The unfulfilled gene is required for the development of mushroom body neuropil in Drosophila

Karen E Bates, Carl S Sung, Steven Robinow*

Abstract

Background: The mushroom bodies (MBs) of Drosophila are required for complex behaviors and consist of three types of neurons, γ, α'/β' and α/β. Previously, roles for transcription factors in MB neuronal differentiation have only been described for a subset of MB neurons. We are investigating the roles of unfulfilled (unf; HR51, CG16801) in MB development. unf encodes a nuclear receptor that is orthologous to the nuclear receptors fasciculation of axons defective 1 (FAX-1) of the nematode and photoreceptor specific nuclear receptor (PNR) of mammals. Based on our previous observations that unf transcripts accumulate in MB neurons at all developmental stages and the presence of axon pathfinding defects in fax-1 mutants, we hypothesized that unf regulates MB axon growth and pathfinding.

Results: We show that unf mutants exhibit a range of highly penetrant axon stalling phenotypes affecting all neurons of the larval and adult MBs. Phenotypic analysis of unf^f1 mutants revealed that α'/β' and α/β neurons initially project axons but stall prior to the formation of medial or dorsal MB lobes. unf^f0001 mutants form medial lobes, although these axons fail to branch, which results in a failure to form the α or α' dorsal lobes. In either mutant background, γ neurons fail to develop larval-specific dorsal projections. These mutant γ neurons undergo normal pruning, but fail to re-extend axons medially during pupal development. unf^PNAi animals displayed phenotypes similar to those seen in unf^f0001 mutants. Unique asymmetrical phenotypes were observed in unf^X1/unf^Z0001 compound heterozygotes. Expression of UAS-unf transgenes in MB neurons rescues the larval and adult unf mutant phenotypes.

Conclusions: These data support the hypothesis that unf plays a common role in the development of all types of MB neurons. Our data indicate that unf is necessary for MB axon extension and branching and that the formation of dorsal collaterals is more sensitive to the loss of unf function than medial projections. The asymmetrical phenotypes observed in compound heterozygotes support the hypothesis that the earliest MB axons may serve as pioneers for the later-born MB neurons, providing evidence for pioneer MB axon guidance in post-embryonic development.

Background

The mushroom bodies (MBs) of Drosophila melanogaster, which are required for olfactory learning and other complex behaviors [1,2], are ideal for studying the transcriptional regulation of interneuronal development because they form discrete axonal projections that are well-characterized [3-5] and easily visualized [4,6-9]. Four neuroblasts in each brain hemisphere sequentially generate three types of Kenyon cells, the γ, α'/β', and α/β MB neurons that begin dividing during embryogenesis and continue to divide through development [10,11]. Each neuron projects dendrites that contribute to a large dendritic field in the calyx, and an axon that travels anteroventrally, forming a tightly bundled peduncle before branching medially to form the γ, β', and β lobes, and dorsally to form the α' and α lobes (Figure 1A). The earliest born γ neurons initially extend axons both medially and dorsally during late embryonic and early larval stages. These larval-specific γ axons are then pruned back to the peduncle by 18 hours after puparium formation (APF) and re-extend medially during

* Correspondence: robinow@hawaii.edu
Department of Zoology, University of Hawaii, Honolulu, HI 96822, USA

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pupal remodeling; the late larval-born α'/β' and pupal-born α/β neurons do not remodel their axonal projection patterns during metamorphosis [3-5].

Since the three different classes of MB neurons are born sequentially, generate a single dendritic field, project axons that fasciculate prior to branching medially and/or dorsally to form type-specific lobes, it is interesting to consider whether any differentiative events of the γ, α'/β', and α/β neurons are regulated by a common set of genes or whether they utilize independent transcriptional networks. Existing data on the role of transcription factors in MB differentiation provide little insight into this question. The genes eyeless [12-14], tramtrak [15], mushroom body miniature [16,17], chinmo [18], polyhomeotic [19], and tailless [20] regulate proliferation, specification, and viability of MB neurons, events that precede differentiation. dachshund (dac), ecysone receptor B1 (EcR-B1), ultraspiracle (usp), and dSmad2 act in subtype-specific pathways [12,13,21-23], consistent with the hypothesis that the differentiation of the γ, α'/β', and α/β neurons utilize independent transcriptional pathways. dac mutants display axonal branching and pathfinding defects in subsets of α'/β' and α/β MB neurons [12,21]. EcR-B1 and its heterodimeric partner, Ultraspireacle (USP'), both members of the nuclear receptor superfamily, form an ecysone-regulated transcription factor that is required for the pruning of MB γ neurons at the outset of metamorphosis [22]. dSmad2 regulates the transcription of EcR-B1 in MB γ neurons during neuronal remodeling [24]. Thus, whether any

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**Figure 1 unf is required for mushroom body lobe formation.** In the adult brain, the mushroom body (MB) is a paired neuropil structure that comprises five axonal lobes, γ, α'/β', and α/β. Each neuron projects dendrites that contribute to a large dendritic field (calyx), and an axon that travels anteroventrally. MB axons fasciculate with other MB axons forming a peduncle (Ped) before projecting axons medially and dorsally. α' and α axons project dorsally, whereas γ, β', and β axons project medially, forming five distinctive lobes. To visualize the MB lobes, OK107-GAL4 was used to drive expression of the UAS-mCD8GFP transgene in all MB neurons (Kenyon cells) and their axons. Lobes were distinguished by using anti-Fasciclin II (anti-Fas II) to label α and β lobes and anti-Trio to label α', β', and γ lobes. (A) In adult UAS-mCD8GFP;OK107-GAL4 control animals labeled with anti-Fas II (red), all MB lobes have formed. (B) In unf<sup>−/+</sup> mutants, MB axons have formed a peduncle (arrowhead), but have spread out and stalled prior to lobe formation (arrow). (C) In unf<sup>10001</sup> mutants, γ, β', and β axons projected medially, but were disorganized. No dorsal lobes were formed (star). (D) In unf<sup>−/+</sup>;Df<sup>2426;UAS-unfRNAi</sup>;OK107-GAL4 adult displayed dramatically reduced dorsal lobes in one brain hemisphere (arrow). (E, F) In unf<sup>−/+</sup> and unf<sup>10001</sup> rescue animals, in which a wild-type unf transgene was expressed in all MB neurons in an otherwise mutant background, all MB lobes were present. It is interesting to note that in rescued flies, MB lobes may have fewer axons, and that some medially projecting axons have extended past the midline (arrow). Eb, ellipsoid body; Meb, median bundle; Ped, peduncle. Scale bars = 10 μm.
differentiative events of the $\gamma$, $\alpha'/{\beta}'$, and $\alpha/\beta$ neurons are regulated by a common set of genes has not been previously reported.

In this study we show that the gene unfilled is required for the development of all three types of MB neurons, supporting the hypothesis that some differentiative events of the three types of MB neurons are regulated by a common set of genes. The unfilled gene (unf; HR51, CG16801) encodes the Drosophila NR2E3 member of the nuclear receptor superfamily [25]. UNF, like all classical nuclear receptors, contains an amino-terminal transactivational domain, a DNA-binding domain, a hinge region, and a carboxy-terminal ligand-binding domain [26]. unf is an ortholog of the Caenorhabditis elegans gene fascilitation of axons defective (fax-1) and the human gene photoreceptor specific nuclear receptor (PNR) [27]. Both fax-1 and PNR mutations disrupt developmental events in a limited number of neurons and result in behavioral or sensory deficits. fax-1 mutants are uncoordinated and display axon pathfinding and neurotransmitter defects [28-30]. The observed axon pathfinding defects are inferred to be due to the misregulation of fax-1 target genes. PNR impacts neuronal identity of vertebrate photoreceptors, functions as a dimer, and acts as a dual function transcriptional regulator, able to act as a transcriptional activator and a transcriptional repressor [31-37].

Based on our previous observations that robust levels of unf transcripts accumulate in MB neurons at all developmental stages [25] and the axon pathfinding defects of fax-1 mutants [28-30], we hypothesized that unf regulates MB axon growth and pathfinding. Phenotypic analysis of unf mutants revealed that MB axons stall prior to the formation of the lobes with the exception of the larval-specific $\gamma$ neurons, which project axons medially, but fail to project dorsally. These axons are pruned appropriately but fail to re-extend during pupal stages. Expression of an unf transgene in the MBs in a mutant background rescued the unf mutant phenotypes, demonstrating that MB defects of unf mutants are due to loss of unf function in the MB neurons. These data demonstrate that unf is required for the proper formation of $\gamma$, $\alpha'/{\beta}'$, and $\alpha/\beta$ lobes, consistent with the hypothesis that at least some differentiative events of the $\gamma$, $\alpha'/{\beta}'$, and $\alpha/\beta$ neurons are regulated by a common set of genes.

Results

unf mutants show a reduction or complete loss of mushroom body lobes

To test the hypothesis that unf regulates MB neuron development, flies of various mutant genotypes (unf$^{X1}$/Df2426, unf$^{X1}$/unfenv, unf$^{env}$/Df2426, unf$^{20001}$/unf$^{env}$, unf$^{X1}$/unf$^{20001}$, unf$^{MB05909}$/Df2426, +/Df2426;UAS-unfRNAi;OK107-GAL4) were analyzed for aberrant MB phenotypes. All MB axons were visualized by expressing the UAS-mCD8GFP (UAS-GFP) reporter [9] using the OK107-GAL4 transgene, which expresses GAL4-driven green fluorescent protein (GFP) in all MB neurons [38]. Specific lobes were unambiguously identified immunohistochemically using anti-Fasciclin II (anti-Fas II) to label the $\alpha/\beta$ lobes [6] or anti-Trio to label the $\alpha'/{\beta}'$ lobes; both antibodies weakly label the $\gamma$ lobes [39]. The unf$^{X1}$ and unf$^{20001}$ alleles have been characterized previously [25], while the unf$^{MB05909}$ allele has been recently identified (FlyBase; Figure 2). All five MB lobes were present and morphologically normal in adult control animals: $\alpha$ and $\alpha'$ lobes projected dorsally, whereas $\gamma$, $\beta$, and $\beta'$ axons projected medially stopping at the median bundle (Figure 1A). MB axons of adult unf$^{X1}$/Df2426 hemizygous (unf$^{X1}$, UAS-GFP/Df2426;OK107-GAL4; Figure 1B) and unf$^{20001}$/unf$^{X1}$ homozygous (unf$^{20001}$/ unf$^{X1}$, UAS-GFP;OK107-GAL4) mutants labeled with anti-Fas II projected anteroventrally, forming a peduncle, but stalled prior to the formation of discrete lobes. Axons that reached the heel region of the MB tended to spread out, but did not extend axons medially or dorsally (Figure 1B; compare Additional file 1 to Additional files 2 and 3). unf$^{X1}$/Df2426 hemizygous labeled with anti-Trio rather than anti-Fas II confirmed that $\gamma$ and $\alpha'/{\beta}'$ unf mutant MB neurons initially projected axons forming a peduncle, but that these axons stalled prior to the formation of lobes (data not shown). MB lobes were never observed in unf$^{X1}$/Df2426 hemizygotes or unf$^{X1}$/ unf$^{X1}$ homozygotes (Table 1, rows 8 and 9). In contrast, all MB lobes were observed in all control genotypes (w$^{118}$, unf$^{X1}$/+, Df2426/CyOGFP, UAS-GFP;OK107-GAL4 controls, and unf$^{X1}$, UAS-GFP;CyOGFP;OK107-GAL4 control siblings; Table 1, rows 1, 2, 4, 5, and 6). We did not observe a reduced number of MB neurons in unf mutants (compare Additional file 1 to Additional files 2 to 5).

unf$^{20001}$/Df2426 hemizygous (unf$^{20001}$/UAS-GFP/ Df2426;OK107-GAL4; Figure 1C) adults formed only medial lobes and displayed $\gamma$ axons that splayed as they approached the midline compared to the compact bulb-like organization of $\gamma$ lobes in wild-type animals (Figure 1C; Table 1, row 10; Additional files 4 and 5). $\alpha'/{\beta}'$ and $\alpha/\beta$ neurons of unf$^{20001}$/Df2426 hemizygotes projected axons medially, but rarely projected dorsal collateral axons (data not shown). Dorsal lobes were observed more frequently in unf$^{20001}$/unf$^{20001}$ homozygotes (unf$^{20001}$/unf$^{20001}$/UAS-GFP/Df2426;OK107-GAL4; Table 1, row 11). The observation that dorsal lobes were observed more frequently in unf$^{20001}$/unf$^{20001}$ homozygotes than in unf$^{X1}$/Df2426 hemizygotes supports the hypothesis that the unf$^{20001}$ allele is a hypomorph [25]. All MB lobes were observed in control genotypes (w$^{118}$,
controls); however, missing dorsal lobes were observed in 1 of 10 unfZ0001, UAS-GFP/Cyo GFP;OK107-GAL4 control siblings (Table 1, rows 1, 3, 4, 5, and 7). unfMB05909; Df2426 hemizygotes (unfMB05909/Df2426, UAS-GFP;OK107-GAL4) displayed all MB lobes and did not display any abnormal MB phenotypes (Table 1, row 12), suggesting that the GAL4 insertion in the unfMB05909 line does not disrupt unf gene function.

To independently test whether unf plays a role in MB neuron development, a UAS-unfRNAi line was crossed to the OK107-GAL4 line to generate animals in which unf levels were reduced in the MBs via RNA interference (RNAi). Adult brains were double-labeled with anti-Fas II and anti-Trio to visualize the five MB lobes. When unfZ0001, UAS-GFP/Df2426; OK107-GAL4 was used to drive UAS-unfRNAi in a wild-type background, normal MBs were observed (data not shown). However, when OK107-GAL4 was used to drive UAS-unfRNAi in unf/Df2426 hemizygotes (unf/Df2426; UAS-unfRNAi;OK107-GAL4) 50% of brains displayed dramatically reduced dorsal lobes or were missing dorsal lobes bilaterally or unilaterally (Figure 1D; Table 1, row 13). These RNAi data are consistent with the analyses of unfX1; and unfZ0001 mutants, supporting the hypothesis that unf is necessary for axon extension and branching in all MB neurons and that dorsal collaterals are more sensitive to loss of unf function than medial projections.

unf expression in the mushroom bodies rescues lobe formation

We tested whether expression of unf in the MBs was sufficient to rescue the phenotypes observed in unf mutants by driving expression of a UAS-unf transgene with the OK107-GAL4 transgene. Interestingly, all UAS-unfΔRF, OK107-GAL4 flies developed to late pupal stages, but failed to eclose. The failure to eclose is probably due to OK107-GAL4-driven expression of unf in regions other than the MBs causing a disruption that prevents further development. We therefore assessed the MBs of rescued animals at late pupal stages, 72 to 96 hours APF. At this late developmental time in wild-type pupae, the MBs are indistinguishable from those of adult MBs [4]. Medial and dorsal MB lobes were observed in all unfX1/Df2426 rescued (unfX1, UAS-GFP/Df2426; UAS-unfΔRF/+; OK107-GAL4; Figure 1E) and unfZ0001/Df2426 (unfZ0001, UAS-GFP/Df2426; UAS-unfΔRF/+; OK107-GAL4; Figure 1F) rescued pupae. In contrast, MB lobes were not observed in unfX1/Df2426 control siblings (unfX1, UAS-GFP/Df2426; TM3/++; OK107-GAL4) that lacked the UAS-unfΔRF transgene (Table 1, compare rows 15 and 18). Similarly, only medial lobes were observed in unfZ0001/Df2426 control siblings (unfZ0001, UAS-GFP/Df2426; TM3/++; OK107-GAL4) (Table 1, compare rows 16 and 23) that lacked the UAS-unfΔRF transgene. All MB lobes were observed in all other control pupae (Table 1, rows 19 to 22). MB lobes appeared thin and less robust in some unfX1 and unfZ0001 rescued animals, suggesting that the rescue was imperfect. Nonetheless, MB axons contributing to each of the five MB lobes could be identified in all rescued animals. An independent rescue line, UAS-unfΔRF, was tested with OK107-GAL4 to express unf in the MBs of unfX1/Df2426 hemizygotes. All MB lobes were observed in six of seven rescued unfX1/Df2426 pupae with the UAS-unfΔRF transgene (unfX1, UAS-GFP/Df2426; UAS-unfΔRF/+; OK107-GAL4). In the seventh pupa, medial lobes were observed bilaterally, while dorsal lobes were observed only in one hemisphere (Table 1, row 17). MB lobes were not detected in any unfX1/Df2426 control siblings that lacked the UAS-unfΔRF transgene (unfX1, UAS-GFP/Df2426; TM3/++; OK107-GAL4; Table 1, row 24). To confirm that the rescue depended upon the expression of an unf open reading frame, we tested the ability of a UAS-lacZ transgene to rescue unfX1/Df2426 hemizygotes. As expected, MB lobes were not observed in unfX1/Df2426 pupae containing the UAS-lacZ transgene (unfX1, UAS-GFP/Df2426; UAS-lacZ/++; OK107-GAL4; Table 1, row 25). These data demonstrate that the axonal defects observed in unfX1 mutants are due to the lack of UNF function.

**Figure 2** Summary of unf alleles: The unfX1 allele disrupts the 5’ donor splice site of intron 2, whereas the unfZ0001 allele has a missense mutation due to a guanine to adenine transition at base 312 of exon 2, resulting in a glycine to arginine substitution (G120R) [25]. The unfMB05909 line contains a GAL4 insertion in intron 1 of the unf gene (FlyBase). DBD, DNA-binding domain.
Table 1 Mushroom body phenotypes in *unf* mutants and rescue animals.

| Row | Genotype                              | All lobes present adult/larvae (%) | All lobes missing adult/larvae (%) | Dorsal lobes missing adult/larvae (%) | Missing a dorsal or medial lobe adult/larvae (%) | n adult/larvae |
|-----|---------------------------------------|-----------------------------------|-----------------------------------|--------------------------------------|------------------------------------------------|-------------|
| 1   | *w*1118                               | 100                               | 0                                 | 0                                    | 0                                               | 10          |
| 2   | unf*1/+                               | 100/100                           | 0/0                               | 0/0                                  | 0/0                                            | 12/8        |
| 3   | unf*1000/+                            | 100/100                           | 0/0                               | 0/0                                  | 0/0                                            | 7/15        |
| 4   | Df/CyOGFP                             | 100/100                           | 0/0                               | 0/0                                  | 0/0                                            | 8/8         |
| 5   | UAS-GFP;OK107                         | 100                               | 0                                 | 0                                    | 0                                              | 10          |
| 6   | unf*1/, UAS-GFP;CyOGFP;OK107          | 100                               | 0                                 | 0                                    | 0                                              | 15          |
| 7   | unf*1000/, UAS-GFP;CyOGFP;OK107       | 90                                | 0                                 | 10                                   | 0                                              | 10          |

**unf** mutants and gene knock down

| Row | Genotype                              | All lobes present adult/larvae (%) | All lobes missing adult/larvae (%) | Dorsal lobes missing adult/larvae (%) | Missing a dorsal or medial lobe adult/larvae (%) | n adult/larvae |
|-----|---------------------------------------|-----------------------------------|-----------------------------------|--------------------------------------|------------------------------------------------|-------------|
| 8   | unf*1/, UAS-GFP/Df;CyO;OK107          | 0/0                               | 100*0                             | 0/90*                                | 0/10                                           | 15/11       |
| 9   | unf*1/, UAS-GFP/unf*1;CyO;OK107       | 0                                 | 100*                              | 0                                    | 0                                              | 7           |
| 10  | unf*10000/;Df; UAS-GFP;CyO;OK107      | 0/0                               | 0/0                               | 100*/100*                            | 0/0                                            | 13/6        |
| 11  | unf*100000/;Df; UAS-GFP;CyO;OK107     | 37.5                              | 0                                 | 37.5                                 | 25                                             | 8           |
| 12  | unf*+Df; UAS-unf+/;CyO;OK107          | 100                               | 0                                 | 0                                    | 0                                              | 14          |
| 13  | unf*1/, UAS-GFP;unf*100000/+;OK107    | 0/0                               | 100*                              | 0                                    | 0                                              | 10          |
| 14  | unf*1/, UAS-GFP;unf*100000/+;OK107    | 0                                 | 13                                | 47                                   | 40                                             | 15          |

**unf** transgenic rescues

| Row | Genotype                              | All lobes present adult/larvae (%) | All lobes missing adult/larvae (%) | Dorsal lobes missing adult/larvae (%) | Missing a dorsal or medial lobe adult/larvae (%) | n adult/larvae |
|-----|---------------------------------------|-----------------------------------|-----------------------------------|--------------------------------------|------------------------------------------------|-------------|
| 15a | unf*1/, UAS-GFP;Df; UAS-unf*100000/+;CyO;OK107 | 100*/83*                         | 0/0                               | 0/0                                  | 0/17                                           | 11/6        |
| 16b | unf*100000/;Df; UAS-GFP; UAS-unf*10000;CyO;OK107 | 100*                             | 0                                 | 0                                    | 0                                              | 8           |
| 17c | unf*1/, UAS-GFP;Df; UAS-unf*1000000/+;CyO;OK107 | 86*                             | 0                                 | 0                                    | 14                                             | 7           |

**unf** rescue controls

| Row | Genotype                              | All lobes present adult/larvae (%) | All lobes missing adult/larvae (%) | Dorsal lobes missing adult/larvae (%) | Missing a dorsal or medial lobe adult/larvae (%) | n adult/larvae |
|-----|---------------------------------------|-----------------------------------|-----------------------------------|--------------------------------------|------------------------------------------------|-------------|
| 18a | unf*1/, UAS-GFP;Df;TM3Sb/+;CyO;OK107  | 0                                 | 100                               | 0                                    | 0                                              | 6           |
| 19a | unf*1/, UAS-GFP;CyO;UAS-unf*100000/+;CyO;OK107 | 100                              | 0                                 | 0                                    | 0                                              | 9           |
| 20a | unf*1/, UAS-GFP;CyO;TM3Sb/+;CyO;OK107 | 100                              | 0                                 | 0                                    | 0                                              | 4           |
| 21a | Df/CyOGFP; UAS-unf*1000000/+;OK107    | 100                              | 0                                 | 0                                    | 0                                              | 5           |
| 22a | Df/CyOGFP;TM3Sb/+;CyO;OK107           | 100                              | 0                                 | 0                                    | 0                                              | 7           |
| 23b | unf*1000000/;Df; UAS-GFP; TM3Sb/+;CyO;OK107 | 0                                | 0                                 | 100                                  | 0                                              | 7           |
| 24c | unf*1/, UAS-GFP;Df;TM3Sb/+;CyO;OK107  | 100                              | 0                                 | 0                                    | 0                                              | 9           |
| 25  | unf*1/, UAS-GFP;Df; UAS-lacZ;CyO;OK107 | 100                              | 0                                 | 0                                    | 0                                              | 9           |
| 26  | UAS-GFP; UAS-unf*1000000/+;CyO;OK107  | 100                              | 0                                 | 0                                    | 0                                              | 5           |

Data are presented as percentages of whole brains that exhibit the phenotype. Control and mutant animals are 0- to 2-day adults, whereas rescue and rescue control animals are 72- to 96-hour pupae. All larvae are third instars. Single entries are adult or late pupae. Rescues and the corresponding control siblings are noted by matching subscript letters in rows. Asterisks indicate P-values of < 0.01 from the Fisher exact test. Abbreviations: Df, Df(2R)ED2426; UAS-GFP; UAS-mCD8GFP; OK107, OK107-GAL4.
It is interesting to note that the medially projecting axons of any genotype carrying the *UAS-unf*^ras^GFP and *OK107-GAL4* transgenes failed to stop appropriately, extending axons past the midline. Midline crossing was observed in mutants carrying *UAS-unf*^ras^GFP and *OK107-GAL4* (Figure 1E) as well as controls carrying these two transgenes. Midline crossing was observed approximately 50% of the time: *unf*^X1^/*OK107-GAL4* rescue animals (55%, n = 11), *unf*^X1^/*UAS-GFP/CyO; UAS-unf*^ras^GFP/+; *OK107-GAL4* control siblings (44%, n = 9), and *UAS-GFP/+; UAS-unf*^ras^GFP/+; *OK107-GAL4* controls (60%, n = 5). This observation suggests that MB axons may be sensitive to levels of UNF expression.

**unf*^X1^/*unf*^Z0001^ compound heterozygotes exhibit a range of mushroom body phenotypes**

Phenotypic analysis of *unf*^X1^/*unf*^Z0001^ compound heterozygotes (*unf*^X1^/*UAS-GFP/unf*^Z0001^; *OK107-GAL4*) revealed a range of aberrant MB phenotypes. Thirteen percent of *unf*^X1^/*unf*^Z0001^ compound heterozygotes lacked all MB lobes (Figure 3A), similar to the *unf*^X1^ mutant phenotype. Forty-seven percent of the *unf*^X1^/*unf*^Z0001^ compound heterozygotes developed only medial lobes and were missing dorsal lobes (Figure 3B, C), similar to the *unf*^Z0001^ mutant phenotype. These *unf*^X1^/*unf*^Z0001^ compound heterozygotes occasionally displayed a thin fascicle of dorsally projecting α' axons (Figure 3B, C). Interestingly, 40% of *unf*^X1^/*unf*^Z0001^ compound heterozygotes exhibited asymmetrical phenotypes in which a dorsal and/or medial lobe were present in one hemisphere but missing in the other (Table 1, row 14). In some cases, medial axons misprojected or extended past the midline (Figure 3B, C, E), γ neurons were also variably affected in *unf*^X1^/*unf*^Z0001^ compound heterozygotes and often appeared defasciculated, and stalled at various points along their medial trajectory (Figure 3C, D, F; Additional files 6, 7 and 8). The novel phenotypes of *unf*^X1^/*unf*^Z0001^ compound heterozygotes that are different from either *unf*^X1^/*Df2426* or *unf*^Z0001^/*Df2426* hemizygotes demonstrate that the *unf*^X1^ and *unf*^Z0001^ alleles interact.

**unf** is required for larval-specific γ dorsal collapsars and the re-extension of γ axons during metamorphosis

The axon stalling phenotypes of *unf*^X1^/*Df2426* hemizygotes suggested that *unf* is required in all MB neurons for axons to extend in any direction beyond the heel region of the MB. This region is the branching point for dorsal collateral projections from medially projecting axons. The MB axons of adult *unf*^X1^/*Df2426* hemizygotes may have all stalled during the initial phase of their outgrowth, either during larval or pupal development. Alternatively, it is possible that MB neurons may initially project axons medially and dorsally, but these axons may not be maintained into the adult. To determine whether *unf* is required for the initial projection patterns of all MB neurons, the MBs of experimental and control animals were analyzed at various larval and pupal stages.

During late embryonic development, γ neurons normally begin to extend axons both medially and dorsally. These medial and dorsal projections persist throughout larval development. By 18 hours APF, γ axons have been pruned to the branching point and they subsequently re-extend medially only [3-5]. *unf*^X1^/*Df2426* (*unf*^X1^/*UAS-GFP/Df2426; *OK107-GAL4*) early-, mid-, and late-third instar larvae displayed only medial axons (*n* = 17), whereas control (*UAS-GFP; *OK107-GAL4*) third instar larvae displayed normal bifurcated larval-specific γ projection patterns (*n* = 5) (compare Figure 4A and Figure 4B). Examination of *unf*^X1^/*Df2426* hemizygotes (*n* = 4) and control (*n* = 6) pupae at 18 hours APF revealed that both medial and dorsal γ axons had been pruned (compare Figure 4D and Figure 4E). At 48 hours APF, MB lobes were not observed in *unf*^X1^/*Df2426* hemizygotes (*n* = 3), whereas control pupae exhibited γ axons that had re-extended medially, forming the adult γ lobe (*n* = 5) (compare Figure 4F and Figure 4G). These data suggest that γ neurons extend axons medially but not dorsally in *unf*^X1^/*Df2426* larvae and that they undergo pruning like wild-type axons at approximately 16 hours APF but that they fail to re-extend during pupal development.

Expression of the *UAS-unf rasF* transgene in the MBs in *unf*^X1^/*Df2426* hemizygotes (*unf*^X1^/*UAS-GFP/Df2426; *UAS-unf rasF/+; *OK107-GAL4*) rescued the dorsal collars of the larval γ neurons in five of six third instar larvae (Figure 4C; Table 1, row 15) and supported the re-extension of γ medial axon projections in pupae (Figure 1E, 1F). These data confirm that the *unf* function in MBs is necessary for the formation of dorsal collars in γ neurons during larval development.

**unf** is required during the early development of α'/β' and α/β mushroom body neurons

The α'/β' neurons develop medially and dorsal projections between mid-third instar and puparium formation. The α/β neurons develop during early pupal stages [3-5]. α'/β' medial or dorsal projections were never observed in *unf*^X1^/*Df2426* (*unf*^X1^/*UAS-GFP/Df2426; *OK107-GAL4*) late-third instar larvae (*n* = 5) or pupae at 18 hours APF (*n* = 6; Figure 4E). Similarly, α'/β' and α/β projections were never observed in *unf*^X1^/*Df2426* pupae at 48 hours APF (*n* = 3; Figure 4G). MB axon projections were normal in control (*UAS-GFP; *OK107-GAL4*) late-third instar larvae (*n* = 5), pupae at 18 hours APF (*n* = 6; Figure 4D), and pupae at 48 hours APF (*n* = 5; Figure 4F). These data indicate that *unf* is necessary for the
differentiation of all $\alpha'/\beta'$ and $\alpha/\beta$ MB neurons early during their development.

201Y-GAL4 and c739-GAL4 driven GFP expression in the mushroom bodies is $\text{unf}^\text{Df2426}$-dependent

To independently confirm that the axon stalling phenotypes of $\text{unf}^{X1}/\text{Df2426}$ hemizygotes were not an artifact associated with the $\text{OK107-GAL4}$ transgene (Figure 1), we examined UAS-GFP expression in the MBs of $\text{unf}^{X1}/\text{Df2426}$ hemizygotes using three other GAL4 drivers known to express in all or subsets of MB neurons, 201Y-GAL4, c739-GAL4, and c747-GAL4 [7,40]. In each case, adult brains were counterstained with fluorescently labeled phalloidin to visualize actin-rich structures, including the extensive dendritic arbors of MB neurons that fill the calyces. The $c747$-GAL4 line showed GFP expression in MB neurons in control and $\text{unf}^{X1}/\text{Df2426}$ MB neurons (Figure 5A, 5B). The $\text{unf}^{X1}/\text{Df2426}$ hemizygotes ($\text{unf}^{X1}$/UAS-GFP/\text{Df2426}, c747-GAL4) displayed stalled axons and failed to develop any MB lobes, confirming the $\text{unf}^{X1}$ mutant phenotypes described using the $\text{OK107-GAL4}$ transgene to drive the reporter transgene in MB neurons. Surprisingly, $\text{unf}^{X1}/\text{Df2426}$ hemizygotes carrying the 201Y-GAL4 transgene failed to express GFP in MB neurons (compare Figure 5C and Figure 5D), and GFP expression in $\text{unf}^{X1}/\text{Df2426}$

![Image of mushroom body phenotypes](image-url)

**Figure 3** $\text{unf}^{X1}/\text{unf}^{20001}$ compound heterozygotes display a range of aberrant mushroom body phenotypes, suggesting that $\text{unf}^{X1}$ and $\text{unf}^{20001}$ alleles interact. **OK107-GAL4** was used to drive expression of the UAS-mCD8GFP transgene in all MB neurons and their axons (green), and anti-Fas II (red) to label the $\alpha$ and $\beta$ axons. (A) In this $\text{unf}^{X1}/\text{unf}^{20001}$ compound heterozygote all MB axons stall (arrows), similar to the $\text{unf}^{X1}$ mutant phenotype. (B) In this $\text{unf}^{X1}/\text{unf}^{20001}$ heterozygote only medial lobes are present, similar to the $\text{unf}^{20001}$ mutant phenotype; a thin fascicle of $\alpha'$ axons is present in the right hemisphere (star). (C) In this $\text{unf}^{X1}/\text{unf}^{20001}$ heterozygote, left hemisphere $\beta'$ and $\beta$ axons extend medially beyond the midline (arrow), whereas $\gamma$ axons appear to stall; right hemisphere $\beta$ and $\beta'$ axons misproject ventrally (arrowhead) and $\gamma$ axons are highly disorganized, only a few $\alpha'$ dorsal axon projections are present in either hemisphere (stars). (D) In this $\text{unf}^{X1}/\text{unf}^{20001}$ heterozygote $\alpha$ and $\alpha'$ dorsal axon projections are present in the left hemisphere, whereas only a thin fascicle of $\alpha'$ axons is present in the right hemisphere (star); $\beta$ axons are present in the right hemisphere and appear to stall (arrow), whereas they are completely absent in the left hemisphere. (E) In this $\text{unf}^{X1}/\text{unf}^{20001}$ heterozygote $\beta$, $\beta'$, and $\gamma$ axons project medially and cross the midline (arrow), but dorsal axons are missing in the left hemisphere; in the right hemisphere $\gamma$ axons project medially and $\alpha$ and $\alpha'$ axons project dorsally but appear to stall (arrowhead). (F) In this $\text{unf}^{X1}/\text{unf}^{20001}$ heterozygote, $\alpha$ and $\alpha'$ dorsal axon projections are present in the left hemisphere, whereas only $\alpha'$ dorsal axons are present in the right hemisphere; medial axon projections are disorganized and stall before reaching the midline in both right and left hemispheres (arrows). Eb, ellipsoid body; Meb, median bundle; Ped, peduncle. Scale bars = 25 μm.
hemizygotes carrying the c739-GAL4 transgene was greatly diminished (compare Figure 5E and Figure 5F). These observations indicate that 201Y-GAL4 and c739-GAL4 expression is unf-dependent. The 201Y-GAL4 transgene is an insertion in the TAK1-associated binding protein 2 (Tab2) gene (FlyBase). Inverse PCR revealed that the c739-GAL4 transgene is inserted in the second intron of hormone receptor-like in 39 (HR39; data not shown).

Discussion
The unfX1 and unfX0001 alleles interact showing that both alleles are at least partially functional

unf mutants exhibit a range of highly penetrant axon stalling phenotypes affecting all neurons (γ, α'/β' and α/β) of the larval and adult MBs. Similar phenotypes have been observed in unf microRNA knockdown animals [41]. unfX1/Df2426 hemizygotes and unfX1/unfX1 homozygotes fail to project larval-specific γ dorsal collaterals,
fail to re-extend γ axons medially during metamorphosis, and fail to project any medial and dorsal axons of α'/β' and α/β neurons. The γ, α'/β' and α/β axons of unf$^{Z0001}$/Df2426 hemizygotes only project medially, whereas MBs were normal in some unf$^{Z0001}$/unf$^{Z0001}$ homozygotes. These data together with previous observations [25] would seem to support the hypothesis that the unf$^{X1}$ allele is an amorph, a null allele, and that the unf$^{Z0001}$ allele is a hypomorph, a partial loss of function allele. However, while the unf$^{Z0001}$ allele behaves as a hypomorph with respect to sterility, it displays dominant properties with respect to wing expansion [25]. Interestingly, the G56R allele of PNR, which displays dominant properties [42], is structurally equivalent to the unf$^{Z0001}$ allele (G120R) [25]. The observation that the unf$^{X1}$/unf$^{Z0001}$ compound heterozygotes display unique phenotypes was unexpected and demonstrates that these alleles interact, compelling us to conclude that the unf$^{X1}$ allele is not a null allele. These data strongly suggest that the unf$^{X1}$ allele encodes a unique isoform of the UNF protein, UNFX1, which is predicted to contain the 110 residue amino-terminal domain and the complete first zinc finger of the DNA-binding domain [25]. These data do not allow us to infer the functional nature of the unf$^{X1}$ allele or the mechanism of this genetic interaction.

Asymmetrical phenotypes suggest a role for unf$^{X1}$ in pioneer axon guidance

The phenotypic variation and asymmetry observed in the MBs of unf$^{X1}$/unf$^{Z0001}$ compound heterozygotes supports the hypothesis that pioneer axons are established early during MB development and that the pathfinding of these pioneers is unf-dependent. The observation that the β lobe axons in one hemisphere project medially while the β lobe axons in the other hemisphere project ventromedially (Figure 3C, 45° angle down, arrowhead) demonstrates that the projection of the β lobes in these unf$^{X1}$/unf$^{Z0001}$ compound heterozygotes is independent of their genotype. In an independent, yet genetically identical animal, unf$^{X1}$/unf$^{Z0001}$ α/β MB neurons assume a different fate and fail to project any medially projecting β axons (Figure 3F). Similar observations can be made for all unf$^{X1}$/unf$^{Z0001}$ MB lobe axons when these and other samples are examined (Figure 3). The fact that the axons of the later-born α/β neurons consistently stall or misproject whenever the axons of the earlier-born α'/β' neurons stall or misproject suggests that the α'/β' axons may be acting as pioneers for the α/β axons. These data support a model of MB lobe formation in which unf is required for MB pioneer axons to navigate to their targets, and that later-born MB neurons project axons that fasciculate along these established axons. We
propose that the variable and asymmetric phenotypes observed in unfX1/unf20001 compound heterozygotes are due to inappropriate targeting of pioneer axons of the MB or the stalling of pioneer axons prematurely as a result of insufficient unf function in α'/β' pioneer axons. Thus, the asymmetric β lobe projection in Figure 3C may be due to asymmetric projections of pioneer axons, while the lack of β lobes in Figure 3F may be due to the stalling of these pioneer axons in the peduncle. These data are supported by an analysis of non-autonomous effects of Dscam mutant clones, which suggests that the α'/β' axons may be acting as pioneers for the α/β axons at least some of the time [43].

unf plays a common role in the early development of all mushroom body neurons

The data presented here demonstrate that unf plays a common role in the early development of all three subtypes of MB neurons by regulating axon extension and branching. While we cannot rule out the possibility that single axons, which normally project dorsally, may be misguided and project medially, our analysis is consistent with the hypothesis that unf mutant γ, α'/β', and α/β neurons fail to project dorsal axon branches. Our observations that unf mutant MB neurons express subtype-specific epitopes such as Fas II and Trio suggest that unf does not impact MB neuronal subtype identity. Interestingly, Lin et al. [41] disagree and conclude that unf does regulate MB neuronal subtype identity based on a series of unf RNAi knockdown experiments. We argue that until the transcriptional codes that distinguish MB neuronal subtypes are defined, one cannot conclusively determine whether the identity of these neurons has been impacted [44-47].

Conclusions

These data support the hypothesis that unf plays a common role in the early development of all three subtypes of MB neurons, γ, α'/β', and α/β, by regulating axon extension and branching during the initial phases of larval and pupal outgrowth. Expression of a UAS-unf transgene in MB neurons of unf mutants rescues the unf mutant MB phenotypes, demonstrating that the MB defects are due to the lack of unf. The phenotypic variation and asymmetry observed in the MBs of unfX1/unf20001 compound heterozygotes suggests a role for unf in the targeting of pioneer axons.

Materials and methods

Drosophila stocks

All stocks were raised on standard cornmeal and sugar medium. The unfX1 and unf20001 stocks have been characterized previously: the unfX1 allele disrupts the 5' donor splice site of intron 2, whereas the unf20001 allele has a missense mutation due to a guanine to adenine transition at base 312 of exon 2 resulting in a glycine to arginine substitution (G120R) [25]. The Df(2R)ED2426 (Df2426) chromosome carries a deletion of 482,016 bp...
on the second chromosome that removes 57 genes or annotated genes in their entirety, including unf [52]. The Hr51^MB05909 (unf^MB05909) line contains a GAL4 insertion in intron 1 of the unf gene (FlyBase). The Df(2R)ED2426, Hr51^MB05909 (unf^MB05909), UAS-CD8:GFP; OK107-GAL4, 201Y-GAL4, c747-GAL4, and c739-GAL4 lines were obtained from Bloomington Stock Center. The UAS-unfRNAi line was obtained from Vienna Drosophila RNAi Center (VDRC). All mutant or transgenic stocks were maintained over GFP-marked chromosomes to facilitate genotyping. Animals were reared at 25°C with the exception of UAS-unfRNAi crosses, which were reared at 29°C.

Transgenic flies and rescue experiment

The transgenic rescue constructs UAS-unfGFP and UAS-unf3ac are two independent isolates of the same transgene, which was generated by cloning the unf cDNA [25] into the vector pUAST [40]. Transgenic flies were generated by P-element-mediated transformation [53]. A homozygous w^1118 stock was used for all P-element-mediated transformations. Df2426/CyO;UAS-unf^GFP/TM3Sb flies were crossed to unf^X1, UAS-GFP/CyOGFP; OK107-GAL4 or unf^G0001, UAS-GFP/CyOGFP;OK107-GAL4 flies to generate rescues and control siblings. Df2426/CyO;UAS-lacZ/TM3Sb flies were crossed to unf^X1, UAS-GFP/CyOGFP;OK107-GAL4 flies for additional rescue controls. All rescue and control larvae and pupae were genotyped using PCR. The following primers were used to detect the presence of unf^X1, unf^G0001, Df2426, and UAS-unf^GFP, unf^X1, 5’ CAGCGGCATTGCTACACTC 3’ (fx1b1) and 5’ GGAAAATTCCGGATTCCGATGAGCTTTGACCACAC 3’ (R974) followed by XbaI digest; unf^G0001, 5’ CTGAGCTGGAACATCAGCTG 3’ (L150Z1) and 5’ GGATTCGCTAGTGTTCTTCT 3’ (R330Z1); Df2426, 5’ TCAATTAATTTAGTGGGCGGA 3’ (2426A) and 5’ CAATCATATCGCTGTCTCACTCA 3’ (R974); UAS-unf^GFP, 5’ CAGCGGCATTGCTACACTC 3’ (fx1b1) and 5’ GATTCCGATGACTCTGTCCACCACAC 3’ (XR941).

Immunohistochemistry and microscopy

Third instar larvae and pupae were staged as described [54,55]. The central nervous system of third instar larvae, pupae, and 0- to 2-day adults were collected, fixed in 4% paraformaldehyde, and processed using standard protocols [9]. mAb1D4 [56] (anti-Fas II: 1:10) and mAb9.4A [39] (anti-Trio: 1:4) were obtained from the Developmental Studies Hybridoma Bank (DSHB). The rabbit anti-Fas II (1:2,000) was a gift from Dr Vivian Budnik (University of Massachusetts). Biotinylated antimouse and anti-rabbit IgG (1:200) were obtained from Vector Labs (Burlingame, CA USA). Streptavidin Alexa Fluor 488, 546 (1:200), and Alexa Fluor 546 phalloidin (1:40) were obtained from Invitrogen Molecular Probes (Carlsbad, CA USA). Preparations were examined and imaged using an Olympus Fluoview FV-1000 laser scanning confocal system mounted on an Olympus IX-81 inverted microscope. Images were processed using Image J and Adobe Photoshop. Movies of z stacks were processed using QuickTime. During z stack collections for Additional files 1 to 3, the photomultiplier tube was manually adjusted for optimal brightness at different focal planes.

Statistics

The Fisher exact test was used to determine whether the frequency of defects in experimental animals was significantly different from the frequency of defects in control animals. Relevant genotypes were tested in pair-wise combinations. P-values less than 0.01 were considered significant.

Additional file 1: Figure S1. Movie of a z stack of an adult unf^X1;UAS-mCD8GFP/CyO;OK107-GAL4 control animal labeled with anti-Fas II (red), in which all MB lobes are properly formed. OK107-GAL4 driven GFP expression is visible in the Kenyon cells (green) and all MB lobes. Images depict the right hemisphere at 90x magnification. Click here for file

Additional file 2: Figure S2. Movie of a z stack of an adult unf^G0001;UAS-mCD8GFP/DD2426;OK107-GAL4 mutant labeled with anti-Fas II (red). The Kenyon cells (green) are visible in posterior planes. A rarely observed medial projection is visible in the region of the calyx. Axons travel anteroventrally then spread out, and stall prior to lobe formation. Images depict the left hemisphere at 90x magnification. Click here for file

Additional file 3: Figure S3. Movie of a z stack of an adult unf^G0001;UAS-mCD8GFP/DD2426;OK107-GAL4 mutant labeled with anti-Fas II (red). Axons project anteroventrally in a poorly organized peduncle, spread out, and stall at the end of the peduncle. The median bundle (green) is to the left. Images depict the right hemisphere at 90x magnification. Click here for file

Additional file 4: Figure S4. Movie of a z stack of an adult unf^G0001;UAS-mCD8GFP/DD2426;OK107-GAL4 mutant labeled with anti-Fas II (red). Axons travel anteroventrally. γ, β, and α axes projected medially but were disorganized. The γ axes occupy the most superficial plane and can be distinguished from the more posterior β (green) and Fas II-positive β (yellow) axes. Images depict the right hemisphere at 90x magnification. Click here for file

Additional file 5: Figure S5. Movie of a z stack of an adult unf^G0001;UAS-mCD8GFP/unf^G0001;OK107-GAL4 mutant labeled with anti-Fas II (red). Axons travel anteroventrally in a tightly organized peduncle. γ, β, and α axes projected medially. A thin fascicle of axons projects dorsally but stalls. Images depict the left hemisphere at 90x magnification. Click here for file
2. Zars T: Behavioral functions of the insect mushroom bodies.

References

1. Davis RL: Olfactory memory formation in Drosophila: from molecular to systems neuroscience. Annu Rev Neurosci 2005, 28:275-302.

2. Zars T: Behavioral functions of the insect mushroom bodies. Curr Opin Neurobiol 2000, 10:790-795.

3. Armstrong JD, de Belle JS, Wang Z, Kaiser K: Metamorphosis of the mushroom bodies; large-scale rearrangements of the neural substrates for associative learning and memory in Drosophila. Learn Mem 1998, 5:102-114.

4. Lee T, A. Luo L: Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. Development 1999, 126:4065-4076.

5. Technau G, Heisenberg M: Neural reorganization during metamorphosis of the corpora pedunculata in Drosophila melanogaster. Nature 1982, 295:405-407.

6. Crimpen-JR, Toussaint EM, Han KA, Kalderon D, Davis RL: Tripartite mushroom body architecture revealed by antigenic markers. Learn Mem 1998, 5:38-51.

7. Yang MY, Armstrong JD, Vilinsky I, Straussfeld NJ, Kaiser K: Subdivision of the Drosophila mushroom bodies by enhancer-trap expression patterns. Neuron 1995, 15:45-54.

8. Ito K, Awanio W, Suzuki K, Hiromi Y, Yamamoto D: The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurons and glial cells. Development 1997, 124:761-771.

9. Lee T, Luo L: Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuor 1999, 22:451-461.

10. Truman JW, Bate M: Spatial and temporal patterns of neurogenesis in the central nervous system of Drosophila melanogaster. Dev Biol 1968, 125:145-157.

11. Ito K, Hotta Y: Proliferation pattern of postembryonic neuroblasts in the brain of Drosophila melanogaster. Dev Biol 1992, 149:134-148.

12. Martini SR, Roman G, Meuser S, Mardon G, Davis RL: The retinal determination gene, dachshund, is required for mushroom body cell differentiation. Development 2000, 127:3663-3672.

13. Kurusu M, Nagao T, Walldorf U, Fliesser S, Gehring WJ, Furukubo-Tokunaga K: Genetic control of development of the mushroom bodies, the associative learning centers in the Drosophila brain, by the eyeless, twin of eyeless, and Dachshund genes. Proc Natl Acad Sci USA 2000, 97:2140-2144.

14. Noveen A, Daniel A, Hartenstein V: Early development of the Drosophila mushroom body: the roles of eyeless and dachshund. Development 2000, 127:3475-3488.

15. Nicolai M, Lasbleiz C, Dura JM: Gain-of-function screen identifies a role of the Srp64 oncogene in Drosophila mushroom body development. J Neurobiol 2003, 57:291-302.

16. Raabe T, Clemens-Richter S, Twardzik T, Ebert A, Gramlich G, Heisenberg M: Identification of mushroom body miniature, a zinc-finger protein implicated in brain development of Drosophila. Proc Natl Acad Sci USA 2004, 101:14276-14281.

17. de Belle JS, Heisenberg M: Expression of Drosophila mushroom body mutations in alternative genetic backgrounds: a case study of the mushroom body miniature gene (mbm). Proc Natl Acad Sci USA 1996, 93:9875-9880.

18. Zhu S, Lin S, Kao CF, Avaskas T, Chiang AS, Lee T: Gradients of the Drosophila Chimo BTB-zinc finger protein govern neuronal temporal identity. Cell 2006, 127:409-422.

19. Wang J, Lee CH, Lin S, Lee T: Steroid hormone-dependent transformation of polyheteromic mutant neurons in the Drosophila brain. Development 2006, 133:1231-1240.

20. Kurusu M, Maruyama Y, Adachi Y, Okabe M, Suzuki E, Furukubo-Tokunaga K: A conserved nuclear receptor, Tailless, is required for efficient proliferation and prolonged maintenance of mushroom body progenitors in the Drosophila brain. Dev Biol 2009, 326:224-236.

21. Martini SR, Davis RL: The dachshund gene is required for the proper guidance and branching of mushroom body axons in Drosophila melanogaster. J Neurobiol 2005, 64:133-144.

22. Lee T, Marticke S, Sung C, Robinow S, Luo L: Cell-autonomous requirement of the USP/Ecr-B edysone receptor for mushroom body neuronal remodeling in Drosophila. Neuron 2000, 28:807-818.

23. Zheng X, Wang J, Haenny TE, Wu Y, Martin J, O'Connor MB, Lee CH, Lee T: TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the Drosophila brain. Cell 2003, 112:303-315.
24. Zheng J, Edelman SW, Thamararajah G, Walker DW, Fletcher SD, Seroude L: Differential patterns of apoptosis in response to aging in Drosophila. Proc Natl Acad Sci USA 2005, 102:12083-12088.

25. Sung C, Wong LE, Chang Sen LG, Nguyen E, Laazga N, Ganzer G, McNabb SJ, Robinson S. The unfulfilled/DRR51 gene of Drosophila melanogaster modulates wing expansion and fertility. Dev Dyn 2009, 238:171-182.

26. Laudet V,AGH. The Nuclear Receptor Factsbook San Diego: Academic Press 2002.

27. DeMeo SD, Lomber RM, Cronin M, Smith EL, Snowflack DR, Reinitz K, Clever S, Wightman B. Specificity of DNA-binding by the FAX-1 and NHR-67 nuclear receptors of Caenorhabditis elegans is partially mediated via a subclass-specific P-box residue. BMC Mol Biol 2008, 9:2.

28. Wightman B, Baran R, Garriga G. Genes that guide growth cones along the C. elegans ventral nerve cord. Development 1997, 124:2571-2580.

29. Much JW, Slade DJ, Kamptt K, Garriga G, Wightman B. The fax-1 nuclear hormone receptor regulates axon pathfinding and neurotransmitter expression. Development 2000, 127:703-712.

30. Wightman B, Ebert B, Carneman N, Weber K, Clever S. The C. elegans nuclear receptor gene fax-1 and homeobox gene unc-42 coordinate interneuron identity by regulating the expression of glutamate receptor subunits and other neuron-specific genes. Dev Biol 2005, 287:74-85.

31. Haider NB, Jacobson SV, Swiderski R, Streb LM, Seabury C, Beck G, Hake R, Hannon DB, Gorman S, Duhl D, Cermi R, Bennett J, Welleher RG, Fishman GA, Wright AF, Stone EM, Sheffeld VC. Mutation of a nuclear receptor gene, NR2E3, causes enhanced s cone syndrome, a disorder of retinal cell fate. Nat Genet 2000, 24:127-131.

32. Haider NB, Molelina N, Gaule M, Yuan Y, Sachs AJ, Nystuen AM, Naggett JK, Nishina PM. Nr2e3-directed transcriptional regulation of genes involved in photoreceptor development and cell-type specific phototransduction. Exp Eye Res 2009, 89:365-372.

33. Peng GH, Ahmad O, Ahmad F, Liu J, Chen S. The photoreceptor-specific nuclear receptor NR2e3 interacts with Crx and exerts opposing effects on the transcription of rod versus cone genes. Hum Mol Genet 2005, 14:747-764.

34. Chen F, Figueroa DJ, Marmorstein AD, Zhang Q, Petrukhin K, Caskey CT, Liang IH, Lee CH, Lee T: The rod photoreceptor-specific nuclear receptor activity Nr2e3-directed transcriptional regulation of genes involved in retinal rod photoreceptor development and cell-type specific phototransduction. Exp Eye Res 2009, 89:365-372.

35. Chen J, Ratter R, Nathans J. The rod photoreceptor-specific nuclear receptor gene Nr2e3 represses transcription of multiple cone-specific genes. J Neurosci 2005, 25:118-129.

36. Cheng H, Kehana H, Oh EC, Hicks D, Mitton KP, Swope A.Photoreceptor-specific nuclear receptor NR2E3 functions as a transcriptional activator in rod photoreceptors. Hum Mol Genet 2004, 13:1563-1575.

37. Kobayashi M, Takezawa S, Hasa K, Yu RT, Umesono Y, Agata K, Tanikawa M, Masuda K, Umesono K. Identification of a photoreceptor cell-specific nuclear receptor, Proc Natl Acad Sci USA 1999, 96:4614-4619.

38. Connolly JB, Roberts U, Armstrong JD, Kaiser K, Forte M, Tully T, O’Kane CJ. Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. Science 1996, 274:2104-2107.

39. Awasaki T, Saito M, Sone M, Suzuki E, Sakai H, Hama C. The Drosophila trio plays an essential role in patterning of axons by regulating their directional extension. Neuron 2001, 26:119-131.

40. Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 1993, 118:401-415.

41. Lin S, Huang Y, Lee T. Nuclear receptor unfulfilled regulates axonal guidance and cell identity of Drosophila mushroom body neurons. PLoS One 2009, 4:e8392.

42. Coppieters F, Leroy BP, Beyeren D, Hellerman J, De Bosscher K, Hagegern G, Robberecht K, Wuyts W, Coucke PJ, De Baere E. Recurrent mutation in the first zinc finger of the orphan nuclear receptor NR2E3 causes autosomal dominant retinitis pigmentosa. Am J Hum Genet 2007, 81:147-157.

43. Wang J, Zuges CT, Liang H, Lee CH, Lee T. Drosophila Dscam is required for divergent segregation of sister branches and suppresses ectopic bifurcation of axons. Neuron 2002, 33:559-571.

44. Brody T, Odenwald WF. Cellular diversity in the developing nervous system: a temporal view from Drosophila. Development 2002, 129:3763-3770.

45. Pearson BJ, Doe CQ. Specification of temporal identity in the developing nervous system. Annu Rev Cell Dev Biol 2004, 20:619-647.

46. Kao CF, Lee T. Birth time/order-dependent neuron type specification. Curr Opin Neurobiol 2009.

47. Yu HH, Lee T. Neuronal temporal identity in post-embryonic Drosophila brain. Trends Neurosci 2007, 30:520-526.

48. Palanker L, Necakos AV, Sampson HM, Ni R, Hu C, Thummel CS, Krause HM. Dynamic regulation of Drosophila nuclear receptor activity in vivo. Development 2006, 133:3549-3562.

49. Kobayashi M, Michaut L, Ino A, Honjo K, Nakajima T, Manayama Y, Mochuzki H, Ando M, Changreker T, Takahashi K, Saigo K, Ueda R, Gehring WJ, Furukubo-Tokunaga K. Differential microarray analysis of Drosophila mushroom body transcripts using chemical ablation. Proc Natl Acad Sci USA 2006, 103:14417-14422.

50. Boyle M, Nighorn A, Thomas JB: Drosophila Eph receptor guides specific axon branches of mushroom body neurons. Development 2006, 133:1845-1854.

51. Wang J, Ma X, Yang JS, Zheng X, Zuges CT, Lee CH, Lee T. Transmembrane/junctamembrane domain-dependent Dscam distribution and function during mushroom body neuronal morphogenesis. Neuron 2004, 43:663-672.

52. Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, Drummond J, Webster J, Gubb D, Gunton N, Johnson G, O’Kane CJ, Huen D, Sharma P, Asztalos Z, Baisch H, Schuler J, Kube M, Kittlaus K, Reuter G, Maroy P, Szondy J, Rasmussen-Lestander A, Ekstrom D, bishop B, Hugentobler C, Stocker H, Hafen E, Leipsent JA, Pflugfelder G, Heisenberg M, et al. The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster. Genetics 2004, 167:797-813.

53. Rubin GM, Spradling AC. Genetic transformation of Drosophila with transposable element vectors. Science 1982, 218:348-353.

54. Barnidge SP, Boxines M. Staging the metamorphosis of Drosophila melanogaster. J Embryol Exp Morphol 1981, 66:37-40.

55. Andres AJ, Thummel CS. Methods for quantitative analysis of transcription in larvae and prepupae. Methods Cell Biol 1994, 44:565-573.

56. Van Vactor D, Stocker H, Hafen E, Leipsent JA, Pflugfelder G, Heisenberg M, et al. The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster. Genetics 2004, 167:797-813.

Rubin GM, Spradling AC. Genetic transformation of Drosophila with transposable element vectors. Science 1982, 218:348-353.

Barnidge SP, Boxines M. Staging the metamorphosis of Drosophila melanogaster. J Embryol Exp Morphol 1981, 66:37-40.

Andres AJ, Thummel CS. Methods for quantitative analysis of transcription in larvae and prepupae. Methods Cell Biol 1994, 44:565-573.

Van Vactor D, Stocker H, Fambrough D, Tsoo R, Goodman CS. Genes that control neuromuscular specificity in Drosophila. Cell 1993, 73:1137-1153.