Development of the *Drosophila* mushroom bodies: elaboration, remodeling and spatial organization of dendrites in the calyx

Sijun Zhu[1], Ann-Shyn Chiang[2] and Tzumin Lee[3],*

[1]Department of Molecular and Integrative Physiology, University of Illinois, Urbana, IL 61801, USA
[2]Department of Life Science, National Tsing Hua University, Hsinchu, 30043, Taiwan
[3]Department of Cell and Structural Biology, University of Illinois, Urbana, IL 61801, USA
*Author for correspondence (e-mail: tzumin@life.uiuc.edu)

SUMMARY

One *Drosophila* mushroom body (MB) is derived from four indistinguishable cell lineages, development of which involves sequential generation of multiple distinct types of neurons. Differential labeling of distinct MB clones reveals that MB dendrites of different clonal origins are well mixed at the larval stage but become restricted to distinct spaces in adults. Interestingly, a small dendritic domain in the adult MB calyx remains as a fourfold structure that, similar to the entire larval calyx, receives dendritic inputs from all four MB clones. Mosaic analysis of single neurons demonstrates that MB neurons, which are born around pupal formation, acquire unique dendritic branching patterns and consistently project their primary dendrites into the fourfold dendritic domain. Distinct dendrite distribution patterns are also observed for other subtypes of MB neurons. In addition, pruning of larval dendrites during metamorphosis allows for establishment of adult-specific dendrite elaboration/distribution patterns. Taken together, subregional differences exist in the adult *Drosophila* MB calyx, where processing and integration of distinct types of sensory information begin.

Key words: *Drosophila*, Mushroom body, Dendritic elaboration, Mosaic analysis, Remodeling

INTRODUCTION

The *Drosophila* olfactory system has gradually become a convenient model system for studying many interesting neurological phenomena, such as odor coding (reviewed by Warr et al., 2001) and conditioned learning (reviewed by Waddell and Quinn, 2001). Formation of olfactory associative memory involves the mushroom bodies (MBs), paired neuropils in the protocerebrum (reviewed by Roman and Davis, 2001). One *Drosophila* MB is composed of ~2000 Kenyon cells that all project dendrites into the calyx where distinct olfactory inputs are received and may be further processed/integrated (reviewed by Jefferis et al., 2002). Investigating whether and how numerous dendritic processes are orderly arranged in the MB calyx is essential for describing the MB-centered olfactory learning and memory circuitry at a single-cell level.

One *Drosophila* MB is derived from four neuroblasts (Nbs) (Ito et al., 1997), each of which sequentially generates three distinct types of MB neurons that can be distinguished based on their axon projection/fasciculation patterns (Lee et al., 1999). The cell bodies of MB neurons are clustered on the dorsal posterior surface of the protocerebrum. Each cell body sends out one primary neurite that gives rise to dendritic branches and then extends ventrally and anteriorly through the peduncle (e.g. Fig. 4A-C). The peduncle ends near to the anterior surface of the protocerebrum, where intrinsic MB axons are finally segregated into three distinct sets of lobes (Fig. 2A). The γ lobe extends medially toward the midline and consists of the axons derived from the MB neurons (γ neurons) that are born before the mid-3rd instar stage (Lee et al., 1999). The α′ and β′ lobes, which project perpendicularly from each other, are derived from bifurcation of the axons of late larval-born MB neurons (α′/β′ neurons) (Lee et al., 1999). The other pair of perpendicular lobes, the α and β lobes, are composed of segregated axonal branches that are derived from pupal-born MB neurons (α/β neurons) (Lee et al., 1999). As implicated by the fact that newly deposited MB axons are centrally localized, individual subtypes of axons are probably organized into concentric longitudinal compartments based on orders of birth within the peduncle and most lobe regions (Kurusu et al., 2002). In addition, four lineages simultaneously produce identical MB axons that get fasciculated together way before reaching the peduncle end, so distal parts of the peduncle as well as all the entire MB lobes are fourfold structures (Ito et al., 1997; Yang et al., 1995). In contrast with what is known about MB axon fasciculation patterns, very little has been learned regarding how MB dendrites are spatially organized in the calyx, mainly because of a lack of recognizable landmarks.

The MB calyx receives olfactory inputs from antennal lobe projection neurons (PNs). Distinct PNs, prespecified by lineage and birth order, consistently form synapses with different specific olfactory receptor neurons (ORNs) (Jefferis et al., 2001). Olfactory information is then relayed through PNs to higher brain

centers, including the MBs and the lateral horns. Interestingly, individual PNs acquire distinct but invariant axon arborization patterns in the lateral horn, supporting the presence of an odor map in the higher brain center (Marin et al., 2002; Wong et al., 2002). Although variations exist in the patterns of PNs' axon collaterals to the MB calyx, certain PN axons often give rise to similar numbers of MB collaterals that project in analogous directions in various organisms (Marin et al., 2002; Wong et al., 2002). PN axon collaterals rarely branch and normally end with a terminal varicosity (S.Z., A.-S.C. and T.L., unpublished). Ultrastructural characterization of synaptic organization in the *Drosophila* MB calyx reveals that most PN varicosities are encircled by multiple tiny MB dendrites and several GABAergic terminals (Yusuyama et al., 2002). Further investigation into whether various PNs make stereotyped connections with different subtypes of MB neurons depends on better understanding of how numerous MB dendritic processes are packed in the calyx. It is unclear whether MB dendrites are distributed according to their clonal origins. It also remains to be determined whether distinct subtypes of MB neurons elaborate their dendrites in different calycal domains. In addition, the MB calyx exhibits great structural plasticity (Technau, 1984). The MB calyces are on average 21% larger after flies are reared in populated cages for weeks, as compared with being cultured singly in small isolation vials (Heisenberg et al., 1995). Characterizing individual MB neurons' dendrite arborization patterns is important for elucidating the cellular basis of the calyx's structural plasticity.

To examine how numerous dendrites are organized in the MB calyx, we selectively labeled various subsets of MB dendrites based on clonal origins and/or cell types. We identified distinct clonal dendritic territories in the adult calyx. Interestingly, various subtypes of MB dendrites contribute differentially to different calycal regions. Single-neuron analysis further revealed that pioneer α/β neurons acquire unique dendritic elaboration patterns and consistently project their primary dendrites into a small common space, the only fourfold adult calycal domain that receives dendritic inputs from all four MB clonal units. In addition, analysis of single neurons allowed us to identify distinct stage-specific dendritic arborization patterns in the MB γ neurons that regenerate dendrites after pruning of larval processes during metamorphosis. Taken together, the adult MB calyx is probably composed of multiple functionally distinct compartments in which synaptic connections can be made between specific subtypes of MB neurons and certain antennal lobe PNs.

MATERIALS AND METHODS

Fly strains

Hs-FLP;UAS>rCD2,y+>mCD8-GFP was used in conjunction with one of the three GAL4 lines, GAL4-OK107, GAL4-201Y and GAL4-c739, for creation of flip-out clones. Hs-FLP;FRTG13,tubP-GAL80/CyO was crossed with FRTG13,UAS-mCD8-GFP;GAL4-OK107 and FRTG13,babo[9],UAS-mCD8-GFP,GAL4-201Y/CyO for generating wild-type and babo mutant MARCM clones, respectively.

Induction of flip-out clones

Induction of the Flipase activity was performed by heat shocking newly hatched larvae, which carry Hs-FLP, UAS>rCD2,y+>mCD8-GFP, and one MB GAL4, at 38°C for 30 minutes. Flip-out clones were examined with confocal microscopy after immunostaining.

MARCM analysis of dendritic elaboration patterns of subtypes of MB neurons

Single-cell/two-cell MARCM clones of γ, α'/β' and α/β neurons were generated by applying 10 minutes of heat shock at various developmental stages as described previously (Lee et al., 1999). Around pupal formation, organisms were re-synchronized using white pupae as the reference point. A 10-minute heat shock was applied before or after formation of white pupae at 2-hour intervals. MARCM clones were examined with confocal microscopy after immunostaining.

Immunohistochemistry and confocal microscopy

Fly brains were fixed and subjected to immunostaining following the procedures as described previously (Lee et al., 1999). Primary antibodies used in this study include rat anti-mouse CD8 mAb (1:100; Caltag), mouse anti-rat CD2 mAb (1:100; Caltag), mouse anti-FAS II mAb 1D4 (1:50; a gift from C. Goodman), and mouse mAb nc82 that recognizes a synaptic antigen (1:20; a gift from E. Buchner). FITC- and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch) were used at 1:100 and 1:500, respectively. Confocal images were collected using a Zeiss LSM510 confocal microscope and processed with Adobe PhotoShop.

RESULTS

Stage-specific spatial organization of the MB dendrites that are derived from different clonal units

Because one *Drosophila* MB is composed of four similar clonal units (Ito et al., 1997), we wondered whether the entire MB calyx, similar to all MB lobes, is a complete fourfold structure. Alternatively, subregional differences might exist such that various spatial domains within the calyx receive dendrites from different clonal units. As a first step to characterize spatial organization of the MB dendrites, we examined how one clone of MB dendrites is distributed with respect to the other three clones of MB dendrites.

Using UAS>rCD2,y+>mCD8-GFP as a reporter gene, one can label GAL4-positive cells with either rCD2 or mCD8-GFP depending on whether the FRT flip-out cassette is excised (Wong et al., 2002). Therefore, in the presence of GAL4-OK107 that selectively labels most MB neurons (Connolly et al., 1996), we could mark one clone of MB neurons differentially from the other three clones by inducing excision of the FRT cassette in one of the four MB neuroblasts at early developmental stages. Flip-out was induced randomly in newly hatched larvae, and reporter gene expression patterns were examined at the wandering larval stage or in adult flies. Our initial analysis was focused on the brain lobes that display expression of mCD8-GFP in only one clone of MB neurons. We found that one clone of MB dendrites can occupy the entire calyx in wandering 3rd-instar larvae, as evidenced by complete overlapping between the mCD8-GFP-positive dendrites and the rCD2-labeled dendrites (Fig. 1A-C). This observation indicates that the larval MB calyx is a perfect fourfold structure. In contrast, after eclosion, the mCD8-GFP-positive dendrites that are derived from a single clone fail to project throughout the entire calyx and appear to occupy a smaller and distinct spatial domain, as compared with the other three clones of rCD2-marked dendrites (Fig. 1D-F). These results suggest that the adult MB calyx, unlike its larval counterpart, exhibits
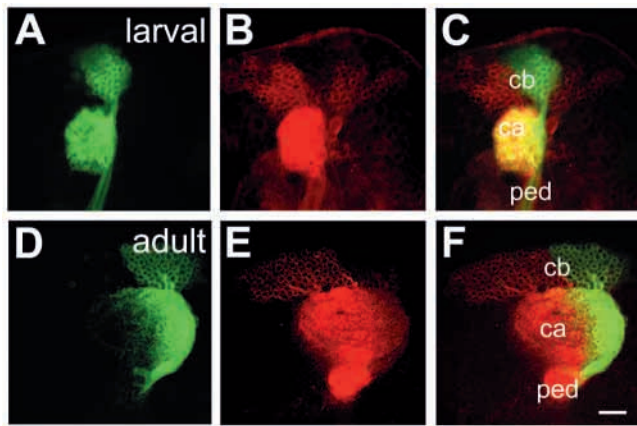
Fig. 1. Larval and adult MB calyces show distinct dendritic organization. Shown are representative confocal sections of larval (A-C) and adult (D-F) MB calyces with flip-out Nb clones generated in newly hatched larvae (NHL). Flip-out clones express mCD8-GFP (green), whereas other MB neurons express rCD2 (red). C,F are merged from A and B, and D and E, respectively. ca, calyx; cb, cell bodies; ped, peduncle. Genotype: *Hs-FLP/+;UAS>rCD2,y+ >mCD8-GFP/+;GAL4-OK107/+*. Scale bar: 20 μm.

regional differences in the distribution of dendrites that are derived from different clonal units.

Mapping of individual clonal dendritic territories in the adult calyx

To better describe how MB dendrites of different clonal origins are spatially organized to form the adult MB calyx,

we tried to outline dendrite domains for individual clonal units in the calyx. Analysis of clone-oriented dendrite distribution patterns was performed in the MBs where clones of mCD8-GFP-postive dendrites happened to alternate with clones of rCD2-labeled dendrites (e.g. Fig. 2C). When the MBs were photo-sectioned from the cell body region to the lobes (Fig. 2B), we noticed that two front clones (AM and AL) flank the two back clones (PM and PL) on most coronal sections (Fig. 2C). Dendritic fields can be easily recognized by the presence of densely packed circular structures (e.g. Fig. 2C, part f; Fig. 3), reminiscent of microglomerular organization of the synapses between PNs and MB neurons (Yusuyama et al., 2002). In summary, two back clonal dendritic fields appear first, then coexist with the two front clonal dendritic fields, and finally vanish when the two front clonal dendritic fields gradually meet each other (Fig. 2C). We also observe that the anteromedial clonal dendritic field always disappears last (Fig. 2C, part g). These observations are consistent with the model that four fascicles of primary neurites roughly cover the posterior hemisphere of the calyx in the following lateral-to-medial order: the anterolateral (AL) clone, the posterolateral (PL) clone, the posteromedial (PM) clone, and the anteromedial (AM) clone (see Discussion; Fig. 7). The primary neurites, which are probably arranged based on birth orders within individual clonal regions (arrows and arrowhead in Fig. 3C; see Discussion; Fig. 7), repeatedly send out dendritic branches toward the anterior surface of the calyx before merging together into the peduncle around the ventral pole. Therefore, on a hypothetical horizontal section, the adult calyx can be roughly divided into four wedged areas whose pointed ends converge at the anterior middle spot (see Discussion; Fig. 7).

In addition, it appears that small subsets of dendritic processes extend further than others and constitute the most anterior tip of the calyx (arrow in Fig. 2C, part h).

Although adult MB dendrites of different clonal origins occupy distinct spatial domains, significant overlap does exist between adjacent clonal dendrite territories. When two neighboring clones are differentially labeled, we observe mixing of mCD8-GFP-marked dendrites with rCD2-labeled dendrites along the entire clonal junction (Fig. 2C, parts d,e). Having different
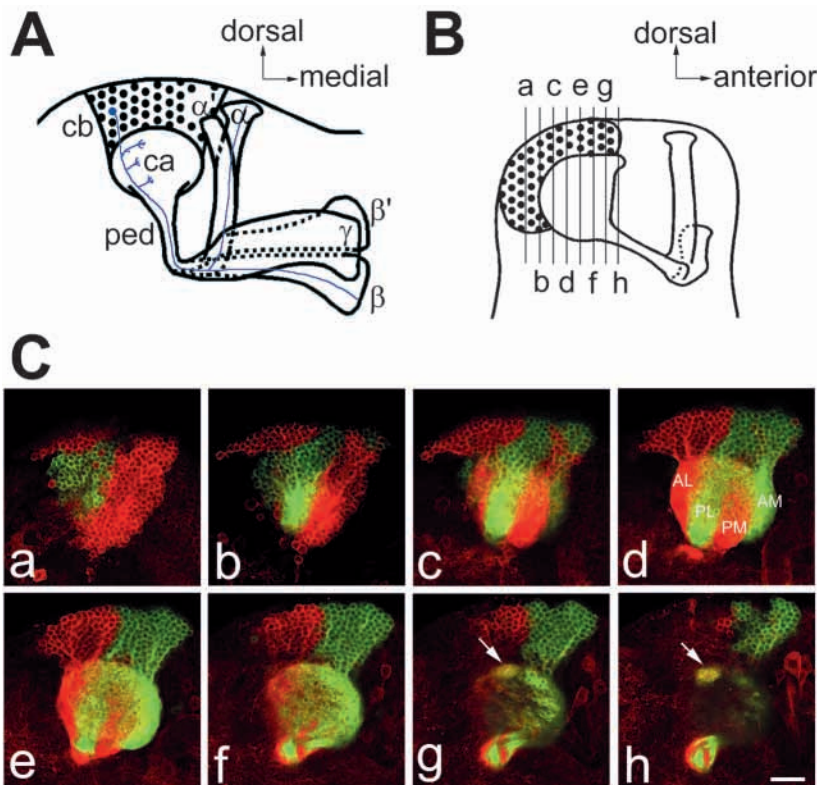

Fig. 2. Each clonal unit has its characteristic dendritic territory in the adult MB calyx. (A) A diagram of the front view of the adult MB in the right hemisphere. One notional α/β neuron is presented. (B) A sagittal view of the MB showing the rough position (a-h) of each confocal image in (C). (C) Serial representative coronal sections of one adult MB calyx in the left hemisphere from posterior to anterior. GFP-positive clones (green) were generated by induction of flip out in newly hatched larvae (NHL). AL, PL, PM and AM represent the anterolateral, posterolateral, posteromedial and anteromedial clonal unit, respectively. Arrows indicate the fourfold structure at the anterodorsal side of the calyx. Genotype: *Hs-FLP/+;UAS>rCD2,y+ >mCD8-GFP/+;GAL4-OK107/+*. Scale bar: 20 μm.

Fig. 3. Various subtypes of MB neurons show different dendritic distribution patterns. (A-F) Mid-coronal confocal sections of adult MB calyces that contain a newly hatched larvae (NHL)-generated, flip-out Nb clone (green). Various subtypes of MB neurons are selectively labeled using GAL4-201Y (A-C) or GAL4-c739 (D-F). Note gaps (arrows in F) present between adjacent GAL4-c739-positive dendritic areas (outlined by broken lines). Mixing of dendrites across clonal boundaries is specifically seen in the upper central subregion (arrowheads in D-F). For a given clone, the primary neurites of γ neurons are localized on the borders (arrows in C) flanking later-derived MB neurites whereas the primary neurites of last-born α/β neurons lie in the center (arrowhead in C). (G-I) Dendrites of GAL4-c739-positive α/β neurons label the calycal fourfold structure (arrowheads), as revealed on the most anterior coronal confocal section of the adult MB calyx. C,F,I are merged from A and B, D and E, and G and H, respectively. Genotypes: (A-C) *Hs-FLP/+;UAS>rCD2, y+>mCD8-GFP/GAL4-201Y*; and (D-I) *Hs-FLP/+; UAS>rCD2,y+>mCD8-GFP/GAL4-c739*. Scale bar: 20 μm.
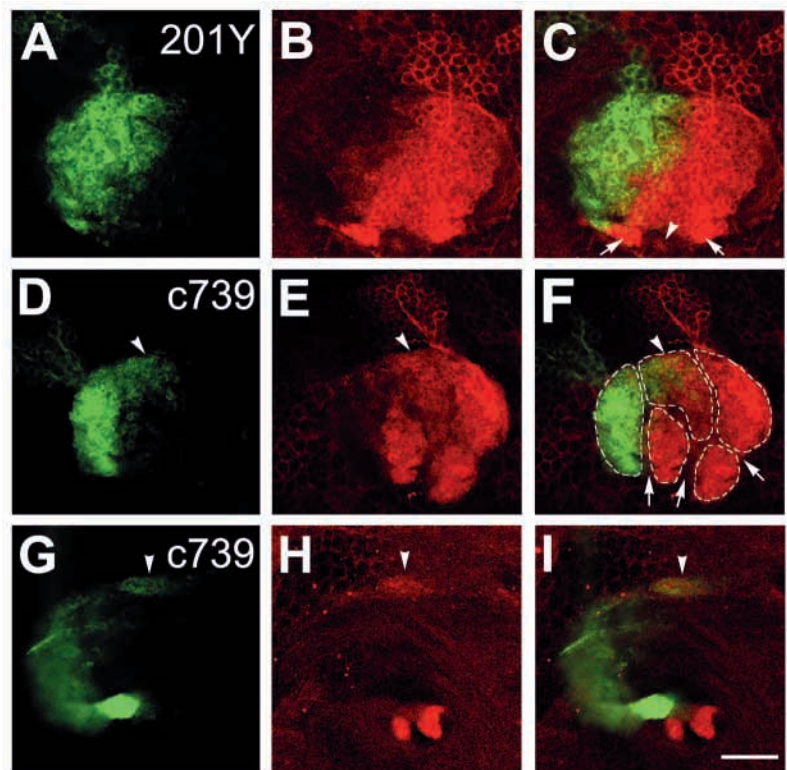
characteristic dendritic territories, grossly indistinguishable MB Nb clones potentially receive distinct clone-specific sensory inputs.

Identification of a small fourfold dendritic domain in the adult calyx

Through analysis of series of coronal focal planes, we further identify a small fourfold spatial domain that receives dendritic inputs from all of the four MB clones at the adult stage (arrows in Fig. 2C, parts g,h). This unique fourfold dendritic domain corresponds to the most anterior tip of the calyx and is probably equidistant from every clone. When one clone of MB dendrites is specifically marked, regardless of its clonal identity, subsets of the marked dendrites consistently reach and label a common dorsal-anterior calycal region that is always shown on the most frontal coronal section of the calyx (data not shown; similar to Fig. 3G). In addition, when two far-apart clonal units, for example the AL and AM clones, are selectively labeled, we observe invading of dendritic processes from distinct clones into each other's territories exclusively at the dorsal-anterior spot (data not shown). It is apparent that only the dorsal-anterior calycal subregion remains as a fourfold dendritic domain in the adult MBs. In addition, we observe restriction of large degrees of mixing of the MB dendrites among neighboring clones to the crescent area immediately around the fourfold dendritic domain (Fig. 2C, part e; arrowhead in Fig. 3F), supporting possible presence of specific threefold dendritic fields (see Discussion; Fig. 7).

Subsets of α/β dendrites, but not γ dendrites, are exuberantly mixed between neighboring clones

Each MB clone consists of a similar set of distinct types of neurons that are sequentially generated from a neuroblast

during development (Lee et al., 1999). Given that the adult calyx contains multiple clone-specific domains plus a fourfold region shared by all the clones, we wondered whether distinct subtypes of MB neurons elaborate their dendrites in different calycal subregions. With GAL4-201Y (Yang et al., 1995), we selectively labeled all γ neurons and a small number of late-born α/β neurons in the mosaic MBs in which distinct clonal units are differentially marked using the UAS>rCD2,y+>mCD8-GFP transgene. We found that GAL4-201Y-positive dendrites extend throughout the clonal-dependent regions (Fig. 3A-C), but fail to reach the dorsal-anterior surface of the calyx such that the fourfold dendritic domain is undetectable (data not shown). This result indicates that neither γ neurons nor late-born α/β neurons normally project their dendrites into the fourfold calycal subregion. In contrast, when α/β neurons are selectively labeled using GAL4-c739 (Yang et al., 1995), the fourfold calycal region appears fully marked (arrowheads in Fig. 3G-I), suggesting that GAL4-201Y-negative α/β neurons specifically mediate formation of the fourfold dendritic domain in the adult calyx.

In addition to making no contribution to the fourfold dendritic domain, GAL4-201Y-positive dendrites seldom cross the clonal boundaries, as evidenced by presence of minimal mixing between differentially labeled clones of GAL4-201Y-positive dendrites (Fig. 3A-C). In contrast, significant overlap exists even outside the fourfold dendritic domain when two neighboring clones of GAL4-c739-positive dendrites are differentially labeled (arrowheads in Fig. 3D-F). Interestingly, GAL4-739-labeled dendrites, but not GAL4-201Y-positive dendrites, are selectively concentrated in five separate spaces – four clone-specific domains plus one shared domain in which dendrites of various clonal origins are significantly mixed (Fig.
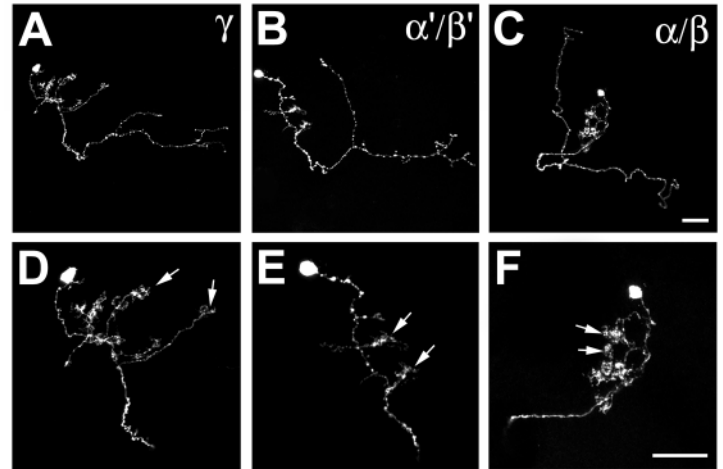
Fig. 4. Single-cell analysis of dendritic elaboration patterns of three distinct types of MB neurons. Shown are single-cell MARCM clones of γ (A,D), α′/β′ (B,E) and α/β (C,F) neurons. (D-F) Enlarged views of the dendritic areas of the single MB cells shown in (A), (B) and (C), respectively. Arrows indicate the claw-like structures at the ends of most dendritic branches. Genotype: *Hs-FLP/+;FRTG13,UAS-mCD8-GFP/FRTG13,tubP-GAL80;GAL4-OK107/+*. Scale bars: 20 μm.

3D-F). These phenomena again suggest that distinct subtypes of MB dendrites are differentially distributed in the adult calyx.

α/β neurons acquire birth order-dependent dendritic elaboration patterns and pioneer α/β neurons contribute to the only fourfold dendritic domain within the adult calyx

To identify which subtype of MB neurons elaborates their dendrites in the fourfold calycal region, we re-examined individual MB neurons' dendritic branching/projecting patterns. Using GAL4-OK107 in the MARCM system (Lee and Luo, 1999), we had systematically labeled single MB neurons based on their birth orders by inducing mitotic recombination at different developmental stages (Lee et al., 1999). We reviewed multiple isolated single-cell clones of MB neurons that were generated at various larval and pupal stages. Although individual neurons acquire morphologically variable dendritic branches, quantitative analysis reveals several common features in their dendrite elaboration patterns (Fig. 4). First, all the neurons have three to six primary dendritic branches that often lack secondary branches and directly end with 'claw-like structures' (referring to clustered fine dendritic arbors; e.g. arrows in Fig. 4). Second, primary branching points are spaced regularly with an average interval of ~7 μm along individual primary neurites. Third, individual dendrites rarely cross each other even in the presence of multiple single-cell clones (e.g. Fig. 6E,F). All the dendrites appear to be distributed locally, making them unlikely to be involved in formation of the fourfold dendritic domain.

If MB neurons that project dendrites into the fourfold calycal region are born over a very narrow window of time, they may not be recovered from our earlier collection of single-cell MARCM clones that are twice-a-day chronological snapshot samples. MB neurons that are positive for GAL4-c739 but negative for GAL4-201Y are born during the first half of the pupal life and have been implicated in mediating formation of the MB fourfold dendritic domain. In order to sample this subset of α/β neurons exhaustively, we collected additional single-cell clones of MB neurons that were generated at contiguous 2-hour windows through the late larval and early pupal stages. Interestingly, we identify unique dendrite branching/projecting patterns in a small subset of MB neurons

that are born around pupal formation and over a period of ~10 hours. They are all characterized by having only one major dendritic process that projects into or toward the fourfold calycal region (Fig. 5A,B,E,F). Pioneer α/β neurons (born over a period of ~6 hours) consistently target their dendrites to the fourfold dendritic domain (arrows in Fig. 5E,I). Extensive elaboration of these dendrites, in which, notably, we observe no typical claw-like structures (Fig. 5E), is restricted to the fourfold dendritic domain (arrow in Fig. 5I). In contrast, subsequently born α/β neurons (born over a period of ~4 hours) appear to terminate their dendrites right outside the fourfold domain (Fig. 5B,F). In this case, short side branches are observed along the primary dendrites that also end with intricate elaboration (Fig. 5F). Remarkably, the fourfold calycal region is selectively revealed after generation of multiple single-cell clones of pioneer α/β neurons by inducing mitotic recombination briefly between 4 hours before pupal formation and 2 hours after pupal formation (Fig. 5I). In contrast, if mitotic recombination is induced within the following 4 hours to generate multiple single-cell clones of α/β neurons, we observe surrounding but no invasion of the fourfold dendritic domain by the labeled dendrites (Fig. 5J). Interestingly, primary dendrites of these early-born α/β neurons appear to project along the top surface of the calyx (Fig. 5I,J); and pioneer α/β neurites often send out short branches that roughly outline the calyx base (arrowheads in Fig. 5E,I). In addition, single-cell clones of α/β neurons, which are generated over the next 2-hour window, possess multiple short dendritic processes that are derived as secondary branches immediately around the primary branching point (Fig. 5C,G,K). All later-born α/β neurons resume typical dendritic branching patterns, which are characterized by presence of multiple primary dendritic processes that project locally (Fig. 5D,H,L). Taken together, our observations confirm that pioneer α/β dendrites contribute to formation of the fourfold dendritic domain in the adult MB calyx, and further demonstrate possible presence of sequentially formed multiple layers of α/β dendritic termini outside the fourfold domain.

In addition, we notice acquisition of different characteristic axon projection patterns in pioneer α/β neurons. Pioneer α/β axons consistently extend their straight medial branches along

Fig. 5. α/β neurons show birth order-dependent dendritic elaboration patterns. Adult single-cell MARCM clones (green) were generated from ~4 hours before white pupa formation to 2 hours after white pupa formation (A,E,I), 2-6 hours after white pupa formation (B,F,J), 6-8 hours after white pupa formation (C,G,K), or later (D,H,L). γ and α/β lobes were counterstained using mAb 1D4 (red) in A-D. Note the pioneer β axonal branch (arrow in A) extends along the dorsal surface of the β lobe and stops short of the β lobe end. (E-H) Enlarged views of the dendritic areas of the single-cell clones in A-D. Calycal regions of multiple single-cell/two-cell clones are shown in I-L. The entire MB calyx was counterstained using mAb nc82 (red) in I. Note that pioneer α/β neurons project long dendritic processes into the fourfold domain (arrows in E,I), while extending short branches around the calyx base (arrowhead). Genotype: *Hs-FLP/+;FRTG13,UAS-mCD8-GFP/FRTG13,tubP-GAL80;GAL4-OK107/+*. Scale bars: 20 μm.
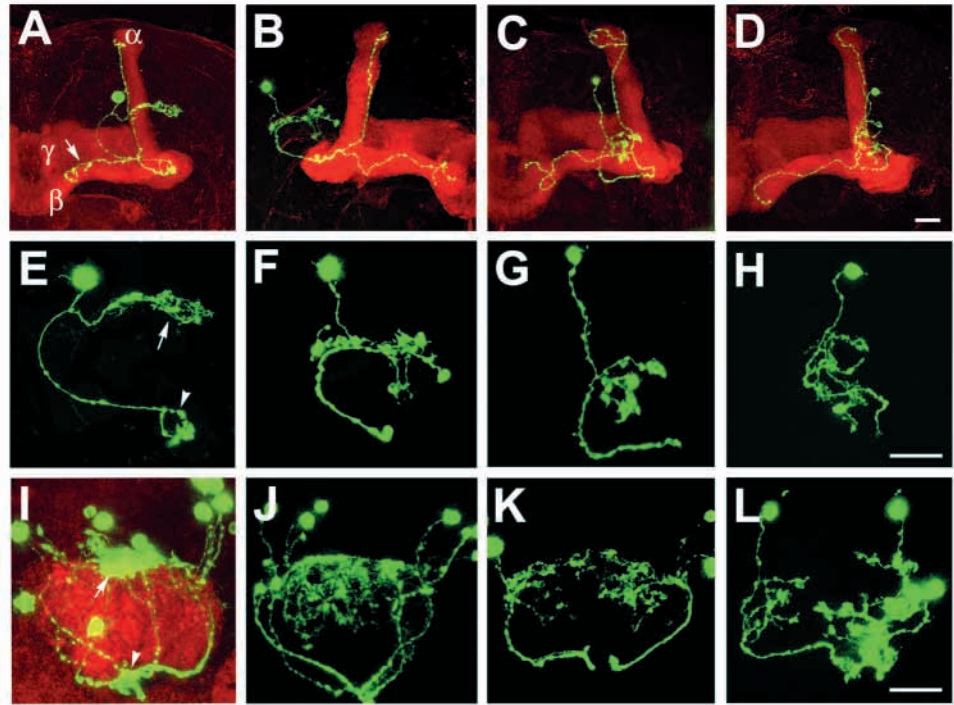
the dorsal surface of the β lobe whereas their dorsal branches, similar to other early-derived processes, are simply at the periphery of the α lobe (Fig. 5A). Moreover, the medial axon branches end prematurely and fail to reach the tip of the β lobe in the pioneer α/β neurons (arrow in Fig. 5A), whose dendrites constitute the fourfold calycal region. Therefore, pioneer α/β neurons could be unambiguously distinguished from other types of MB neurons based on dendrite elaboration and axon projection patterns.

Pruning of larval dendrites allows γ neurons to alter dendritic elaboration patterns during metamorphosis

Metamorphosis of MB γ neurons involves pruning of all larval dendritic processes as well as larval-specific axonal branches (Lee et al., 1999). Although the entire larval calyx, mainly consisting of γ dendrites, is a fourfold structure, γ neurons are not involved in formation of the fourfold dendritic domain in the adult calyx. To elucidate how pruning of larval dendrites contributes to γ neurons' final, mature dendritic arborization patterns, we examined whether γ neurons acquire different dendrite branching/projecting patterns at the adult stage if their dendrites fail to be pruned during metamorphosis. Single-cell clones of MB γ neurons that are homozygous for a loss-of-function mutation in *ultraspiracle*, *baboon* or *dSmad2*, have been shown to be defective in pruning of both larval dendrites and axonal branches at the pupal stage (Lee et al., 2000; Zheng et al., 2003). We characterized dendritic elaboration patterns in isolated single-cell clones of such mutant γ neurons, and detected similar abnormal dendritic arborization patterns in all remodeling-defective mutant γ neurons (Fig. 6A-C). First, all wild-type γ neurons have three or more primary dendritic branches (average=4.9±1.1; *n*=12) (Fig. 6D-F), whereas 50% of mutant neurons have only one primary branch (average=1.4±0.5; *n*=20) (Fig. 6A-C). Second, primary branching points are

distributed widely over the primary neurites in wild-type γ neurons (Fig. 6D-F), whereas most mutant dendrites appear to originate within a narrow circumferential region above the equator of the adult calyx (Fig. 6A-C). Third, wild-type dendrites usually elaborate in a non-overlapping manner and end with distinct claw-like structures; and individual terminal branches appear to occupy discrete territories of their own (Fig. 6D-F). Therefore, when only one or two γ neurons within each of the four MB clonal units are simultaneously labeled, their dendrites are widely distributed and roughly outline the entire calyx (Fig. 6F). In contrast, mutant dendrites often project toward a common area and tangle with one another (arrow in Fig. 6B). As a consequence, multiple mutant dendrites that are derived from different clonal units become extensively intermingled and occupy a relatively restricted space close to the central anterior surface of the calyx, forming an ectopic fourfold dendritic domain (arrow in Fig. 6C).

Interestingly, primary branching patterns are basically preserved between larval γ neurons and the adult γ neurons that have retained their larval processes into the adult stage. Single-cell analysis of larval γ neurons reveals that individual γ neurites generate one to two primary dendritic branches over a narrow 7-μm range at the larval stage (Fig. 6G-I). However, larval dendrites undergo immediate arborization (Fig. 6G-I); but retained larval dendrites in the mutant γ neurons remain relatively unelaborated until reaching the ectopic fourfold dendritic domain at the adult stage (Fig. 6A-C). These phenomena underscore the physiological significance for pruning of larval dendrites in establishing MB γ neurons' mature dendritic arborization/distribution patterns.

DISCUSSION

Analysis of various subsets of dendrites allows us to reveal how

Fig. 6. Remodeling allows γ neurons to alter dendritic elaboration patterns. (A-C) Single-cell MARCM clones of adult γ neurons homozygous for a *babo* mutation. The entire calyx (circled by a broken line) was counterstained using mAb nc82 (red) in C. Note that dendrites of mutant γ neurons from different clonal units tangle together at the center of the calyx (arrows). (D-F) Wild-type single-cell MARCM clones of adult γ neurons. (G-I) Wild-type single-cell MARCM clones of larval γ neurons. Note that larval dendrites of various clonal origins elaborate in the same dendritic field (H, I). Genotype: (A-C) *Hs-FLP/+;FRTG13,babo[9],UAS-mCD8-GFP,GAL4-201Y/FRTG13,tubP-GAL80*; (D-I) *Hs-FLP/+;FRTG13,UAS-mCD8-GFP/FRTG13,tubP-GAL80;GAL4-OK107/+*. Scale bar: 20 μm.
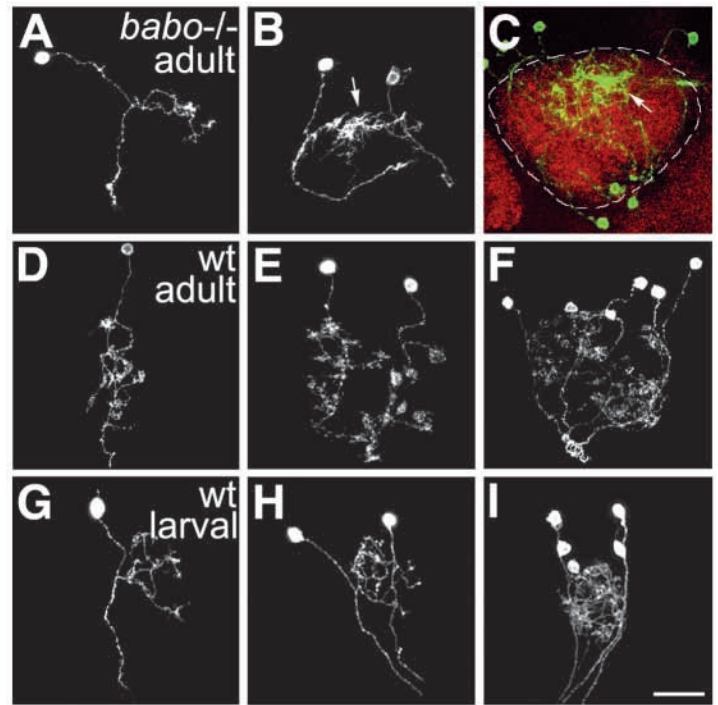
numerous dendritic processes are organized in the *Drosophila* adult MB calyx. One MB consists of four clonal units (Ito et al., 1997). Similar shapes of dendritic fields are observed for different clones of MB neurons. At the adult stage, four clonal dendritic fields are arranged into one calyx in a stereotyped, partially overlapping manner such that different calycal regions receive dendrites from different subsets of MB clones (Fig. 7). Peripheral calycal regions probably receive dendrites from one to two local clones whereas the most anterior calycal tip consists of dendrites derived from all four MB clones. In addition, most intermediate calycal zones, in particular the crescent area immediately outside the fourfold dendritic domain, are probably innervated by two to three clones. Interestingly, distinct subtypes of MB neurons contribute differentially to different calycal regions (Fig. 7). For instance,
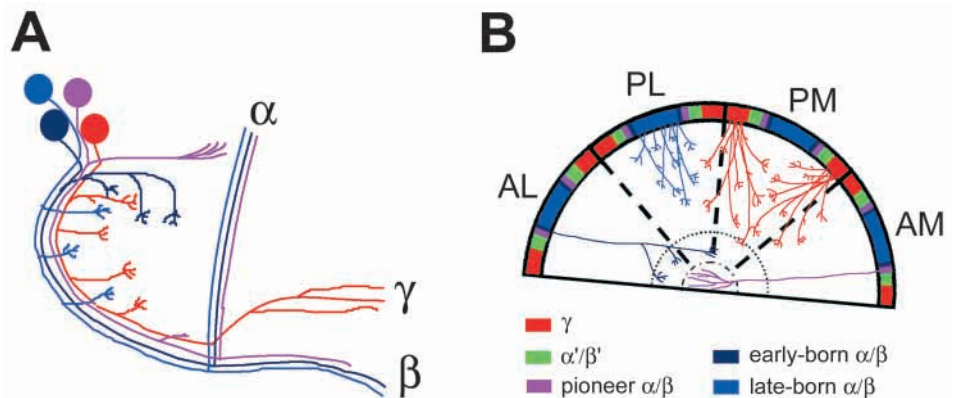
GAL4-201Y-positive dendrites are rather uniformly distributed but never invade the fourfold calycal domain whereas GAL4-c739-positive dendrites are selectively concentrated in five well-separated regions in addition to the fourfold dendritic domain. Therefore, the adult MB calyx exhibits regional differences, raising the possibility that various PNs can relay distinct olfactory information to different characteristic subtypes of MB neurons through targeting their axon arbors to different calycal subregions. In addition, distinct PNs potentially communicate with different clones of MB neurons at the adult stage, because the adult calyx, unlike the larval calyx, contains various clone-specific dendritic domains.

Single-neuron analysis further reveals that pupal-born, adult-specific α/β neurons acquire birth order-dependent dendritic elaboration/distribution patterns. Pioneer α/β neurons

Fig. 7. Schematic models of MB dendritic elaboration/distribution patterns. (A) Frontal view of various representative MB neurons in the anterolateral clone. (B) An oblique section of the MB calyx from posteroventral to anterodorsal surface. The primary neurites of MB neurons are aligned in order on the posterior surface of the calyx such that late-deposited primary neurites are centrally localized within individual clones, as indicated by patterned arrangement of color patches in the curved strip. Broken lines show the junctions between neighboring clonal dendritic fields. The fourfold domain

contributed by the dendrites of pioneer α/β neurons is outlined by the dash-dotted line. The space between the dotted line and dash-dotted line represents the two- to threefold domain that is exclusively innervated by early-born α/β neurons. For simplicity, one subtype of MB dendrites is shown in each clonal unit. Note that late-derived α/β dendrites, but not γ dendrites, are well restricted to individual clonal territories. The calyx is normally tilted forward on the medial side. AM, PM, PL and AL represent the anteromedial, posteromedial, posterolateral and anterolateral clonal units, respectively.

that are born around pupal formation consistently send one long dendritic process along the upper surface of the calyx into the fourfold anterior calycal tip. Interestingly, the same neurons give rise to short branches that roughly cover the calyx base. These pioneer α/β dendrites, together with primary neurites, outline the entire adult calyx. Given that larval dendrites are largely pruned when pioneer α/β neurons are born (Lee et al., 1999), these first-derived adult MB dendrites might play important roles in guiding elaboration of later-derived dendritic processes. Pioneer α/β neurons also acquire unique axonal branches that are probably involved in initial formation of the MB α and β lobes. In addition, as implicated from analysis of subsequently born early α/β neurons, sequentially derived α/β dendrites end shorter and shorter, potentially forming concentric layers of dendritic termini outside the fourfold domain. Partitioning of the calyces into several concentric subdivisions has been documented in other insects with well-developed MBs (Gronenberg, 2001; Strausfeld and Li, 1999). In contrast with orderly organization of the α/β dendrites, no special pattern has been observed for the distribution of γ dendrites. In the *Drosophila* brain, α/β neurons are selectively involved in olfactory associative long-term memory (Pascual and Preat, 2001). It remains to be examined whether and how orderly organization of the α/β dendrites contributes to processing and integration of distinct olfactory information in the MBs.

Patterns of elaboration and distribution of adult MB dendrites are probably established through sequential deposition of different subtypes of dendrites. This scenario may explain why α/β neurons, but not γ neurons, acquire distinct dendrite elaboration/distribution patterns depending on when they are born. Although γ and α/β neurons are both sequentially generated during development, all γ neurons simultaneously remodel their dendritic processes during early metamorphosis whereas α/β neurons sequentially deposit their dendrites through the pupal stage (Lee et al., 1999). Given that all mature-looking larval dendrites are pruned around pupal formation (Lee et al., 1999), pioneer adult MB dendrites are probably derived from the first-born α/β neurons whose dendritic processes, in fact, outline the adult calyx. In addition, regeneration of γ dendrites occurs around 24 hours after pupal formation (Lee et al., 1999), which roughly coincides with the transition in α/β neurons' dendritic elaboration from birth order-dependent unique patterns to the universal adult-specific pattern that is characterized by the presence of multiple, regularly separated, simple primary branches. Interestingly, late-born α/β neurons appear to restrict their dendritic growth to small individual clone-specific regions, which may simply be because of the fact that limited space is left after full elaboration of adult γ dendrites. All of these phenomena support that the adult MB calyx consists of region-specific stereotyped dendrites, orderly organization of which possibly involves sequential elaboration of distinct types of adult dendrites during the pupal stage.

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