Fasciclin II and Beaten path modulate intercellular adhesion in Drosophila larval visual organ development

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SUMMARY

Previous studies demonstrated that Fasciclin II and Beaten path are necessary for regulating cell adhesion events that are important for motoneuron development in Drosophila. We observe that the cell adhesion molecule Fasciclin II and the secreted anti-adhesion molecule Beaten path have additional critical roles in the development of at least one set of sensory organs, the larval visual organs. Taken together, phenotypic analysis, genetic interactions, expression studies and rescue experiments suggest that, in normal development, secretion of Beaten path by cells of the optic lobes allows the Fasciclin II-expressing larval visual organ cells to detach from the optic lobes as a cohesive cell cluster. Our results also demonstrate that mechanisms guiding neuronal development may be shared between motoneurons and sensory organs, and provide evidence that titration of adhesion and anti-adhesion is critical for early steps in development of the larval visual system.

Key words: Intercellular adhesion, Bolwig’s Organ, Neuronal development, Sensory organ, Drosophila

INTRODUCTION

Establishment of neuronal connections is a dynamic process requiring temporal and spatial coordination of sequential developmental events. These events include specification of neuronal and target cell identity, cell migration, extension of axonal projections and maintenance of final connections between a neuron and its appropriate target cell. The success of each of these steps involves coordination of intercellular associations, some temporary and some permanent, which are facilitated in part by the selective expression of cell adhesion molecules.

Complementary genetic (Seeger et al., 1993; Van Vactor et al., 1993; Salzberg et al., 1994; Kania et al., 1995; Kolodziej et al., 1995; Martin et al., 1995; Schmucker et al., 1997; Holmes et al., 1998), biochemical (Bonhoeffer and Huf, 1985; Luo et al., 1993; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995; Drescher et al., 1995) and molecular (Patel et al., 1987; Bieber et al., 1989; Grenningloh et al., 1991; Kolodkin et al., 1993; Harris et al., 1996; Mitchell et al., 1996) analyses have revealed some of the genes and molecules that direct neuronal pathfinding and connectivity. The success of each of these steps involves coordination of intercellular associations, some temporary and some permanent, which are facilitated in part by the selective expression of cell adhesion molecules.

Analysis of development of the motoneurons of the Drosophila larva has proved particularly revealing and has enabled the characterization of an array of proteins including those involved in adhesion, anti-adhesion, attraction and repulsion, as well as signal transduction molecules with specific roles in neuronal development (reviewed in Goodman and Shatz, 1993; Chiba and Keshishian, 1996; Garrity and Zipursky, 1996; Goodman, 1996; Kolodkin, 1996; Tessier-Lavigne and Goodman, 1996; Brunner and O’Kane, 1997). Homologs of many of these molecules are found in vertebrates (Harrelson and Goodman, 1988; Bier et al., 1989; Grenningloh et al., 1990; Kolodkin et al., 1993; Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996; Thor and Thomas, 1997; Kidd et al., 1998). Of the different classes of proteins involved in neuronal development, those mediating intercellular adhesion have been most thoroughly studied. Experiments initially performed in grasshopper demonstrated that growth cones of developing nerves distinguish between different bundles of existing axons and selectively fasciculate only with axons following the correct path (Raper et al., 1983a,b,c; Bastiani et al., 1984, 1986; Doe et al., 1986; du Lac et al., 1986). These experiments led to the proposal that developing nerves follow ‘labeled pathways’ and that the molecular ‘labels’ promote or discourage fasciculation (Raper et al., 1983a,b,c, 1984). Identification of the labels may provide a molecular basis for understanding the mechanisms of neuronal pathfinding. Studies in grasshopper led to the identification of one such molecule, Fasciclin II (Fas II), a cell surface protein expressed on a subset of fasciculated axons in the embryo (Bastiani et al., 1987). Fas II is a member of the immunoglobulin (Ig) superfamily of proteins and is related to vertebrate neural cell adhesion molecule (NCAM) (Harrelson and Goodman, 1988; Snow et al., 1988; Grenningloh et al., 1991).

Analyses of fas II expression in Drosophila indicate that it
is expressed by a subset of CNS axons and by all motoneurons in the PNS (Grenningloh et al., 1991; Van Vactor et al., 1993; Lin et al., 1994). It is also expressed by some non-neuronal tissues. Intriguingly, Fas II is often expressed in non-neuronal cells as Fas II-expressing axons grow over them (Van Vactor et al., 1993). Cumulatively, these experimental studies suggest that Fas II may be directly involved in selective fasciculation and pathfinding. The role of Fas II in the development of specific motoneurons was further explored by manipulating the levels of fas II expression (Lin et al., 1994; Lin and Goodman, 1994). Phenotypic analyses of both loss-of-function fas II mutant strains and ectopic expression studies support a role for Fas II in selective fasciculation during CNS development and suggest that the process is sensitive to timing, location and levels of Fas II expression (Lin et al., 1994; Lin and Goodman, 1994). Fas II was also demonstrated to be important later in neuronal development for establishment of synapses (Schuster et al., 1996a,b; Davis et al., 1997; Thomas et al., 1997; Zito et al., 1997).

Fas II participates in homophilic adhesion (Grenningloh et al., 1990), suggesting an obvious mechanism for its role in fasciculation. Analysis of the role of Fas II in motoneuron development indicates that a mechanism must exist to modulate Fas II-mediated adhesion. During development of specific motor neuron pathways, Fas II-expressing axons fasciculate with preexisting neuron tracks, and at defined choice points, defasciculate and innervate their target cells (Grenningloh et al., 1991; Van Vactor et al., 1993; Lin et al., 1994). A recent advance in understanding Fas II activity has come from analysis of the beaten path (beat) gene (Fambrough and Goodman, 1996). Mutations in beat prevent defasciculation in a subset of motoneurons (Van Vactor et al., 1993; Fambrough and Goodman, 1996). The beat loss-of-function phenotype is similar to the Fas II overexpression phenotype, and mutations in fas II suppress the beat phenotype. Fambrough and Goodman propose that Beat is a secreted anti-adhesion molecule that promotes defasciculation of Fas II-expressing axons at appropriate choice points.

To complement the understanding of neuronal development gained from analysis of the motoneurons, we have studied development of a sensory organ, the larval visual system. The larval visual system (LVS) is a relatively simple sensory system composed of two clusters of 12 photoreceptor cells, known as the Bolwig’s Organs (BOs), from which axons extend in a single fascicle to the brain. Development of the LVS differs in several significant ways from that of the motoneurons (Schmucker et al., 1992, 1997; Green et al., 1993; Holmes et al., 1998). Notably, the axon tracks of the Drosophila CNS and PNS develop through axonal outgrowth and migration, characterized by growth cone extension and selective fasciculation. In contrast, the initial connections of the pioneer axons of the LVS are established when the BOs are close to their target cells, the developing larval brain (Green et al., 1993). Thus, it is neuronal cell bodies, the BOs, that move through a complex environment during LVS development, rather than the axons as in development of the CNS and PNS. It is currently unclear whether the anterior relocation of the BOs occurs as a passive consequence of head involution, or whether an active migratory process is involved. Preliminary analysis of two mutations we have identified, not enough anterior extension and out of place, which result in the failure of the BOs to attain their proper location, revealed that, despite failures in BO development, head involution appears to occur normally (Holmes et al., 1998). The apparent genetic separation of BO movement and head involution suggests the processes may also be mechanistically separable. However, the developmental mechanism driving BO migration remains to be determined.

The BOs arise from cells of the optic lobe placodes (OLs), ectodermal tissues that will go on to form the optic lobes of the adult fly. The BOs detach from the OLs and remain at the periphery of the embryo when the OLs invaginate (Green et al., 1993). As the BOs detach from the OLs, axons extend from the BOs and establish initial connections with the brain, which is adjacent to the OLs. Therefore, the axons need only navigate a relatively short distance to reach their targets in the brain at this early stage of development. The BO cells remain clustered for the remainder of development, during which they migrate anteriorly and the Bolwig’s Nerves (BNs) increase significantly in length. Therefore, proper development of the LVS requires that the BO precursors detach from the OLs, that the pioneer connections between the BOs and the brain be established and maintained, that axons properly fasciculate with the pioneer axons, and that the BOs migrate correctly to their final positions.

By genetic analysis of LVS development, we identified a mutant allele of beat that disrupts early stages of BO development (Holmes et al., 1998). beat encodes a putative secreted protein of 401 amino acids (Fambrough and Goodman, 1996) that is predicted to contain two Ig domains in its amino terminus (Bazan and Goodman, 1997; Mushegian, 1997). The Beat protein also contains six Cys residues in its carboxyl terminus that are loosely similar to a cysteine knot domain and may function in dimerization of the Beat protein (Bazan and Goodman, 1997). To explore the role of beat in LVS development, we determined that it is expressed in a cluster of cells in the OLs before the BOs become distinct and that in LVS development, as in motor neuron development, beat interacts genetically with fas II. We have also shown that a mutation in fas II disrupts LVS development, resulting in a phenotype that is, in some ways, the opposite of that resulting from mutations in beat. Conversely, overexpression of fas II in the BO causes defects in BO development resembling those that result from mutations in beat. By directing expression of beat to either the BOs or the OLs we rescued the LVS phenotype of beat mutations. Together, these results demonstrate that detachment of the BO precursors from the OL may involve interaction between Beat and Fas II. Thus, at least some of the mechanisms important for regulating intercellular interactions during motor axon development also function in guiding development of sensory organs, suggesting that some of the general principles of neuronal development may overlap in these two different neuronal systems.

MATERIALS AND METHODS

Genetics and fly stocks

The beat

allele was isolated in a screen for mutations disrupting LVS development (Holmes et al., 1998). The beat

and beat

alleles were obtained from D. Fambrough and C. Goodman. The beat

and beat

alleles were induced in a fas II

mutant background (Van
Vactor et al., 1993). However, *fas III* and *beat**tric* complement one another, indicating that *fas III* does not contribute to the LVS phenotypes in *beat* mutants. Phenotypic rescue experiments were performed by crossing *P[omb-GAL4]; beat**tric*, *P[gl-lacZ] or P[Kr-GAL4], beat**tric* to beat**tric*, P[gl-lacZ]; P[UAS-beat], and inspecting the LVS.

**Histology**

Embryos were prepared for antibody staining by standard methods (Mitchison and Sedat, 1983). 0.2% saponin or 0.1% Triton X-100 was added to all solutions after fixation. Primary antibodies anti-β-galactosidase (Promega) and anti-Krüppel (gift of U. Gaul) were used at 1:500 dilution, and mAb 1D4 (anti-Fas II) (gift of C. Goodman) at 1:5 dilution. HRP-conjugated secondary antibodies, goat anti-mouse (BioRad) and goat anti-rabbit (Pierce) were preabsorbed and used at 1:500 dilution. β-galactosidase activity was detected by standard methods (Lis et al., 1983; Simon et al., 1985).

**RNA in situ hybridization**

RNA in situ hybridizations were performed as described by Lehmann and Tautz (1994). *beat* cDNA used to generate beat antisense RNA probes was a gift of D. Fambrough, T. Pipes and C. Goodman.

### RESULTS

**Mutations in *beaten path* disrupt the larval visual system**

In a screen for mutations that disrupt the larval visual system (LVS), we identified one gene that we originally called *triclops* (*tric*) to reflect the presence of supernumerary Bolwig’s Organs (BOs) in a subset of mutant embryos (Holmes et al., 1998). Genetic mapping of *tric* revealed that the mutant phenotype is uncovered by Df(2L)R10, a deficiency that also uncovers the *beaten path* (*beat*) phenotype (Van Vactor et al., 1993). Mutations in *tric* and *beat* fail to complement one another for the LVS phenotype, indicating that *tric* is allelic with *beat*. The allele we recovered will be referred to as *beat**tric*.

We examined the LVS phenotypes caused by three mutant alleles of *beat: beat**tric*, beat2 and beat4. We also examined embryos heteroallelic for *beat**tric*/beat2 and *beat**tric*/beat4 and embryos hemizygous for *beat**tric* and beat4 over various deletions of the region. The LVS defects resulting from all of these combinations of *beat* mutations are similar and indicate that all alleles examined behave as amorphic or hypomorphic alleles with regard to their effects on the LVS. However, although the LVS phenotypes of the three *beat* alleles are indistinguishable, *beat**tric*, unlike beat2 and beat3, is viable when homozygous or in combination with any other available *beat* allele. Furthermore, *beat**tric* does not affect motoneuron development (H. Wan and C. Goodman, personal communication). The basis for these differences in motoneuron phenotypes of the *beat* alleles is not known.

In normal development, the precursor cells of the BOs can first be identified at embryonic stage 12. The BO cells are distinguished morphologically in the ventral tip of the optic lobe placodes (OLs) (Green et al., 1993) and biochemically by expression of Krüppel (Schmucker et al., 1992), Fasciclin II (Grenningloh et al., 1991; Schmucker et al., 1997), and Glass (Moses et al., 1989) proteins, all of which are detectable in BOs of late stage-12 embryos. Because glass expression is diagnostic for photoreceptor cells, we used a lacZ reporter gene driven by the glass regulatory elements (gl-lacZ) (Moses and Rubin, 1991) to identify the BOs in most of the studies reported here. By stage 13 the BOs can be identified as discrete clusters of cells located at the periphery of the embryo (Fig. 1A). The BOs migrate anteriorly during head involution (Fig. 1B), and achieve their final positions in the embryo by stage 16, where they remain for the duration of embryogenesis (Fig. 1C,D). In embryos, the mature LVS consists of two BOs located near the anterior end, from which the BNs project posteriorly. The trajectory of the BNs in wild-type embryos is characterized by a sharp ventral turn where it dives around the anterior portion of the brain (Fig. 1C). This turn causes the BN to go out of the focal plane when examined in dorsal views (Fig. 1D). The BNs terminate in the posterior region of the brain (Fig. 1C,D).

In *beat* mutant embryos the cells of the BOs form, but LVS morphology is severely disrupted and increased numbers of photoreceptor cells are apparent from the earliest stages of LVS development. In stage-13 embryos, in addition to photoreceptor cells at the normal location of the BO clusters, extra photoreceptor cells are dispersed between the two clusters (Fig. 1E). Axonal projections from these cells suggest that they are neuronal cells (Fig. 1E). The OLs begin to invaginate during stage 13, and in *beat* mutants the abnormal glass-expressing cells are often located near the invaginating optic lobes. Precise quantitation of the number of extra photoreceptor cells in *beat* mutants is difficult with currently available reagents. However, using an antibody to Krüppel (Gaul et al., 1987), which stains the nuclei of the BOs, we concluded that there are more photoreceptor cells in *beat**tric* mutants than in wild-type (not shown). For example, in the embryo shown in Fig. 1E, BO clusters of approximately normal size are located at the periphery of the embryo, the normal location at this stage of development. However, approximately 15 additional cells are distributed between the two BO clusters. Frequently in *beat* mutant embryos, a BO fails to separate properly (Fig. 1G), suggesting that mutations in *beat* may affect this early step of LVS development.

The LVS is abnormal throughout development in *beat* mutant embryos and a range of defects is seen in the BOs and BNs at later stages. Although no single defect in LVS morphology is predominant, defects are readily apparent in greater than 65% of mutant embryos. The frequency with which we see each major class of defects is shown in Table 1. One particularly striking phenotype is the presence of three BOs (Fig. 1H); in rare embryos four BO clusters are seen (not shown). Although migration of the BOs appears relatively normal in some *beat* mutant embryos, in others it is disrupted. Often the BO does not achieve its proper location (Fig. 1I), although head involution appears normal, or the BO is elongated (Fig. 1J). The morphology of the major BO clusters in mutant embryos ranges from essentially normal in appearance (Fig. 1H) to moderately disrupted (Fig. 1I,J) or severely disrupted (Fig. 1K). The most severe defects in BO morphology result from the presence of too many photoreceptor cells and a failure of these cells to form tightly associated clusters (Fig. 1K). Because of the range of dispersed BO cell phenotypes seen in *beat* mutant embryos it is difficult to know if BO cells that were initially clustered dissociate, or if the cells were never tightly associated. Further analysis will distinguish whether defects seen at later stages are a consequence of disruption of early development or if
they reflect additional roles for beat throughout LVS development.

**beat is expressed in the optic lobes**

Using digoxigenin-labeled anti-sense RNA probes for in situ hybridization analysis we determined the mRNA expression in wild-type embryos. Fambrough and Goodman (1996) noted that, in addition to being expressed in the motoneurons, beat is expressed in unidentified cells in the embryonic brain. Our analysis focused on resolving the identity of these cells. In the brain, beat expression is first detected at stage 12 in a small cluster of cells located in the vicinity of the OLs (Fig. 2A). To determine the identity of the beat-expressing cells, we looked at the simultaneous expression of beat mRNA and gl-lacZ. We performed X-gal staining followed by in situ hybridization using a beat anti-sense RNA probe on embryos carrying gl-lacZ. When expression of beat and lacZ is examined in these embryos, beat mRNA is never detected in the BOs or BNs once these structures become distinguishable from the OLs (Fig. 2B,C). In stage-13 embryos, the pattern of beat expression, although distinct from the BO, is close to the developing BOs (Fig. 2B). Expression of beat mRNA in the brain persists through stage 16, where beat is clearly expressed in the embryonic brain (Fig. 2C). Thus, beat is expressed in a subset of cells in the OL that are close to, but that do not overlap with, the BO.

**fasciclin II is expressed in the LVS and is required for its development**

The proposed role of Beat in motoneuron development is to promote defasciculation of axons expressing Fas II (Fambrough and Goodman, 1996). Because Fas II is expressed in the BOs, BNs and OLs (Fig. 3A-C and Grenningloh et al., 1991; Schmucker et al., 1997) it was important to determine whether it is also required for their development. Using mAb 1D4 (Grenningloh et al., 1991; Van Vactor et al., 1993), which recognizes Fas II, we detected Fas II expression in the OL at least as early as stage 12 (Fig. 3A) before the BOs become morphologically distinct. Fas II expression in the BOs, the BNs and the optic lobes persists for the remainder of LVS development (Fig. 3B,C).

We examined embryos hemizygous for the fas $^{Ir}\text{76}$ mutation, which is reported to result in greater than 90% reduction in Fas II protein expression (Grenningloh et al., 1991). In a majority of fas $^{Ir}\text{76}$ embryos, the LVS is morphologically defective (Table 1), although the defects are more subtle than those seen in beat mutant embryos. Disruption of BO development can be seen as early as stage 13 in fas $^{Ir}\text{76}$ embryos, in which unclustered photoreceptor cells are located near the normal BOs (Fig. 3D). Defects seen

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Fig. 1. Larval visual system (LVS) development in wild-type and beat mutant embryos. (A-D) LVS development in wild-type embryos carrying P[gl-lacZ]. (E, H-K) LVS development in beat mutant embryos carrying P[gl-lacZ]. All embryos are oriented with anterior to the left; dorsal is up in lateral views. (A) Stage 13, dorsal view. The developing BOs are ectodermal (arrowheads) (B) Stage 14, lateral view. BO (arrowhead) has begun to migrate and BN extends toward brain (arrow). (C) Stage 16. LVS is fully developed. BO (arrowhead) is located anteriorly, and BN has assumed its characteristic path, turning ventrally around the brain (arrow) and terminating in the posterior of the brain (double arrow). The other BO and BN are out of focal plane. (D) Stage 16 embryo. In dorsal view, the BNs extend from the BOs (arrowheads) and leave the focal plane where they project ventrally around the brain (arrows). Non-neuronal gl-lacZ expressing tissue between the BOs is unidentified (Moses et al., 1989), gl-lacZ is also expressed in the embryonic brain (C and D, double arrowheads). (E) Stage-13 beat$^{+}$ embry. BOs are located normally (arrowheads); however, additional gl-lacZ-expressing cells with axon-like projections are distributed throughout the head region (arrow). (F) Higher magnification of stage-13 wild-type embryo stained with mAb 1D4 to show Fas II expression. BO (arrowhead) is seen separating normally from the invaginating OL. (G) Higher magnification of stage-13 beat mutant embryo stained with mAb 1D4. No distinct cluster is apparent. (H-K) Stage-16 beat mutant embryos. (H) Three clusters of BO cells are present (arrowheads). (I) Hemizygous beat$^{+}$ in which one BO has failed to migrate normally (asterisk). The other BO is in the normal location (arrowhead). A node of gl-lacZ-expressing cells is located midway on the path of the BN (double arrowhead). (J) beat$^{+}$ embryo in which one BO is elongated (double arrowhead). (K) beat$^{+}$beat$^{+}$ embryo with severely misshapen BO (arrowhead). The BO is composed of more than 12 loosely clustered photoreceptor cells, and the BN extending from it follows an abnormal path, bifurcating near BO (arrow). Bar, 50 μm.
at later stages in development of fas II	extsuperscript{e76} mutants include BOs that are smaller than normal (Fig. 3E), which could result from fewer than 12 cells in the BO cluster. In a subset of late stage fas II	extsuperscript{e76} embryos the BOs do not migrate properly (Fig. 3F and Table 1), and nodules are present along the nerve in a subset of mutant embryos (Fig. 3G and Table 1). Head involution appears normal in all fas II	extsuperscript{e76} mutants. It is noteworthy that mutations in fas II	extsuperscript{e76} and beat disrupt BO relocation without apparently affecting head involution. The defects in BO migration could be due to disruption of the coordination of BO movement and head involution, or they could reflect a role for these molecules in BO migration. Especially intriguing are embryos such as that shown in Fig. 3F, in which a small cluster of photoreceptor cells has continued to migrate beyond the point at which the majority is located.

Table 1. Larval visual system defects resulting from mutations in, or altered expression of, beat and fas II

<table>
<thead>
<tr>
<th>Phenotypes	extsuperscript{a}</th>
<th>Stage 13</th>
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<tbody>
<tr>
<td></td>
<td>n (PR)</td>
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<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Expressivity</th>
<th>Three BOs</th>
<th>‘beat’ unclustered BOs	extsuperscript{c}</th>
<th>‘fas II’ unclustered BOs	extsuperscript{c}</th>
<th>Nodules</th>
<th>Misshapen BOs</th>
<th>Misplaced BOs</th>
<th>Bifurcated nerves</th>
<th>Scattered PR cells</th>
</tr>
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<tbody>
<tr>
<td>beat</td>
<td>649</td>
<td>65%</td>
<td>8</td>
<td>21</td>
<td>–</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>7</td>
<td>&gt;50</td>
</tr>
<tr>
<td>fasII	extsuperscript{e76}</td>
<td>69</td>
<td>82%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>56</td>
<td>21</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>fasII	extsuperscript{e76} beat	extsuperscript{tric}</td>
<td>203</td>
<td>33%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>33</td>
<td>4</td>
<td>12</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td>Kr-GALA4; UAS-fas II</td>
<td>170</td>
<td>6%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>beat	extsuperscript{tric}; omb-GALA4; UAS-beat</td>
<td>&gt;100</td>
<td>0%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>&gt;50</td>
</tr>
<tr>
<td>beat	extsuperscript{tric}</td>
<td>43</td>
<td>7%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>2</td>
<td>–</td>
<td>&gt;20</td>
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Embryos were collected from stocks of the indicated genotype. All stocks contained P[gl-lacZ] and the LVS phenotype was assessed using anti-β-galactosidase antibody. The frequency with which each phenotype was observed is indicated. Stage 13 embryos were assessed independently of later stage embryos.

BO, Bolwig’s Organ; PR, photoreceptor; –, phenotype not seen.

The proportion of embryos expressing a particular LVS phenotype is reported as a percentage of all embryos of that genotype. Some embryos display more than one class of phenotype.

The individual phenotypes occur with the same frequencies in embryos carrying any of the beat mutant alleles. The percentages indicated reflect results obtained from inspection of all combinations of beat	extsuperscript{tric}, beat	extsuperscript{2} and beat	extsuperscript{1}, and a deletion of the beat genomic region.

The ‘unclustered’ BO phenotype differs in fas II	extsuperscript{e76} and beat mutants. ‘beat’ unclustered BOs are larger than wild type, whereas ‘fas II’ unclustered BOs are smaller.
molecule are important for normal development and that concurrent reduction of both restores normal LVS development in the double mutants.

**Overexpression of Fas II phenocopies beat mutant defects**

To further explore the importance of relative levels of Beat and Fas II, we determined whether elevating expression of Fas II in the BOs would affect LVS development. Increasing expression of Fas II in the BO precursor cells could strengthen adhesion between the BOs and OLs. This could in turn result in interference with the coordinated separation of the BOs from the OLs. To determine the consequences of overexpression of Fas II we examined embryos of the genotype gl-lacZ; Kr-GAL4, UAS-fasII. In these embryos, fas II expression is increased above endogenous levels in the BOs due to expression of UAS-fas II driven by Kr-GAL4. In the majority of stage-13 embryos in which Fas II is overexpressed, extra photoreceptor cells are seen distributed between the two normal BO clusters (Table 1). These embryos very closely resemble beat- embryos of the same stage (Fig. 1E). Despite the similarity of defects in early stages of LVS development, later stages are less affected by Fas II overexpression when compared to development in the absence of Beat. Overexpression of Fas II appears to have only minimal effects on anterior migration of the BOs. The BOs are located in the normal position in greater than 90% of stage-15 embryos carrying Kr-GAL4 and UAS-fas II. Subtle disruptions in the morphology of the BOs and BNs do result from overexpression of Fas II (not shown), but these are neither as frequent nor as severe as defects caused by mutations in beat (Table 1). The similarity of defects in early LVS developmental resulting from reduced expression of Beat and overexpression of Fas II bolsters the proposal that interaction between Beat and FasII is important for separation of the BOs from the OLs. That the effects of these two alterations in adhesion are different in later stages of LVS development may reflect differing roles of the molecules at different times. Alternatively, because Fas II is normally expressed in the mature LVS, we cannot determine whether expression of UAS-fasII using Kr-GAL4 has any effect on the amount of Fas II present in the LVS after stage 13.

**Ectopically expressing beat in the optic lobes or the Bolwig’s Organs rescues the beat-ic LVS phenotype**

To confirm that the absence of the wild-type beat gene product is responsible for the LVS defects in beat- mutant embryos and to determine when and where beat expression is required for wild-type development, we tested whether expression of a beat cDNA could rescue the beat- mutant LVS phenotype. By expressing beat in the OLs, we were also able to determine whether beat expression in the OLs is necessary and sufficient for normal LVS development. We used optomotoblind-GAL4 (omb-GAL4) to direct expression of UAS-beat (Fambrough and Goodman, 1996) to the OLs. In embryos homozygous for beat-ic in which UAS-beat was expressed in the omb-GAL4-expressing cells, LVS development occurred normally in 93% of the embryos (Table 1). Therefore, ectopic expression of wild-type beat in the optic lobes is sufficient to allow normal LVS development (Fig. 5).

Although expression of beat in the omb-GAL4-expressing
cells is able to substitute for the absence of normal beat expression, an unusually large number of omb-GAL4; beat<sup>tric</sup>, gl-lacZ; UAS-beat embryos failed to develop, the majority dying prior to the onset of gl-lacZ expression. This is similar to the effects on motor axon development caused by misexpression of beat in muscles (Fambrough and Goodman, 1996). Our results are therefore consistent with those of Fambrough and Goodman, whereby ectopic expression of beat in certain cells can have detrimental effects. Fas II is expressed in the embryonic brain and ventral nerve cord, and the high rate of death in embryos expressing beat in omb-GAL4-expressing cells may reflect disruption of other Fas II-mediated adhesion events resulting from inappropriate expression of beat. Furthermore, these results reinforce the idea that the timing and dosage of beat expression are critical for normal development of the LVS.

Having demonstrated that expression of beat in the optic lobes can restore normal LVS development in beat mutant embryos, we wanted to test whether expression of beat elsewhere in the LVS could also rescue the mutant phenotype. Beat is probably required early in LVS development, perhaps before the BOs have separated from the OLs. If Beat facilitates this early developmental step and Beat is a secreted protein, we reasoned that expression of beat by the BO cells may also provide Beat protein at the appropriate time and location to allow normal LVS development. We used Kr-GAL4 (Castelli-Gair et al., 1994) to direct expression of UAS-beat in the BO cells from stage 12 onward. Embryos homozygous for beat<sup>tric</sup> and carrying Kr-GAL4, UAS-beat and gl-lacZ were evaluated by in situ hybridization to beat mRNA and by X-gal staining. In 100% of beat<sup>tric</sup> embryos in which we detected beat mRNA expression in the BOs, the BOs appeared normal (compare Fig. 5C,D and Table 1). Because beat mRNA is not normally detectable in the BOs, those embryos in which we detected beat in the BOs must have carried Kr-GAL4 and UAS-beat.

These results indicate that ectopic expression of beat by cells in the vicinity of normal Beat expression can provide sufficient Beat to restore normal LVS development in beat mutant embryos. This further supports a role for secreted Beat in facilitating separation of the Fas II-expressing BO cells from the OLs.

We conducted similar experiments using GMR-GAL4 to direct UAS-beat expression to the BOs and obtained identical results to those with Kr-GAL4 (not shown). We also determined that expression of beat in the BOs rescues the LVS phenotype of beat<sup>tric</sup>/beat<sup>+</sup> embryos, indicating that the result is not allele specific (not shown). In contrast to the reduced viability we observed in omb-GAL4; UAS-beat embryos, expression of beat in the BOs and other Krüppel-expressing cells has no discernible effect on the embryos other than to allow the LVS to develop properly. Thus, although ectopic expression of beat in some tissues results in detrimental effects, in other tissues ectopic beat expression does not disrupt normal development.

**DISCUSSION**

We have demonstrated that beat and fas II have roles in development of the LVS, revealing overlap in the molecules important for development of motoneurons and sensory organs. In LVS development, beat and fas II appear to be required early, perhaps for the separation of photoreceptor cell bodies from surrounding cells, whereas in motoneuron development, beat and fas II are required in later stages for selective fasciculation and defasciculation of specific subsets of axons (Fambrough and Goodman, 1996).

The beat and fas II mutant phenotypes reveal roles in early LVS development

We observe defects early in LVS development in beat and fas II mutant embryos, suggesting that beat and fas II are required for the initial steps in BO development. Although early steps in BO development have been described (Green et al., 1993), little is known mechanistically about recruitment of photoreceptor cells into the BO clusters or how the BOs become induced to separate from the invaginating OLs. The importance of these two early events in LVS development is reflected in the likelihood that their disruption will affect all subsequent events and prevent establishment of normal LVS morphology. It is possible that these two events are themselves interrelated and that integrity of the BO cluster contributes to regulation of BO cell number. Schmucker and colleagues (1994) proposed that a lateral inhibition mechanism may be involved in establishment of BO cell number, based on data showing that a reduction in functional Patched protein results in excess BO cells with a coincident reduction in the size of the optic lobes. If such a mechanism is involved in restricting BO cell number, disrupting other aspects of intercellular communication could also lead to development of excessive numbers of BO cells. It is unlikely that beat and fas II are directly involved in a lateral inhibition mechanism that regulates cell numbers; however, ample evidence indicates that they can modulate intercellular adhesion. Loss of intercellular adhesion could, in turn, lead to disruption of intercellular signaling. Coordination of inductive and inhibitory signals

![Fig. 4](image-url) Reducing levels of Fas II expression rescues the beat LVS phenotype. Embryos of genotype fas<sup>1P76</sup>; beat<sup>tric</sup>, P[gl-lacZ] stained with anti-β-galactosidase. (A) Stage 13. Early BO development (arrowheads) appears normal. No extra gl-lacZ-expressing cells are present (compare to single mutants, Figs 1E, 3D). (B) Stage 16. BOs (arrowheads) and BNs (arrows) resemble wild type. One BO (asterisk) may be less tightly clustered, a characteristic of a subset of fas<sup>1P76</sup> single mutant embryos. Bar, 50 µm.
could explain regulation of photoreceptor cell number. When separation of the BO from the OL is delayed, more photoreceptor cells may be induced, resulting in the increased number of cells seen in beat mutant embryos. Conversely, premature separation of the BO and OL in fas II mutants may disrupt the inductive signal, resulting in fewer cells and smaller BOs. Defects in timing of BO separation from the OL may also disrupt coordination of LVS development and other morphological events of head involution, resulting in magnification of early BO developmental defects as embryogenesis progresses. Thus, mutations in beat or fas II that compromise the integrity of the BO cell cluster early in development, or that interrupt the timing of BO dissociation from the OL, could account for all of the morphological defects seen in mutant embryos.

**Beat tempers Fas II-mediated adhesion in early LVS development**

Consistent with its homophilic adhesion activity (Grenningloh et al., 1991), loss of function mutations in fas II result in failures in motoneuron fasciculation and disorganization of the CNS, whereas overexpression of fas II leads to inappropriate fasciculation and failure of appropriate branching in the PNS (Lin et al., 1994; Lin and Goodman, 1994). Mutations in beat result in failure of some Fas II-expressing axons to defasciculate at appropriate choice points. Reducing the level of Fas II expression in beat mutants reduces the severity of the branching defects. The effects of beat and fasII mutations on LVS development also reveal complimentary phenotypes. In beat mutants, excess photoreceptor cells develop, whereas in fas II mutants the BOs are reduced in size. In embryos with a mutation in either gene, defects at later stages of LVS development, including a failure to maintain integrity of the BOs, may be a consequence of earlier developmental defects or may reflect roles for beat and fas II throughout LVS development. We propose that in LVS development, Beat and Fas II play roles parallel to those they serve in motoneuron development and that the consequence of normal Beat-Fas II interaction during LVS development is coordinated detachment of the BOs from surrounding cells of the optic lobe placodes.

Fig. 6 illustrates how Beat and Fas II might interact early in LVS development to facilitate BO formation. The proposed interaction is consistent with defects in LVS development that result from mutations in beat and fas II. In wild-type embryos (Fig. 6A), beat is expressed in the optic lobe placodes, and Fas II is expressed in the BOs and optic lobe placodes. Before the BOs become distinct from the OLs, Fas II protein can be detected in the OLs in cells that include precursors of the BOs (Fig. 6AII), suggesting that for the BOs to separate from the OLs, Fas II-mediated intercellular adhesion must be reduced. Secretion of Beat by cells of the OL provides the anti-adhesion activity required to reduce the strength of Fas II adhesion and allow the BOs to detach (Fig. 6AII). Fas II may also be required to maintain the integrity of the BO clusters after they have separated from the optic lobes (Fig. 6AIII,IV).

In embryos homozygous for a loss-of-function mutation in beat (Fig. 6B), Fas II is expressed normally and the absence of Beat prevents efficient separation of the BO cells (Fig. 6BII). As a result, the BO cells may not separate at the correct time or as a single cluster, since they may be equally adherent to each other and other non-BO cells. The consequences of this include dissolution of the BO clusters and to production of additional BO cells due to disruption of a lateral inhibition mechanism or an inductive mechanism that ordinarily defines the number of photoreceptors through short range, intercellular signals (Fig. 6BIII,IV). When Fas II expression is increased in the BOs, a similar situation is seen, despite the presence of normal levels of Beat. An imbalance in the levels of Beat and FasII prevents efficient separation of the BO cells (Fig. 6B). Thus, overexpression of FasII disrupts early stages of BO development effectively in the same way as loss-of function mutations in beat. This further reveals the importance of

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**Fig. 5.** Expressing beat in the OL or the BOs rescues the beat LVS phenotype. (A) Stage-13 P[omb-Gal4]; P[UAS-lacZ] embryo stained with X-gal. Arrow indicates omb-GAL 4-expressing cells in the OL. (B) Stage-16 P[omb-Gal4]; beat[ger]; P[UAS-lacZ]; P[UAS-beat] embryo stained with anti-β-galactosidase. The BOs (arrowheads) and BNs (arrows) appear to have developed normally. (C) Stage-16 P[Kr-GAL4]; beat[erg]; P[UAS-beat]; P[gl-lacZ]; P[UAS-lacZ]; P[UAS-beat] embryo stained with X-gal to detect the BOs and subjected to in situ hybridization with a beat cDNA probe. Only one BO is clearly present in the embryo (double arrowhead) and it is located in an abnormal position. (D) Sibling embryo to that in C. This embryo also carries P[UAS-beat]. The darker staining in the BOs results from the product of X-gal staining and in situ staining, indicating that beat is expressed in the BOs. The LVS appears wild type in these beat[erg] embryos in which beat is expressed in the BOs. Bar, 50 μm.
balancing expression levels of these interacting molecules to properly modulate intercellular adhesion and coordinate BO development.

When \textit{fas II} expression is reduced (Fig. 6C), the strength of association of the BO cells and their neighbors is weakened, allowing the BOs to separate from the optic lobes with a reduced requirement for Beat (Fig. 6CII,III). Early separation of the BOs from the OLs from which photoreceptor cells are recruited may result in too few cells in the BO clusters, perhaps due to premature termination of photoreceptor cell recruitment into the BO cluster. Reduction of Fas II may also compromise the integrity of the BOs and permit some cells to separate from the BO cluster (Fig. 6CIV).

Perturbing the coordinated separation of the BOs from the OLs by altering the relative concentrations of Beat and Fas II may cause disruptions of the LVS that would be reflected later in development. The separation of the BOs occurs shortly before, or coincident with, the onset of head involution. Head involution is characterized by drastic morphogenic movements and results in remodeling of the anterior region of the embryo. Anterior migration of the BOs occurs during head involution and may require synchronization of these concurrent events. Thus, if BO cells separate from the OLs too early, as may occur in \textit{fas II} mutants, or too late, as may occur in \textit{beat} mutants, the nascent BOs may be inappropriately located and may lose the ability to properly coordinate with head involution. In both \textit{beat} and \textit{fas II} mutant embryos, defects in late-stage LVS morphology are detected at a greater frequency than are defects in early development, such as dispersed BO cells. This may reflect a role for \textit{beat} and \textit{fas II} later in development or may indicate that dispersed BO cells are only one consequence of disrupted timing of BO separation, and one that is most apparent early in LVS development, whereas more subtle defects in BO separation are manifested only at later stages.

In double-mutant embryos, simultaneous reduction in levels of \textit{fas II} and \textit{Beat} allows the BOs to form normally. This perhaps surprising result suggests that the relative levels of Beat and Fas II are more important than the absolute level of either protein. That the BOs form normally in the majority of double-mutant embryos indicates that other adhesion molecules are also likely to be important for BO formation and that the absence of both \textit{beat} and \textit{fas II} is less disruptive to development than the absence of either one alone. In \textit{fas II}^\textit{e76}, \textit{beat}^{\textit{tric}} double mutants, subtle defects in BO development are seen that resemble a subset of the defects found in \textit{fas II} single mutants: small BOs or BOs that have not migrated as a cohesive cluster. Together with the fact that Fas II is expressed in the BOs throughout development, this observation may suggest a role for Fas II in later stages of LVS development. Thus, a dynamic titration of adhesion and anti-adhesion molecules is key for multiple stages of normal neuronal development.

\textbf{Multiple roles for Beat-Fas II interaction}

Our results demonstrating that expression of \textit{beat} by cells in either the OL or the BO can restore normal LVS development to \textit{beat} mutants supports the proposal that Beat is a secreted protein acting at an early stage in development when these two groups of cells are still adjacent. These results also suggest that Beat diffuses to its target cells and need not be specifically targeted. Although Fas II is expressed throughout the LVS and OLs, expression of \textit{beat} by cells that do not normally express it does not necessarily disrupt LVS development. Similarly, when \textit{beat} is expressed on all CNS and PNS neurons in \textit{beat} mutants, two adjacent groups of cells that are normally separated by the head involution may come into closer contact, releasing the BOs from their neighbors and allowing them to separate from the OLs.

\textit{Beat} and \textit{Fas II} are both expressed in the LVS during development, and their relative levels appear to be important for the coordinated separation of the BOs from the OLs. The spatial and temporal expression patterns of these proteins suggest that they may act at different stages of development, with \textit{Beat} playing a role in early stages and \textit{Fas II} later on. The dynamic regulation of these molecules is crucial for proper neuronal development, and understanding their interactions may provide insights into the mechanisms underlying normal and abnormal development.
mutants, normal motoneuron development is restored and the CNS develops normally (Fambrough and Goodman, 1996). Together, these experiments reveal that Beat does not disrupt adhesion of all Fas II-expressing cells, but is instead specific for subsets of Fas II-expressing neurons in both the CNS and LVS. One explanation for these results, as suggested by Fambrough and Goodman (1996), is that subsets of Fas II-expressing neurons also express a Beat receptor that renders them sensitive to Beat-induced defasciculation. No Beat receptor has yet been identified in either class of neurons in which Beat is known to be important, nor do such receptors need to be expressed exclusively on Fas II-expressing neurons. Genetic interactions between beat and connectin, which encodes another neuronally expressed homophilic adhesion molecule (Nose et al., 1992) have been demonstrated in motoneuron development (Fambrough and Goodman, 1996), and sidestep has been proposed to encode a potential receptor for Beat (Fambrough and Goodman, 1996). We examined embryos with mutations in connectin and sidestep and saw no defects in LVS development (not shown). Furthermore, a mutation in connectin that reduces the severity of the beat mutant motoneuron defects has no effect on the LVS defects in beat mutants (not shown). Thus, although there is overlap in Beat-mediated modulation of adhesion used to direct motoneuron and LVS development, some interactions are not shared between motoneurons and the LVS. Presumably additional elements of regulation of adhesion may be unique to the LVS. Although not yet identified, candidates for these molecules may be revealed by analysis of recently characterized mutations that also affect LVS development (Schmucker et al., 1997; Holmes et al., 1998).

Modulation of intercellular adhesion provides a mechanism to allow reorganization of neuronal connections during invertebrate and vertebrate development, and NCAM-related molecules have been implicated as mediators of differential adhesion (Rutishauser et al., 1988; Harrelson and Goodman, 1988). Modification of NCAM in chick by addition of polysialic acid (PSA) leads to refinement of motoneuron connections (Rutishauser and Landmesser, 1991), resembling those seen in the fly that result from interaction of Beat with Fas II-expressing axons. The consequences of perturbations in the relative levels of PSA and NCAM in the chick are reminiscent of those resulting from modulating levels of Beat and Fas II in that removal of PSA disrupts normal innervation patterns in avian motoneurons (Tang et al., 1992), and concurrent reduction of NCAM suppresses these effects (Tang et al., 1994). Our results demonstrate parallel roles for Beat and Fas II in sensory organ development and motoneuron development, thus extending the correspondence in NCAM function seen in vertebrates and invertebrates. We observed that Beat and Fas II are required for separation of the BO cell bodies from the OL, in addition to their previously demonstrated roles in guidance of motor axon growth cones.

In vertebrates, modulation of NCAMs affects both growth cone and neuronal cell body migration. In mice, mutations in NCAM cause defects in neuronal migration, including preventing normal migration of the precursor cells of the olfactory bulbs, a sensory organ (Tomasiwicz et al., 1993; Cremer et al., 1994; Ono et al., 1994). Modification of NCAM by PSA, which presumably modifies the avidity of intercellular adhesion, is important for normal migration of the olfactory precursors (Ono et al., 1994). Likewise, Kallmann syndrome, a complex X-linked disorder of humans that includes defects in migration of olfactory neurons and gonadotropin-releasing hormone neurons, results from mutations in a gene whose product resembles NCAM and other cell adhesion molecules (Franco et al., 1991; Legouis et al., 1991; Hardelin et al., 1992). Continued studies of modulation of intercellular adhesion during CNS and LVS development in Drosophila are likely to reveal additional parallels, both mechanistic and molecular, to corresponding processes in vertebrates and provide complementary routes to understanding defective neuronal development.

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