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From Lineage to Wiring Specificity: POU Domain Transcription Factors Control Precise Connections of *Drosophila* Olfactory Projection Neurons

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Summary

Axonal selection of synaptic partners is generally believed to determine wiring specificity in the nervous system. However, we have recently found evidence for specific dendritic targeting in the olfactory system of *Drosophila*: second order olfactory neurons (Projection Neurons) from the anterodorsal (adPN) and lateral (lPN) lineages send their dendrites to stereotypical, intercalating but non-overlapping glomeruli. Here we show that POU domain transcription factors, Acj6 and Drifter, are expressed in adPNs and lPNs respectively, and are required for their dendritic targeting. Moreover, misexpression of Acj6 in lPNs, or Drifter in adPNs, results in dendritic targeting to glomeruli normally reserved for the other PN lineage. Thus, Acj6 and Drifter translate PN lineage information into distinct dendritic targeting specificity. Acj6 also controls stereotypical axon terminal arborization of PNs in a central target, suggesting that the connectivity of PN axons and dendrites in different brain centers is coordinately regulated.

Introduction

The proper functioning of nervous systems depends on the development of specific connections between up to a trillion neurons. Understanding how information in the genome is translated into specific neuronal connections remains a fundamental challenge of biology. Precise temporal and spatial regulation of gene expression by transcription factors makes a significant contribution to wiring specificity. Transcription factors can act on at least three levels: specification of neuronal fates, selection of specific axonal pathways, and selection of specific synaptic partners. This is exemplified by study of the circuitry of the vertebrate spinal cord, especially at the first two levels (reviewed by Jessell, 2000; Shirasaki and Pfaff, 2002). For example, the axonal pathways adopted by different motor neuron populations are regulated by the combinatorial action of the LIM homeodomain proteins (Kania et al., 2000; Sharma et al., 2000). Remarkably, an earlier study in *Drosophila* identified a similar LIM code in motor axon pathway selection (Thor et al., 1999). At the third level, specific synaptic connectivity, ETS domain transcription factors have been proposed to specify the connections of distinct populations of sensory and motor neurons based initially on expression pattern (Lin et al., 1998); recent genetic experiments provide support for this proposal (Arber et al., 2000; Livet et al., 2002).

Selection of synaptic partners involves the interaction of presynaptic axons and postsynaptic dendrites. While axons have traditionally been regarded as the active partner determining connection specificity, dendrites may also contribute to the selection of synaptic partners. Moreover, most neurons in the central nervous system have specific dendritic inputs as well as specific axonal outputs as they collect, transform, and transmit information between different parts of the nervous system. Thus, two questions arise: first, is there a transcriptional program for dendritic targeting? Second, how are the axonal and dendritic connections of any particular neuron coordinated? Here we address these general questions in the wiring of the *Drosophila* central olfactory circuit.

The ordered axonal projections of olfactory receptor neurons (ORNs) bearing the same olfactory receptor to specific glomeruli is an example of extremely precise neuronal wiring (Mombaerts et al., 1996; Vosshall et al., 2000). No less remarkable is the finding that projection neurons (PNs), the second order neurons of the *Drosophila* olfactory system (equivalent to vertebrate mitral/tufted cells: Figure 1A), show a similar degree of precision in their dendritic targeting to specific glomeruli (Jefferis et al., 2001) along with highly stereotyped axonal branching patterns in higher olfactory centers specific to each glomerular class (Marin et al., 2002; Wong et al., 2002). Present evidence suggests that both connections are likely to develop independent of olfactory input and that a genetic program is responsible for building a highly stereotyped neural network linking ORNs to higher olfactory centers (Jefferis et al., 2001; Marin et al., 2002; Wong et al., 2002). *Drosophila* PNs thus provide an excellent system to address the genetic origins of highly specific dendritic and axonal connectivity.

We have previously shown that the two major lineages of PNs, anterodorsal PNs (adPNs) and lateral PNs (lPNs), send dendrites to stereotyped and mutually exclusive sets of glomeruli in the antennal lobe (Figure 1B), where they synapse with ORN axons. Within the adPN lineage, birth order further specifies the identity of these neurons' future dendritic and axonal targets (Jefferis et al., 2001). Here we address the molecular mechanisms that control these specific and coordinated patterns of dendritic and axonal connectivity. We show that two POU domain transcription factors, Acj6 and Drifter, are differentially expressed in adPNs and lPNs, respectively. Both are required for the distinct dendritic connectivity of PNs in their respective lineages and cause mistargeting when misexpressed in PNs of the alternate lineage. Furthermore, *acj6* mutant PNs exhibit a highly specific axon terminal arborization defect that can be modified by Drifter misexpression. We propose that Acj6 and Drifter

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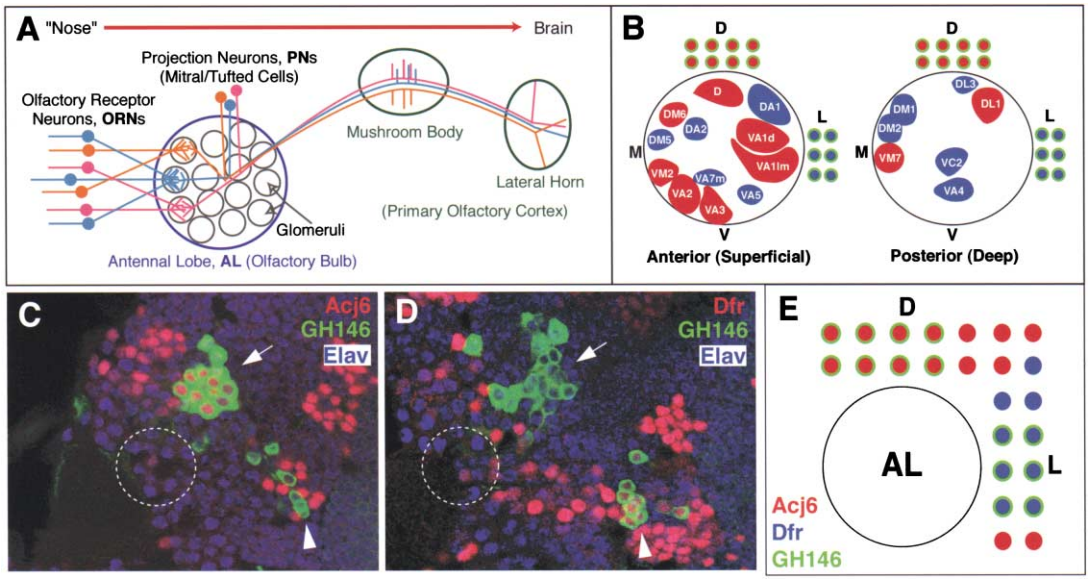


Figure 1. Acj6 and Drifter Are Expressed in Two Distinct PN Lineages Whose Dendrites Innervate Intercalating but Non-Overlapping Glomeruli

(A) A schematic of the organization of the *Drosophila* olfactory system, with mammalian counterparts in parentheses. Olfactory receptor neurons (ORNs) expressing the same receptor (represented by the same color) project their axons to the same glomeruli in the antennal lobe (AL). Projection neurons (PNs) send dendrites to glomeruli and axons to the mushroom body and the lateral horn, two higher olfactory centers approximately analogous to vertebrate primary olfactory cortex.

(B) A schematic of the mutually exclusive patterns of glomerular innervation of adPNs and lPNs. adPNs (red with green outline) innervate red glomeruli, and lPNs (blue with green outline) innervate blue glomeruli (Jefferis et al., 2001).

(C and D) Expression of Acj6 (C) and Dfr (D) at 0 hr APF examined in a single confocal section. Among GH146 positive PNs (green), adPNs (arrows) express Acj6 (red channel in C) but not Dfr (red channel in D), and lPNs (arrowheads) express Dfr but not Acj6. The approximate position of the developing antennal lobe is shown with dotted circles.

(E) A schematic of Acj6 (red), Dfr (blue), and GH146 (green) expression pattern around the antennal lobe (AL). GH146-positive adPNs express Acj6 and lPNs express Dfr.

In this and all subsequent figures, the right side of the brain is shown. Medial is to the left, dorsal is up.

translate lineage information into wiring specificity: they regulate dendritic targeting and coordinate dendritic and axonal connectivity of PNs to ensure the highly stereotyped acquisition and delivery of olfactory information by these central olfactory neurons.

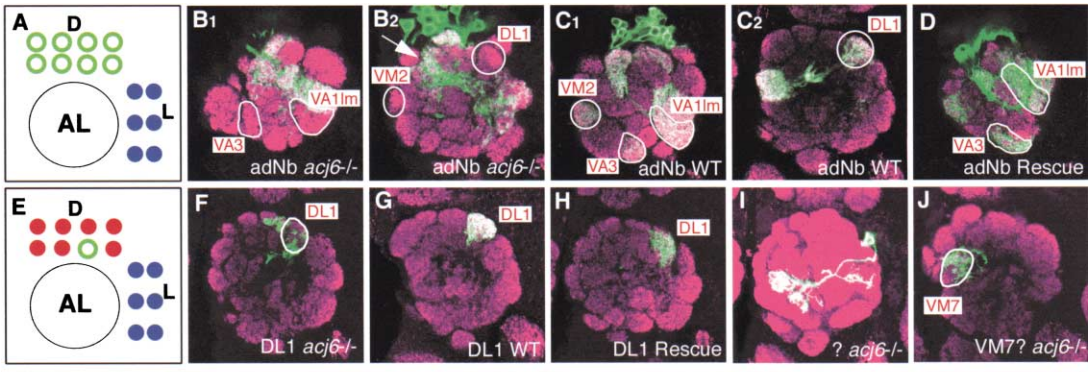
Results

In this study, we focus on PNs that express the enhancer trap line *GAL4-GH146* (hereafter referred to as *GH146*), which labels one half to two thirds of all PNs (Stocker et al., 1997). This includes two major groups of PNs whose cell bodies are anterodorsal and lateral to the antennal lobe (adPNs and lPNs, respectively) and which originate from two separate neuroblasts (Jefferis et al., 2001). For genetic analysis, we used the MARCM system (Lee and Luo, 1999) to generate labeled PN clones (green in Figures 2–7) that are homozygous mutant for a gene, or express a transgene, or both at once. Unless otherwise noted, all clones were induced by heat shock applied in newly hatched larvae; therefore, our genetic analyses are restricted to larval-born adPNs (~32) innervating ~13 glomeruli, and lPNs (~33) innervating ~12 glomeruli (Jefferis et al., 2001). We further restricted our glomerular analysis to "landmark" glomeruli (Laissue et al., 1999) that are highly stereotyped and unequivocally identifiable by staining with a monoclonal antibody nc82 that recognizes a synaptic antigen (magenta in Figures 2–7).

Acj6 Is Expressed in adPNs but Not in lPNs

acj6 (abnormal chemosensory jump 6) was identified in an olfactory behavioral screen (McKenna et al., 1989) and shown to have a peripheral olfactory defect (Ayer and Carlson, 1991). It encodes a POU domain transcription factor, Acj6, which is expressed in all ORNs and is required for expression of a subset of the OR genes (Clyne et al., 1999a, 1999b). It remains to be determined whether Acj6 also plays a role in ORN axon targeting. Acj6 is also highly expressed in a number of central brain neurons, notably in the vicinity of the antennal lobe (Certel et al., 2000a), so we decided to test a potential role of Acj6 in PN development.

To identify in which cell populations Acj6 is expressed, we stained brains for Acj6, *GH146*, and Elav (expressed in all post-mitotic neurons: Robinow and White, 1988). We carried out this expression analysis at 8 different developmental stages: 48 and 72 hr after larval hatching (ALH); wandering 3rd instar larvae; 0, 18, 30, and 50 hr after puparium formation (APF); and in adults. Consistent with previous observations (Certel et al., 2000a), we found that in the brain Acj6 is exclusively expressed in the nuclei of post-mitotic neurons at all stages examined (Figure 1C and data not shown).



K

	DL1	D	VA3	VA1d	VM7	VM2	DM6*	VA1lm
acj6 -/-	11/19	15/19	0/19	19/19	19/19	2/19	3/19	12/19
Rescue	19/21	20/21	11/21	21/21	21/21	19/21	5/21	19/21

Figure 2. The Dendrites of *acj6*$^{-/-}$ adPNs Fail to Innervate Specific Glomeruli

(A and E) Schematics of experiments described in this figure. Anterodorsal neuroblast (adNb) clone (A) and single-cell clone (E) are schematized. Only the labeled adPNs (green outline) are *acj6*$^{-/-}$, while the rest of the adPNs have at least one copy of wild-type *acj6* (red nuclei); the lPNs still express *dfr* (blue nuclei).

(B and C) Antennal lobe dendritic innervation pattern of *acj6*$^{-/-}$ (B) and wild-type (C) adNb clones shown in anterior (B_1, C_1) or posterior (B_2, C_2) confocal sections. The *acj6*$^{-/-}$ clone failed to innervate VA1lm, VA3 (B_1), DL1, and VM2 (B_2). Brains are unavoidably mounted at slightly different angles explaining the missing dorsomedial portion of the antennal lobe in B_1, which is in more posterior sections.

(F and G) Antennal lobe dendritic innervation pattern of *acj6*$^{-/-}$ (F) and wild-type (G) DL1 single-cell clones. The glomerulus DL1 is circled in (F).

(D and H) *acj6*$^{-/-}$ phenotypes were rescued by *acj6* transgene expression in an adNb clone (D, anterior confocal section is shown) or in a DL1 single-cell clone (H).

(I and J) Antennal lobe dendritic innervation pattern of two *acj6*$^{-/-}$ single-cell clones induced by late heat shock in stacks of confocal sections (I) and a confocal section (J). (I) is an example with very wide dendritic projections without having a focus, and (J) mostly innervates the glomerulus VM7.

(K) Quantification of *acj6*$^{-/-}$ and rescue adNb clone phenotypes (n = 19 and 21, respectively). The numbers indicate instances when each glomerulus is properly innervated. Yellow highlights glomeruli with defective innervation. The glomerulus DM6 (*) was occasionally absent, which was scored as uninnervated.

In this and all subsequent images, green represents mCD8-GFP, a cell marker for labeled MARCM clones; magenta represents mAb nc82, a synaptic marker highlighting all glomeruli in the antennal lobe.

Strikingly, we find that Acj6 is expressed in a lineage-specific fashion in *GH146*-positive PNs. It is highly expressed in all adPNs at all developmental stages examined, but not expressed in lPNs with the exception of two neurons (Figure 1C and data not shown). These two lPNs are absent in clones induced by early larval heat shock (data not shown), so they are either embryonically born or belong to a different neuroblast lineage. Therefore, these two neurons are excluded from our genetic analysis (see above). Notably, we found some Acj6-positive/*GH146*-negative neurons next to the *GH146*-positive adPNs and lPNs (Figure 1C); we do not know whether these cells are *GH146* negative PNs or other neurons such as local interneurons.

Acj6 Is Required for Proper Dendritic Targeting of adPNs

Since Acj6 is expressed in adPNs but not in lPNs, we hypothesized that Acj6 may contribute to the lineage-specific connectivity of adPNs. To test this hypothesis, we studied the consequences of removing Acj6 activity exclusively from adPNs using the MARCM system (Lee and Luo, 1999). In this strategy, we generated neuroblast (Nb) or single-cell clones that are both labeled with membrane-targeted mCD8-GFP and homozygous for an *acj6* null mutation (*acj6*^{6}) in an unlabeled heterozygous background (Figures 2A and 2E; see Experimental Procedures for details). We found no significant change in cell numbers in *acj6*$^{-/-}$ adNb or lNb clones (neuroblast clones of adPN and lPN lineage, respectively) compared to wild-type clones (data not shown); thus, Acj6 is not required for proliferation or survival of PNs. In addition, mutant neurons retained dendritic innervation of the antennal lobe and axonal projections to the mushroom body and lateral horn (see below), so they still acquired the broad fate of PNs. We then focused on the dendritic targeting specificity of mutant adPNs in the antennal lobe.

acj6$^{-/-}$ adNb clones exhibited significant dendritic targeting defects. First, we found non-specific accumulation of dendrites (accounting for perhaps a quarter of the total dendritic mass) spanning a portion of the dorsal side of the antennal lobe (Figure 2B, arrow); furthermore, the organization of the glomeruli in this region, as visualized by nc82 staining, is disrupted. Second, outside of the dorsal area where nonspecific dendritic accumulation was observed, no specific mistargeting to lateral landmark glomeruli or non-landmark glomeruli was detected (Figure 2B and data not shown). Third and most notably, *acj6*$^{-/-}$ adNb clones innervate significantly fewer dorsal landmark glomeruli. We focused our analysis on 8 landmark glomeruli whose organization as visualized by nc82 staining remained largely unaffected: DL1, D, VA3, VA1d, VM7, VM2, DM6, and VA1lm. These 8

glomeruli are always fully innervated by wild-type adNb clones (Figure 2C). Significantly, 6 of these 8 glomeruli showed defects in *acj6*$^{-/-}$ adNb clones (Figure 2B, quantified in 2K). VA3, VM2, and DM6 were frequently (>80%) weakly innervated or completely uninnervated, while the loss of dendritic innervation of DL1, D, and VA1lm was less frequent (20%–50%). VA1d and VM7 showed no detectable phenotype in adNb clones, although this does not preclude an Acj6 requirement in these neurons (see below). This differential dependence on Acj6 does not correlate with the birth order of adPNs innervating these glomeruli (Jefferis et al., 2001) or with the position of these glomeruli in the antennal lobe (see Figure 1B). For instance, VM2 and VM7 are next to each other and are at a similar distance from adPN cell bodies, but in *acj6*$^{-/-}$ adNb clones, VM2 is one of the most affected glomeruli while VM7 is least affected. Thus, these phenotypes do not simply reflect a defect in general dendritic growth and elaboration; rather, they suggest a defect in specific targeting.

To understand this phenotype at higher resolution, we analyzed the dendritic projection pattern of single-cell clones (Figure 2E), which specifically innervate the DL1 glomerulus in wild-type (Figure 2G). This particular class was chosen for analysis since adPN single-cell clones induced by early larval heat shock (0–36 hr) invariably innervate DL1 (Jefferis et al., 2001). This is currently the only PN class we can identify independent of its dendritic pattern in the antennal lobe, which is of course essential for studying mutants that could affect dendritic targeting specificity. All 11 examples of *acj6*$^{-/-}$ DL1 single-cell clones showed abnormal dendritic innervation. They all weakly innervate only part of DL1 when compared to wild-type and possess additional dendritic projections in the vicinity of DL1 (Figure 2F). Thus, uniglomerular dendritic targeting is disrupted in *acj6*$^{-/-}$ DL1 neurons.

To extend the DL1 finding to other PN classes, we also studied adPN single-cell clones induced by a heat shock at several later stages (ranging between 50–100 hr ALH). Wild-type adPNs have predominantly uniglomerular innervation regardless of their birth time (Jefferis et al., 2001). However, at least 17 out of 18 late-born *acj6*$^{-/-}$ adPN single-cell clones had diffuse dendrites, which did not target one specific glomerulus, making it difficult for us to identify the original glomerular classes of these clones in majority of the cases (e.g., see Figure 2I). Figure 2J shows one of the least affected clones, a PN which partially innervates VM7 and has diffuse dendrites outside this glomerulus. Interestingly, VM7 targeting defects were not detected in our neuroblast clone analysis (Figure 2K), which is likely to have a lower resolution in detecting phenotypes.

These data demonstrate that Acj6 is required for proper dendritic targeting of a large subset of adPNs, perhaps even all. As expected from the lack of Acj6 expression in lPNs, no dendritic targeting defects were detected in lNb clones (data not shown).

Post-Mitotic Expression of an *acj6* Transgene Rescues Dendritic Targeting Phenotypes of *acj6*$^{-/-}$ Clones

To confirm that the dendritic targeting phenotypes were specifically due to loss of *acj6* activity, we carried out a rescue experiment using MARCM: an *acj6* transgene was expressed only in labeled *acj6*$^{-/-}$ clones.

Five of the six glomeruli that were defective in *acj6*$^{-/-}$ adNb clones were significantly rescued by the expression of *UAS-Acj6(1,4)*, a transgene coding for one of the alternatively spliced Acj6 isoforms (Certel et al., 2000a) (Figure 2D, quantified in 2K). The dendritic phenotype of *acj6*$^{-/-}$ DL1 single-cell clones was also rescued (Figure 2H, n = 11). These results demonstrate that Acj6 is required cell autonomously for proper dendritic targeting of adPNs. Furthermore, since *GH146* is only expressed in post-mitotic neurons (our unpublished data), Acj6 is only required in post-mitotic neurons; this is consistent with the observation that Acj6 protein is not detected in neuroblasts or ganglion mother cells.

Misexpression of Acj6 in lPNs Causes Dendritic Mistargeting

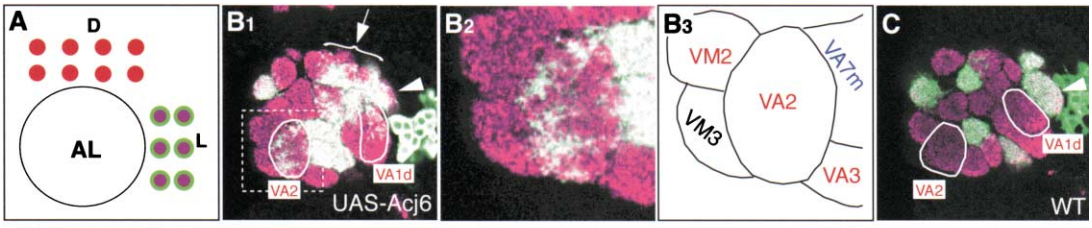
The expression pattern and loss-of-function phenotypes of *acj6* suggest a role in specifying the dendritic targeting patterns of adPNs. We might therefore expect that misexpression of Acj6 in lPNs that normally do not express Acj6 could affect their dendritic targeting specificity. To test this, we used MARCM to misexpress a *UAS-Acj6* transgene only in labeled lNb clones (Figure 3A). Transgene expression was confirmed by immunostaining for Acj6 (data not shown).

The dendrites of lPNs misexpressing *UAS-Acj6(1,4)* showed three phenotypes: reduced innervation of appropriate glomeruli, non-specific accumulation and specific mistargeting to inappropriate glomeruli. First, lPNs misexpressing *UAS-Acj6* still innervate the same 10 lateral landmark glomeruli (DA1, DL3, DA2, DM5, DM2, DM1, VA7m, VA5, VA4, and VC2) as wild-type, though they occasionally innervate some glomeruli more weakly or partially (Figures 3B versus 3C, arrowhead). Secondly, they exhibit diffuse dendritic projections that span part of the lateral side of the antennal lobe (Figure 3B, arrow), with associated glomerular abnormalities as revealed by nc82 staining. Thirdly and most significantly, lPNs misexpressing Acj6 also mistarget their dendrites to well-developed dorsal landmark glomeruli that are normally reserved for adPNs (compare circles in Figures 3B and 3C; quantified in 3D). While this mistargeting often leads to only partial occupation of these inappropriate glomeruli, it is rather specific, since we frequently observe a glomerulus with mistargeted dendrites surrounded by uninnervated glomeruli. For instance, VA2 is always mistargeted whereas its two neighbors VA3 and VM2 are rarely mistargeted (Figure 3D, see also Figures $3B_2$ and $3B_3$). This apparent specificity argues against the possibility that mistargeting is simply a random dendritic spillover.

These results indicate that misexpression of Acj6 in lPNs compromises the specificity of their dendritic targeting and suggest that at least some lPNs acquire characteristics of adPNs.

Drifter Is Expressed in lPNs but Not in adPNs

Since Acj6 is expressed only in adPNs, one of the two major *GH146*-positive PN lineages, we suspected that there was another factor(s) with analogous functions in lPNs, the other major PN lineage. We tested a second



D

VA2*	DL1	D	VA3	VA1d	VM7	VM2	DM6	VA1lm
8/8	0/8	2/8	1/8	8/8	0/8	0/8	8/8	2/8

Figure 3. Acj6 Misexpression in lPNs Causes Dendritic Innervation of Ectopic Glomeruli Including adPN Targets

(A) A schematic of experiments in this figure. The labeled lPNs express an *acj6* transgene (red) in addition to endogenous *dfr* (blue), making their nuclei purple.

(B and C) Compared with control (C), lateral neuroblast clones misexpressing Acj6 (B_1) innervate some lateral landmark glomeruli only partially (arrowhead), exhibit accumulation of dendrites in the lateral part of the antennal lobe (arrow), and innervate additional glomeruli that are normal targets of adPNs, including VA2 and VA1d. The dotted square in B_1 is magnified in B_2, whose glomerular composition is schematized in B_3. In addition to a lateral landmark glomerulus VA7m, VA2 is specifically mistargeted while VM2, VM3, and VA3 remain uninnervated.

(D) Quantification of Acj6 misexpression experiments. All glomeruli listed are normally targets of adPNs, so they are never innervated by wild-type lPNs. The numbers indicate the incidents that these glomeruli are now innervated by lPNs due to Acj6 misexpression (highlighted in yellow). VA2(*) is innervated by embryonically born adPNs, so it was excluded from the loss of function analysis in Figure 2.

POU domain transcription factor, Drifter (Dfr), as a candidate lPN factor. Dfr has previously been shown to be expressed both in neurons and widely outside of the nervous system and is required for differentiation and migration of tracheal cells, the development of midline glia, embryonic central nervous systems, and wing (Anderson et al., 1995; Certel et al., 2000b).

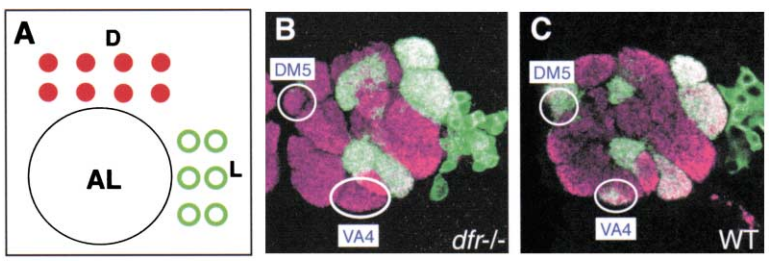
Systematic developmental expression analysis revealed that Dfr is expressed in lPNs but not in adPNs (Figure 1D; data not shown). All lPNs except for the two early-born Acj6-positive lPNs express a variable level of Dfr at larval and early pupal stages. The number of Dfr positive lPNs starts to decrease at 18 hr APF, and in the adult only a few lPNs maintain Dfr expression (data not shown). Unlike Acj6 whose expression is only detectable in post-mitotic neurons, we found that Dfr is also expressed during development in Elav-negative cells likely to be neuroblasts (data not shown). Double-labeling of Acj6 and Dfr in wandering 3rd instar larvae showed no overlap of Acj6 and Dfr expression in *GH146* positive PNs or cells near the antennal lobe (data not shown).

In summary, Acj6 and Dfr are expressed in mutually exclusive *GH146*-positive PNs, with Acj6 in adPNs and Dfr in lPNs (Figure 1E). All adPNs and most, if not all, lPNs that we study here express one and only one of these two transcription factors during larval and early pupal stages when these PNs are being born and targeting dendrites to the antennal lobe.

Dfr Is Required for Proper Dendritic Targeting of lPNs

To test whether Dfr has functions in lPNs analogous to those of Acj6 in adPNs, we removed *dfr* from lPNs by generating MARCM neuroblast clones using a *dfr* null allele, dfr^{B129} (Figure 4A). Like $acj6^{-/-}$ clones, $dfr^{-/-}$ Nb clones showed no significant alteration in cell numbers or in the presence of dendrites and axons in the antennal lobe and mushroom body/lateral horn. However, $dfr^{-/-}$ lNb clones showed a decrease in the number of lateral landmark glomeruli innervated. Eight out of the ten lateral landmark glomeruli were still innervated by $dfr^{-/-}$ lNb clones, but VA4 was never innervated in our twelve examples, and DM5 was missing about half of the time (Figure 4B compared with 4C; quantified in 4D). Notably, no specific mistargeting to dorsal landmark glomeruli or non-landmark glomeruli was detected (Figure 4B and data not shown). The same experiment using dfr^{E82}, a strong hypomorphic allele generated in a different genetic background, showed a qualitatively similar phenotype (data not shown).

The defects of specific glomerular targeting in $dfr^{-/-}$ Nb clones, although qualitatively similar to $acj6^{-/-}$ clones, are much milder. One possible account for this



D

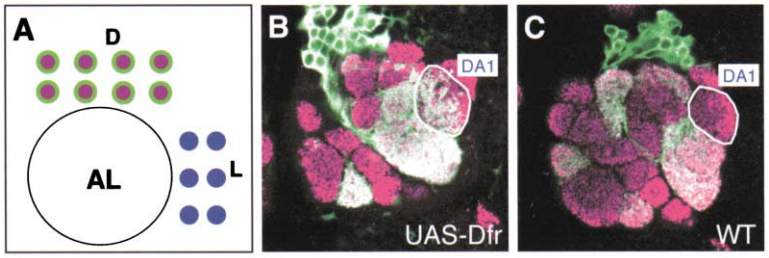
DA1	DL3	DA2	DM5	DM2	DM1	VA7m	VA5	VA4	VC2
12/12	12/12	12/12	7/12	12/12	12/12	12/12	12/12	0/12	12/12

Figure 4. $dfr^{-/-}$ lPNs Fail to Send Dendrites to at Least Two Glomeruli

(A) A schematic of experiments in this figure. *dfr* was homozygous mutant only in the labeled lPNs.

(B and C) Antennal lobe dendritic innervation patterns of $dfr^{-/-}$ lNb clone (B) and a wild-type control (C). The $dfr^{-/-}$ clone failed to innervate DM5 and VA4.

(D) Quantification of $dfr^{-/-}$ lPNs innervation defects (highlighted in yellow).



D

DA1	DL3	DA2	DM5	DM2	DM1	VA7m	VA5	VA4	VC2
6/9	0/9	3/9	0/9	0/9	0/9	0/9	0/9	1/9	0/9

Figure 5. Dfr Misexpression in adPNs Causes Additional Innervation of lPN Targets

(A) A schematic of experiments. Only the labeled adPNs express a *dfr* transgene in addition to endogenous *acj6*, making their nuclei purple.

(B and C) Compared with control (C), adNb clones misexpressing Dfr (B) have additional innervation of DA1, a normal target of lPNs.

(D) Quantification of adPN mistargeting defects due to Dfr misexpression (highlighted in yellow).

difference is perdurance. Unlike Acj6, whose expression is only detected in post-mitotic neurons, Dfr is also expressed in neuroblasts. The reduced severity of the $dfr^{-/-}$ phenotype compared with *acj6* could be due to perdurance of Dfr mRNA or protein inherited from the parental neuroblast in the MARCM analysis. Alternatively, it is possible that *dfr* is more redundant with other lPN factors (see Discussion).

Misexpression of Dfr in adPNs Causes Dendritic Mistargeting

Next, we tested whether Dfr misexpression in adPNs (Figure 5A)—which normally express Acj6 but not Dfr—can alter their dendritic targeting in a manner analogous to Acj6 misexpression in lPNs. We found that in adNb clones misexpressing Dfr (expression confirmed by immunostaining; data not shown), the innervation of dorsal landmark glomeruli was largely unaffected, and loss of dendritic projections to these glomeruli was rarely observed (2 out of 9 clones lacked VA3 innervation and other glomeruli were always innervated). Significantly, however, these adPNs also mistargeted their dendrites to 3 (out of 10) lateral landmark glomeruli, DA1, DA2, and VA4 (Figure 5B, compared with 5C; quantified in 5D).

Thus, both loss-of-function and misexpression phenotypes suggest that *dfr* controls dendritic targeting specificity of lPNs.

Novel Phenotypes Generated by Simultaneous Loss of Acj6 and Gain of Drifter

So far, we have created situations in which PNs are either devoid of both POU domain proteins (loss-of-function clones) or simultaneously express both POU factors (misexpression clones). We now tested the effect of simultaneous loss of Acj6 and misexpression of Dfr in adPNs (Figure 6A). Because of the complexity of the resulting phenotypes, we focused our analysis on single-cell DL1 clones, which give us a higher resolution.

DL1 single-cell clones misexpressing Dfr did not show detectable phenotypes (Figures $6C_1$ and $6C_2$), perhaps because the level of Dfr misexpression was unable to overcome the endogenous Acj6 activity. However, all DL1 single-cell clones that are both *acj6* mutant and misexpress Dfr completely failed to innervate the glomerulus DL1. Most of their dendrites partially innervated one or two glomeruli anterior to DL1, with some wandering dendrites within the dorsal half of the antennal lobe (Figures $6B_1$ and $6B_2$, $n = 10$). This is in clear contrast to $acj6^{-/-}$ DL1 single-cell clones, which always weakly innervate part of DL1 as well as nearby regions (Figure 2F). Thus, the residual ability of $acj6^{-/-}$ clones to target correctly was further disrupted by Dfr misexpression.

Acj6 Regulates Axon Terminal Arborization

Finally we tested whether Acj6 and Dfr also regulate the highly stereotyped PN axon terminal arborizations in the lateral horn, one of the two central targets for PN axons (Figure 1A). These studies made use of single-cell clones to resolve stereotypical axonal branching patterns (Marin et al., 2002), initially concentrating on DL1 PNs since these are the only class that can be unequivocally identified.

Wild-type DL1 PNs have a stereotyped axon branching pattern and terminal field. DL1 axons bifurcate as soon as they enter the lateral horn; the main lateral branch is a smooth continuation of the axon proj-

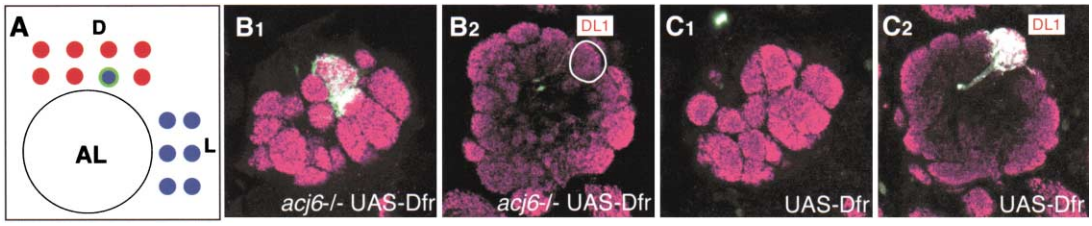


Figure 6. DL1 Single Cells which Are $acj6^{-/-}$ as Well as Misexpressing Dfr Completely Fail to Innervate DL1

(A) A schematic of the $acj6^{-/-}$ UAS-Dfr experiment. The singly labeled DL1 adPN expresses Dfr instead of Acj6; since it has also lost Acj6 its nucleus is blue rather than purple.

(B and C) A DL1 adPN misexpressing Dfr alone has no obvious defects (C_1, C_2); however when combined with $acj6^{-/-}$ (B), it completely fails to innervate DL1 (circled in B_2), but instead innervates the anterior surface of the antennal lobe (B_1).

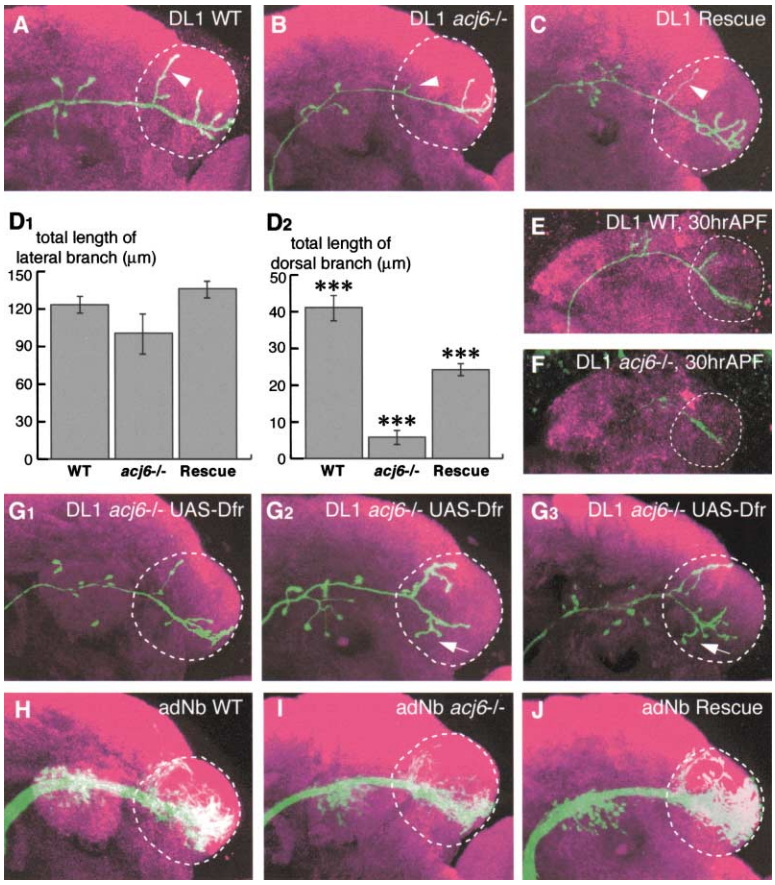


Figure 7. *acj6* Mutant adPNs Have Specific Axon Terminal Arborization Phenotypes which Can Be Modified by Dfr Misexpression

(A–C) The axon arborization pattern of DL1 single-cell clones in wild-type (A), $acj6^{-/-}$ (B) and $acj6^{-/-}$ + UAS-Acj6 (Rescue, C). The characteristic dorsal branch is substantially shorter in the $acj6^{-/-}$ clone; this is partially rescued by *acj6* transgene expression (arrowheads). Dotted circles represent the outline of the lateral horn as judged by the nc82 counterstaining.

(D) Quantification of DL1 single-cell clone axon phenotypes. The total axon length (including collateral branches) of the lateral (D_1) and dorsal (D_2) branches is compared (WT, n = 15; $acj6^{-/-}$, n = 8; rescue, n = 11). The $acj6^{-/-}$ phenotype was highly specific to the dorsal branch, and was significantly rescued by the Acj6 transgene (t test: ***, $p < 0.001$).

(E and F) Axons of DL1 single-cell clones of wild-type (E) or $acj6^{-/-}$ (F) examined at 30 hr after puparium formation.

(G) The axon patterns of $acj6^{-/-}$ UAS-Dfr DL1 single-cell clones. Six out of ten (6/10) showed a wild-type-like pattern (G_1), while four out of ten (4/10) showed a novel pattern (G_2, G_3).

(H–J) The axon pattern of adNb clones. $acj6^{-/-}$ clone has a smaller area of innervation (I versus H), which is rescued by *acj6* transgene expression (J).

ecting from the mushroom body and there is also a perpendicular dorsal branch (Figure 7A; Marin et al., 2002). In $acj6^{-/-}$ DL1 PNs, axons always reach the lateral horn, indicating that Acj6 is not required for general axon outgrowth and guidance. However, the dorsal branch of $acj6^{-/-}$ PNs failed to extend properly (Figures 7B versus 7A, arrowheads, n = 11), while the lateral branch was largely unaffected. We measured the total axon length of the entire dorsal and lateral branches, respectively, after three-dimensional tracing (see Experimental Procedures). $acj6^{-/-}$ DL1 axons exhibit an 8-fold reduction in the total axon length of the dorsal branch compared with wild-type (Figure $7D_2$). The lateral branch was only slightly reduced and this reduction was not statistically significantly (Figure $7D_1$). This result suggests that *acj6* selectively controls one specific aspect of axon terminal arborization of DL1 PN.

The axon phenotype of $acj6^{-/-}$ single-cell clones was significantly rescued by *UAS-Acj6(1,4)* expression only within those labeled single-mutant cells (Figure 7C, n = 13; quantified in 7D), demonstrating that Acj6 has a cell-autonomous and post-mitotic function in controlling the specificity of axon terminal arborization.

Does the selective disruption of the dorsal branch result from a requirement for Acj6 in outgrowth or maintenance? In wild-type, at 30 hr APF, the dorsal branch has just extended, creating the stereotyped branching pattern (Marin et al., 2002; Figure 7E). In $acj6^{-/-}$ clones, the dorsal branch was always either completely missing or significantly shorter than wild-type (Figure 7F, n = 6). This observation strongly suggests that *acj6* is required for extension of the dorsal branch as it connects with appropriate targets, rather than for stabilization of the branch after it has extended to the correct area.

Dfr is not expressed in adPNs, so making *dfr* loss-of-function clones of the model DL1 adPN is not informative. Nor did DL1 single-cell clones misexpressing Dfr have obvious defects (data not shown). However, surprisingly, in all 10 single-cell DL1 clones simultaneously mutant for *acj6* and misexpressing Dfr (Figure 6A), the axon pattern differed from $acj6^{-/-}$ clones. While 6 resembled wild-type DL1 arborization (Figure $7G_1$), the other 4 appeared to have a novel, stereotyped phenotype not seen in any wild-type or $acj6^{-/-}$ DL1 PNs. The dorsal branch has additional side branches; the lateral branch stops well short of the lateral edge of the lateral horn and instead curves and branches to innervate the ventral area of the lateral horn never innervated by wild-type DL1 axons (arrows in Figures $7G_2$ and $7G_3$, compared with Figures 7A and 7B) nor any larval-born adPN axons (Figure 7H). This novel axon branching and terminal field pattern did not appear to fall into any of the 15 PN classes which we have previously analyzed (Marin et al., 2002), so we do not know if this corresponds to a PN class which we did not analyze (there are 25∼35 more classes), or if this pattern does not exist in wild-type animals. These observations suggest that Dfr could also regulate specificity of axon arborization in post-mitotic PNs.

We extended the analysis of axon branching patterns

beyond DL1 single-cell clones with two additional experiments. First, we examined single-cell clones generated in late larval stages and found that axons of all *acj6*$^{-/-}$ PNs follow the correct axonal tract leaving the antennal lobe and extended to the mushroom body and the lateral horn; Acj6 therefore does not play a role in general axon growth and guidance. The dendritic targeting defects (see Figures 2I and 2J) prevented us from identifying the glomerular class for a given single-cell clone and thus from performing detailed analysis of their axon terminal arborization. In a second experiment, we found that *acj6*$^{-/-}$ adNb clones had a significantly smaller axonal innervation area than wild-type, leaving the dorsal part of the lateral horn largely uninnervated (Figures 7I versus 7H). This phenotype was also rescued by expression of a *UAS-Acj6* transgene (Figure 7J). We conclude that Acj6 regulation of axon terminal arborization is not restricted to DL1 adPNs.

Discussion

Prior to our study of *Drosophila* PNs, it was generally believed that synaptic connection specificity is conferred by selection of synaptic partners by presynaptic axons. Systematic lineage analysis (Jefferis et al., 2001) strongly suggested that PN dendrites play an active role in establishing connection specificity. Specifically, a given PN's lineage and birth order predicts its glomerular target. However, the position of a given PN's target glomerulus is correlated neither with its neuroblast lineage nor with birth order. Thus, it is unclear how a PN's lineage contributes to its dendritic targeting specificity.

We now provide molecular genetic evidence that this active dendritic targeting is controlled by transcriptional programs within PNs. Our data suggest that the observed dendritic targeting specificity could be achieved in two steps: specification of a particular lineage and further intra-lineage specification. The POU domain transcription factors Acj6 and Dfr play critical roles in the first step.

Acj6 and Drifter Regulate Lineage-Specific PN Dendrite Targeting

Several lines of evidence support the idea that Acj6 and Drifter play analogous roles in translating lineage information into dendritic targeting specificity of adPNs and lPNs. First, Acj6 and Dfr are mutually exclusively expressed in adPNs and lPNs; this lineage-specific expression could be used to regulate the distinct wiring specificity of these two PN lineages.

Second, loss-of-function phenotypes in neuroblast clones demonstrated that Acj6 and Dfr are required for proper dendritic targeting of at least a subset of PNs in their respective lineages. The neuroblast clone phenotypes likely underestimate the requirement of Acj6 or Dfr in PN dendritic targeting. Since each glomerulus is innervated by an average of 3 PNs (Jefferis et al., 2001), we may not be able to detect inappropriate targeting if 1 or 2 PNs in the same class still innervate the glomerulus properly. This possibility was supported by our study of DL1 PNs. In neuroblast clone analysis, 11 out of 19 *acj6*$^{-/-}$ clones exhibited no detectable defects in DL1 glomerular innervation; in single-cell clone analysis with a higher resolution, each of the 11 clones showed significant phenotypes. Results from single-cell clone analysis of other PN classes support the generality of the DL1 phenotype—failure to innervate one specific glomerulus.

Third, misexpression of Acj6 in lPNs, or Dfr in adPNs, leads to dendritic targeting defects. In the case of Acj6 misexpression in lPNs, where the phenotypes are stronger (possibly due to a higher ratio of transgene to endogenous Acj6 expression than could be observed for Dfr transgene/endogenous Dfr; data not shown), there are two qualitatively different mistargeting phenotypes. The first is non-specific accumulation of dendrites in the lateral part of the antennal lobe with associated glomerular organization defects. This phenotype is analogous to the non-specific accumulation of adPN dendrites in the dorsal part of the antennal lobe in *acj6*$^{-/-}$ adPN clones and may reflect a default response of dendrites deprived of targeting information. The second class of phenotypes is more revealing. In this case, lPN dendrites are mistargeted to well-defined dorsal landmark glomeruli distant from lPN cell bodies and areas of non-specific accumulation. Certain inappropriate glomeruli are specifically targeted, while their neighbors remain uninnervated; this observation argues against the alternative interpretation that misexpression simply causes non-specific dendritic spillover. The specificity of the mistargeting phenotypes caused by misexpression is further supported by the following two observations: (1) overexpression of Acj6 in adPNs, or Dfr in lPNs, never results in any phenotypes (data not shown); and (2) specific mistargeting is not observed in loss-of-function mutants.

Taken together, these results strongly suggest that Acj6 and Dfr participate in instructing adPNs and lPNs to innervate a set of glomeruli appropriate to each lineage. At present, it remains probable that other transcription factors act in concert with Acj6 and Dfr to completely specify these lineage-dependent wiring programs. The existence of these other factors—in addition to the likely underestimation of phenotypes in our neuroblast clone analysis discussed above, or perdurance in the case of Dfr—may explain why both loss-of-function and gain-of-function experiments affect only specific subsets of glomeruli.

Intra-Lineage Specification

It is important to note that Acj6 and Dfr alone cannot specify a particular PN to target its dendrites to a particular glomerulus. All adPNs express Acj6, yet they project their dendrites to a series of different glomeruli according to their birth order (Jefferis et al., 2001). There must be timing factors, probably also transcription factors, which further distinguish PNs within the same lineage based on their birth order. An elegant mechanism to specify different progeny from a common neuroblast has recently been described in the *Drosophila* embryonic CNS (Isshiki et al., 2001), where neuroblasts exhibit asymmetric cell division patterns similar to those giving rise to PNs. In the embryonic CNS, the neuroblast changes its transcription factor profile as a function of time, thereby specifying the fate of neurons born at different stages. We suspect that analogous timing fac-

tors might exist in PN lineages. These timing factors, in collaboration with lineage-specific factors we describe here, will ultimately specify the expression of a repertoire of cell surface molecules that allow PNs to target their dendrites precisely to specific glomeruli.

Could the same hypothetical timing factors be used in both lineages? We tested this by attempting to switch the DL1 class of adPN to its lPN equivalent by simultaneously removing Acj6 and misexpressing Dfr. If the only differences between the DL1 adPN and its lPN equivalent are the POU domain lineage factors, we might expect that the DL1 PNs lacking Acj6 but expressing Dfr now would target to a novel glomerulus. We found that these PNs indeed acquire novel features compared to simple loss of Acj6. They no longer even partially innervate DL1. In a subset of clones, their axons also acquired novel branching patterns and terminal fields. However, a clear switch is not observed based both on these dendritic or axonal phenotypes. This could be due to inappropriate level and/or timing of transgene expression; it could also be because: (1) the hypothetical timing factors are not exactly the same in adPNs and lPNs, (2) Acj6 and Dfr are not the only factors distinguishing these two lineages, or (3) cell-cell interaction among PNs from the same lineage may play a role in determining targeting specificity.

Coordination of PN Dendritic and Axonal Connection Specificity

Acj6 is necessary not only for PN dendritic targeting, but also for establishing highly stereotyped PN axon branching patterns and terminal fields in a higher olfactory center. This is best exemplified by the analysis of DL1 single-cell clones. *acj6*$^{-/-}$ DL1 PNs are defective specifically in the dorsal branch without affecting general axon growth and guidance. This specific phenotype suggests that Acj6 plays a role in selecting synaptic connections with specific third order neurons. Axon terminal arborizations of other classes of PNs are also likely to be regulated by Acj6, as revealed by phenotypes from neuroblast clones containing ~13 classes of adPNs. As for Dfr, we do not have evidence from loss-of-function studies that it plays a role in PN axon terminal arborization because we do not have any equivalent in the lateral lineage to the DL1 PN, which we can unambiguously identify independent of its dendritic innervation. However, the fact that simultaneous loss of Acj6 and gain of Dfr in DL1 clones result in qualitatively different axonal phenotypes compared with simple loss of Acj6 suggests that Dfr also plays a role in regulating axon terminal arborization in the lateral horn.

These observations bring back the question of why PNs are prespecified (Jefferis et al., 2001) to project their dendrites to specific glomeruli and thereby receive specific olfactory input, and to have axons exhibiting specific branching patterns and terminal fields, presumably allowing stereotyped connections with third order neurons. By making PNs genetically distinct at the outset, it is possible to coordinate the dendritic choices of different glomeruli and the specific connections made by axons in higher centers. This coordination may contribute to innate behavioral responses to odorant stimuli by allowing a highly stereotyped relaying of olfactory information from the periphery to higher olfactory centers. Mechanistically, it is possible that PNs use similar cell surface molecules, whose expression depends on specific transcription factors such as Acj6 and Dfr, to guide both dendrites and axons to appropriate targets. The dual Acj6 phenotypes (both axonal and dendritic) provide support for this hypothesis. In our ongoing forward genetic screens and candidate tests to identify genes necessary for PN dendritic and axonal connectivity, we have found additional mutants with simultaneous defects in dendritic targeting and axonal arborization (H. Zhu, T. Chihara, L.L., unpublished data).

In theory, the dual phenotypes in dendrites and axons could be caused by primary defects in dendritic targeting, with axon arborization defects as a secondary consequence, or vice versa. However, two lines of evidence argue against such possibilities. First, developmental studies indicate that there is not a sequential development of dendritic and axonal arborization (G.S.X.E.J. et al., unpublished data). Second, different mutants exhibit different ranges and specificity in their axonal and dendritic phenotypes (this study; H. Zhu, T. Chihara, L.L., unpublished data); even for individual PNs with the same mutant genotype, there was no clear correlation between the severity of dendritic and axonal phenotypes. We thus favor the possibility that the correct targeting of PN axons and dendrites are both directly regulated events rather than a sequential process in which e.g., the correct targeting of dendrites then instructs the corresponding axonal arborization.

Functions of POU Domain Proteins in Neural Development

POU domain transcription factors are used widely in *C. elegans*, *Drosophila*, and mammalian development. In particular, classes III and IV POU domain proteins play a variety of important roles in neural development. *C. elegans* UNC-86, the founding member of the POU IV class, is expressed shortly after asymmetric division in one of the two daughter cells. In *unc-86* mutants, the daughter neuroblast that usually expresses UNC-86 now acquires the fate of its parental neuroblast, resulting in reiterations of cell lineage. UNC-86 also regulates differentiation of a number of neuronal classes such as touch sensory neurons or HSN motor neurons (Chalfie et al., 1981; Desai et al., 1988; Finney et al., 1988; Finney and Ruvkun, 1990). In mammals, 3 class IV and 4 class III POU domain proteins are widely expressed in the nervous system during development (reviewed in Ryan and Rosenfeld, 1997). Knockout experiments demonstrate their important functions in different developmental processes (e.g., Erkman et al., 2000, McEvilly et al., 2002). Because there is genetic redundancy between members of the same class, however, phenotypes resulting from single gene knockouts tend to reflect defects in cells that uniquely express that particular POU domain protein (Ryan and Rosenfeld, 1997).

Acj6 and Dfr are respectively the single existing members of the class IV and class III POU domain proteins in *Drosophila*. Both genes have been shown to play a variety of roles in development. In particular, photoreceptor axon targeting is disrupted in *acj6* mutants, however this phenotype is not cell autonomous (Acj6 is not

expressed in photoreceptors) and is probably due to a requirement for Acj6 in the target lamina neurons (Certel et al., 2000a). By restricting genetic manipulations to a small subset of neurons with well-defined connection specificity, we bypass the requirement of Acj6 and Dfr in other developmental events and focus on their function in olfactory projection neurons. We assign, to the best of our knowledge, a new function for POU domain proteins: regulating lineage-dependent wiring specificity down to specific synapse formation. Interestingly, PNs from two lineages utilize two POU domain proteins of different classes for analogous functions. It remains to be seen whether the large number of mammalian POU domain proteins (He et al., 1989) could be used in this way to regulate the specificity of numerous connections necessary to assemble the mammalian nervous system.

Lastly, as we have already noted, Acj6 functions in a subset of ORs to regulate the expression of olfactory receptors (Clyne et al., 1999b); it is possible that it also regulates other molecules including putative ORN axon targeting molecules (which are likely to be distinct from the ORs themselves; see Jefferis et al., 2002). Our demonstration that Acj6 is necessary for dendritic targeting specificity of a subset of PNs raises an intriguing possibility that Acj6 may regulate matching ORNs and PNs destined to form synaptic connections. In fact, Acj6 is also expressed in a subset of neurons whose cell bodies are located near the lateral horn (our unpublished data), one of the two central targets of PN axons. Thus, it is even feasible that Acj6 also regulates matching of synaptic partners in the next olfactory center. We are currently developing specific molecular markers and other genetic tools to test these intriguing possibilities.

Experimental Procedures

Clonal Analysis

In the MARCM strategy, a cell marker is under the control of a UAS promoter, which is activated by GAL4 and repressed by GAL80 (Lee and Luo, 1999). The GAL80 transgene is driven by a ubiquitous promoter and placed distal to an FLP-mediated recombination site. Flies heterozygous for the GAL80 transgene and carrying appropriate GAL4 and UAS-marker transgenes initially do not express the marker due to repression by GAL80. Only after FLP-mediated mitotic recombination will the marker be expressed in cells that have lost the GAL80 transgene and express the GAL4. In some experiments described in this paper, a mutation of an endogenous gene was placed in *trans* to GAL80, thus a labeled clone that lost GAL80 is homozygous for the mutation. If a second transgene under UAS control is present, this transgene will also only be expressed in labeled clones along with the UAS-marker.

MARCM clones were induced by 1.5 hr, 37°C heat shocks at approximately 0–24 hr after larval hatching. The null allele *acj6*^{6} (Clyne et al., 1999a), or the *drifter* null allele *dfr*^{B129} (truncation mutation before the POU domain, W.A.J., unpublished data) and the strong hypomorph *dfr*^{E82} (Anderson et al., 1995), were recombined with appropriate FRT chromosomes and used for MARCM analysis. The genotypes for various experiments are as follows: (1) *acj6* loss-of-function, *acj6*^{6} *FRT*^{19A}*/hs-FLP tubP-GAL80 FRT*^{19A}*; GAL4-GH146 UAS-mCD8-GFP*; (2) *acj6* misexpression, *y w hs-FLP UAS-mCD8-GFP/+; FRT*^{G13} *tubP-GAL80/FRT*^{G13} *GAL4-GH146 UAS-mCD8-GFP; UAS-Acj6(1,4)/+*; (3) *acj6* rescue: *acj6*^{6} *FRT*^{19A}*/hs-FLP tubP-GAL80 FRT*^{19A}*; GAL4-GH146 UAS-mCD8-GFP; UAS-Acj6(1,4)/+*; (4) *dfr* loss-of-function, *y w hs-FLP UAS-mCD8-GFP/+; GAL4-GH146 UAS-mCD8-GFP/+; FRT*^{2A} *dfr/ FRT*^{2A} *tubP-GAL80*; (5) *dfr* misexpression, *y w hs-FLP UAS-mCD8-GFP/+; FRT*^{G13} *GAL4-GH146 UAS-mCD8-GFP/ FRT*^{G13} *tubP-GAL80; UAS-Dfr/+*; and (6) *acj6* loss-of-function and *dfr* misexpression: *acj6*^{6} *FRT*^{19A}*/hs-FLP tubP-GAL80 FRT*^{19A}*; GAL4-GH146 UAS-mCD8-GFP; UAS-Dfr/+*.

Immunochemistry

Fixation, immunochemistry, and imaging were carried out as described (Jefferis et al., 2001). Additional antibodies used in this study were mouse monoclonal anti-Acj6, 1:5 (Certel et al., 2000a); rat anti-Drifter, 1:3000 (Anderson et al., 1995); rabbit anti-GFP, 1:500 (Molecular Probes); mouse monoclonal anti-Elav (9F8A9), 1:10; rat monoclonal anti-Elav (7E8A10), 1:10 (Developmental Studies Hybridoma Bank at the University of Iowa), Cy5 conjugated goat anti-rat/mouse IgG 1:200 (Jackson).

3D Reconstruction

Raw confocal images of DL1 axons were imported into Neurolucida (MicroBrightfield, Colchester, VT) and the GFP signal corresponding to the single-cell clone was manually captured. The two major branches in the lateral horn were manually distinguished and total branch lengths for each were then measured with Neuroexplorer.

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