Developmental Biology **247,** 76–88 (2002)
doi:10.1006/dbio.2002.0702

Reveals Specific Roles of Flamingo in Neuronal Morphogenesis

Neal T. Sweeney,*,†,[1] Wenjun Li,*,[1] and Fen-Biao Gao*,†,‡,[2]

**Gladstone Institute of Neurological Disease, †Neuroscience Program, and ‡Departments of Physiology and Neurology, University of California, San Francisco, California 94141-9100*

To study the roles of intracellular factors in neuronal morphogenesis, we used the mosaic analysis with a repressible cell marker (MARCM) technique to visualize identifiable single multiple dendritic (MD) neurons in living *Drosophila* larvae. We found that individual neurons in the peripheral nervous system (PNS) developed clear morphological polarity and diverse dendritic branching patterns in larval stages. Each MD neuron in the same dorsal cluster developed a unique dendritic field, suggesting that they have specific physiological functions. Single-neuron analysis revealed that Flamingo did not affect the general dendritic branching patterns in postmitotic neurons. Instead, Flamingo limited the extension of one or more dorsal dendrites without grossly affecting lateral branches. The dendritic overextension phenotype was partially conferred by the precocious initiation of dorsal dendrites in *flamingo* mutant embryos. In addition, Flamingo is required cell autonomously to promote axonal growth and to prevent premature axonal branching of PNS neurons. Our molecular analysis also indicated that the amino acid sequence near the first EGF motif is important for the proper localization and function of Flamingo. These results demonstrate that Flamingo plays a role in early neuronal differentiation and exerts specific effects on dendrites and axons.

***Key Words: Drosophila*; dendrites; morphogenesis; Flamingo; EGF motif.**

INTRODUCTION

Neuronal morphogenesis is a critical step in neural development. Many neurons elaborate highly branched dendritic trees that can make thousands of synaptic connections with other neurons (Ramón y Cajal, 1911). The proper formation of dendritic fields and axonal arborizations is crucially important for the assembly of a functional nervous system (Masland, 2001). However, it remains unclear how the morphologies of different neurons are specified during development and to what extent this process is controlled by intrinsic factors or environmental cues.

The formation of dendritic fields is mainly affected by the extent and the direction of dendritic outgrowth and branching. The mechanisms controlling dendrite development have been studied with neuronal cell cultures (reviewed in Craig and Banker, 1994; Higgins *et al.*, 1997; Bradke and Dotti, 2000). In recent years, various *in vivo* approaches have been taken to study neuronal morphology. For instance, different versions of green fluorescent protein (GFP) were used to label a small number of neurons in worms and flies (Roayaie *et al.*, 1998; Lee and Luo, 1999; Gao *et al.*, 1999). Local dye superfusion or virus-mediated GFP expression allowed the visualization of neurons in cultured hippocampal slices (Engert and Bonhoeffer, 1999; Maletic-Savatic *et al.*, 1999). In another approach, transgenic mice were created in which red, green, yellow, or cyan fluorescent proteins were selectively expressed in a small subset of central nervous system (CNS) neurons (Feng *et al.*, 2000). More recently, single-cell electroporation enabled ectopic gene expression in the brain of *Xenopus* tadpoles or rat hippocampal slices (Haas *et al.*, 2001). These advances have greatly enhanced our ability to study neuronal morphogenesis *in vivo* at the molecular level.

Both *in vitro* and *in vivo* approaches have revealed a number of key molecules that regulate dendritic and axonal outgrowth and branching (reviewed in Brose and Tessier-Lavigne, 2000; Jontes and Smith, 2000; Matus, 2000; Wong and Wong, 2000; McAllister, 2000; Redmond and Ghosh,

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed. Fax: (415) 826-6541. E-mail: fgao@gladstone.ucsf.edu.

0012-1606/02 $35.00

2001; Scott and Luo, 2001; Jan and Jan, 2001). For instance, neurotrophins and their receptors are important in controlling axonal growth (reviewed in Reichardt and Farinas, 1997) and in regulating dendritic morphology (McAllister *et al.,* 1995; Lom and Cohen-Cory, 1999; Xu *et al.,* 2000). Semaphorin 3A and Slit1, both of which are involved in axon guidance, also regulate the development of cortical dendrites (Polleux *et al.,* 2000; Whitford *et al.,* 2002). In *Xenopus,* the glycosylphosphatidyl-inositol (GPI)-linked molecule CPG15 promotes both dendritic growth and axonal arborization *in vivo* (Nedivi *et al.,* 1998; Cantallops *et al.,* 2000). Despite the recent progress, the molecular understanding of the intracellular machinery that controls the formation of specific neuronal morphology remains far from complete. For instance, it is not known how dendritic extension and branching are coordinated, nor is the differential regulation of dendritic and axonal development understood.

To study these issues, we used the peripheral nervous system (PNS) of *Drosophila* embryos as a model system in which GFP was expressed in multiple dendritic (MD) neurons to visualize dendritic outgrowth and branching (Gao *et al.,* 1999). The easy visualization of these dendrites *in vivo* and their relatively discrete developmental phases made it feasible to carry out genetic screens to identify molecules that control distinct aspects of dendritic morphogenesis. This approach led to the identification of the *flamingo* mutations that result in the overextension of MD neuron dorsal dendrites (Gao *et al.,* 1999, 2000). The *flamingo* gene, which encodes a G protein-coupled receptor-like protein, also controls planar cell polarity in conjunction with *frizzled* (Usui *et al.,* 1999; Chae *et al.,* 1999; Lu *et al.,* 1999). In mutant embryos, the activity of *flamingo* is altered not only in MD neurons but also in adjacent epithelial cells (Usui *et al.,* 1999; Gao *et al.,* 2000). In addition, Flamingo affects spindle orientation in adult sensory organ precursor cells (Lu *et al.,* 1999). Therefore, it is not clear how Flamingo controls the formation of dendritic fields directly in postmitotic neurons and to what extent Flamingo functions cell-autonomously in this process.

To further understand the roles of Flamingo and other intrinsic factors in neuronal morphogenesis, we labeled single wild-type or mutant MD neurons with GFP in living *Drosophila* larvae using the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo, 1999). Here, we describe an assay system that allows the dendritic morphology of single identifiable MD neurons in the dorsal cluster to be studied *in vivo*. We show that individual PNS sensory neurons, unlike CNS neurons, develop clear morphological polarity and highly diverse dendritic branching patterns. Although located in the same dorsal cluster, individual MD neurons develop highly unique dendritic fields. This finding suggests that intrinsic properties of each MD neuron are important in controlling the development of its unique dendritic field and that each MD neuron fulfills a specific physiological function. Furthermore, our genetic analysis with single-neuron resolution reveals that Flamingo directly controls dendritic fields of different dorsal cluster MD neurons in a cell-autonomous fashion. Surprisingly, Flamingo does not affect the dendritic branching patterns in a global way; instead, it only limits the extension of one or more processes toward the dorsal midline without grossly affecting the extension of lateral branches. Flamingo does so partially by controlling the timing of dorsal dendrite initiation *in vivo*. Flamingo also has a cell-autonomous function in promoting axonal elongation and preventing premature axonal branching of PNS sensory neurons. Mutational analysis indicates that Flamingo function and the proper localization of Flamingo in dendrites and axons depend on the amino acid sequence near the first EGF domain. Altogether, these studies demonstrate that Flamingo controls several aspects of neuronal morphogenesis and exerts differential effects on initial dendritic and axonal growth.

MATERIALS AND METHODS

Fly Stocks and Genetic Crosses

The following fly lines obtained from the Bloomington Stock Center or other laboratories were used in this study: (1) $GAL4^{C155}$, *UAS-mCD8-GFP, hs-FLP/FM7*; (2) y^1 *w*;* FRT^{G13}*/Cyo*; (3) y^1 *w*;* FRT^{G13}*, tubP-GAL80/Cyo*.; (4) y^1 *w*;* $flamingo^{E59}$*/Cyo*; (5) y^1 *w*; GAL4–109(2)80, UAS-GFP,* $flamingo^{72}$*/Cyo*; and (6) y^1 *w*;* FRT^{G13}*, L/Cyo*. The following fly lines were constructed from the lines listed above: (1) $GAL4^{C155}$, *UAS-mCD8-GFP, hs-FLP1/FM7;* FRT^{G13}*, tubP-GAL80/Cyo*; (2) $GAL4^{C155}$, *UAS-mCD8-GFP, hs-FLP1/FM7;* FRT^{G13}*/Cyo*; (3) $GAL4^{C155}$, *UAS-mCD8-GFP, hs-FLP1/FM7;* $flamingo^{E59}$*/Cyo*; and (4) $GAL4^{C155}$, *UAS-mCD8-GFP, hs-FLP1/FM7;* $flamingo^{72}$*/Cyo*.

For MARCM experiments, $GAL4^{C155}$, *UAS-mCD8-GFP, hs-FLP1;* FRT^{G13}, *tubP-GAL80/*FRT^{G13}, *tubP-GAL80* male flies were crossed with $GAL4^{C155}$, *UAS-mCD8-GFP, hs-FLP1/FM7;* FRT^{G13}*/Cyo* or $GAL4^{C155}$, *UAS-mCD8-GFP, hs-FLP1/FM7;* $flamingo^{E59}$*/Cyo* virgin flies for further analysis.

Imaging Dendritic Morphology of the Single GFP-Labeled Neuron in Larvae

Drosophila embryos produced from the crosses described above were collected at 25°C for 2 h in vials containing standard medium. At 4–6 h after egg laying (AEL), embryos were heat-shocked in a 37°C water bath for 30–45 min to induce mitotic recombination. Vials were then kept at 25°C for 2–4 days. Larvae were collected and examined for the presence of a single GFP-labeled PNS neuron. For most of the experiments reported here, third instar larvae were used for image collection. Larvae containing a GFP-labeled neuron account for about 10% of the total larvae screened, depending on the heat shock efficiency. The selected larva was then immersed in 90% glycerol in phosphate-buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 10 mM phosphate buffer, pH 7.2) under a coverslip. Confocal images of dendritic morphology from the single mCD8–GFP-labeled neuron were obtained with a BioRad confocal microscope (Radiance 2000) and processed with Adobe Photoshop on an Apple G4 computer. Although it was thought that $GAL4^{C155}$ drives marker expression in all neurons (Lin and Goodman, 1994), we found that it also drives low-level expression of mCD8–GFP in

epithelial cells adjacent to PNS sensory neurons in embryos and larvae. Therefore, in larvae containing single mCD8–GFP-labeled neuron, we also often found single epithelial cells labeled by mCD8–GFP.

Immunohistochemistry

Flies were kept overnight in bottles with yeast–grape agar plates, and staged embryos were collected, dechorionated with 2.5% sodium hypochlorite, and washed with PBS and 0.1% Triton X-100. The embryos were fixed for 30 min at room temperature in heptane and 4% formaldehyde (1:1) in PBS and incubated for 30 min in blocking solution containing 5% normal goat serum and 0.1% Triton in PBS. Monoclonal antibody 22C10 (1:200) or anti-Flamingo antibody (1:10; gift of T. Uemura) was used as the primary antibody. Cy3-conjugated goat anti-mouse IgG (Jackson Laboratories; used at 1:200) was used as the secondary antibody. The embryos were mounted in 90% glycerol in PBS, and confocal images were obtained and processed as described above.

Identification of flamingo Mutations

Sense and antisense oligonucleotides approximately 1 kb apart were used to amplify a 12-kb genomic *flamingo* sequence by PCR. The PCR products from two independent reactions were cloned into PCR2.1-TOPO vector (Invitrogen), and at least two independent clones were sequenced. Sequences of the two 1-kb fragments were aligned with the *flamingo* cDNA sequence and the genomic sequence in the database to identify each EMS mutation. A nonsense mutation was found in *flamingo*^{72} mutant embryos at amino acid 2343, and a missense mutation was found in *flamingo*^{6} mutant embryos at the amino acid 1464 (cysteine to tyrosine).

RESULTS

GFP Labeling of Single PNS Sensory Neurons in Living Drosophila Larvae

Each abdominal hemisegment of *Drosophila* embryos and larvae contains 44 peripheral neurons that can be grouped into dorsal, lateral, and ventral clusters (Campos-Ortega and Hartenstein, 1985; Ghysen *et al.*, 1986; reviewed in Jan and Jan, 1993) (Fig. 1A). Our studies primarily focus on the dorsal cluster, which contains four external sensory (ES) neurons and eight MD neurons that can be further classified into three groups: one bipolar dendrite (BD) neuron, six dendritic arborization (DA) neurons, and one internal sensory neuron whose dendrites include a spring-like structure that contacts motorneuron axon terminals (Fig. 1B). GAL4 line 109(2) 80 labels all the eight MD neurons but not the four ES neurons in the dorsal cluster (Gao *et al.*, 1999).

To label single MD neurons in *Drosophila* larval PNS with GFP, we used the MARCM technique (Lee and Luo, 1999). This technique allows the labeling of a clone of cells by GFP due to the loss of the GAL80 repressor via FLP recombinase-mediated mitotic recombination in precursor cells. With the UAS-GAL4-targeted expression system (Brand and Perrimon, 1993), all of the larval PNS sensory

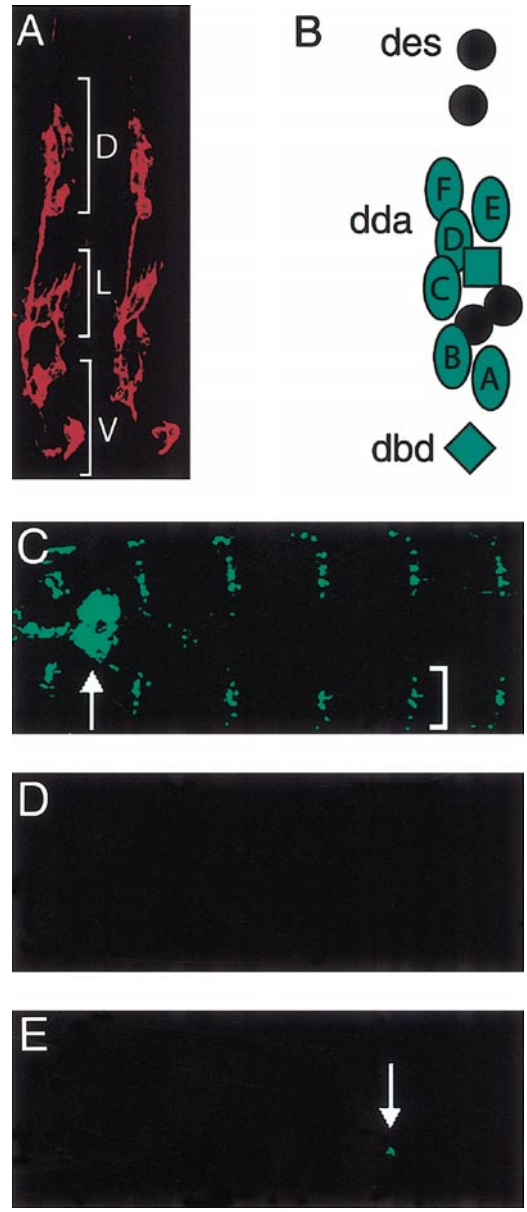

FIG. 1. GFP labeling of single neurons in Drosophila larvae. (A) 22C10 antibody staining of PNS sensory neurons in a wild-type stage 16 embryo. D, dorsal cluster; L, lateral cluster; V, ventral cluster. (B) Schematic representation of all 12 neurons in each dorsal cluster of abdominal hemisegments. Ovals, dda neurons; black circles, des neurons; diamond, dbd neuron; square, the internal sensory neuron whose dendrites contact motorneuron axons. The six dda neurons are ddaA, ddaB, ddaC, ddaD, ddaE, and ddaF. (C) Labeling of all the PNS and CNS neurons with mCD8–GFP. Bracket indicates all the 12 PNS sensory neurons in the dorsal cluster. Arrow indicates the CNS. Only a few segments are shown here. (D) Suppression of mCD8–GFP expression by the *tubP-Gal80* transgene. (E) mCD8–GFP labeling of a single dorsal cluster neuron. Genotypes: (A) *GAL4*^{C155}, *UAS-mCD8-GFP, hs-FLP1/+; FRT*^{G13}*/Cyo.* (B, C) *GAL4*^{C155}, *UAS-mCD8-GFP, hs-FLP1/+; FRT*^{G13}*/FRT*^{G13}, *tubP-GAL80.*

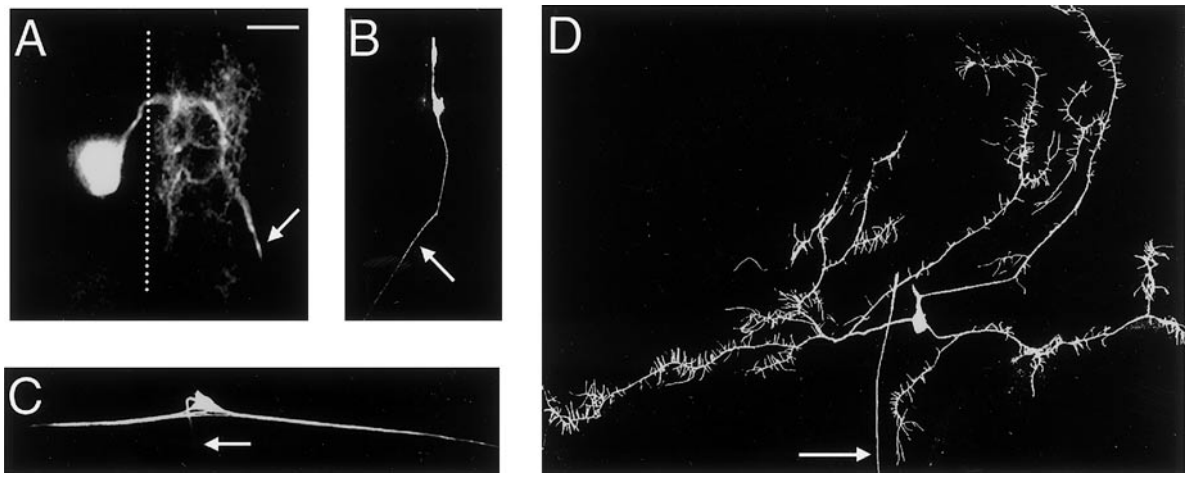

FIG. 2. Neuronal polarity and diversity of neuronal morphology in *Drosophila*. (A) A CNS neuron sends its axon past the ventral midline (dotted line). Its neurites elaborate three-dimensionally in the CNS. (B) An ES neuron in a dorsal cluster has a single dendrite extending dorsally in the opposite direction of axon extension. (C) A BD neuron in a dorsal cluster with two unbranched dendrites running along the anterior–posterior axis. (D) An MD neuron with numerous spine-like protrusions in a lateral cluster. Arrows indicate the axons of mCD8–GFP-labeled neurons. The bar represents 10 μm for (A) and 40 μm for (B–D).

neurons could be labeled by mCD8–GFP under the control of GAL4^{C155}, which allows marker expression in all postmitotic neurons (Lin and Goodman, 1994) (Fig. 1C). mCD8–GFP was targeted to the cell membrane; therefore, the cell body of each sensory neuron in each dorsal cluster could be easily identified in larval stages. In contrast, CNS neurons were tightly packed together and could not be distinguished individually (Fig. 1C). When GAL80 was ubiquitously expressed under the control of the *Drosophila* tubulin 1α promoter, mCD8–GFP expression was suppressed in all PNS sensory neurons (Fig. 1D). Since MD neuron precursor cells divide 5–7 h AEL, we heat-shocked embryos 4–6 h AEL to induce FLP recombinase expression and then examined larvae for the presence of single-neuron clones. We found that about 10% of the larvae contained a single PNS sensory neuron labeled by mCD8–GFP in one of the dorsal clusters (Fig. 1E). At a much lower frequency, we could find larvae in which two neurons were labeled by mCD8–GFP in the same dorsal cluster.

Morphological Diversity of PNS Sensory Neurons

With the MARCM-based single-neuron assay system, recombination events occur in a random fashion at low frequency. As there are only 12 PNS sensory neurons in each dorsal cluster, it is possible to find larvae in which only one of the 12 neurons is labeled by mCD8–GFP. Indeed, we obtained images of the dendritic branching patterns of each subtype of PNS neurons in the dorsal cluster, as well as images of other PNS sensory neurons in the lateral and ventral clusters. Since our genetic studies mainly focus on the dorsal cluster, only the development of dendritic fields of dorsal cluster MD neurons is described here in detail.

We found that, even in the relatively simple *Drosophila* nervous system, different neurons developed strikingly diverse dendritic morphologies. *Drosophila* CNS neurons are unipolar cells similar to those in other insects (Shankland and Goodman, 1982). The CNS neuron in Fig. 2A sends its axon across the ventral midline and elaborates neuronal processes in a three-dimensional manner that makes it difficult to reconstruct the neuron's morphology. This complex morphology also prevents clear differentiation of its dendritic and axonal processes. In contrast, the morphological polarity of PNS sensory neurons is much easier to define. For example, each ES neuron extends a single dendrite without further branching in the direction opposite to that of its axon (Fig. 2B). Dorsal cluster BD (dbd) neurons extend two unbranched dendrites along the anterior–posterior axis and an axon ventrally toward the CNS (Fig. 2C). Most MD neurons develop highly diverse dendritic branching patterns (Fig. 2D) and share morphological similarity with dendrites of mammalian CNS neurons, such as the tapering of dendrites with further branching and extension (Craig and Banker, 1994). The dendritic complexity of some MD neurons is also comparable to that of many mammalian CNS neurons (Ramón y Cajal, 1911).

Some MD neurons (Fig. 2D) have processes similar to the "headless" spines on many developing mammalian neurons (Peters and Kaiserman-Abramof, 1970). These processes are typically 5–10 μm long in third instar larvae, extend to both side of the dendritic branches, and are more numerous on dendrites distal to the cell body. Though the physiological function of these processes in *Drosophila* is unclear, further studies with single-neuron resolution might enhance our understanding of the formation and the maintenance of these fine structures *in vivo*.

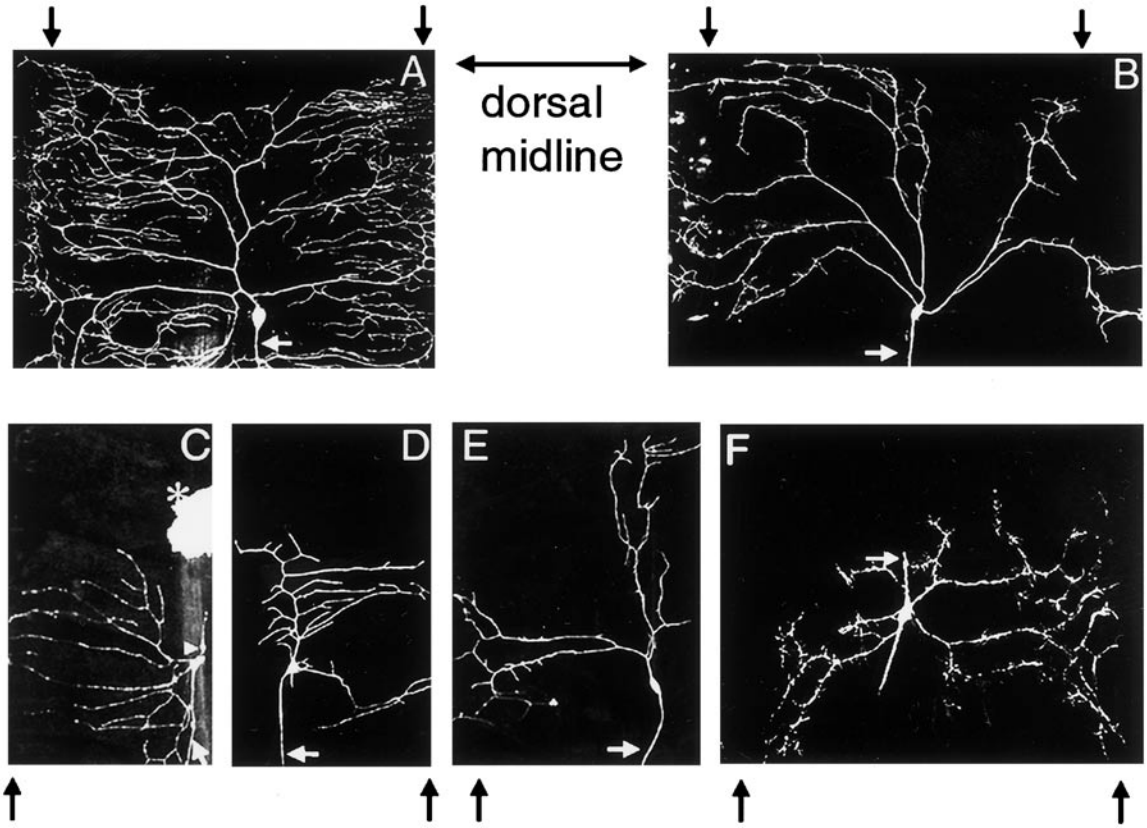

FIG. 3. Dendritic fields of the six dda neurons in the dorsal cluster in wild-type larvae. (A) The ddaC neuron sends out dendrites that cover a large area from the anterior segment boundary to the posterior segment boundary and from the dorsal midline to the lateral cluster. (B) The ddaD neuron has fewer dendritic branches than ddaC neuron. (C) Dendrites of the ddaF neuron only cover the anterior half of the hemisegment. GAL4^{C155} also drives low-level expression of CD8–GFP in epithelial cells. Therefore, CD8–GFP-labeled single epithelial cells can be seen in some larvae (asterisk). The arrowhead indicates an ES neuron. (D) Dendrites of the ddaE neuron only cover the posterior half of the hemisegment. (E) The ddaB neuron extends dendrites to the anterior segment boundary and the dorsal midline. (F) The ddaA neuron extends dendrites along the anteroposterior axis. The arrow indicates an ES neuron. Black vertical arrows indicate segment boundaries.

Development and Organization of MD Neuron Dendritic Fields in the Dorsal Cluster

Most MD neurons in the dorsal cluster, including the BD neuron and the six DA neurons, elaborate their dendrites underneath the epidermis. These neurons probably function as touch receptors or proprioceptors (Bodmer and Jan, 1987). Labeling single PNS neurons in living larvae allowed us to study how the dendritic fields of different MD neurons are formed and organized in the same dorsal cluster, although their cell bodies are close to each other.

Our single-neuron analysis also reveals that each MD neuron has a defined dendritic field. Among larvae at the same stage, the general branching pattern for a particular MD neuron remains the same. For instance, the ddaC neuron sends its primary dendrite dorsally; this dendrite soon branches into secondary and tertiary branches that cover the whole hemisegment from the anterior segment boundary to the posterior segment boundary (Fig. 3A) and from the dorsal midline to the lateral cluster of PNS neurons (not shown). The ddaC neuron also extends many smaller dendritic processes more or less parallel to the dorsal midline. Interestingly, these dendritic processes from the same ddaC neuron never overlap (Fig. 3A), suggesting a "self-avoidance" mechanism, as previously described for axonal branches of a single mechanosensory neuron in leeches (Kramer *et al.*, 1985; Kramer and Stent, 1985; Wang and Macagno, 1998). Similar to the ddaC neuron, the ddaD neuron in the dorsal cluster sends dendrites covering the area between segment boundaries (Fig. 3B). However, it only sends out four or five major branches to cover the area between the dorsal midline and its cell body, with fewer smaller branches. Some terminal branches of the ddaD neuron appear to cross the segment boundaries (Fig. 3B). Unlike the ddaC and ddaD neurons, the four other DA

neurons in the dorsal cluster have more restricted dendritic fields. The ddaF and ddaE neurons send out only a few branches toward either the anterior or the posterior segment boundaries with their most dorsal dendritic branches falling short of the dorsal midline. Strikingly, unlike other MD neurons, none of the dendritic branches of the ddaF and ddaE neurons have any spine-like processes (Figs. 3C and 3D). The ddaB neuron has one or two dendritic branches that reach the dorsal midline, and a few branches that extend toward the anterior segment boundary (Fig. 3E). The ddaA neuron is the most lateral MD neuron in the dorsal cluster and extends its dendrites mainly along the anteroposterior axis (Fig. 3F). The dendritic fields of the ddaC and ddaD neurons overlap with each other and with the dendritic fields of four other MD neurons. However, the dendritic fields of ddaE and ddaF neurons have minimal overlap. The presence of different dendritic fields in the same dorsal cluster raises the possibility that each MD neuron has a defined physiological function and that the dendritic morphology of each MD neuron is largely determined by its intrinsic properties.

Flamingo Has a Cell-Autonomous Function in Postmitotic Neurons to Control the Extension of Dorsal Dendrites of MD Neurons

Previous studies have identified Flamingo as a regulator of dendritic growth (Gao *et al.*, 1999, 2000) and of the spindle orientation in sensory organ precursor (SOP) cells (Lu *et al.*, 1999). To address whether Flamingo has a direct role in controlling neuronal morphogenesis in postmitotic neurons and to assess the cell-autonomous function of Flamingo, we generated single neuron mutant clones homozygous for the *flamingo*^{E59} (Usui *et al.*, 1999) and *flamingo*^{72} (Gao *et al.*, 1999) alleles in wild-type larvae.

When dorsal cluster MD neurons were devoid of *flamingo* gene activity, one or more dendritic processes overextended toward the dorsal midline in about 15% of more than 100 *flamingo*^{E59} mutant neurons (Figs. 4A–4C) and in about 9% of 80 *flamingo*^{72} mutant neurons (Fig. 4D). Surprisingly, the basic architecture of the dendritic branching patterns was not obviously altered. For instance, ddaC neuron still extended a primary dendrite dorsally, which branched into several secondary and tertiary dendrites (Fig. 4A). In addition, the ddaF or ddaE neuron still sent out lateral branches normally toward either the anterior or posterior segment boundaries (Figs. 4B–4D). The total length of the lateral branches of ddaF or ddaE neurons and the dendritic fields covered by these branches also appeared to be normal. For instance, the average total length of the lateral branches of wild-type ddaE neurons is 1.2 ± 0.2 mm (n = 10), which is the same as that of *flamingo* mutant ddaE neurons (1.3 ± 0.2 mm, n = 4). In some cases, two *flamingo* mutant MD neurons in opposite dorsal clusters were simultaneously labeled by mCD8–GFP, and their overextended dorsal dendrites crossed the dorsal midline and invaded the dendritic field of another MD neuron in the opposite hemisegment

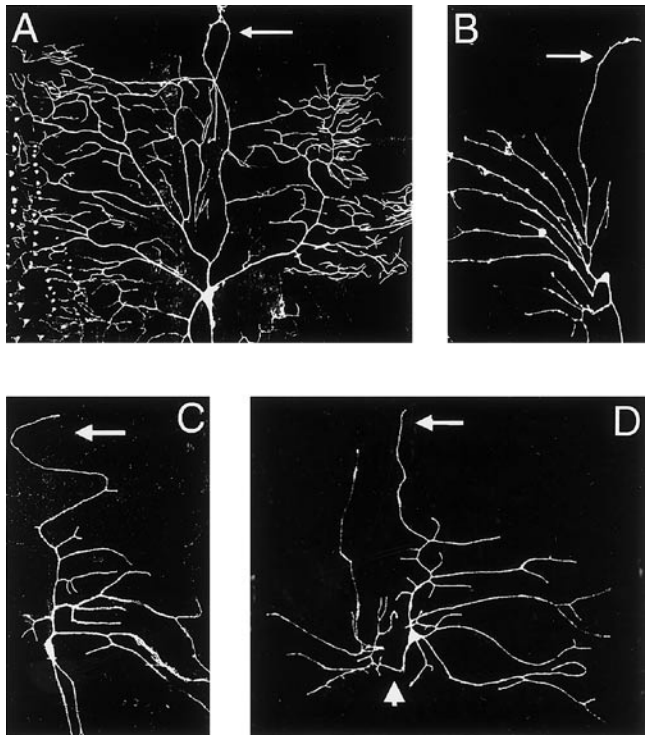

FIG. 4. Dendritic phenotypes of single *flamingo* mutant neurons. (A) A *flamingo*^{E59} mutant ddaC neuron. (B) A *flamingo*^{E59} mutant ddaF neuron. (C) A *flamingo*^{E59} mutant ddaE neuron. (D) A *flamingo*^{72} mutant ddaE neuron. The arrowhead in (D) indicates the axon that fails to develop normally. The axonal phenotype of ddaE neuron is described later together with Fig. 6. Arrows indicate overextended dendritic branches. The general branching patterns of *flamingo* mutant neurons are approximately the same as the wild type neurons in Fig. 3.

(data not shown). These data demonstrate that Flamingo does not control the general dendritic branching patterns of MD neurons. Instead, Flamingo has a cell-autonomous function in limiting the extension of dorsal dendrites with little or no effect on lateral branches. These studies also demonstrate that Flamingo plays a direct role in dendritic morphogenesis in postmitotic neurons, which is independent of its function in precursor cells.

Flamingo Controls the Timing of Dorsal Dendrite Initiation

To further study how Flamingo regulates dorsal dendrite extension with gross effect on lateral dendrites, we investigated the role of Flamingo in the timing of dorsal dendrite initiation. We collected either wild-type or *flamingo* mutant embryos at 11–12 h AEL. Homozygous *flamingo* mutant embryos could be easily identified by the absence of the specific GFP expression pattern driven by *Krüppel GAL4* on the Cyo balancer chromosome. MD neurons in

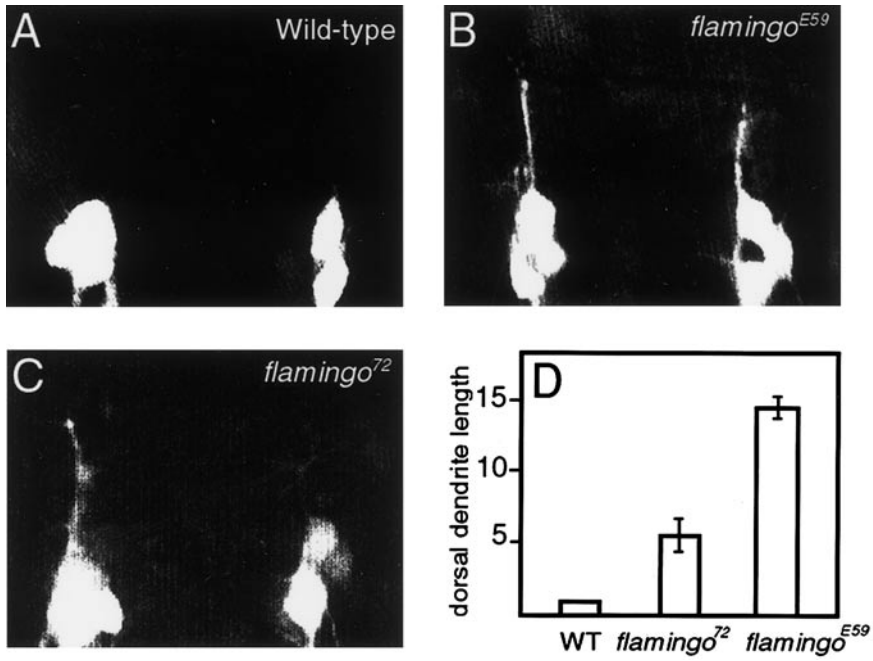

FIG. 5. Flamingo inhibits precocious initiation of dorsal dendrites. (A) A wild-type embryo. (B) A *flamingo*^{E59} mutant embryo. (C) A *flamingo*^{72} mutant embryo. All the embryos are 11–12 h AEL and labeled by GFP under the control of *GAL4 109(2) 80*. (D) The average length (μm) of dorsal dendrites in abdominal hemisegments (A1 to A6) of wild-type or mutant embryos.

these embryos were labeled by GFP under the control of *GAL4 109(2) 80*; therefore, the initiation of their dorsal dendrites could be directly visualized in living embryos. In wild-type embryos at 11–12 h AEL, the dorsal dendrites of MD neurons had not yet extended (Fig. 5A; also in Gao *et al.*, 1999). However, in *flamingo* mutant embryos at the same stage, MD neuron dorsal dendrites in many hemisegments had already initiated (Figs. 5B and 5C). These dendritic processes appeared to be thin. It is possible that the processes extended from one MD neuron in the dorsal cluster. This precocious dendritic initiation phenotype was found in 34, 46, and 74% of abdominal hemisegments (A1 to A6) in *flamingo*^{72}, *flamingo*^{6}, and *flamingo*^{E59} mutant embryos, respectively. The average length of dorsal dendrites per abdominal hemisegment in wild-type or *flamingo* mutant embryos at 11–12 h AEL is presented in Fig. 5D. The early initiation of MD neuron dorsal dendrites in *flamingo* mutant embryos was also confirmed by the immunostaining analysis with 22C10 antibody (data not shown). During embryogenesis, dorsal dendrite extension stops at about 16 h AEL, before lateral branching occurs (Gao *et al.*, 1999). In *flamingo* mutant embryos at 16 h AEL, the dorsal dendrites are longer than that in wild-type embryos at the same stage (data not shown). These findings suggest that Flamingo regulates the length of dorsal dendrites partially by controlling the timing of dorsal dendrite initiation. We observed that the rate of extension at 13–15 h AEL is indistinguishable in wild-type and *flamingo* mutant embryos (data not shown).

Cell-Autonomous Function of Flamingo in Axonal Growth and Guidance

Flamingo is expressed on both dendrites and axons of MD neurons (Gao *et al.*, 2000), suggesting that Flamingo may also regulate axonal morphogenesis. To investigate whether the *flamingo* mutations that affect dendritic initiation and extension also affect axon development, we examined the axons of single MD neurons containing *flamingo*^{E59} mutations in wild-type larvae. In 10% of more than 100 *flamingo*^{E59} mutant neurons, their axons did not fully extend to the CNS (Fig. 6A). This finding was consistent with the axonal phenotype in living *flamingo*^{E59} mutant embryos, in which axonal break points could be found in the axon bundles of dorsal cluster MD neurons (Figs. 6B and 6C). In addition, the axons of PNS neurons failed to fasciculate as tightly as those of wild-type embryos (Fig. 6B). Axonal breaks were previously found in the CNS of *flamingo* mutant embryos (Usui *et al.*, 1999). Interestingly, 70% of the mutant axons that failed to fully extend also branched at their termini (e.g., the ddaF neuron in Fig. 6D), while wild-type PNS axons never branched before reaching the CNS. In some cases, a thin process derived from the axon extended dorsally (Fig. 6A). Similar axonal phenotypes were found in *flamingo*^{72} mutant single neurons (Fig. 4D). These

studies demonstrate that Flamingo has a cell-autonomous function in promoting axonal elongation and in preventing premature branching of axons before reaching their synaptic targets.

Next, we asked whether Flamingo also affects axon guidance. We found that the axons of some *flamingo* mutant ES neurons veered dramatically from the normal path before halting in ectopic locations (Fig. 6E). Similar pathfinding defects were also found for axons of MD neurons. These findings suggest that Flamingo has a cell-autonomous function in controlling axon guidance either directly or indirectly.

Flamingo Domains Essential for Its Function

To understand Flamingo function in neuronal morphogenesis, we carried out molecular analysis on different *flamingo* alleles that all exhibit similar defects in dendritic initiation and extension. Studies on the $flamingo^{E59}$, $flamingo^{72}$, and $flamingo^{6}$ alleles are presented here.

To understand the molecular nature of the mutations in the *flamingo* gene, we sequenced the *flamingo* gene region of the genomic DNA isolated from *flamingo* mutant embryos. We found a nonsense mutation in the $flamingo^{72}$ allele that would result in a truncated Flamingo ectodomain with 2342 amino acids that contains all the nine cadherin motifs, two laminin A globular domains, and four EGF motifs (Fig. 7A). We also identified a missense mutation in the $flamingo^{6}$ allele that resulted in a single amino acid change near the first EGF motif (Fig. 7A). The nonsense mutation in the $flamingo^{E59}$ allele was previously identified by Usui *et al.* (1999).

We used anti-Flamingo N terminus antibody (Usui *et al.*, 1999) to analyze Flamingo expression patterns in stage 14 *flamingo* mutant embryos. The wild-type Flamingo protein was expressed on cell membranes of all dorsal cluster neurons (Fig. 7B; also in Gao *et al.*, 2000). Flamingo was barely detectable on MD neurons in $flamingo^{E59}$ mutant embryos (Fig. 7C). However, we detected Flamingo expression on cell bodies of MD neurons in $flamingo^{72}$ mutant embryos (Fig. 7D), although the level of Flamingo expression was two- to three-fold lower than that in stage 14 wild-type embryos. This finding suggests that the transmembrane domain of Flamingo is required for its normal function.

We also found that Flamingo was expressed on MD neuron cell bodies in $flamingo^{6}$ mutant embryos (Fig. 7E), at a level even higher than that in wild-type embryos. To understand how this mutation affects Flamingo function, we analyzed Flamingo localization in detail. The mutant Flamingo protein failed to localize to the dendrites of MD neurons in $flamingo^{6}$ mutant embryos (Figs. 8A–8C) or to the axons of both PNS and CNS neurons (Figs. 8D and 8E). The mislocalization resulted in the higher level of immunostaining on cell bodies of MD neurons (Fig. 7D). These findings indicate that the amino acid sequence near the first EGF motif affects the normal localization and function of Flamingo.

DISCUSSION

To study how neuronal morphogenesis is controlled by intracellular factors during development, we used the MARCM technique (Lee and Luo, 1999) to visualize single wild-type or mutant PNS neurons in living *Drosophila* larvae. We found that dendritic fields of MD neurons in the dorsal cluster are highly organized. Individual MD neurons have their own specific, well-defined dendritic fields. Genetic manipulation in single neurons revealed that Flamingo controls the formation of MD neuron dendritic fields by limiting the extension of one or more dorsally oriented branches with minimal effects on lateral branches. In addition, Flamingo is required cell autonomously for promoting axonal growth and for preventing premature axonal branching *in vivo*. Our further analyses indicate that Flamingo controls the timing of dorsal dendrite initiation, and that the amino acid sequence near the first EGF motif is required for the proper localization and function of Flamingo.

An Assay System to Study Intrinsic Mechanisms Controlling Neuronal Morphogenesis in Living Drosophila Larvae

Many genes function in several biological processes at multiple developmental stages. Consequently, mutations in these genes often cause pleiotropic developmental defects. An ideal approach for studying the roles of intrinsic factors in neuronal morphogenesis would be to manipulate gene activity in a single postmitotic neuron *in vivo* without disturbing the surrounding environment. Therefore, pleiotropic effects of the gene could be eliminated. Taking advantage of the MARCM technique (Lee and Luo, 1999), we were able to study the dendritic and axonal growth of single identifiable MD neurons in living *Drosophila* larvae (Fig. 1).

MD neurons are an excellent model system because the mechanisms that control their generation have been well studied (Bodmer *et al.*, 1989; Brewster and Bodmer, 1995; Vervoort *et al.*, 1997; Orgogozo *et al.*, 2001; reviewed in Jan and Jan, 2000). Some MD neurons share a common precursor cell and therefore belong to "solo" MD lineages that do not produce other cell types (Bodmer *et al.*, 1989; Brewster and Bodmer, 1995). Other dorsal cluster MD neurons are generated from MD–ES lineages that also give rise to ES neurons and support cells in ES organs (Brewster and Bodmer, 1995); however, the exact order of cell division in MD–ES lineages remains controversial (Vervoort *et al.*, 1997; Orgogozo *et al.*, 2001). If the FLP recombinase-mediated recombination occurs before the formation of the MD neuron precursor cell, then two MD neurons or the MD neuron and the ES neuron in the same lineage will be

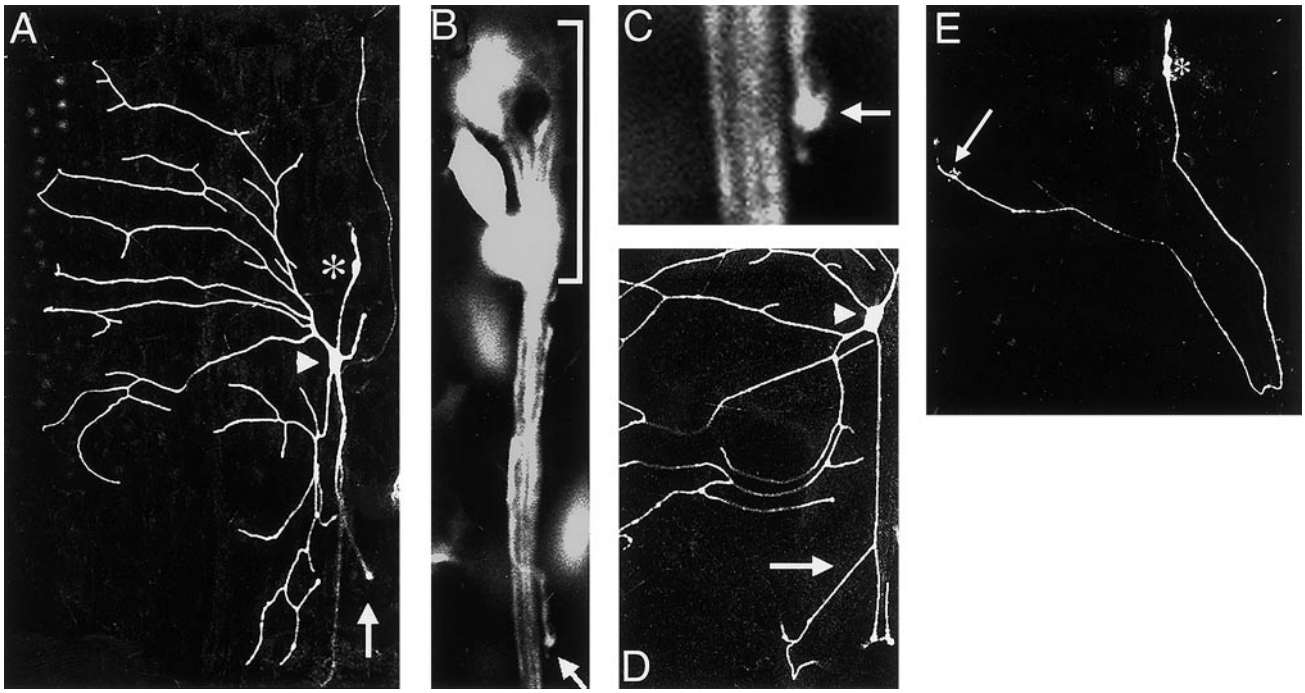

FIG. 6. Axonal defects in *flamingo* mutant neurons. (A) A mCD8–GFP-labeled two-neuron clone in a dorsal cluster. Asterisk indicates the cell body of an ES neuron. The arrowhead indicates the cell body of the ddaF neuron. The arrow shows the axonal break point of the ddaF neuron. A thin process derived from the axon extends dorsally. (B) GFP-labeled dorsal cluster MD neurons in a living *flamingo* mutant embryo. Bracket indicates the cell bodies of MD neurons. Arrow indicates axonal break point of the axon from one dorsal cluster neuron. (C) An enlarged image of the area indicated by the arrow in (B). (D) A mCD8–GFP-labeled ddaF neuron. Arrow indicates premature termination and branching of the axon. Arrowhead indicates the cell body. (E) A *flamingo* mutant ES neuron in the dorsal cluster. Arrow indicates the axon that terminates at the wrong place. Asterisk indicates the cell body.

labeled by GFP. Therefore, the presence of a single mCD8–GFP-labeled MD neuron itself indicates that the somatic recombination occurs during the last cell division that gives rise to the MD neuron. Since most MD neurons except BD neurons are not associated with support cells (Brewster and Bodmer, 1995; Vervoort *et al.*, 1997; Orgogozo *et al.*, 2001), the mCD8–GFP-labeled MD neuron will be the only mutant cell, whereas other cells in the lineage and other neurons in the same cluster remain wild type. If both an MD neuron and an ES neuron are labeled by GFP, it may indicate that the two neurons are derived from the same cell lineage. For instance, our study indicates that ddaF neuron is generated from the MD–ES lineage (Fig. 4).

Using this assay system to study neuronal morphogenesis with single-neuron resolution offers several advantages. First, each dorsal cluster contains only a few neurons, which elaborate their dendrites in a relatively two-dimensional plane. We can study dendritic and axonal growth of the same identifiable MD neuron *in vivo* and compare wild-type and mutant neurons with ease and precision. Second, we can continuously image the dendrites or the spine-like processes of a single wild-type or mutant MD neuron in a living animal over a period of several days. Third, since the FRT-mediated somatic recombination can occur during the last cell division that gives rise to the MD neuron, the cell-autonomous function of a gene in dendritic morphogenesis and axonal growth can be demonstrated independently of effects the gene may have on the proliferation or cell fate of neuronal precursor cells (Lee and Luo, 1999). Such a single-neuron assay system could be used to identify and characterize intracellular factors that affect neuronal morphogenesis. One potential drawback is the perdurance of wild-type protein and mRNA in the GFP-labeled mutant single neurons, which may mask the mutant phenotype during early stages of neuronal differentiation for some genes. This is probably why a low percentage of *flamingo* mutant single neurons show either dendritic or axonal defects.

How Are the Dendritic Fields of Different MD Neurons Specified during Development?

Individual MD neurons in the dorsal cluster have specific and distinct dendritic fields. The dendrites of the ddaC neuron cover the whole area of the hemisegment, whereas other MD neurons have their own unique territories. This

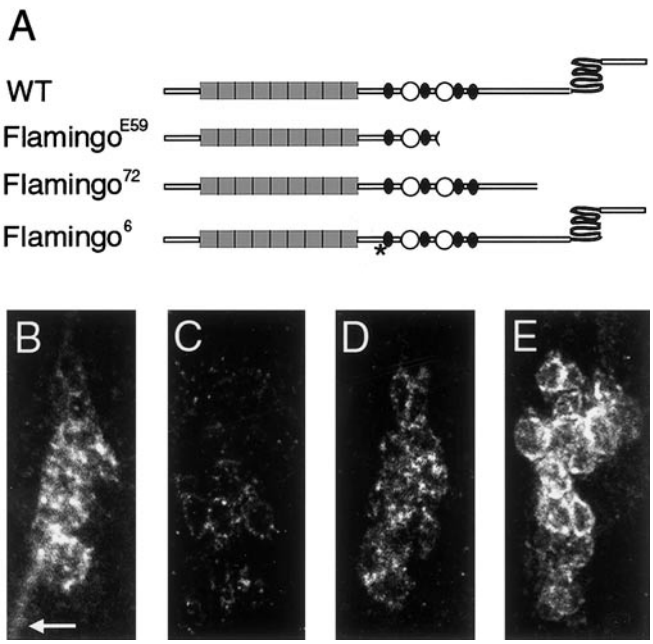

FIG. 7. Expression of mutant Flamingo proteins on dorsal cluster PNS neurons. (A) Mutations in different *flamingo* alleles. Squares, cadherin motifs; open circles, laminin A globular domains; ovals, EGF motifs. The *flamingo*^{E59} allele was characterized by Usui *et al.* (1999). A nonsense mutation was found in the *flamingo*^{72} allele that resulted in a truncated Flamingo fragment with 2343 amino acids, and a missense mutation (asterisk) was found about 18 amino acids away from the first EGF motif in the *flamingo*^{6} allele. Anti-Flamingo N terminus antibody was used to detect Flamingo expression in stage 14 wild-type embryos (B), *flamingo*^{E59} mutant embryos (C), *flamingo*^{72} mutant embryos (D), and *flamingo*^{6} mutant embryos (E). The arrow indicates the axons of dorsal cluster neurons in wild-type embryos.

finding suggests that each MD neuron has a specific physiological function in the *Drosophila* PNS, as in the vertebrate retina where different cell types with unique shapes represent distinct physiological entities (Masland, 2001). Indeed, Pickpocket, a *Drosophila* protein homologous to vertebrate epithelial Na^+ channel molecules, is only expressed in one MD neuron in the dorsal cluster (Adams *et al.*, 1998). It is reasonable to speculate that other unidentified channel molecules may be specifically expressed in different MD neurons to carry out certain sensory functions. In addition, the axons of different MD neurons project into different regions of the CNS, indicating different functions of MD neurons in neuronal circuitry (Schrader and Merritt, 2000).

How are the dendritic fields of MD neurons in *Drosophila* PNS specified during development? A laser ablation study in *Drosophila* suggested that one subtype of MD neurons had no effect on the formation of dendritic field of other MD neurons in the same dorsal cluster (Gao *et al.*, 2000). In addition, competition between dendrites of homologous neurons near the dorsal midline plays a role in defining their dendritic fields (Gao *et al.*, 2000). However, the competition mechanism probably functions as a means to fine tune the mature dendritic territory, as occurs in the retina, where adjacent ganglion cell dendrites compete to define their territories (Perry and Linden, 1982). The close clustering of the MD neuron cell bodies in the same area suggests that intrinsic properties of each MD neuron may play major roles in determining the size and location of its dendritic field. One clue may come from the fact that different MD neurons are derived from different cell lineages (Bodmer *et al.*, 1989; Brewster and Bodmer, 1995; Vervoort *et al.*, 1997; Orgogozo *et al.*, 2001). The lineage of a particular MD neuron may dictate the combination or activity of cell-intrinsic factors it may inherit or express, which in turn may control the development of its specific dendritic field. The mechanisms could be, in principle, similar to neuronal fate determination by a combination of transcriptional factors in the spinal cord (Jessell, 2000). Consistent with the idea, proneural genes *achaete* and *scute* are required for the formation of some MD neurons derived from MD–ES lineages, while *amos* is required for the generation of other MD neurons (Huang *et al.*, 2000). Although individual MD neuron-specific factors have not been identified, it seems that the novel zinc finger protein Sequoia is required for the normal dendrite development of most, if not all, MD neurons (Brenman *et al.*, 2001). The identification of additional intrinsic factors and the differential regulation of the activities of the same factors in individual MD neurons will help us further understand how individual MD neurons respond differentially to the same environment to control the formation of their specific dendritic fields.

Specific Function of Flamingo in Controlling Dendritic Fields

In studies of neuronal morphogenesis, it is important to differentiate the direct and indirect effects of the gene of interest. Similar to other important regulators, Flamingo functions in different cell types and at different developmental stages (Usui *et al.*, 1999; Chae *et al.*, 1999; Lu *et al.*, 1999; Gao *et al.*, 2000). Our studies here provide evidence that Flamingo has a direct role in controlling dorsal dendritic growth in postmitotic neurons.

Although individual MD neurons in the dorsal cluster differ greatly in their dendritic fields, the defects caused by *flamingo* mutations appear to be similar: mostly one process of the mutant neurons overextends toward the dorsal midline. Surprisingly, the general dendritic architecture of these MD neurons is not affected dramatically (Fig. 4). In addition, our findings suggest that Flamingo is not a cell-type-specific regulator of dendritic morphology, nor does it affect dendritic branching patterns in a global way. It seems that Flamingo functions cell autonomously in controlling dendritic fields of different MD neurons by limiting the overextension of their dorsal dendrites. On the contrary,

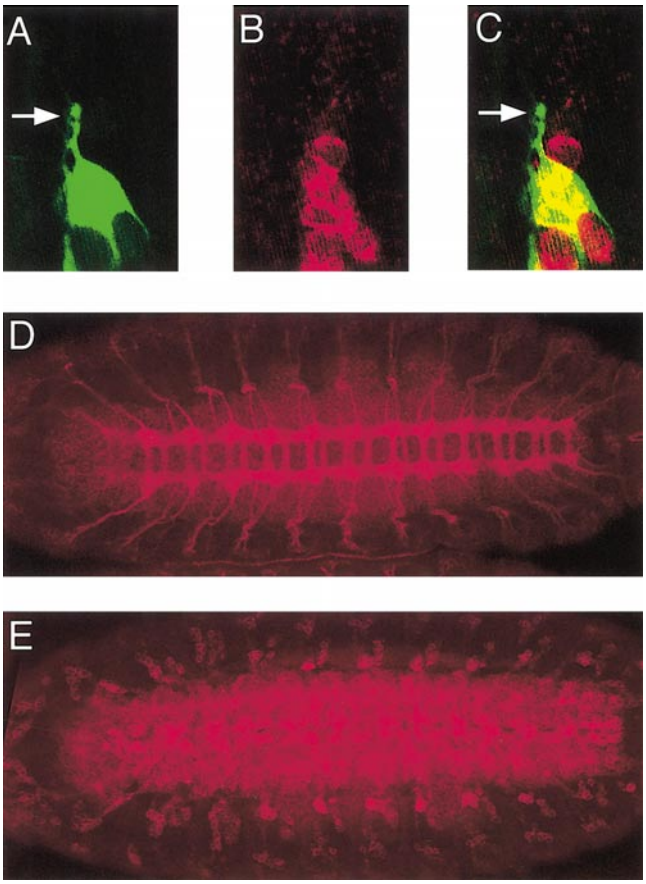

FIG. 8. The amino acid sequence near the first EGF motif is required for the proper localization of Flamingo. MD neurons in stage 14 *flamingo*^{6} mutant embryos extend their dorsal dendrites precociously (A). Flamingo is expressed on MD neurons (B) but is not detectable on dorsal dendrites (C) in *flamingo*^{6} mutant embryos. Similarly, Flamingo is highly localized to axon tracks in the wild-type CNS (D) and to the neuronal cell bodies in *flamingo*^{6} mutant embryos (E). Arrows indicate the precociously extending dendrite.

other mutants identified from the genetic screen (Gao *et al.*, 1999), such as *tumbleweed*, appear to affect both dorsal and lateral dendrites in a more general way. Our studies also demonstrate that Flamingo function in neuronal morphogenesis is independent of its function in precursor cells.

How does Flamingo mainly control dorsal dendrite extension in postmitotic neurons? In a previous study, we found that neuronal morphogenesis of dorsal cluster MD neurons can be separated into relatively discrete developmental phases. These neurons always extend their axons first toward the ventral nerve cord. The extension of dorsal dendrites toward the dorsal midline ceases at 16–17 h AEL, before lateral dendrites extend toward the adjacent segment boundaries (Gao *et al.*, 1999). If different development phases are controlled by different mechanisms, Flamingo may function mainly during dorsal dendrite extension. Indeed, we found that Flamingo also prevents precocious initiation of dorsal dendrites that contributes to the longer dorsal dendrites before 16 h AEL in *flamingo* mutant embryos (Fig. 5). The failure to stop after 17 AEL also contributes to the longer dorsal dendrites in *flamingo* mutant embryos (Gao *et al.*, 2000). Accordingly, the level of *flamingo* mRNA expression decreases during late embryogenesis (Chae *et al.*, 1999), and Flamingo expression also decreases in the first instar larvae (data not shown). The results presented here indicate that Flamingo has a function during early neuronal differentiation to control the initiation and extension of dorsal dendrites.

How does Flamingo function at the molecular level? Flamingo contains seven transmembrane segments homologous to a subset of G protein-coupled receptors, however, whether Flamingo functions through G protein signaling pathway is still unknown. Our finding that the extracellular domain of Flamingo is still present on MD neuron membranes in *flamingo*^{72} mutant embryos indicates that the transmembrane segments are important for Flamingo function. In addition, our mutational analysis suggests that the amino acid sequence near the first EGF motif is required for the proper subcellular localization and function of Flamingo (Fig. 8). Whether the first EGF motif is directly involved in protein–protein interactions or whether the mutation affects the folding and/or trafficking of Flamingo remains to be determined. It would be useful to identify the proteins that interact with the first EGF motif and other domains of Flamingo.

Flamingo Also Functions Cell Autonomously in Controlling Axonal Development

GFP labeling of single mutant neurons provides an opportunity to study whether genes that control dendrite development also affect other aspects of neuronal morphogenesis, such as axon growth. We found that Flamingo is required cell autonomously for promoting axonal growth. Since axons extend several hours earlier than dendrites, it is possible that the perdurance of Flamingo prevents the appearance of axonal phenotypes in some mutant neurons. Indeed, more *flamingo* mutant neurons exhibited dendritic defects than axonal defects.

Different molecules downstream of Flamingo might mediate its differential functions in dendrites and axons; alternatively, the same downstream molecules could transduce different signals in the two compartments (e.g., through the cyclic nucleotide pathway; Song *et al.*, 1998; Polleux *et al.*, 2000). Further dissection of the Flamingo pathway will help to elucidate how dendritic initiation and axonal growth are coordinated during development. Since Flamingo is highly conserved from flies to humans (Usui *et al.*, 1999; Chae *et al.*, 1999), it is likely that Flamingo also plays an essential role in controlling neuronal morphogenesis in mammals.

ACKNOWLEDGMENTS

We thank T. Uemura, Y.-N. Jan, L. Luo, Bloomington Stock Center, and Berkeley *Drosophila* Genome Center for antibodies and fly lines. We thank S. Ordway and G. Howard for editorial assistance, N. Do for technical assistance, and T. Jue for manuscript preparation. We are grateful to C. Bargmann, Y.-M. Chan, and other colleagues for comments on the manuscript. This work is supported by set-up funds from the Gladstone Institute of Neurological Disease and the University of California, San Francisco (F-B.G.). N.T.S. is a recipient of a National Science Foundation Predoctoral Fellowship. F.-B.G. is a recipient of an Alfred P. Sloan Fellowship in Neuroscience.

REFERENCES

Adams, C. M., Andersen, M. G., Motto, D. G., Price, M. P., Johnson, W. A., and Welsh, M. J. (1998). Ripped pocket and pickpocket, novel *Drosophila* DEG/EnaC subunits expressed in early development and in mechanosensory neurons. *J. Cell Biol.* **140,** 143–152.

Bodmer, R., Carretto, R., and Jan, Y. N. (1989). Neurogenesis of the peripheral nervous system in *Drosophila* embryos: DNA replication patterns and cell lineages. *Neuron* **3**, 21–32.

Bodmer, R., and Jan, Y. N. (1987). Morphological differentiation of the embryonic peripheral neurons in *Drosophila*. *Wilhelm Roys's Arch. Dev. Biol.* **196,** 69–77.

Bradke, F., and Dotti, C. G. (2000). Establishment of neuronal polarity: Lessons from cultured hippocampal neurons. *Curr. Opin. Neurobiol.* **10,** 574–581.

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118,** 401–415.

Brenman, J. E., Gao, F.-B., Jan, L. Y., and Jan, Y. N. (2001). Sequoia, a tramtrack-related zinc finger protein, functions as a pan-neural regulator for dendrite and axon morphogenesis in *Drosophila*. *Dev. Cell* **1**, 667–677.

Brewster, R., and Bodmer, R. (1995). Origin and specification of type II sensory neurons in *Drosophila*. *Development* **121**, 2923–2936.

Brose, K., and Tessier-Lavigne, M. (2000). Slit proteins: Key regulators of axon guidance, axonal branching, and cell migration. *Curr. Opin. Neurobiol.* **10,** 95–102.

Campos-Ortega, J. A., and Hartenstein, V. (1985). "The Embryonic Development of *Drosophila melanogaster*," Second edition. Springer-Verlag, New York.

Cantallops, I., Haas, K., and Cline, H. T. (2000). Postsynaptic CPG15 promotes synaptic maturation and presynaptic axon arbor elaboration *in vivo*. *Nat. Neurosci.* **10,** 1004–1011.

Chae, J., Kim, M. J., Goo, J. H., Collier, S., Gubb, D., Charlton, J., Adler, P. N., and Park, W. J. (1999). The *Drosophila* tissue polarity gene starry night encodes a member of the protocadherin family. *Development* **126,** 5421–5429.

Craig, A. M., and Banker, G. (1994). Neuronal polarity. *Annu. Rev. Neurosci.* **17,** 267–310.

Engert, F., and Bonhoeffer, T. (1999). Dendritic spine changes associated with hippocampal long-term synaptic plasticity. *Nature* **399,** 66–70.

Feng, G., Mellor, R. H., Bernstein, M., Keller-Peck, C., Nguyen, Q. T., Wallace, M., Nerbonne, J. M., Lichtman, J. W., and Sanes, J. R. (2000). Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* **28,** 41–51.

Gao, F.-B., Brenman, J. E., Jan, L. Y., and Jan, Y. N. (1999). Genes regulating dendritic outfrowth, branching, and routing in *Drosophila*. *Genes Dev.* **13**, 2549–2561.

Gao, F.-B., Kohwi, M., Brenman, J. E., Jan, L. Y., and Jan, Y. N. (2000). Control of dendritic field formation in *Drosophila*: The roles of Flamingo and competition between homologous neurons. *Neuron* **28**, 91–101.

Ghysen, A., Dambly-Chaudière, C., Aceves, E., Jan, L. Y., and Jan, Y. N. (1986). Sensory neurons and peripheral pathways in *Drosophila* embryos. *Roux's Arch. Dev. Biol.* **195**, 281–289.

Haas, H., Sin, W-C., Javaherian, A., Li, Z., and Cline, H. T. (2001). Single-cell electroporation for *in vivo* neuronal gene expression. *Neuron* **29,** 583–591.

Higgins, D., Burack, M., Lein, P., and Banker, G. (1997). Mechanisms of neuronal polarity. *Curr. Opin. Neurobiol.* **7,** 599–604.

Huang, M.-L., Hsu, C. H., and Chien, C.-T. (2000). The proneural gene *amos* promotes multiple dendritic neuron formation in the *Drosophila* peripheral nervous system. *Neuron* **25,** 57–67.

Jan, Y. N., and Jan, L. Y. (1993). The peripheral nervous system. In "The Development of *Drosophila melanogaster*," pp. 1207–1244. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Jan, Y. N., and Jan, L. Y. (2000). Polarity in cell division: What frames thy fearful asymmetry? *Cell* **100,** 599–602.

Jan, Y. N., and Jan, L. Y. (2001). Dendrites. *Genes Dev.* **15,** 2627–2641.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: Inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1,** 20–29.

Jontes, J. D., and Smith, S. J. (2000). Filopodia, spines and the generation of synaptic diversity. *Neuron* **27,** 11–14.

Kramer, A. P., Goldman, J. R., and Stent, G. S. (1985). Developmental arborization of sensory neurons in the leech *Haementeria ghilianii*. I. Origin of natural variations in the branching pattern. *J. Neurosci.* **5,** 759–767.

Kramer, A. P., and Stent, G. S. (1985). Developmental arborization of sensory neurons in the leech *Haementeria ghilianii*. II. Origin of natural variations in the branching pattern. *J. Neurosci.* **5,** 768–775.

Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22,** 451–461.

Lin, D. M., and Goodman, C. S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron* **13,** 507–523.

Lom, B., and Cohen-Cory S. (1999). Brain-derived neurotrophic factor differentially regulates retinal ganglion cell dendritic and axonal arborization *in vivo*. *J. Neurosci.* **19,** 9928–9938.

Lu, B., Usui, T., Uemura, T., Jan, L. Y., and Jan, Y. N. (1999). Flamingo controls the planar polarity of sensory bristles and asymmetric division of sensory organ precursors in *Drosophila*. *Curr. Biol.* **9**, 1247–1250.

Maletic-Savatic, M., Malinow, R., and Svoboda, K. (1999). Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science* **283,** 1923–1927.

Masland, R. H. (2001). Neuronal diversity in the retina. *Curr. Opin. Neurobiol.* **11,** 431–436.

Matus, A. (2000). Actin-based plasticity in dendritic spines. *Science* **290,** 754–758.

McAllister, A. K., Lo, D. C., and Katz, L. C. (1995). Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron* **15,** 791–803.

McAllister, A. K. (2000). Cellular and molecular mechanisms of dendrite growth. *Cereb. Cortex* **10,** 963–973.

Nedivi, E., Wu, G. Y., and Cline, H. T. (1998). Promotion of dendritic growth by CPG15, an activity-induced signaling molecule. *Science* **281,** 1863–1866.

Orgogozo, V., Schweisguth, F., and Bellaiche, Y. (2001). Lineage, cell polarity and *inscuteable* function in the peripheral nervous system of the *Drosophila* embryo. *Development* **128,** 631–643.

Perry, V. H., and Linden, R. (1982). Evidence for dendritic competition in the developing retina. *Nature* **297,** 683–685.

Peters, A., and Kaiserman-Abramof, I. R. (1970). The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. *Am. J. Anat.* **127,** 321–355.

Polleux, F., Morrow, T., and Ghosh, A. (2000). Semaphorin 3A is a chemoattractant for cortical apical dendrites. *Nature* **404,** 567–573.

Ramón y Cajal, S. (1911). "Histology of the Nervous System of Man and Vertebrates." Oxford University Press, Oxford.

Redmond, L., and Ghosh, A. (2001). The role of Notch and Rho GTPase signaling in the control of dendritic development. *Curr. Opin. Neurobiol.* **11,** 111–117.

Reichardt, L. F., and Farinas, I. (1997). Neurotrophic factors and their receptors: Roles in neuronal development and function. In "Molecular and Cellular Approaches to Neural Development," pp. 220–263. Oxford Univ. Press, New York.

Roayaie, K., Crump, J. G., Sagasti, A., and Bargmann, C. I. (1998). The G alpha protein ODR-3 mediates olfactory and nociceptive function and controls cilium morphogenesis in *C. elegans* olfactory neurons. *Neuron* **20**, 55–67.

Schrader, S., and Merritt, D. J. (2000). Central projections of *Drosophila* sensory neurons in the transition from embryo to larva. *J. Comp. Neurol.* **425,** 34–44.

Scott, E. K., and Luo, L. (2001). How do dendrites take their shape? *Nat. Neurosci.* **4,** 359–365.

Shankland, M., and Goodman, C. S. (1982). Development of the dendritic branching pattern of the medial giant interneuron in the grasshopper embryo. *Dev. Biol.* **92,** 489–506.

Song, H., Ming, G., He, Z., Lehmann, M., McKerracher, L., Tessier-Lavigne, M., and Poo, M. (1998). Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* **281,** 1515–1518.

Usui, T., Shima, Y., Shimada, Y., Hirano, S., Burgess, R. W., Schwarz, T. L., Takeichi, M., and Uemura, T. (1999). Flamingo, a seven-pass transmembrane cadherin, regulates epithelial planar cell polarity under the control of Frizzled. *Cell* **98,** 585–595.

Vervoort, M., Merritt, D. J., Ghysen, A., and Dambly-Chaudière, C. (1997). Genetic basis of the formation and identity of type I and type II neurons in *Drosophila* embryos. *Development* **124**, 2819–2828.

Wang, H., and Macagno, E. R. (1998). A detached branch stops being recognized as self by other branches of a neuron. *J. Neurobiol.* **35,** 53–64.

Whitford, K. L., Marillat, V., Stein, E., Goodman, C. S., Tessier-Lavigne, M., Chedotal, A., and Ghosh A. (2002). Regulation of cortical dendrite development by slit-robo interactions. *Neuron* **33**, 47–66.

Wong, W. T., and Wong, R. O. (2000). Rapid dendritic movement during synapse formation and rearrangement. *Curr. Opin. Neurobiol.* **10,** 118–124.

Xu, B., Zang, K., Ruff, N. L., Zhang, Y. A., McConnell, S. K., Stryker, M. P., and Reichardt, L. F. (2000). Cortical degeneration in the absence of neurotrophin signaling: Dendritic retraction and neuronal loss after removal of the receptor TrkB. *Neuron* **26,** 233–245.

Received for publication January 23, 2002
Revised April 11, 2002
Accepted April 17, 2002
Published online May 24, 2002