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Dock and Pak regulate olfactory axon pathfinding in *Drosophila*

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SUMMARY

The convergence of olfactory axons expressing particular odorant receptor (Or) genes on spatially invariant glomeruli in the brain is one of the most dramatic examples of precise axon targeting in developmental neurobiology. The cellular and molecular mechanisms by which olfactory axons pathfind to their targets are poorly understood. We report here that the SH2/SH3 adapter Dock and the serine/threonine kinase Pak are necessary for the precise guidance of olfactory axons. Using antibody localization, mosaic analyses and cell-type specific rescue, we observed that Dock and Pak are expressed in olfactory axons and function autonomously in olfactory neurons to regulate the precise wiring of the olfactory map. Detailed analyses of the mutant phenotypes in whole mutants and in small multicellular clones indicate that Dock and Pak do not control olfactory neuron (ON) differentiation, but specifically regulate multiple aspects of axon trajectories to guide them to their cognate glomeruli. Structure/function studies show that Dock and Pak form a signaling pathway that mediates the response of olfactory axons to guidance cues in the developing antennal lobe (AL). Our findings therefore identify a central signaling module that is used by ONs to project to their cognate glomeruli.

Supplemental figures and tables available online

Key words: *Drosophila*, Dock, Pak, Olfactory, Axon Pathfinding, Signaling, Antennal lobe, Glomeruli

INTRODUCTION

The survival and reproduction of many animal species are critically dependent on their keen sense of smell. In mammals, the detection of diverse odors in the environment is mediated by ~10^3 different seven transmembrane odorant receptors, expressed by ~10^6 olfactory neurons (ONs) arrayed in the olfactory epithelium (reviewed by Firestein, 2001). The perception of odor requires that the odor information be encoded in a spatial map by the precise targeting of olfactory axons in the brain (reviewed by Mombaerts, 2001).

The exquisite connectivity of the odor map raises questions about its development. What are the molecular mechanisms that allow olfactory axons to connect precisely with their targets? A number of guidance molecules have been found to be expressed in the developing olfactory system in vertebrates (reviewed by Key and St John, 2002). Targeted disruptions of the neuropilin and ephrin A5 genes in mouse show that they direct the projections of olfactory axons to broad zones in the olfactory bulbs (Cloutier et al., 2002; Knoll et al., 2001; Schwarting et al., 2000). Furthermore, mutations of the odorant receptor genes reveal that they control the convergence of olfactory axons on specific glomeruli (Mombaerts et al., 1996; Wang et al., 1998). Little is known about the molecular mechanisms by which these proteins coordinately guide olfactory axons to their correct glomerular targets (Key and St John, 2002).

Drosophila provides an excellent system with which to unravel odor map development at the molecular and cellular levels. The anatomy and development of the fly odor map not only bear close resemblance to those of mammals, it is also simpler, containing only ~60 Or genes (Clyne et al., 1999; Gao and Chess, 1999; Scott et al., 2001; Vosshall et al., 1999) and ~40 uniquely identifiable glomeruli (Laissue et al., 1999). In *Drosophila*, ONs differentiate in the antennal disc and send their axons to the antennal lobe (AL), the fly equivalent of the mammalian olfactory bulb (Jhaveri et al., 2000; Stocker et al., 1990). Within the AL, olfactory axons synapse on the dendrites of projection neurons (PNs) in distinct glomeruli. As in mammals, each glomerulus is innervated by olfactory axons expressing the same Or gene (Gao et al., 2000; Scott et al., 2001; Vosshall et al., 2000). Remarkably, each glomerulus is also innervated by PNs born at a specific time in development (Jefferis et al., 2001). Thus, in *Drosophila*, there is a precise pairing of ONs expressing a given Or gene with PNs of a specific identity.

Previous studies have identified Dock and Pak (p21-activated Kinase) as components of a signaling cascade that regulates the projection of photoreceptor axons (Garrity et al., 1996; Hing et al., 1999; Rao and Zipursky, 1998). Dock is the fly homolog of the human Nck protein, consisting entirely of three SH3 domains and a single C-terminal SH2 domain (Garrity et al., 1996). The domain structure of Dock suggests that it acts as an adapter, linking receptors to downstream signaling proteins (Li et al., 2001). Pak is a serine/threonine kinase that is also highly conserved in evolution. Kinases of

this family are defined by the ability to bind, and be activated by Cdc42 and Rac, key regulators of the actin cytoskeleton (Bagrodia and Cerione, 1999; Daniels and Bokoch, 1999). Experiments, in both *Drosophila* and mammalian cells, show that Dock and Pak function in a signaling cascade to regulate cell motility (Hing et al., 1999; Sells et al., 1999).

In this study, we show that *Drosophila* olfactory axons take stereotyped pathways in an outer nerve layer to find and innervate their cognate glomeruli. We found that *dock* and *Pak* are necessary for the precise wiring of the olfactory map. Using cell-type specific cDNA rescue, antibody localizations and clonal analyses, we observed that *dock* and *Pak* function autonomously in olfactory axons to control various choice points in their trajectories to the cognate glomeruli. Finally, structure/function studies show that Dock and Pak function in a signaling pathway in ON axon guidance. Thus, Dock and Pak may form a core signaling cascade employed by different ON subclasses to pathfind to their respective glomeruli.

MATERIALS AND METHODS

Experimental animals and transgenes

All fly lines were obtained from the *Drosophila* stock center except for the following. *981-Gal4* and *SG18.1-Gal4* (Jhaveri et al., 2000) were kindly provided by V. Rodrigues. *Or22a-Gal4*, *Or47a-Gal4* and *Or47b-Gal4* (Vosshall et al., 2000) were gifts from L. Vosshall. *GH146-Gal4* (Stocker et al., 1997) was from R. Stocker, *UAS-nsyb::GFP* (Estes et al., 2000) was from M. Ramaswami and *ey-FLP; FRT82 Pak^{16}/TM6B* (Newsome et al., 2000b) was from B. Dickson. *UAS-dock* (Rao and Zipursky, 1998) was provided by Y. Rao, while *UAS-Pak* was constructed by ligating a Myc-tagged *Pak* cDNA into *pUAST* and then transforming into the fly using standard germline transformation technique.

Clonal analysis

MARCM (Lee and Luo, 1999) was carried out by heat-shocking third-instar larvae of the following genotypes: *hs-FLP/+; dock Or47a-Gal4/dock* (or +)*; FRT82 Gal80/FRT82 UAS-mCD8::GFP* and *hs-FLP/+; dock Or47b-Gal4/dock* (or +)*; FRT82 Gal80/FRT82 UAS-mCD8::GFP* at 37ºC for 40 minutes. Adult brains were dissected and processed as described below.

Immunohistochemistry

Adult brains (from 1- to 2-day-old animals), pupal antennae and larval imaginal discs were dissected in phosphate buffered saline (PBS). Tissues were fixed in PLP (2% paraformaldehyde, 0.25% sodium periodate, 75 mM lysine-HCl and 37 mM sodium phosphate pH 7.4), washed with PBST (PBS with 0.5% Triton X-100) and subjected to antibody staining. nc82 mAb (1:20) (A. Hofbauer, PhD thesis, University of Wurzburg, 1991) was a gift from A. Hofbauer. Rabbit anti-Dock 1:500 (Clemens et al., 1996) was a gift from J. Dixon. Mouse 22C10 mAb (1:20) and rat anti-ELAV, 7E8A10 (1:20) were from Developmental Studies Hybridoma Bank. Rabbit anti-GFP polyclonal antibody (1:100) was from Clontech, and rat anti-CD8 α subunit mAb (1:100) was from Caltag. The secondary antibodies, FITC-conjugated goat anti-rabbit, Cy3-conjugated goat anti-mouse and FITC-conjugated goat anti-rat, were purchased from Jackson Laboratories and used at 1:200 dilutions.

Cuticle preparations

Adult antennae from animals expressing *Or-Gal4/UAS-lacZ^{nuclear}* were fixed in 25% gluteraldehyde for 1 hour, washed in PBST and stained in 0.2% X-Gal solution (Ashburner, 1989). The antennae were then cleared in Faure's mountant (34% v/v chloral hydrate, 13% glycerol, 20 mg/ml gum Arabic) and photographed with the SPOT-RT cooled CCD camera. Sensilla on the third antennal segment were counted by projecting images on a video monitor.

RESULTS

dock and *Pak* regulate the precise projection of olfactory axons to their targets

We first noticed that the prominent paired protuberances at the anterior of the brain, the ALs, are severely reduced in *dock* and *Pak* homozygous escapers compared with wild type. To probe the structure of the ALs, we stained the *dock^{P1}/dock^{P1}* and *Pak^{6}/Pak^{11}* mutant brains (see Table 1 for descriptions of the mutant alleles) with the nc82 mAb, which stains the AL neuropil (A. Hofbauer, PhD thesis, University of Wurzburg, 1991). In wild type, ALs are rounded (~90 μm diameters) and exhibit distinct anatomical subdivisions: the glomeruli (Fig. 1A). In *dock* and *Pak* mutants however, ALs are smaller (50 to 60 μm), mis-shapen and have an amorphous neuropil, suggesting that glomeruli development is defective (Fig. 1B,C). The loss of well-defined glomeruli in *dock* and *Pak* mutants suggests that guidance and/or synaptogenesis of ON axons are disrupted.

To ascertain the fate of the individual glomeruli, we expressed the synaptobrevin::GFP fusion protein (encoded by the *UAS-nsyb::GFP* gene) (Estes et al., 2000) under the control of specific odorant receptor promoters (*Or-Gal4* drivers) (Vosshall et al., 2000). The presynaptic varicosities of the DM2, DM3 and VA1lm glomeruli (Laissue et al., 1999), which are located at various positions in the AL, were surveyed using the *Or22a-Gal4*, *Or47a-Gal4* and *Or47b-Gal4* drivers, respectively. In wild-type controls, DM2 and DM3 are spheroidal neuropils of ~10 μm diameters and are located in the mediodorsal surface of the lobe (Fig. 1D,G). VA1lm is crescent in shape, ~40 μm long and 10 μm wide, and is located on the anterolateral surface, close to the nerve entry point (Fig. 1J). In both *dock^{P1}/dock^{P1}* and *Pak^{6}/Pak^{11}* mutants, DM2, DM3 and VA1lm are severely mis-shapen or split into smaller structures that scattered randomly in the AL (Fig. 1). In *dock*, 42% of DM2 (n=26) and 75% of DM3 (n=36) are ectopically located or splinter into smaller structures, scattered in the neuropil (Fig. 1E,H). Although the integrity and position of VA1lm remain relatively unchanged in both *dock* and *Pak* mutants, it is enlarged and appears to extend into the domains of surrounding glomeruli. In 95% of *dock* (n=20) and 95% of *Pak* (n=18) ALs it is seen to engulf the adjacent VA1d glomerulus completely (Fig. 1K,L). There does not appear to be any consistent pattern in the distribution of the ectopic glomeruli, which are scattered randomly and differ from one

Table 1. Mutant alleles of *dock* and *Pak* used in the analyses

Alleles	Molecular lesions	Refs
dock^{P1}	P element insertion in first intron	Garrity et al., 1996
Pak^{4}	P9L, disrupts Dock interaction motif (PAPPVR)	Hing et al., 1999
Pak^{6}	R113Stop, truncation in CRIB domain	Hing et al., 1999
Pak^{11}	Q227Stop, deletes kinase domain	Hing et al., 1999
Pak^{16}	Q230Stop, deletes kinase domain	Newsome et al., 2000b

AL to another. As assessed by the randomness in the positions of DM2 and DM3, and the aberrant structure of VA1lm, the phenotype of *Pak* is indistinguishable from that of *dock*, although defects occur at a slightly lower penetrance (for example, 66% of DM3, n=18; Fig. 1I).

dock and *Pak* function autonomously in olfactory neurons

The severe defects in glomerular development of *dock* and *Pak* mutants lead to the critical question of the focus of *dock* and *Pak* actions in the developing AL. The precise formation of the olfactory map requires multiple interactions between ingrowing olfactory fibers and major cell types of the AL, such as the PNs and AL glia (Oland and Tolbert, 1996). In theory, *dock* and *Pak* may act in any of these cells to direct the wiring of the AL.

Staining with the anti-Dock antibody showed that Dock is highly expressed in the dendrites and axons of ONs during the period of axon pathfinding to the AL (see supplemental figures at http://dev.biologists.org/supplemental/). Staining with the anti-Pak antibody showed that Pak is similarly localized to ON axons (data not shown), although the lack of a Pak protein null mutation prevents us from assessing the specificity of the staining. The presence of Dock and Pak proteins in ON axons is consistent with a role in ON axon guidance. To test the notion that the proteins are needed in ONs, we asked if removal of *dock* and *Pak* functions specifically from ONs would disrupt their glomerular targeting. We found that the *ey-FLP/FRT* method of site-specific recombination (Newsome et al., 2000a) induced the formation of large patches of homozygous tissues in the eye-antennal disc but not in the brain (see supplemental figures at http://dev.biologists.org/supplemental/). We used the *ey-FLP/FRT* system to test whether *dock* and *Pak* are required in the antenna for the precise targeting of ON axons. Clones of wild-type, $dock^{P1}$ and Pak^{16} mutant tissues were induced in the antennae of animals carrying either *Or47a-Gal4* or *Or47b-Gal4* transgenes (see Fig. 2 legend for the respective genotypes). The mosaic animals were allowed to develop to adulthood and their brains were stained with nc82 mAb and anti-GFP to probe glomerular development. In animals with wild-type antennae, the AL neuropil is subdivided into well-defined glomeruli and ON axons converged precisely on their target glomeruli (Fig. 2A). By contrast, in animals with $dock^{P1}/dock^{P1}$ or Pak^{16}/Pak^{16} antennae, the ALs are severely mis-shapen and aglomerular, and ON axons terminated in ectopic locations (Fig. 2B,C). We conclude from these observations that *dock* and *Pak* function within the antennae to regulate the precise targeting of ON axons.

The mosaic experiments do not preclude an additional requirement for *dock* and *Pak* in the brain or in nonneuronal cells of the antenna. To test the hypothesis that *dock* and *Pak* function specifically in ONs for AL development, we asked if the expression of wild-type *dock* and *Pak* cDNAs in ONs would rescue the *dock* and *Pak* mutant phenotypes. The enhancer trap line *SG18.1-Gal4* (Jhaveri et al., 2000) was chosen to drive the expression of the cDNAs because we observed that it is preferentially expressed in a subpopulation of ONs with little expression in the brain. Examination of antennae from *SG18.1-Gal4/UAS-mCD8::GFP* animals at 30 hours after puparium formation (hAPF) showed that GFP is expressed only in cells with a bipolar morphology characteristic of ONs (Fig. 2D). Examination of the brain in pupae at 30 and 55 hAPF, and in the adult, showed that GFP is specifically localized to the nerve layer of the AL and in numerous glomeruli, with little staining elsewhere (Fig. 2E, Fig. 3A). Removal of both the antennae and maxillary palps abolished GFP expression in the ALs, showing that the AL staining is contributed by olfactory afferents from these two organs (Fig. 2F). Hence, *SG18.1-Gal4* is expressed preferentially in ONs of the antenna and the maxillary palp. Expression of *dock* and *Pak* cDNAs under the control of *SG18.1-Gal4* strongly rescued the glomerular structures of the AL (Fig. 2G-J). We monitored four landmark glomeruli (VA1d, VA6, DA4 and DM6) as an indicator of normal AL development (Fig. 2K). Expression of cDNAs under the control of *SG18.1-Gal4* increased the average

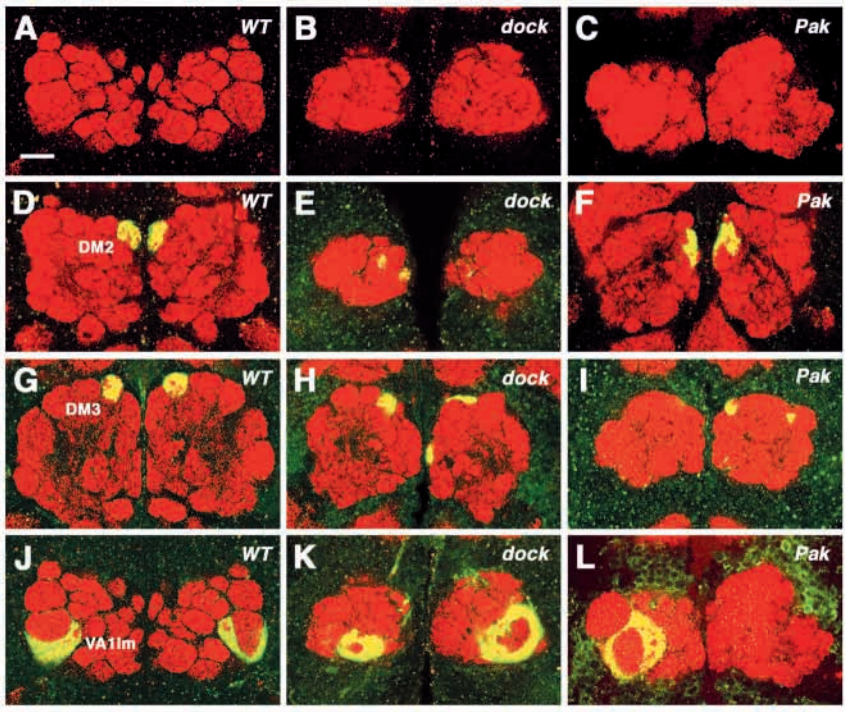

Fig. 1. DM2, DM3 and VA1lm glomeruli are mis-shapen and ectopically localized in *dock* and *Pak* mutants. Confocal micrographs of adult ALs stained with the nc82 (red) and anti-GFP (green) antibodies, revealing the neuropil structure. (A) Wild-type ALs showing distinct anatomical subdivisions: the glomeruli. (B) A pair of $dock^{P1}/dock^{P1}$ ALs and (C) a pair of Pak^{6}/Pak^{11} ALs showing an amorphous neuropil structure. (D-F) DM2, (G-I) DM3 and (J-L) VA1lm glomeruli were visualized by expressing *UAS-nsyb::GFP* with *Or22a-Gal4*, *Or47a-Gal4* and *Or47b-Gal4*, respectively. In the wild type (D,G,J), DM2, DM3 and VA1lm have characteristic shapes and are located in invariant positions in the AL. In $dock^{P1}/dock^{P1}$ (E,H,K), and Pak^{6}/Pak^{11} (F,I,L) mutants, DM2 and DM3 glomerular structures are smaller and scattered randomly in the AL. While VA1lm is located approximately in the right position, it is severely mis-shapen and frequently engulfs the adjacent VA1d glomerulus. Scale bar: 20 μm.

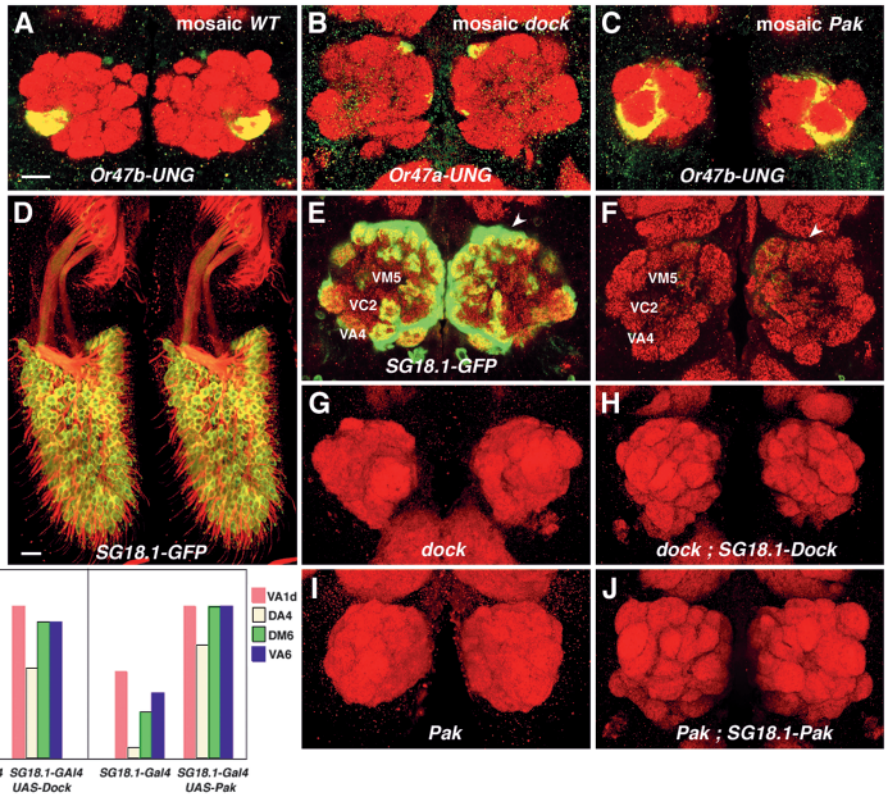

Fig. 2. *dock* and *Pak* function autonomously in ONs for AL development. (A) ALs from animals with mosaic wild-type antennae: *ey-FLP/+; Or47b-Gal4/+; FRT82/FRT82 M(3) arm-lacZ UNG*. *UNG* stands for *UAS-nysb::GFP*. (B) ALs from animal with mosaic $dock^{P1}$ antennae: *ey-FLP/+;* $dock^{P1}$ *FRT40/cycE FRT40; Or47a-Gal4 UNG/+*. (C) ALs from animal with mosaic Pak^{16} antennae: *ey-FLP/+; Or47b-Gal4/+; FRT82* Pak^{16} *UNG/FRT82 M(3) arm-lacZ*. (D-F) Animals expressing *UAS-mCD8::GFP* under the control of *SG18.1-Gal4*. (D) Confocal stereo pair of a 30 hAPF antenna double-stained with anti-mCD8 (green) and 22C10 mAb (red). Cells with a bipolar morphology, characteristic of ONs, express both GFP and Futsch. (E) Optical section of the adult ALs, showing GFP expression in the outer nerve layer (arrowhead) and in many glomeruli. Little GFP staining is observed outside of the AL. (F) Optical section at an equivalent plane as in E of an animal from which both the third segments of the antennae and the maxillary palps were surgically removed 6 days earlier. GFP staining in the outer nerve layer (arrowhead) and glomeruli is lost. (G-J) Expression of *UAS-dock* and *UAS-Pak* under the control of *SG18.1-Gal4* rescues the mutant AL phenotypes. 3D reconstructions of ALs from *dock* (G) and *Pak* (I) mutants show that their ALs are mis-shapen and aglomerular. (H) Expression of the *dock* cDNA in $dock^{P1}$ homozygotes using *SG18.1-Gal4* rescues the development of most of the glomeruli in the mutant. (J) Similarly, expression of *Pak* cDNA using *SG18.1-Gal4* in Pak^{6}/Pak^{11} mutants strongly rescues the development of most of the glomeruli. (K) Quantification of the rescue of the *dock* and *Pak* AL phenotype by wild-type *dock* and *Pak* cDNAs. The frequencies with which four indicator glomeruli (VA1d, DA4, DM6 and VA6) were observed in the different genotypes are presented. Scale bars: in A, 25 μm for A-C,E-J; in D, 25 μm for D.

frequency of distinctly identifiable glomeruli from 14% to 85% in $dock^{P1}/dock^{P1}$ ALs and 35% to 94% in Pak^{6}/Pak^{11} ALs. Although the rescue was strong, we noticed that not all glomeruli were restored. The incomplete rescue is probably due to the lack of *SG18.1-Gal4* expression in some glomeruli (Fig. 2E). Nonetheless, the ability of *SG18.1-Gal4* to support the substantial rescue of the mutant phenotypes indicates that it is suitably active during olfactory axon pathfinding. We conclude from these experiments that *dock* and *Pak* genes are specifically required in the ONs to direct the guidance of the ON axons.

dock and *Pak* regulate the precise pathfinding of olfactory axons

We infer from the ectopic glomeruli that ON axons fail to project to their correct destinations in *dock* and *Pak* mutants. To assess whether ON axons are misprojecting in *dock* and *Pak* mutants, we probed fiber pathways from the antenna to the AL. During development, ON axons leave the antenna and travel to the AL between 20 and ~50 hAPF (Jhaveri et al., 2000). Axon pathways in the pupal antenna were visualized by staining 30 hAPF antennae with 22C10, a mAb against Futsch (see Fig. 6A), while pathways in the adult AL were labeled with either the mCD8::GFP or GAP::GFP fusion proteins (encoded by *UAS-mCD8::GFP* or *UAS-GAP::GFP*) expressed under the control of various *Gal4* drivers. Axon trajectories were examined with standard fluorescence microscopy or after three-dimensional (3D) reconstructions of 2D confocal sections. Fig. 3A shows a representative reconstruction of a wild-type adult AL labeled by the *SG18.1-Gal4* driver, which is expressed in coeloconic and trichoid sensilla (Jhaveri and Rodrigues, 2002). The overall pattern of ON projection in the AL is bilaterally symmetric and relatively invariant between individuals (see Fig. $3B_1$ for an interpretive drawing). GFP-expressing fibers extend through the antennal nerve and enter the AL at its anterolateral point (close to VA1lm). Thereafter, the axons radiate over the AL surface in characteristic tracks within a fibrous nerve layer, to target their cognate glomeruli. Contralateral axons course through a prominent commissure that connects the opposite ALs. That the individual ON classes project in stereotyped pathways was confirmed by labeling ONs with the *Or22a-Gal4*, *Or47a-Gal4* and *Or47b-Gal4* drivers. In confocal reconstructions, *Or47a* axons can be seen to take relatively straight paths from the nerve entry point to DM3, their target glomerulus (Fig. 3E,F1). Examination of single *Or47a* axons (n=83) using the MARCM technique (Lee and Luo, 1999) showed that each axon projects determinately to DM3, where it splits into an ipsilateral branch, that terminates in DM3, and a contralateral branch, that projects across the commissure (Fig. 4A,B). Axons appear to extend

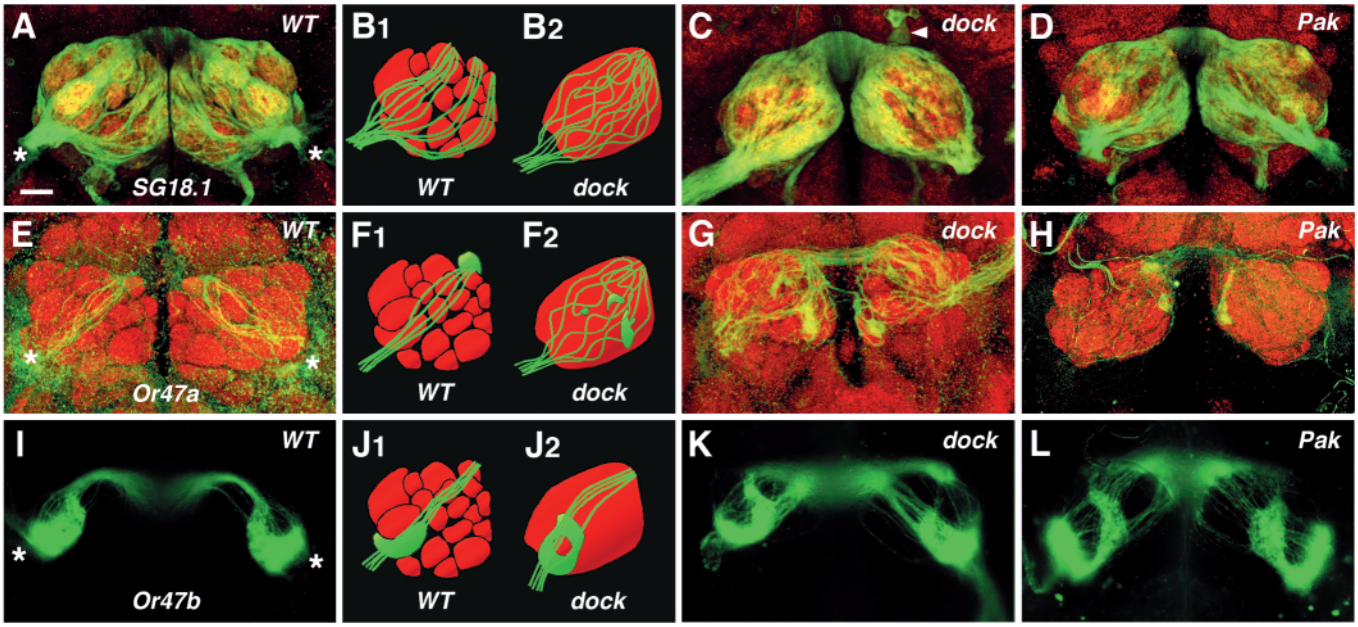

Fig. 3. Olfactory axon trajectories are severely disrupted in *dock* and *Pak* mutants. ALs of animals expressing *GFP* under the control of various *Gal4* drivers were either observed with fluorescence microscopy (I,K,L) or stained with anti-GFP (green) and nc82 mAb (red) and examined by confocal microscopy (A,C-E,G,H). (B,F,J) Interpretive drawings of confocal images (A,B_1) In the wild type, upon entering the AL, *SG18.1-Gal4* expressing axons radiate over its surface in characteristic tracks in search of their target glomeruli. These pathways are bilaterally symmetric and invariant from one lobe to another. (B_2 ,C) In the $dock^{P1}/dock^{P1}$ and (D) Pak^{4}/Pak^{6} mutants, however, *SG18.1-Gal4* axons take circuitous paths in the nerve layer creating a homogenous mat. Some axons project to dorsal brain regions (arrowheads) in $dock^{P1}$ mutants. (E,F_1) In the wild type, *Or47a* axons take direct paths, with little sidetracking, from the entry point to their target, DM3. (F_2,G) However, in $dock^{P1}/dock^{P1}$ and (H) Pak^{4}/Pak^{6} mutants, *Or47a* axons make chaotic and meandering projections, terminating in numerous ectopic glomeruli. (I,J_1) In the wild type, *Or47b* axons terminate immediately on VA1lm upon entering the AL. A distinct fascicle, containing contralateral axons, connects the glomerulus with the commissure. (J_2,K) In $dock^{P1}/dock^{P1}$ and (L) Pak^{6}/Pak^{11} mutants, the VA1lm glomerulus is severely mis-shapen and contralaterally projecting axons enter and exit the glomerulus over its entire surface. Asterisks in A,E,I indicate the antennal nerve entry points at the anterior lateral region of the AL. Scale bar: 25 μm.

directly to DM3 without making substantial changes in direction. Many fibers appear to project individually, although a number of fibers also merge into fascicles as they converge upon their targets (Fig. 3E). *Or47a* axons always remain in the nerve layer throughout the entire trajectory until in the vicinity of their target, whereupon the converging fibers enter the lobe and terminate precisely on DM3. *Or47b* axons, however, terminate immediately upon disembarking from the nerve, establishing the crescent shape VA1lm (Fig. 3I,J_1). A distinct fascicle issues from the medial edge of VA1lm carrying collaterals between the glomerulus and the commissure. Single-axon analysis (n=38) showed that the contralateral axons project in relatively straight paths, seldom straying beyond the confines of a narrow zone, between VA1lm and the commissure (Fig. 4E,F). Our analyses show that in the wild type, olfactory axons make bilaterally symmetric and stereotyped patterns of projections to their glomeruli in the AL.

In antennae of $dock^{P1}/dock^{P1}$ and Pak^{4}/Pak^{6} mutants at 30 hAPF, cells exhibiting a bipolar morphology can be seen, projecting their axons out of the antenna in distinct fascicles, a pattern indistinguishable from the wild type (Fig. 6A-C). However, once in the AL, the pathways are clearly abnormal. In both *dock* and *Pak* mutants, instead of forming characteristic tracks, *SG18.1-Gal4*-expressing fibers interweave to form a dense mat (Fig. 3B_2-D). In 22% (n=32) of the $dock^{P1}/dock^{P1}$ brains, ON axons extend aberrantly to dorsal brain regions (Fig. 3C, right AL). Disruption in the overall projection pattern is reflected in the trajectories of the individual ON subtypes. In 3D confocal reconstruction, *Or47a* axons can be seen to deviate from their stereotyped pathways from the outset, veering to distant part of the AL, including even the core, in chaotic, meandering trajectories (Fig. 3F_2). Visualization of single *Or47a* axons in $dock^{P1}/dock^{P1}$ mutants showed that misrouting affect both the ipsilateral and contralateral branches of an axon (Fig. 4C,D). In 21% of the cases (n=45), both branches remain in the ipsilateral AL. Most of the branches (64%, n=45) ultimately fail to reach their destination, forming mis-shapen glomeruli in ectopic locations. Interestingly, in 9% (n=45) of the cases, three axon branches were observed. However, the extra branch is usually short and not associated with ectopic glomeruli. Although *Or47b* axons terminate in a single large glomerulus as in wild type, it is strongly mis-shapen in *dock* and *Pak* mutants (Fig. 3J_2). Furthermore, the contralaterally projecting *Or47b* axons are severely defasciculated, projecting through a wide area of the AL surface. Examination of single *Or47b* neurons showed that whereas ipsilateral axons terminate normally in VA1lm, contralateral axons are frequently misrouted, either projecting to dorsal brain regions or stopping short of the VA1lm glomerulus (Fig. 4G,H). Our analyses of the *dock* and *Pak* mutant phenotypes therefore show that *dock* and *Pak* are not necessary either for the outgrowth of olfactory axons or for

their projection through the antennal nerve. Instead, *dock* and *Pak* function primarily in the guidance of ON axon within the AL to steer the sensory fibers precisely to their cognate glomeruli.

Interaction between Dock and Pak is critical for proper AL development

The similarity in *dock* and *Pak* olfactory connectivity phenotypes suggests that these genes might function in a signaling cascade to regulate the targeting of ON axons. It has previously been shown that Dock and Pak interacts through the N-terminal PXXP motif of Pak and the second SH3 domain of Dock (Hing et al., 1999). We now observed that the P9L mutation (*Pak*^{4}), which affects the N-terminal PXXP motif of Pak and abolishes its ability to bind to Dock, strongly disrupts ON axon pathfinding (Fig. 3H). To evaluate the hypothesis that Dock-Pak interaction is critical for ON axon guidance, we examined the requirements of the individual Dock domains for AL development. Expression of wild-type *UAS-dock* cDNA (Rao and Zipursky, 1998) under the control of *SG18.1-Gal4* significantly rescued the *dock*^{P1}/*dock*^{P1} aglomerular phenotype (compare Fig. 5A with 5B). Although mutations that disrupt the first SH3 domain, the third SH3 domain or the SH2 domain do not affect the ability of *dock* to rescue the mutant phenotype (Fig. 5C,E,F), a mutation that disrupts the second SH3 domain completely abolished *dock* activity (Fig. 5D). The requirement of both the N-terminal PXXP motif of Pak and the second SH3 domain of Dock suggests that physical interaction between Dock and Pak is necessary for proper AL development. To obtain evidence of a functional relationship between *dock* and *Pak* we tested for genetic interactions between the genes. First, we asked if simultaneous reduction of *dock* and *Pak* functions by half would disrupt AL development. The ALs of *dock*^{P1}/+; *Pak*^{4}/+ animals exhibit normal axon trajectories and glomerular subdivisions similar to those of wild-type or heterozygous animals (see supplemental figures at http://dev.biologists.org/supplemental/). However, the absence of genetic interaction in the compound heterozygotes should not be taken as evidence against *dock* and *Pak* functioning in the same signaling cascade. It is still possible that the decreased levels of *dock* and *Pak* are sufficient for normal functioning of the signaling pathway. We also asked if expression of the constitutively active *Pak*^{myr} (Hing et al., 1999) in the *dock* mutant would correct the mutant phenotype. However, expression of *UAS-Pak*^{myr} with *SG18.1-Gal4* in wild type strongly disrupted the AL structure (L.-H. A and H. H., unpublished). The strong gain-of-function phenotype makes its use in genetic epistasis studies unfeasible.

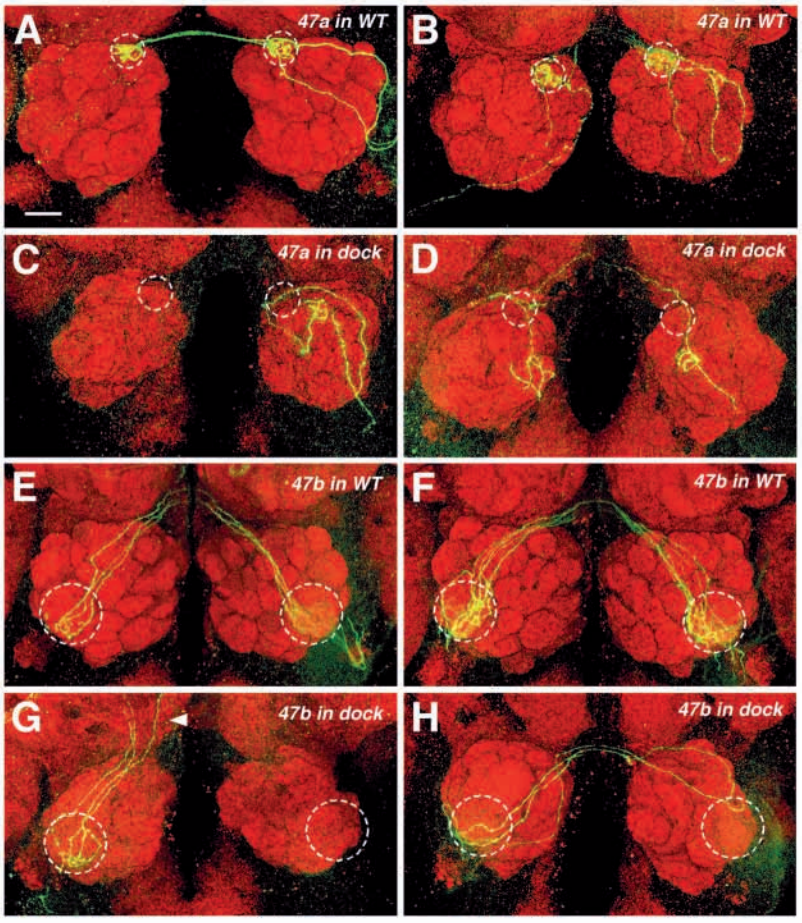

Fig. 4. *Or47a* and *Or47b* axons show strong pathfinding defects and terminate in ectopic glomeruli in *dock* mutants. Antennal lobes, in which single *Or47a* or *Or47b* axons were labeled with mCD8::GFP using the MARCM technique, were stained with anti-CD8 (green) and nc82 mAb (red). See Materials and Methods for the genotypes. (A,B) In wild type, each *Or47a* axon can be seen to synapse on the ipsilateral DM3 and then send a collateral to the contralateral DM3. (C,D) In the *dock* mutant, *Or47a* axons branch normally. However, both axon branches are severely misrouted and arborize away from their normal target area. (E,F) Wild-type *Or47b* axons terminate on VA1lm and extend a collateral across the commissure to the corresponding glomerulus on the contralateral lobe. (G,H) In the *dock* mutant, some *Or47b* axons can be seen extending to dorsal brain regions (arrowhead), while others stop short of VA1lm. Broken outlines in all panels indicate the approximate positions of the glomerular targets. Scale bar: 25 μm.

dock and *Pak* do not function in antennal neuron differentiation

As *dock* and *Pak* are autonomously required in ONs, one explanation for defective targeting of their axons is that cell fate specification of the neurons is disrupted. To explore this possibility, we examined in detail the critical steps of ON differentiation in both mutants. By monitoring ON development at various stages, we demonstrate that ONs differentiate normally in *dock*^{P1}/*dock*^{P1} and *Pak*^{6}/*Pak*^{11} homozygotes. First, antennal discs of *dock* and *Pak* mutants show *lozenge* gene expression in three semi-elliptical domains similar to wild type (see supplemental figures at http://dev.biologists.org/supplemental/). Second, in nascent antennae derived from 30 hAPF mutant pupae, ONs express both the ELAV and Futsch antigens (Fig. 6A-C). Furthermore, axons extend out of the antenna normally. Third, in the mutant antennae, *Or22a*, *Or47a* and *Or47b* genes are expressed by characteristic numbers of cells that are scattered randomly within circumscribed domains as in wild type (Table 2; Fig. 6G-I;

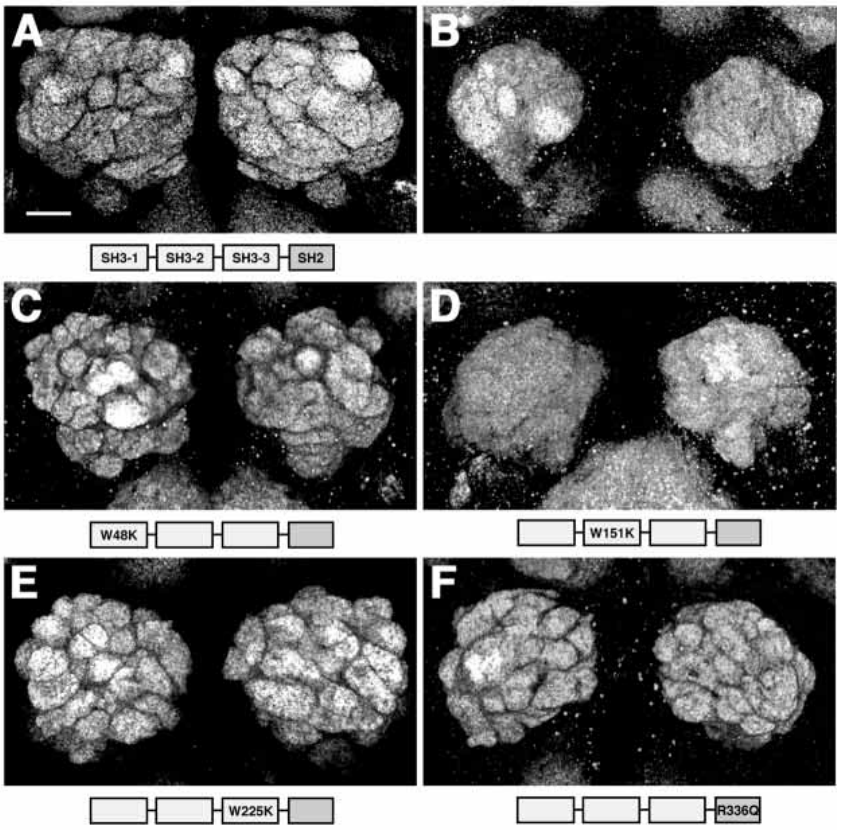

Fig. 5. Interaction between Dock and Pak is necessary for antennal lobe development. ALs of adult $dock^{P1}/dock^{P1}$ mutants, expressing wild-type and various mutant *UAS-dock* cDNA constructs under the control of *SG18.1-Gal4*, were stained with nc82 mAb to assess AL development. (A) Wild-type *dock* cDNA: $UAS\text{-}dock^{WT}$; $dock^{P1}/dock^{P1}$. (B) No cDNA: $dock^{P1}/dock^{P1}$. (C) Mutation in the first SH3 domain: $UAS\text{-}dock^{W48K}$; $dock^{P1}/dock^{P1}$. (D) Mutation in the second SH3 domain: $UAS\text{-}dock^{W151K}$; $dock^{P1}/dock^{P1}$. (E) Mutation in the third SH3 domain: $UAS\text{-}dock^{W225K}$; $dock^{P1}/dock^{P1}$. (F) Mutation in the SH2 domain: $UAS\text{-}dock^{R336Q}$; $dock^{P1}/dock^{P1}$. Scale bar: 25 μm.

see supplemental figures at http://dev.biologists.org/supplemental/) (Vosshall et al., 1999; Vosshall et al., 2000). Fourth, the three olfactory sensilla types (basiconic, trichoid and coeloconic) are found on the mutant antennae and have similar morphology, numbers and distribution as in wild type (Table 2, Fig. 6D-F; see supplemental figures at http://dev.biologists.org/supplemental/) (Shanbhag et al., 1999). Examination of indigenous cells of the AL, such as the PNs and the AL glia (see supplemental figures at http://dev.biologists.org/supplemental/), showed that these cells also differentiate normally in the *dock* and *Pak* mutants. However, in the mutants, the dendritic arborization of PNs are more diffused and the number of glia processes are somewhat reduced. Thus, morphogenetic changes of the cells that accompany normal glomerular development fail to occur properly when the *dock* or *Pak* genes are disrupted.

DISCUSSION

The connectivity of the olfactory map, and thus the logic of olfactory coding, is a direct consequence of the precise targeting of olfactory axons to their cognate glomeruli during development. The cellular and molecular mechanisms that underlie development of the olfactory map are essentially unknown. We have now characterized the pathways that olfactory axons take to their glomeruli and

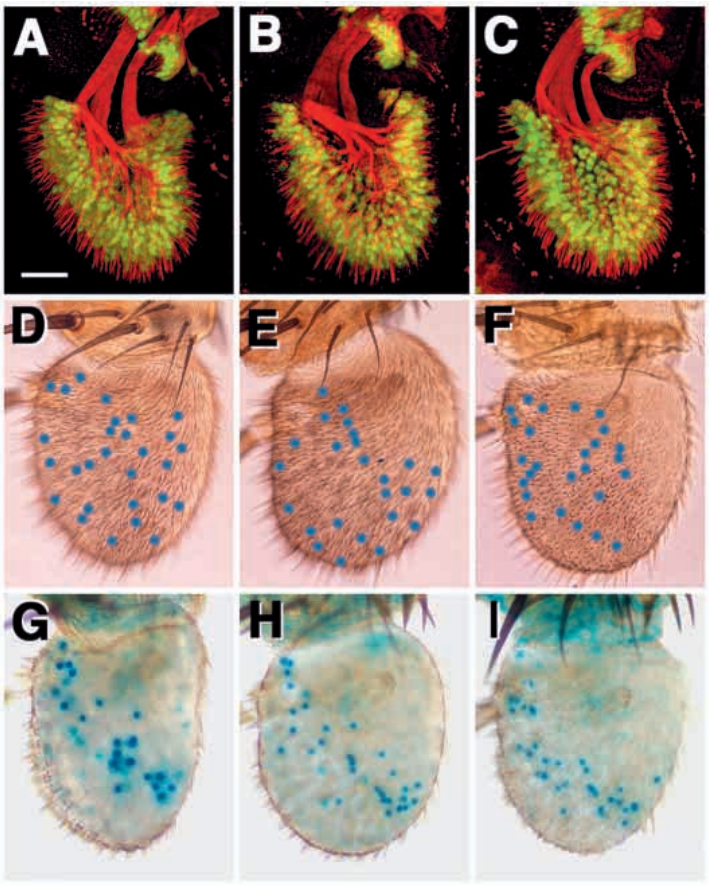

Fig. 6. *dock* and *Pak* do not function in antennal neuron differentiation. (A-C) Confocal micrographs of 30 hAPF pupal antennae double-stained with anti-Elav (green) and 22C10 mAb (red). ONs express Elav and project their axons out of the antenna normally in the *dock* (B) and *Pak* (C) mutants, compared with the wild type (A). (D-F) Adult antennae mounted in Faure's mountant; coeloconic sensilla are highlighted by blue dots. The morphology, number and distribution of sensilla in *dock* (E) and *Pak* (F) mutants are similar to those in wild type (D). See Table 2 for a summary of the results. (G-I) Antennae from $Or47a\text{-}Gal4/UAS\text{-}lacZ^{nuclear}$ stained with X-gal showing the distribution and number of *Or47a* ONs. The number and distribution of *Or47a* neurons, in a diagonal lateral-to-medial stripe, in *dock* (H) and *Pak* (I) mutants are similar to those in wild type (G). Scale bar: 30 μm.

Table 2. Numbers of sensilla and ON cell bodies in *dock* mutant, *Pak* mutant and wild-type antennae

	Genotype		
Sensilla	Wild type	*dock*	*Pak*
Coeloconica	58±4.02	56±3	51±4.76
Basiconica	217±5.24	210±5.59	210±5.09
Trichoidea	123±3.63	121±2.17	115±3.56
Olfactory neurons			
Or22a	16±3.67	18±4.55	15±4.21
Or47a	31±4.26	31±2.87	31±3.91
Or47b	51±5.55	42±7.22	58±1.41

demonstrate that two growth-cone signaling molecules, Dock and Pak, function to regulate the precise convergence of the axons on their targets.

ONs of the antennae and maxillary palps undergo terminal differentiation during early metamorphosis and become predestined to express particular Or genes and synapse in specific glomeruli (Jhaveri et al., 2000; Vosshall et al., 1999). Between 20 and ~50 hAPF, their axons leave the nascent antenna in fascicles and enter the AL in search of their targets. PNs, however, acquire their cell fates, which predetermine their glomerular choice, during larval development (Jefferis et al., 2001). During early pupal development their dendrites enter the AL and become precisely paired with ON axons in specific glomeruli. Thus, ONs expressing a given Or gene rendezvous with PNs of a particular identity within a topographically defined glomerulus in the AL.

We find that in the wild type, olfactory axons take stereotyped paths on the surface of the AL to converge on their cognate glomeruli. Detailed characterization of the axon trajectories, using *Gal4* drivers expressed in different subclasses of ONs shows that, upon arrival at the anterolateral point of the AL, afferents project directly, with little sidetracking to their postsynaptic targets. As in the mouse and moth, these axon pathways are bilaterally symmetric and invariant from AL to AL (Mombaerts et al., 1996; Oland et al., 1998; Wang et al., 1998). How is this precise wiring pattern formed during development? In one model, each ON initially sends collaterals to multiple glomeruli and then withdraws the inappropriate branches in a process requiring odorant-evoked activity. Alternatively, the invariant pattern of connections is the result of directed axon migrations in response to spatially restricted pathfinding cues in the developing AL. A definitive answer to this question will require developmental study or direct observation of the extending axons. However, several observations are consistent with the notion that olfactory axons navigate directly to their cognate glomeruli. First, a temporal lag between early axon pathfinding and subsequent *Or* gene expression (Clyne et al., 1999) indicates that an odorant-evoked activity is unlikely to play an important role. Indeed, activity is neither required for formation nor maintenance of the olfactory map in mouse and moth (Belluscio et al., 1998; Lin et al., 2000; Oland et al., 1996). Second, and importantly, our finding that the growth cone guidance genes, *dock* and *Pak*, are needed for development of the olfactory map, provides strong evidence that directed axon migration plays a key role in the matching of ON axons with their correct glomeruli. Directed navigation of olfactory axons to their targets is also observed in zebrafish and moth (Dynes and Ngai, 1998; Oland et al., 1998).

In *dock* and *Pak* mutants, the stereotyped connectivity of AL neuropil is severely disrupted, leading to an aglomerular phenotype. We present three pieces of evidence indicating that *dock* and *Pak* function in ONs. First, antibody staining shows that Dock and Pak proteins are expressed in antennal axons during the period in which they are projecting to the brain. Consistent with their requirements in ONs, removal of *dock* and *Pak* activities from only the antennae results in ectopic targeting of olfactory axons. Finally, expression of *dock* and *Pak* cDNAs specifically in ONs in otherwise mutant animals leads to strong rescue of the mutant AL phenotype. We noticed that although numerous glomeruli were restored upon the expression of the wild-type cDNAs, some glomeruli were not. We believe that the incomplete rescue is due to the expression of *SG18.1-Gal4* in only a subset of all the ONs. However, it is also possible that the partial rescue reflects an additional requirement of *dock* and *Pak* functions in the brain. A recent study indicates that ONs may be divided into different classes based on the timing of their projections (Jhaveri and Rodrigues, 2002). We did not determine further if *dock* and *Pak* are required in all ONs or in only a specific subset. Although *dock* and *Pak* are specifically required in ONs, our finding of nonautonomous effects on the morphogenetic changes of the PNs and AL glia is in accord with earlier studies in which ONs were physically or genetically ablated (Graziadei and Monti-Graziadei, 1992; Hildebrand et al., 1979; Stocker and Gendre, 1988). Our data therefore show that proper termination of ON axons is also an important step in the sculpting of the AL neuropil into distinct glomeruli.

We provide evidence that the disruption in AL development in *dock* and *Pak* mutants is not an indirect effect of aberrant cell-fate determination or axonogenesis. By contrast, we observed that the precise targeting of ON axons is severely disrupted in *dock* and *Pak* mutants. To identify the cause of the mistargeting, we examined the axon pathways of individual ON classes (*Or47a*, and *Or47b*) at the single-cell level. Although an additional short branch was observed in 9% of *dock* mutant neurons (n=45), the most striking defect observed in single-cell clones (64%, n=45) is the chaotic trajectories exhibited by both the ipsilateral and contralateral axons of the ONs. We conclude that the primary function of *dock* and *Pak* in ONs is axon pathfinding, to steer ON axons precisely to their target glomeruli. In mouse, mutations in the odorant receptor genes abolish the ability of olfactory axons to pathfind in the anteroposterior axis without affecting their migration in the dorsoventral axis, leading to the proposal that odorant receptors participate in the recognition of only anteroposterior guidance cues (O'Leary et al., 1999; Wang et al., 1998). However, after examining several hundred ALs for each *dock* and *Pak* mutant, we did not observe any consistent patterns in the mistargeting of ON axons. We did observe that the ON classes are affected to different degrees by the loss of *dock* and *Pak* activities. Although *Or22a* and *Or47a* axons terminate in numerous ectopic glomeruli, *Or47b* axons terminate in a single glomerulus, albeit mis-shapen, in the approximate position of the wild-type VA1lm. We currently do not know the reason for the differential sensitivity of the ON subtypes to the loss of *dock* and *Pak* functions. One possibility is that *Or47b* axons, which are among the first axons to enter the AL, are confronted

with fewer developing glomeruli (Jhaveri et al., 2000) and hence fewer guidance choices than *Or22a* and *Or47a* axons that enter the AL later. Alternatively, *Or47b* axons may have less need for *dock*- and *Pak*-mediated navigational functions because VA1lm is located near the nerve entry point. Indeed, while the *Or47b* ipsilateral axons frequently terminate accurately on VA1lm, the contralateral axons, which have to project across the entire AL surface, are often misrouted. In contrast to the severe projection defects in the AL, the migration of *dock* and *Pak* mutant axons through the antennal nerve takes place normally. It is possible that the lack of requirement of *dock* and *Pak* functions during this phase of axon growth reflects a different guidance mechanism in the antennal nerve.

The observation that the ON axon trajectories are severely disrupted in *dock* and *Pak* mutants suggests that the genes may mediate the detection or response of the growth cones to guidance cues in the environment. Our results indicate that in these events, *dock* and *Pak* are very likely to act in a signaling pathway. First, loss of either *dock* or *Pak* functions results in olfactory connectivity phenotypes that are indistinguishable. Second, both *dock* or *Pak* function autonomously in ONs. Third, mutations that disrupt the domains of Dock (second SH3 domain) and Pak (N-terminal PXXP domain; Pak^4), which mediate interaction between the two proteins (Hing et al., 1999), disrupt ON axon targeting. We therefore propose that Dock and Pak are part of a signal transduction cascade that allows ONs to find and precisely pair with the correct postsynaptic partners. Although severely disrupted, the guidance of ON axons in *dock* and *Pak* mutants is not completely abolished, indicating that other genes function to steer ON axons to their targets as well.

As the Dock-Pak signaling pathway appears to govern the pathfinding of a number of ON subtypes, it is unlikely to explain the specificity of ON targeting. What transmembrane receptors might control the Dock-Pak signaling pathway and direct the precise pairing between ONs and their postsynaptic targets? Because ONs that target different glomeruli are interspersed in the olfactory epithelium, thus precluding their tagging by simple gradients of molecular cues, it is anticipated that the receptors are molecularly diverse. A highly diverse receptor family is, of course, the odorant receptor family. In mouse, odorant receptors themselves provide the specificity for target selection (Mombaerts et al., 1996; Wang et al., 1998). Odorant receptors are unlikely to play a guidance role in *Drosophila*, however, as expression of these genes begin long after axon migration has taken place (Clyne et al., 1999). Another family of diverse receptors that functions in cell-cell adhesion and is expressed in synapses is the cadherin superfamily (Yagi and Takeichi, 2000). In mouse, some members of the family, the CNRs, are expressed in the olfactory bulb, indicating that cadherin family proteins may also play a role in olfactory map development (Kohmura et al., 1998). Recently, in a biochemical screen for proteins that bind to Dock, Zipursky and colleagues identified the immunoglobulin superfamily transmembrane receptor Dscam (Schmucker et al., 2000). Interestingly, the expression of the *Dscam* gene is regulated by a novel, combinatorial splicing mechanism, which allows *Dscam* to encode up to 38,000 isoforms (Schmucker et al., 2000). Because of the tremendous diversity in its gene products, *Dscam* has been proposed to play a role in encoding synaptic specificity. Our discovery that the Dock-Pak signaling pathway regulate the projection of ON axons to their cognate glomeruli now presents us with a unique opportunity to begin to assess the possible roles of these receptors in the development of the *Drosophila* olfactory map.

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