFP) could have resulted from one of the following scenarios. (1) In some mutant cells, GAL80 protein concentration may still be too high to allow GAL4-induced marker expression. (2) $GAL4$ expression may not be uniform. Indeed, even with
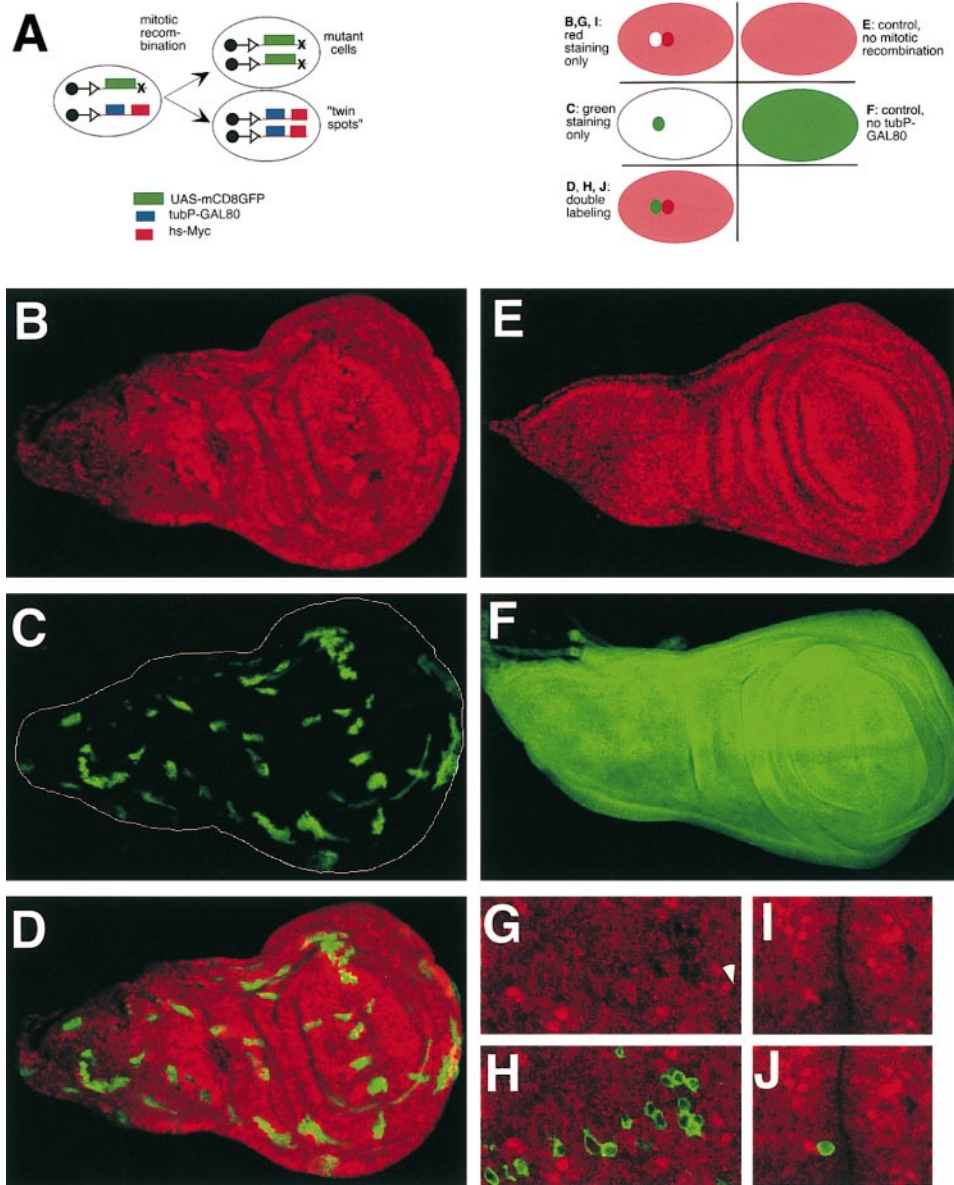
Figure 4. Specificity and Sensitivity of the MARCM System as Revealed by Marking Simultaneously with the Conventional and MARCM Systems

(A) Schematic representation of experiments that allow simultaneous marking of mutant clones with both conventional and MARCM systems (see text). The right part of (A) serves as a guide for (B)–(F).

(B–D and G–J) Single section confocal images of third instar wing discs, double labeled with anti-MYC (red) and anti-mCD8 (green) mAbs, revealed mosaic clones as detected by the conventional method (B, G, and I), the MARCM method (C), and both (D, H, and J). All mCD8-GFP-positive clones coincide with MYC-negative holes (D, H, and J) in a mosaic disc. Almost all MYC-negative holes are filled with mCD8-GFP-positive cells when viewed at low magnification (B–D). At high magnification, some potential MYC-negative single-cell clones (arrowhead in [G]) are not filled with mCD8-GFP. Note that because of the limited $GAL4^{109-68}$ expression pattern, most red holes are not expected to be filled in (I)–(J). As controls, (E) shows the MYC staining of a disc without induction of mitotic recombination and (F) shows the $tubP\text{-}GAL4$ driven expression of $mCD8\text{-}GFP$ in the absence of $tubP\text{-}GAL80$.

Genotypes: (B–E and G–H), $hs\text{-}FLP/+$; $FRT^{G13},UAS\text{-}mCD8\text{-}GFP/FRT^{G13},hs\text{-}\pi MYC,tubP\text{-}GAL80;\ tubP\text{-}GAL4/+$; (F), $hs\text{-}FLP/+$; $FRT^{G13},UAS\text{-}mCD8\text{-}GFP/CyO;\ tubP\text{-}GAL4/+$; (I and J), $hs\text{-}FLP/+$; $FRT^{G13},GAL4^{109-68},UAS\text{-}mCD8\text{-}GFP/\ FRT^{G13},hs\text{-}\pi MYC,tubP\text{-}GAL80$. Mitotic recombinations were induced by a 30 min heat shock 48 hr before (in all cases except for [E]), and πMYC expression was induced by a 30 min heat shock 1 hr before the dissection.

the ubiquitous tubulin 1α promoter driven $GAL4$, one observes heterogeneity in the level of marker expression (Figure 4F). (3) The level of MYC expression is not uniform even in the absence of mitotic recombination (Figure 4E). Especially at high magnification, one can not always distinguish at the single cell level cells that express zero, one, or two copies of the $hs\text{-}\pi MYC$ transgene (Figures 4G and 4H). This problem underscores the importance of marking mutant cells in a positive way.

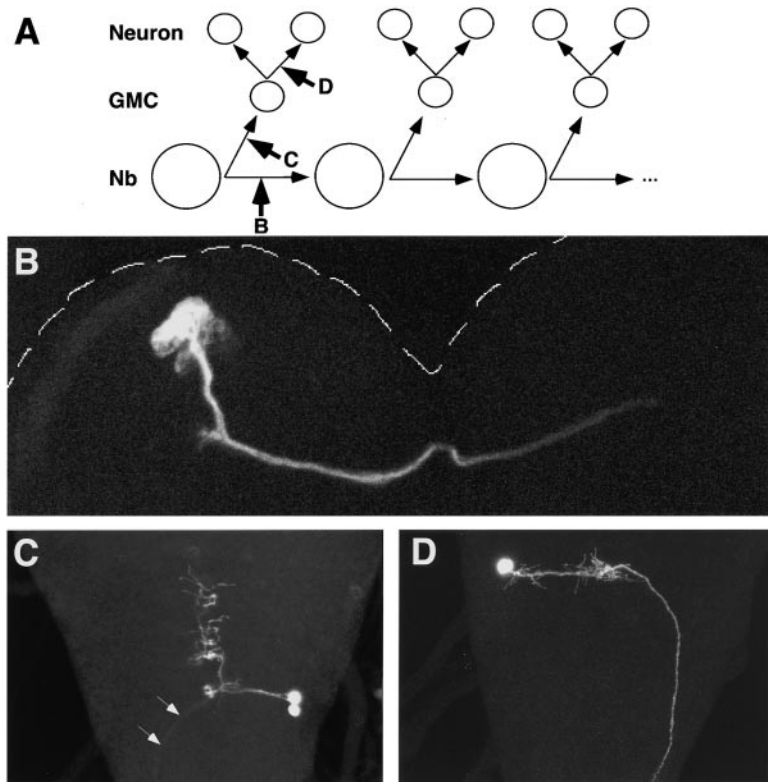On many occasions, we observe clearly marked single

Figure 5. Generation of Neuronal Clones in the *Drosophila* CNS

(A) Schematic drawing of CNS neuroblast division pattern illustrates how neuronal clones of various sizes can be generated in the MARCM system (see text).

(B, C, and D) Composite confocal images of neuronal clones in third instar CNSs marked with the MARCM system revealed a brain lobe neuroblast clone sending an axonal bundle across the midline ([B], the brain lobes are outlined by a white broken line), a two-cell clone with anterior and posterior (indicated by arrows) processes in a ventral nerve cord (C), and a single motor neuron clone sending its axon out of the ventral nerve cord (D). Anterior is up.

The genotype is *hs-FLP/GAL4^C155^; FRT^G13^, UAS-mCD8-GFP/FRT^G13^,tubP-GAL80.* Mosaic clones were generated by a 30 min heat shock in first instar larvae.

mutant cells. One example in wing disc is illustrated in Figures 4I and 4J, where a single hole in red staining (Figure 4I), next to a brightly labeled "twin spot," was strongly stained with the green marker. We use wing disc as a model system to assess the specificity and sensitivity of the MARCM system, because the thin epithelia allow clear visualization of the cells by both markers (especially the MYC marker). Results from analogous experiments in CNS and the eye disc are consistent with our assessment of the specificity and sensitivity derived from experiments in the wing disc. In particular, we encounter many strongly labeled two-cell and single-cell clones (see Figure 5), as soon as 24 hr after heat shock induction of FLP.

In summary, the experiments described in this section indicate that the MARCM system is highly specific—all cells expressing the marker protein are homozygous mutant for *x*. The sensitivity is time dependent: after 48 hr, most mutant cells are positively marked. The remaining unmarked "mutant" cells could be due to residual GAL80 activity, patchy GAL4 activity, or simply our inability to confirm the nature of mutant cells by other means.

Harrison and Perrimon (1993) described an interesting system designed for generating marked clones in *Drosophila*, in which a ubiquitous promoter and the open reading frame (ORF) of a marker gene are separately placed on homologous chromosomes at adjacent positions. An FRT site is placed after the promoter on one chromosome, and before the marker ORF on the other. The marker gene is expressed only when the promoter is fused with the ORF following site-specific recombination between the FRT sites. Compared to the MARCM system described here, this system has higher sensitivity because the marker is immediately expressed after mitotic recombination. However, in this system one daughter cell will always be labeled after mitotic recombination, regardless of its genotype (which depends on chromotid segregation pattern). Although there is a bias against the assortment of two recombinant-type chromatids, it does occur at a significant frequency (Pimpinelli and Ripoll, 1986). In these cases, marked cells remain heterozygous for the chromosome arm distal to the recombination site, limiting the use of this system for mosaic analysis due to low specificity. A new version of this technique may have overcome these limitations (E. Spana and N. Perrimon, personal communication).

Mosaic Analysis in *Drosophila* CNS

The typical neuroblast division pattern in the CNS is illustrated in Figure 5A (reviewed in Goodman and Doe, 1993). A neuroblast (Nb) divides and gives rise to another neuroblast and a ganglion mother cell (GMC). The GMC subsequently divides to generate two postmitotic neurons. This division pattern is repeated as many as several hundred times for certain Nbs. Given this cell division pattern, the MARCM system allows for the generation of clones of different sizes. If mitotic recombination occurs prior to the neuroblast division to generate mosaic daughter cells, half of the time the new neuroblast will be devoid of the repressor transgene and positively labeled (referred hereafter as Nb clones). All the subsequent progeny are labeled as long as the GAL4 protein is expressed continuously in all neurons. Thus, a large clone of labeled neurons can be generated, with
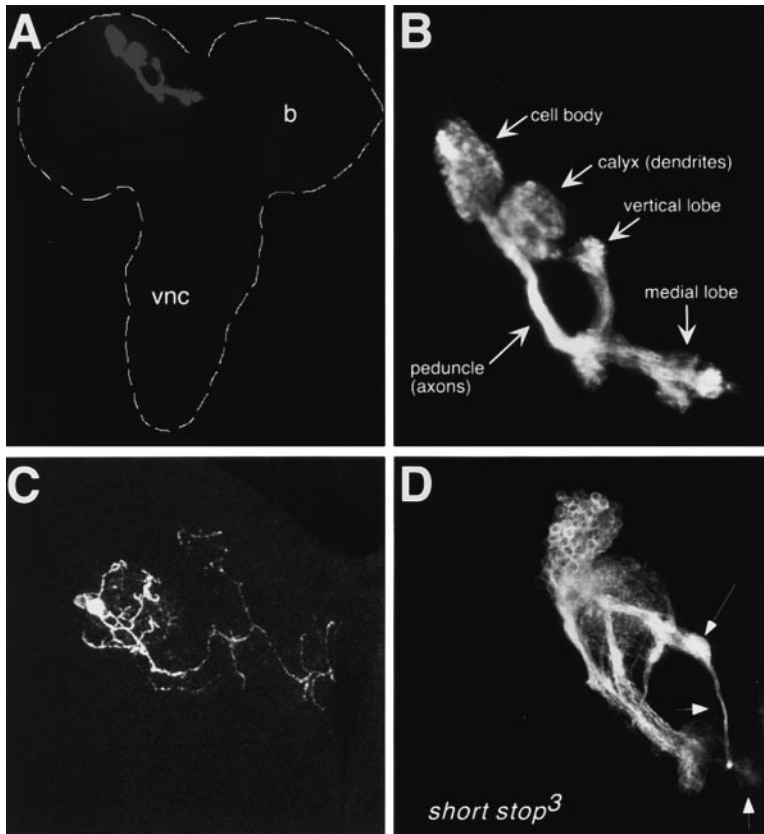
Figure 6. Selectively Generating Wild-Type or *short stop³* Mushroom Body Clones by Inducing Mitotic Recombination in Newly Hatched Larvae

(A–C) Composite confocal images of wild-type mushroom body clones, generated by inducing mitotic recombinations during the first 4 hr of larval life, revealed a neuroblast clone in a third instar CNS ([A]: b, brain lobes; vnc, ventral nerve cord; and CNS is outlined by a broken white line), which is shown at a higher magnification in (B). The salient features of the mushroom body are indicated in (B). With the same magnification as (B), a clone composed of two mushroom body neurons, one of which more intensely stained, is shown in (C).

(D) Compared with the wild-type MBNb clone (B), the *shot³* MBNb clone revealed misguided axonal bundles (long arrows) and significantly reduced lobes (vertical and medial lobes are indicated by horizontal and vertical arrows, respectively).

Anterior is up. Genotypes: (A–C), *hs-FLP/ GAL4^{C155}; FRT^{G13},UAS-mCD8-GFP/FRT^{G13},tubP-GAL80*; (D), *hs-FLP/GAL4^{C155},UAS-mCD8-GFP; FRT^{G13},shot³/FRT^{G13},tubP-GAL80*. Mitotic recombination was induced by a 40 min heat shock during the first 4 hr of larval life.

their dendritic arborizations and axonal projections revealed in an otherwise unstained brain (Figure 5B). The other half of the time, the GMC is devoid of the repressor transgene. This results in the labeling of a two-cell clone (Figure 5C). If mitotic recombination occurs before the division of the GMC, then one of the two postmitotic neurons is labeled, effectively creating "single neuron mosaic" (Figure 5D). Using all three types of clones, one can analyze the function of gene *x* in neuroblast or GMC cell divisions and in the morphogenesis of postmitotic neurons including the elaboration of dendrites and the pathfinding of axons.

Restricting Mitotic Recombination to the Mushroom Body

In order to compare organisms of different genotypes for phenotypic analysis, it is essential to target identifiable cells for mitotic recombination so that the wild-type cell division and axon projection pattern is defined. This can be achieved by spatially or temporally restricting the FLP recombinase activity. As an example, we show here that temporal regulation of FLP activity using the *hs-FLP* transgene (Golic and Lindquist, 1989) can restrict mitotic recombination to the Kenyon cells of the brain lobe that give rise to mushroom body structures (hereafter referred to as MB neurons).

In the first few hours of *Drosophila* larval life, there are only five actively dividing neuroblasts per brain lobe, four of which give rise to the MB neurons (Ito and Hotta, 1992). Furthermore, each of the four mushroom body

neuroblasts (MBNb) per brain lobe generates MB neurons with virtually identical projection patterns (Ito et al., 1997). We reason that for efficient mitotic recombination to occur, the FLP activity may need to coincide with active proliferation of cells. If we limit FLP activity to the first few hours of larval life, we may selectively generate mitotic recombination in MB precursors. (In theory, all G2/M-arrested neuroblasts also have the capability of mitotic recombination and thus generating homozygous clones. The fact that we do not observe such events [see below] indicates that either most neuroblasts are arrested at G1/S transition as was found for lamina precursors [Selleck et al., 1992], or G2/M-arrested neuroblasts are not sensitive to heat shock induction of FLP activity.)

Indeed, when we induce FLP activity through heat shock at 0–4 hr after larval hatching and examine at wandering third instar, the most frequent clones we obtain are those of MB neurons. In both neuroblast clones (Figures 6A and 6B) and two-cell clones (Figure 6C), the entire neuronal morphology, including the cell body, the dendritic projection in the calyx, the axon peduncle, and the subsequent branched projections to the vertical lobe (analogous to the α/α' lobe in the adult) and medial lobe (analogous to the β/γ lobe in the adult), can be visualized by the mCD8-GFP marker. The pattern of projection is consistent with previous Golgi staining and mushroom body specific enhancer trap lines expression pattern (Yang et al., 1995) and with staining patterns of antibodies against antigens highly enriched in mushroom bodies (Crittenden et al., 1998).

Table 1. Phenotypic Analysis of *shot³* Mushroom Body Clones

Geno-type	No. of MB Clones[a]	MBs with Reduced Density of Lobes[b]	MBs with Misguided Axons[c]	MBs with Misguided Axons Passing through the Calyx	MBs with Misguided Axons Approaching the Midline
Wild type	>100	0	0	0	0
shot³	21	21	20	18	15

[a] Only mushroom body neuroblast clones with normal number of neurons were considered. For reasons that are unclear to us, 4 out of 25 *shot³* MB neuroblast clones we obtained contained much fewer neurons compared to wild type.
[b] The density of vertical and medial lobes was examined on merged confocal images.
[c] Only grossly misguided bundles of axons were considered.

Assuming the typical neuroblast division pattern (Figure 5A) applies to MB neuroblasts, one should have equal chance of generating Nb clones (Figure 5B) and two-cell clones (Figure 5C) because both are products of the same mitosis. However, we have found many more Nb clones (Figures 6A and 6B) than two-cell clones (Figure 6C) in the MB lineage. One explanation is that in two-cell clones the GAL80 protein is not diluted sufficiently to allow marker expression, whereas in the Nb clones the rapid division and dilution of the GAL80 protein allow efficient marker expression. However, we did obtain two-cell clones of MB neurons that were strongly stained as early as 30 hr after heat shock in early second instar larvae. This makes it unlikely that GAL80 perdurance is a major factor, given that in most studies we dissected third instar larvae more than 100 hr after heat shock. Other possibilities that could account for the bias of Nb clones over two-cell clones include selective physiological changes in early born MB neurons such as downregulation of GAL4 activity and selective cell death. We rarely observe single-cell clones in the MB lineage. Along with the same problems as the two-cell clones, this may suggest that the GMC division in the early MB lineage is less likely to be targeted for mitotic recombination.

Analysis of Gene Function in Neuronal Morphogenesis: *short stop* Is Required in Mushroom Body Neurons for Their Correct Axonal Pathfinding

As a test of the MARCM system for the analysis of gene function in neuronal morphogenesis, we chose to study a gene, *short stop* (*shot*), that was previously identified for its requirement in embryonic motor and sensory axon extension. In embryos homozygous for *shot¹*, ISN and ISNb motor axons fail to extend beyond choice points between neural and mesodermal substrates (Van Vactor et al., 1993), and Ich chordotonal sensory neurons stall at various points along their path to the CNS (Kolodziej et al., 1995). In embryos homozygous for an apparently more severe allele, *shot³*, axons are largely absent in Ich neurons and severely truncated in other PNS neurons (Kolodziej et al., 1995). No misguidance defects were found in either the sensory or motor axons, so the gene was categorized as affecting axon "growth" (Kolodziej et al., 1995). It was not known whether the defects in sensory or motor axons are due to absence of *shot* activity in neurons or in their environment. Cloning of the gene has not been reported yet, therefore no molecular information is available.

Mushroom body Nb clones of *shot³* generated using the MARCM system exhibit drastic axon projection defects compared with wild-type controls (Figure 6D, compared with Figure 6B; Table 1). In most cases (21 out of 25), there are no obvious changes in the numbers of MB neurons compared with wild type. However, in every mutant clone examined, a very small fraction of axons reached the branching point and projected to the vertical and medial lobes (Figure 6D, horizontal and vertical arrows, respectively). In all but one case, abnormally guided axon bundles were observed, most of them passing through the calyx where the dendritic field of the MB neurons is located (Figure 6D, long arrows). The origin of axon pathfinding errors appears to be near the dendritic projection area, where in wild type all axons form a tight fascicle. In *shot³* clones, this fascicle disintegrates into several bundles (Figure 6D; data not shown). Interestingly, in the majority of cases (Table 1) we observe that the abnormal axon bundles extending out from the calyx appear to project toward the midline region where the normal medial lobe is, and in some cases, they eventually fuse with the medial lobe when traced using confocal microscopy. This observation suggests that these abnormal axons, mutant for *shot* activity, may retain some ability to be attracted to the final destination even though the original pathway selection is abnormal. In normal as well as aberrant pathways taken by *shot³* mutant neurons, a large fraction of axons must stop along the pathways because the immunoreactivity grows progressively weaker (Figure 6D). Such axon extension defects are consistent with findings in embryonic motor and sensory axon projection (Van Vactor et al., 1993; Kolodziej et al., 1995).

These axon pathfinding defects are likely caused by the *shot* gene rather than a background chromosome mutation because *shot¹*, which is generated in a different chromosome background, gave qualitatively similar yet milder phenotypes when mutant clones were analyzed in analogous experiments (data not shown).

The only cells that are homozygous mutant for *shot* are the progeny of a MBNb, including the MB neurons and possibly cell body glia (Ito et al., 1997). Even if cell body glia are included in these larval clones, they are spatially restricted to the cell body region and do not play a role in axon guidance. The fact that abnormalities in the axonal outgrowth were observed outside the cell body region in spite of no apparent defects in the organization of cell bodies indicates that *shot* is required by the MB neurons for their correct axon pathfinding. Due to the difficulties in generating large numbers of two-cell or single-cell clones, as well as the difficulties of assigning them as mushroom body clones with certainty

Table 2. List of Transgenes and Recombinant Chromosomes Used in This Study

List of transgenes used in this study

Names	Purposes	References
tubP-GAL80 in pCaSpeR4	Allow ubiquitous expression of GAL80	This study
tubP-GAL4 in pCaSpeR4	Allow ubiquitous expression of GAL4	This study
mCD8-GFP in pUAST	Allow expression of membrane targeted GFP under the control of GAL4	This study
UAS-tau-lacZ in pY.E.S.	Allow expression of microtubule targeted β-galactosidase under the control of GAL4	This study
GAL4^{C155}	Drive expression of UAS transgenes in neurons	Lin and Goodman, 1994
GAL4$^{109-68}$	Drive expression of UAS transgenes in sensory organ precursors and the R8 photoreceptors	Guo et al., 1996
GAL4^{54}	Drive expression of UAS transgenes in visual pigment cells	This study
UAS-lacZ	Allow expression of β-galactosidase under the control of GAL4	Brand and Perrimon, 1993
UAS-Drac1$^{L89.6}$	Allow expression of a dominant-negative Drac1 transgene under the control of GAL4	Luo et al., 1994
hs-πMYC	Allow heat shock-induced expression of πMYC	Xu and Rubin, 1993
hs-FLP	Allow heat shock-induced expression of FLP recombinase	Golic and Linquist, 1989
FRT^{G13}	Allow FLP-mediated site specific recombination on the chromosome arm 2R	Chou and Perrimon, 1996
FRT^{19A*}	Allow FLP-mediated site specific recombination on the X chromosome	Xu and Rubin, 1993
FRT^{82B*}	Allow FLP-mediated site specific recombination on the chromosome arm 3R	Xu and Rubin, 1993

List of recombinant chromosomes constructed in this study

UAS-lacZ,FRT^{G13}, GAL4$^{109-68}$	2nd chromosome
FRT^{G13},UAS-tau-lacZ,GAL4$^{109-68}$	2nd chromosome
FRT^{G13},tubP-GAL80	2nd chromosome
FRT^{G13},hs-πMYC,tubP-GAL80	2nd chromosome
FRT^{G13},UAS-mCD8-GFP	2nd chromosome
FRT^{19A},tubP-GAL80,hs-FLP*	X chromosome
FRT^{82B},tubP-GAL80*	3rd chromosome
FRT^{G13},shot^3	2nd chromosome
FRT^{G13},shot^1	2nd chromosome
GAL4^{C155},UAS-mCD8-GFP	X chromosome
GAL4^{C155},UAS-mCD8-GFP,hs-FLP	X chromosome
GAL4^{54},UAS-Drac1$^{L89.6}$	2nd chromosome

* Although not used in this study, these recombinant chromosomes are available for mosaic analyses of genes that are located on the X and 3R chromosome arms.

List of additional fly stocks generated for the MARCM system

FRT^{19A},tubP-GAL80,hs-FLP; Pin/Cyo
FRT^{19A},tubP-GAL80,hs-FLP; UAS-mCD8-GFP
FRT^{19A},GAL4^{C155}; BcElp/Cyo
FRT^{19A},GAL4^{C155}; Ly/Tm6B
GAL4^{C155}; FRT^{G13},tubP-GAL80
GAL4^{C155},hs-FLP; FRT^{G13},tubP-GAL80
FRT^{G13},hs-πMYC,tubP-GAL80; tubP-GAL4/Tm6B
hs-FLP; FRT^{G13},UAS-mCD8-GFP

if the axon projection is grossly abnormal, we have not been able to determine the cell autonomy of *shot* with single neuron resolution.

In summary, we demonstrate that *shot* gene is required not only for extension, but also for fasciculation and correct guidance of mushroom body axons. Our experiments also indicate that *shot* acts within neurons. *shot* may encode a cell surface receptor or a cytoplasmic factor that is generally required for axon guidance decisions in a wide variety of neurons. Further insight into the mechanisms of function awaits the cloning and molecular characterization of the gene.

Limitations and Other Applications of the MARCM System

Because of the perdurance of the GAL80 protein (see the section on specificity and sensitivity), the MARCM system can only be used reliably for mosaic analysis of gene function 48 hr after the induction of mitotic recombination. In order to use the system for mosaic analysis sooner after mitotic recombination, a more labile version or temperature-sensitive GAL80 protein needs to be developed to replace the wild-type GAL80 protein. The choice of GAL4 line is also very important to ensure that all the mutant cells would express the marker protein. In this regard, the ubiquitous *tubP-GAL4* we have generated should be of general use, and the panneuronal *GAL4^{C155}* may be useful in the nervous system. Another potential limitation, which applies to all mosaic analysis, is that the gene of interest may be highly expressed in precursor cells, and the protein product may be very stable, such that perdurance of the protein of interest could present a challenge to study its requirement in early events of morphogenesis right after clones are made.

Since expression of the marker gene is driven by GAL4 in our design, one can drive simultaneous expression of multiple UAS transgenes in mosaic clones generated

by the MARCM system. By coexpressing a transgene of interest and the marker gene in mosaic clones, one can introduce a dominant transgene into a wild-type or mutant clone, or a rescuing construct into a mutant clone. These studies will allow one to perform genetic interaction experiments and to perform structure–function analysis of a gene of interest.

The MARCM method is also ideal for lineage tracing during development. If mitotic recombination is controlled so that it occurs at a low enough frequency, one can be confident that the event is due to recombination in a single precursor cell in any given microenvironment. One can then examine the progeny from such a precursor at any later developmental stages, as the progeny will be the only cells labeled. This should be particularly useful in the nervous system since the marker system we introduced allows the visualization of the entire neurons, including axons and dendrites.

Although especially useful in studying gene functions in neuronal morphogenesis, the MARCM system can be used for analysis of gene function in other tissues. In many cases, it is useful to visualize only the mutant cells for morphological studies or simply to better trace the mutant tissues at different stages of development. Lastly, because both the UAS/GAL4 expression system (Ornitz et al., 1991) and the FLP/FRT (Dymecki, 1996) or CRE/LOX mitotic recombination systems (Gu et al., 1994; Van Deursen et al., 1995) function in mice, it may be possible to adapt the MARCM system for use in mice.

Experimental Procedures

New Transgenes
tubulin 1αP-GAL80 in pCaSpeR4
The GAL80 open reading frame (ORF) (Ma and Ptashne, 1987) was amplified by PCR with the primers ATTAAGCGGCCGCAACATGGAC TACAACAAGAGATC and GCGTGTCTAGATTATAAACTATAATGCG AGATATTG and subcloned into the pCaSpeR4 that has been modified to carry the tubulin 1α promoter (O'Donnell et al., 1994) between EcoRI and NotI and the SV40 poly A region between XbaI and StuI, with NotI and XbaI as the cloning sites.
tubulin 1αP-GAL4 in pCaSpeR4
The GAL4 gene containing the hsp70 polyA region was cut out with NotI from pGaTN (Brand and Perrimon, 1993) and inserted into the pCaSpeR4 carrying the tubulin 1α promoter.
mCD8-GFP in pUAST
The green fluorescence protein (GFP) ORF bearing F64L, S65T mutations was amplified by PCR and cloned into Bluescript (pBS, Stratagene) with BamHI and XbaI as the cloning sites. Then a PCR product encoding the mouse CD8 (mCD8) gene (Liaw et al., 1986) was subcloned into the GFP-containing pBS with XhoI and BamHI as the cloning sites, generating a new ORF with GFP fused in frame to the 3′ of mCD8. Finally, mCD8-GFP was subcloned into pUAST (Brand and Perrimon, 1993) with XhoI and XbaI as the cloning sites.
UAS-tau-lacZ in pY.E.S.
An EcoRI-cut DNA fragment containing the tau-lacZ ORF (Callahan and Thomas, 1994) and the SV40 polyA region was subcloned into the pBS modified to have the UAS promoter between BamHI and EcoRI. Then the UAS-tau-lacZ was subcloned into pY.E.S. (Patton et al., 1992) with XbaI and XhoI as the cloning sites.

All constructs were injected into Df(1)w strain to generate transgenic flies according to standard procedure (Spradling and Rubin, 1982).

Construction of Fly Strains Used in This Study
Recombinant chromosomes having multiple transgenes were generated and verified by bio-assays for particular transgenes. For instance, the presence of tubP-GAL80 was confirmed by the ability to suppress the eye phenotype caused by $GAL4^{54}, UAS\text{-}Drac1^{L89.6}$ (Figures 2A and 2B); the presence of histological marker genes was assayed by marker expression under appropriate conditions; and the presence of FLP or FRT was verified by the ability to generate mitotic recombinations given other components. Individual recombinant chromosomes were combined with other transgene-containing chromosomes using standard genetic methods to generate new strains of transgenic flies. Table 2 lists the transgenes, recombinant chromosomes, and their combinations used in this study, as well as other available strains for the use of the MARCM system on different chromosome arms.

Fly Culture, Histology, and Microscopy
Drosophila melanogaster were grown on standard media at 25°C. To induce mitotic recombination, staged larvae were heat shocked in a 37°C water bath for 30–40 min at the specified time and then returned to 25°C. The nervous systems from wandering third instar larvae or young adults were dissected in 100 mM Na$_2$HPO4/ NaH$_2$PO4 (pH 7.2) (PB) and fixed in PB containing 4% formaldehyde (Electron Microscopy Sciences) for 20 min at room temperature. For GFP visualization, samples were washed in PBT (PB + 0.3% Triton) several times for a total time of 30 min, further dissected, and mounted on slides in 80% glycerol. For immunofluorescence, samples were blocked in 5% normal goat serum for 30 min in PBT, incubated with primary antibody (rat anti-mCD8 mAb from Caltag: 1:100; rabbit anti-β-gal from Cappel, 1:10,000; mouse anti-MYC mAb from Santa Cruz, 1:50) at 4°C overnight, washed 6 × 10 min in PBT, incubated with secondary antibody (Jackson lab, 1:200) for 2 hr at room temperature, further washed 6 × 10 min in PBT, and further dissected and mounted on slides in Slow-Fade (Molecular Probes). X-GAL staining was done as described (Giniger et al., 1993). Samples were viewed and images were taken either under a Nikon E-600 microscope with a cooled CCD camera (Diagnostic Instruments) or a Bio-Rad MRC 1024 laser scanning confocal microscope using the Laser Sharp image collection program. Images were processed using Adobe Photoshop.

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