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even-skipped Determines the Dorsal Growth of Motor Axons in *Drosophila*

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Summary

Axon pathfinding and target choice are governed by cell type–specific responses to external cues. Here, we show that in the *Drosophila* embryo, motorneurons with targets in the dorsal muscle field express the homeobox gene *even-skipped* and that this expression is necessary and sufficient to direct motor axons into the dorsal muscle field. Previously, it was shown that motorneurons projecting to ventral targets express the LIM homeobox gene *islet*, which is sufficient to direct axons to the ventral muscle field. Thus, *even-skipped* complements the function of *islet*, and together these two genes constitute a bimodal switch regulating axonal growth and directing motor axons to ventral or to dorsal regions of the muscle field.

Introduction

The analysis of growth cone guidance and targeting has concentrated on a few accessible systems (such as the retinotopic projection of visual axons or the growth and guidance of motor axons in the periphery [for reviews see Nieto, 1996; Tessier-Lavigne and Goodman, 1996]). Such studies have led to the general conclusion that axon pathfinding and target choice are governed by cell-specific responses to external guidance cues. While the landscape of guidance cues that outgrowing axons traverse is increasingly better defined, little is known about the mechanisms that underlie the intrinsic characteristics of neurons that lead to the development of individual patterns of nerve growth.

So far as motorneurons are concerned, it seems likely that their growth properties are specified in a combinatorial fashion that leads to a progressive segregation of motor axons as they explore the muscle field that they will ultimately innervate (Tsuchida et al., 1994; Appel et al., 1995; Chu-LaGraff et al., 1995). The best evidence for such a system of combinatorial specification comes from studies of vertebrate embryos where it has been possible (in the chick and the zebrafish) to establish a correlation between the expression of particular combinations of the LIM homeobox genes *islet-1*, *islet-2*, *Lim-1*, and *Lim-3* and the projection patterns of these neurons in the muscle field (Tsuchida et al., 1994; Appel et al., 1995). LIM homeobox genes encode putative transcriptional activators that presumably regulate the expression of downstream genes that control the differentiation of neuronal properties. However, loss of function studies in vertebrates have so far failed to uncover a role for LIM homeobox genes in regulating motorneuron choice of axon pathways and target areas, as those genes tested turn out to be essential for motorneuron formation and embryonic viability (Shawlot and Behringer, 1995; Pfaff et al., 1996; Sheng et al., 1996).

The *Drosophila* embryo is a good model system with which to study the diversification of neurons that lies behind these individual patterns of growth. In each abdominal half segment (A_2–A_7), 30 muscles form and are specifically and invariantly innervated by 30–40 motorneurons (Bate, 1990; Sink and Whitington, 1991; Landgraf et al., 1997). Motor axons are channeled into one or the other of two principle nerve trunks in each hemisegment, the segmental (SN) and the intersegmental nerve (ISN; Thomas et al., 1984; Jacobs and Goodman, 1989b). From these nerves, secondary branches diverge at specific points in the periphery, each innervating a discrete muscle set (Johansen et al., 1989; Van Vactor et al., 1993). The formation of the SN and ISN is independent of targets in the periphery, although secondary nerve branches only form in response to the presence of target muscles (Landgraf, 1996; Prokop et al., 1996). Thus, motorneurons appear to have intrinsically determined patterns of growth, which deliver their axons into one of the two main nerve trunks and facilitate the recognition of a specific set of target muscles.

This simple pattern of growth provides us with the means to make a genetic and experimental analysis of the factors operating within motorneurons to distribute their axons in the muscle field. Here, we have focused on one part of the nerve pattern, namely the ISN, which delivers motor axons to dorsal and ventral sectors of the muscle field (Landgraf et al., 1997). The axons of motorneurons that innervate ventral muscles defasciculate at the ISNb/d nerve branch, while those innervating dorsal muscles remain fasciculated along the ISN path. We find that motorneurons with dorsal destinations express the homeobox gene *even-skipped* (*eve*). Furthermore, we show that loss of *eve* leads to a truncation of ISN, so that it no longer reaches the dorsal regions of the muscle field, while ectopic *eve* expresssion in all motorneurons diverts their axons into the ISN and to dorsal sectors of the field. This function for *eve* in directing dorsal growth of motor axons complements the previously described function of *islet* (Thor and Thomas, 1997) in directing motor axons to ventral muscles in the *Drosophila* embryo. The complementary roles and expression patterns of *eve* and *islet* provide us with a

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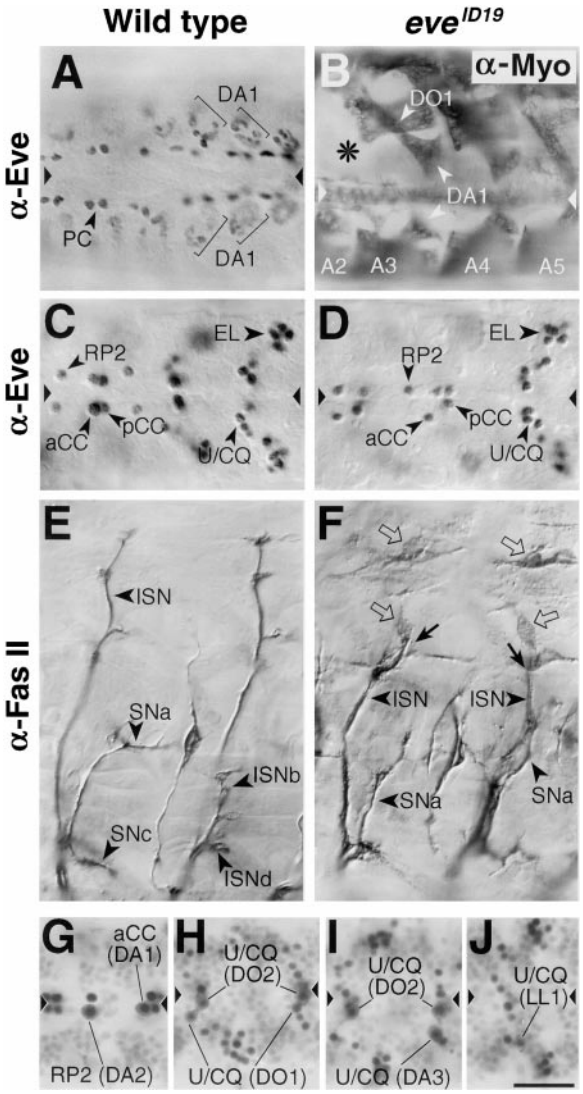

Figure 1. Loss of Eve Function in Muscles and Neurons

(A, C, and E) Wild-type and (B, D, and F) *eve*^{ID19} mutant embryos where Eve function was removed at 5 hr AEL; specimens are late stage 16 (B–E and I) or stage 15 (A).

(A) anti-Eve staining of a stage 15 wild-type embryo. Eve labels nuclei of a subset of pericardial cells (PC) and muscle DA1 [1] (brackets) either side of the dorsal midline (triangles).

(B) Anti-Myosin staining in *eve*^{ID19} embryos reveals that muscle DA1 [1] is usually formed in abdominal segments A_3–A_5. Segments A_2 and A_6 frequently show muscle defects (asterisk) even at the permissive temperature and are excluded from analyses (see Experimental Procedures). Triangles denote the dorsal midline (heart).

(C and D) Eve-expressing neurons in the ventral nerve cord of late stage 16 wild-type (C) and *eve*^{ID19} mutant (D) embryos. In this *eve*^{ID19} mutant embryo, all Eve-expressing motorneurons have formed with the exception of the central segment where one RP2 neuron is missing and one aCC cell is misplaced. Triangles indicate the ventral midline.

(E and F) Peripheral motor projections visualized with anti-Fas II in late stage 16 wild-type (E) and *eve*^{ID19} mutant (F) embryos where the ISN terminates prematurely at the level of the lateral connective (arrows). Open arrows point to adult muscle precursor cells, which in the wild type would no longer express high levels of Fas II at this stage.

(G–J) The motorneurons (aCC, RP2, and U/CQ neurons) that innervate dorsal muscles DA1–3 [1–3], DO1–2 [9 and 10], and LL1 [4] in wild-type abdominal segments were retrogradely labeled with DiI (brown/lighter plasma membrane stain). Double staining with anti-Eve antibody (purple/darker nuclear stain) shows that these muscles are innervated by Eve-expressing motorneurons. All other Eve-positive neurons (pCC, fpCC, and EL neurons) are interneurons (Bossing et al., 1996; Schmidt et al., 1997). Morphologically, the CQ neurons correspond to the U neurons (see text). The ventral midline is indicated by triangles. Dorsal is up and anterior is left.

Scale bar: (A)–(F) = 20 μm; (G)–(J) = 15 μm.

model system with which to investigate the way in which such transcription factors act to determine patterns of nerve growth.

Results

The Dorsal ISN Is a Common Path for Eve-Positive Motorneurons

After segmentation is complete, Eve expression in the wild-type embryo is very restricted. In the mesoderm, Eve is expressed in a subset of pericardial cells and muscle DA1 [1] (Figure 1A; Frasch et al., 1987; Bate, 1993). In the CNS, Eve is expressed in approximately 16 cells per abdominal hemineuromere: medially, the pCC and fpCC interneurons and the aCC and RP2 motorneurons; mediolaterally, four CQ neurons; and laterally, the eight to ten EL interneurons (Figure 1C; Patel et al., 1989; Bossing et al., 1996; Schmidt et al., 1997). Of the Eve-expressing motorneurons, aCC and RP2 innervate dorsal muscles DA1 [1] and DA2 [2], respectively (Sink and Whitington, 1991), and the four CQ neurons are thought to project to other dorsal muscles (Doe, 1992; Goodman and Doe, 1993; Bossing et al., 1996).

The fact that Eve is expressed in several dorsally projecting motorneurons and that aCC and RP2 have been shown to require Eve function to project to their dorsal targets (Doe et al., 1988b) suggests that Eve expression might be a common property of motorneurons that project through the ISN into the dorsal muscle field.

To test this idea, we established the pattern of innervation in late stage 16 embryos by retrogradely labeling motorneurons with DiI (Landgraf et al., 1997) and subsequently double labeling with anti-Eve antibody. We find that six motorneurons express Eve, and these innervate exclusively dorsal to dorsolateral muscles. As previously shown, aCC and RP2 innervate muscles DA1 [1] and DA2 [2], respectively (Sink and Whitington, 1991; Bossing et al., 1996). In addition, muscles DO1–2 [9 and 10], DA3 [3], and LL1 [4] are innervated by the four ventral mediolateral CQ neurons (Figures 1G–1J; Patel et al., 1989; Doe, 1992). We find that the CQ neurons correspond to the independently identified so-called U neurons by position, morphology, and muscle target (Sink and Whitington, 1991; Landgraf et al., 1997). While Eve-expressing motorneurons innervate exclusively the most dorsal and two dorsolateral targets, there is also an array of four dorsolateral muscles, DO3–5 [11, 19, and 20] and DT1 [18], that are innervated by non-Eve-expressing motorneurons (Landgraf et al., 1997).

Eve Is Necessary for Motor Axon Projections into the Dorsal ISN

To investigate the requirement for Eve for the formation and projection patterns of the Eve-expressing neurons,

we used the temperature-sensitive *eve*^{lD19} allele to remove Eve protein function at different times of development. Eve is expressed at 5 hr after egg laying (AEL) in the ganglion mother cells that give rise to the Eve-positive neurons (Broadus et al., 1995; Weigmann and Lehner, 1995), of which aCC and pCC are the first born at 6 hr AEL (Doe et al., 1988a). At 9.45 to 10 hr AEL, aCC generates an axon that pioneers the ISN, closely followed by the axons of the U/CQ and RP2 neurons (Jacobs and Goodman, 1989b).

When Eve function is removed from 5 hr AEL onward, most Eve-expressing neurons form (85% of RP2; 88% of aCC; 95% of U/CQs; 84% of ELs; n = 60 hemisegments; Figures 1C and 1D; see also Doe et al., 1988b), suggesting that Eve function at this stage is not essential for the generation of the Eve-positive neurons. However, the dorsal projections of motor axons in these embryos are always abnormal. The ISN, through which aCC, RP2, and the U/CQ axons project, is arrested prematurely in the ventral or dorsolateral region of the muscle field, leaving dorsal muscles without innervation (100%; n = 108; Figures 1E and 1F). Removing Eve function at progressively later times, we find that the occurrence of dorsolateral ISN truncation is increasingly less frequent. By 9–10 hr AEL, that is as the axons of aCC, RP2, and the U/CQ neurons exit the CNS (Jacobs and Goodman, 1989a; Lin et al., 1995), removal of Eve function rarely affects formation of the ISN. In such embryos, the dorsal-most muscles are reliably contacted by ISN axons at late stage 16 (14.5 hr AEL) in 93% of all segments (n = 108; data not shown). Furthermore, staining such embryos with anti-Synaptotagmin (Littleton et al., 1993) suggests that by late stage 17 (24 hr AEL) these contacts have formed normal synapses despite the lack of Eve function in motorneurons or muscle DA1 [1] (n = 70 hemisegments; data not shown). In our experiments, dorsal muscles, including the single Eve-expressing muscle DA1 [1], form normally (Figure 1B). Moreover, neither removal of all dorsal targets nor ectopic expression of Eve in ventral and lateral muscles affects the projection of the ISN into the dorsal muscle field (data not shown).

We conclude that Eve function is required in motorneurons (but not their targets) prior to or during the early phase of ISN formation if their axons are to grow dorsally.

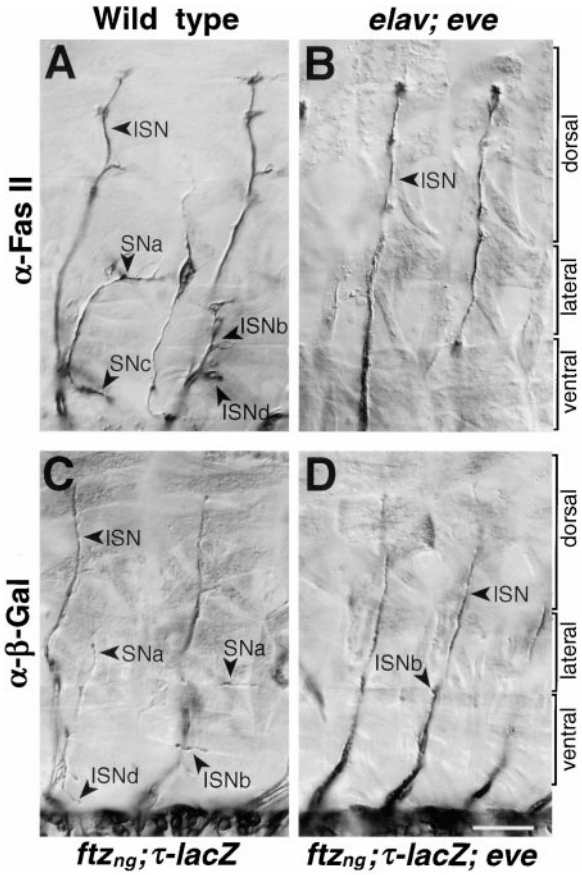

Figure 2. Eve Is Sufficient to Direct Motor Axons via the ISN to Dorsal Muscles

Peripheral motor projections visualized with anti-Fas II (all motor axons) of late stage 16 wild-type (A) and *elav*-GAL4; UAS-*eve* (B) embryos.

(A and B) All peripheral nerve branches fuse with the ISN in *elav*-GAL4; UAS-*eve* embryos (B) and project to dorsal muscles, leaving ventral muscles without innervation.

(C and D) Late stage 16 *ftz*$_{ng}$-GAL4; UAS-*tau-lacZ* (C) and *ftz*$_{ng}$-GAL4; UAS-*eve*; UAS-*tau-lacZ* (D) embryos stained with anti-β-Gal to reveal the projections of motorneurons that ectopically express Eve under control of the *ftz*$_{ng}$-GAL4 driver.

(C) *ftz*$_{ng}$-GAL4 is expressed in motorneurons of all peripheral nerve branches.

(D) When these ectopically express Eve, their axons, including those of SNa and SNc (not in this focal plane) are rerouted to the dorsal ISN, leaving ventral muscles without innervation. In the central segment, the fusion is incomplete and ISNb can be seen running alongside the ISN. Anterior is left and dorsal is up.

Scale bar: 20 μm.

Eve Is Sufficient to Direct Motor Axons Into the ISN Path toward Dorsal Muscles

To test the idea that Eve functions to direct motorneuron axons to the dorsal sector of the muscle field, we used neural GAL4 drivers to express Eve ectopically, either in all neurons (using the driver line *elav*-GAL4; Figures 2A and 2B; Lin and Goodman, 1994) or in a small subset that consists mostly of motorneurons (using *ftz*$_{ng}$-GAL4 as the driver line; Figures 2C and 2D; Lin and Goodman, 1994). Both driver lines express high levels of ectopic Eve in the CNS from at least stage 12 (7.5 hr AEL) until late stage 16 (data not shown). The projections of motorneurons in these embryos are dramatically and consistently altered: all peripheral nerve branches (SN and ISN) are now fused and follow the ISN path as revealed by anti-Fas II (Figures 2A and 2B) and anti-Connectin stainings (data not shown). The penetrance of this phenotype is dose-dependent: at 29°C ISN and SN always fuse before or immediately after exiting the CNS (100%, n = 100), but when using weaker UAS-*eve* responders or temperatures lower than 29°C to reduce GAL4 activity (Brand et al., 1995), the fusion of nerve branches is less frequent and less complete. We coexpressed UAS-*tau-lacZ* and stained *ftz*$_{ng}$-GAL4; UAS-*eve*; UAS-*tau-lacZ* embryos with anti-β-galactosidase and thus confirmed that the axons of motorneurons that express Eve fuse with the ISN and project into dorsal regions of the muscle

field (Figures 2C and 2D). Unlike the situation with motorneurons, ectopic Eve expression does not redirect the axons of interneurons into the muscle field (tested with other Gal4 drivers; not shown). This presumably reflects the fact that in the wild type sets of interneurons (pCC, fpCC, and the EL neurons) express Eve and that their axons remain within the CNS (Doe et al., 1988b; Bossing et al., 1996; Schmidt et al., 1997). Thus, misexpression of Eve in motorneurons, which do not normally express it, is sufficient to alter dramatically their patterns of pathfinding and fasciculation so as to direct their axons via the ISN to the dorsal muscle field.

To assay the consequences of ectopic Eve for the formation and projections of motoneurons that normally do not express Eve, we used anti-Connectin and anti-Fas III to identify subsets of such cells. In wild-type embryos, Fas III is expressed by the three medial RP1, 3, and 4 motorneurons whose axons exit the CNS via the anterior root of the ISN (Halpern et al., 1991). In *elav*-GAL4; UAS-*eve* embryos, 91% of the Fas III positive RP motorneurons are formed, and their axons exit the CNS via the anterior part of the fused ISN/SN (n = 192 hemisegments; not shown). In contrast, Connectin labels almost exclusively those motorneurons that project via the SN in wild type. In *elav*-GAL4; UAS-*eve* embryos, Connectin-positive neurons form, but their axons exit the CNS via the posterior part of the fused ISN/SN (data not shown). Thus, ectopic Eve only weakly interferes with the formation of these motorneurons but leads to a drastic rerouting of SN axons into the ISN.

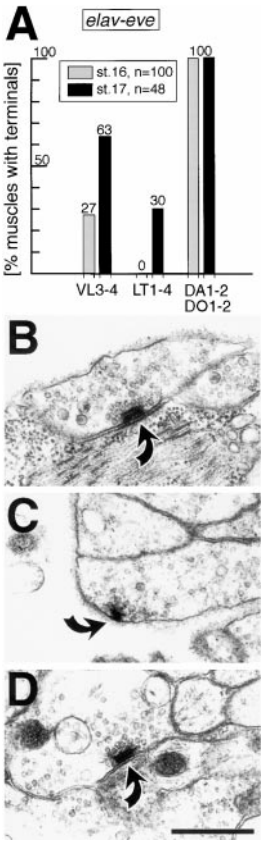

Figure 3. Synaptic Release Sites Form when Eve Is Ectopically Expressed in the CNS

(A) Innervation of ventral and lateral muscles by misrouted motor axons is delayed and occurs largely during stage 17 (n = 48 for VL3–4 [6 and 7], n = 81 for LT1–4 [21–24], n = 48 for DA1–2 [1 and 2], DO1–2 [9 and 10]).

(B–D) Examples of different types of release sites (arrows) found in late stage 17 *elav*-GAL4; UAS-*eve* embryos: (B) neuromuscular, (C) neurohemal, and (D) neuroneuronal release site.

Scale bar: 700 nm.

Neuromuscular Junctions Form on Ventral and Lateral Muscles when Eve Is Misexpressed in the Nervous System

Because ectopic Eve expression causes a dramatic rerouting of those motor axons that would normally innervate ventral or lateral muscles, the development of motorneuron projections onto ventral and lateral targets is disrupted. At late stage 16, only 27% of muscles VL3–4 [6 and 7] and 0% of muscles LT1–4 [21–24] (wild type = 99%; n = 100) receive innervation. However, there is ample evidence that in the *Drosophila* embryo innervation can take place despite misrouting of motor axons, although often with a delay (Nose et al., 1994; Desai et al., 1996; Krueger et al., 1996; Nose et al., 1997; Rhaghavan and White, 1997).

To determine whether these uninnervated muscles receive innervation at later stages, we stained late stage 17 (24 hr AEL) *elav*-GAL4; UAS-*eve* embryos with anti-Synaptotagmin to visualize sites of presynaptic differentiation (data not shown; Littleton et al., 1993). The frequency of neuromuscular junctions (NMJs) on ventral and lateral muscles assayed in this fashion increases dramatically during the time from late stage 16 to late stage 17 (from 27% to 63% for VL3–4, [6 and 7] and from 0% to 30% for LT1–4 [21–24]; n = 48 and n = 81 hemisegments, respectively; Figure 3A). While morphologically normal NMJs form on muscles DA1–2 [1 and 2] and DO1–2 [9 and 10], the delayed ventral and lateral NMJs are often located at abnormal positions on the muscles (data not shown).

Because it is known that presynaptic release sites can form in the absence of muscle contact (Prokop et al., 1996), we investigated the ultrastructure of the junctions identified by anti-Synaptotagmin staining. We were able to confirm that NMJs formed in the lateral or ventral muscle field in late stage 17 *elav*-GAL4;UAS-*eve* embryos have all the characteristic features of functional sites of release (Figures 3B). When analyzed region specifically, the fraction of neuromuscular synapses (as opposed to neural release sites into the hemolymph or onto other neurons or glia cells [Figures 3C and 3D]) is much higher in dorsal (80%, n = 65) and ventral (79%, n = 33) areas but strongly reduced laterally (25%, n = 135). Thus overall, NMJs are formed on ventral and lateral muscles, though with a delay, despite ectopic Eve and concomitant axon misrouting.

Eve Regulates Pathfinding but Not Target Recognition Properties in Motorneurons

To distinguish between random opportunistic innervation of uninnervated muscles and correct but delayed innervation by appropriate motorneurons in *elav*-GAL4;

UAS-*eve* embryos, we used Connectin and Fas III as markers for subsets of motorneurons and their target muscles that normally do not express Eve. In the wild type, Connectin-positive muscles are exclusively innervated by Connectin-expressing motorneurons (Nose et al., 1992; Meadows et al., 1994). In *elav*-GAL4; UAS-*eve* embryos, neuromuscular contacts onto Connectin-expressing muscles form after late stage 16 and in late stage 17 *elav*-GAL4; UAS-*eve* embryos, the Connectin-positive motoraxons almost exclusively contact their normal set of target muscles. Nontarget muscles such as muscles VL1–4 [12, 13, 6, and 7] are never contacted by Connectin-positive axons at stage 16 (n = 96) and only rarely contacted at late stage 17 (3%, n = 101 hemisegments; data not shown). Fas III is expressed by the RP3 motorneuron, which normally innervates the ventral longitudinal muscles VL3–4 [6 and 7] (Halpern et al., 1991). In late stage 16 *elav*-GAL4; UAS-*eve* embryos 27% of muscles VL3–4 [6 and 7] receive motorneuronal contacts, of which 85% are Fas III-positive (n = 100; data not shown).

To be more specific, we used DiI retrograde labeling to identify the type of motorneuron innervating different muscles in *elav*-GAL4; UAS-*eve* embryos (Landgraf et al., 1997). Although the CNS is grossly disrupted and the dendritic arbors of motorneurons are deranged in these embryos, neurons can still be identified by their morphology, size, and position of cell bodies. As expected, in late stage 16 *elav*-GAL4; UAS-*eve* embryos, muscles DA1–2 [1 and 2] and DO1–2 [9 and 10] are properly innervated by aCC, RP2, and the U/CQ neurons (Figures 4A and 4B). When assaying the innervation of muscles VL3–4 [6 and 7] in late stage 17 *elav*-GAL4; UAS-*eve* embryos, we find that of those muscles VL3–4 [6 and 7], which do receive innervation, 85% are innervated by "RP-like" motorneurons (large cell bodies at a dorsal and medial position; n = 20; Figures 4C and 4D). Strikingly, in most instances (82%; n = 20), the "RP-like" motorneuron first projects its axon into the lateral to dorsal ISN. An axonal side branch then comes off the dorsally projecting one, reaching back ventrally to contact its normal muscles VL3–4 [6 and 7] (Figure 4D).

Thus, while Eve expression dictates the growth properties of motorneurons so that they direct their axons via the ISN into the dorsal muscle field, the properties that are required for target recognition appear to be unaffected by ectopic Eve.

Eve Suppresses Islet Expression in Motorneurons

Thor and Thomas (1997) have recently shown that the LIM homeobox gene *islet* is sufficient to direct motor axons via the ventral branch of the ISN (ISNb/d) into the ventral muscle field. The implications of our findings are that together *eve* and *islet* might constitute a bimodal switch that directs motor axon growth either to ventral (*islet*) or dorsal (*eve*) regions of the muscle field. One prediction of such an interpretation would be that the expression patterns of these two genes in motorneurons are mutually exclusive. In the wild type, this is the case (Thor and Thomas, 1997; this paper). Moreover, while the expression pattern of Eve remains unchanged when Islet is either absent or ectopically expressed (data not shown; Thor and Thomas, 1997), we find that ectopic Eve expression throughout the CNS suppresses Islet expression in most motorneurons. In the wild type, Islet is expressed medially in the dorsal RP1, 3, and 4 neurons (Figure 5A) and one ventral VUM motorneuron (Figure 5B), and laterally, in approximately four to five motorneurons (Thor and Thomas, 1997). In stage 16 *elav*-GAL4; UAS-*eve* embryos, the Islet expression pattern is markedly reduced: medially, Islet expression is consistently lost from the VUM (Figure 5D) and from two of the three RP motorneurons (Figure 5C), and laterally, from a further four to six cells (n = 182). However, the Islet expression pattern does not expand when Eve function is removed (data not shown).

Thus, together *islet* and *eve* constitute a bimodal switch that governs patterns of motor axon growth: while *islet* directs axons to the ventral muscle field, *eve* antagonizes *islet* by suppressing its expression and by directing axons into the dorsal sector of the muscle field.

Eve Regulates Adhesive Properties of Motorneurons

How do transcriptional regulators such as *eve* and *islet* direct patterns of axonal growth? The phenotype that we observe in *elav*-GAL4; UAS-*eve* embryos (fusion of the main nerve trunks and failure of secondary nerve branching) is similar to, though more severe than, phenotypes produced in embryos where general interaxonal adhesion is increased either by overexpression of the homophilic CAM Fas II (Lin and Goodman, 1994) or by removal of its antagonist *beaten path* (*beat*; Fambrough and Goodman, 1996). In such embryos, the two main nerve trunks (SN and ISN) form, but secondary nerves fail to branch off. First, we tested if ectopic Eve increased interaxonal adhesion by downregulating the antiadhesive neural CAM antagonist *beat*. We do not detect any significant changes in the overall pattern or relative levels of *beat* mRNA expression in *elav*-GAL4; UAS-*eve* embryos (Figures 5E and 5F). Next, we analyzed the expression patterns of the major neural CAMs Fas II, Fas III, and Connectin in *elav*-GAL4; UAS-*eve* or eve^{ID19} mutant embryos, but we were unable to detect any changes in their expression patterns in our experimental embryos. To test if Eve might regulate the expression of another (as yet unidentified) neural CAM, we reasoned that *beat* might antagonize interaxonal adhesion mediated by such a CAM, just as *beat* antagonizes adhesion mediated by Fas II and Connectin (Fambrough and Goodman, 1996). We find that when we ectopically coexpress Eve and *beat*, the *elav*-GAL4; UAS-*eve* phenotype of excessive axonal fasciculation is partially rescued (Figures 5G and 5H). Specifically, the complete fusion of ISN and SN nerves is reduced (at the level of the CNS exit points from 63% to 56% and in the periphery from 86% to 55%, n = 252). Furthermore, the SNa never forms in late stage 16 *elav*-GAL4; UAS-*eve* embryos, but forms in 16% of hemisegments when *beat* is coexpressed (Figures 5G and 5H; n = 252).

Thus, Eve directs motor axons to the dorsal region of the muscle field by suppressing expression of the ventrally directing *islet* gene and by promoting adhesion to the ISN.

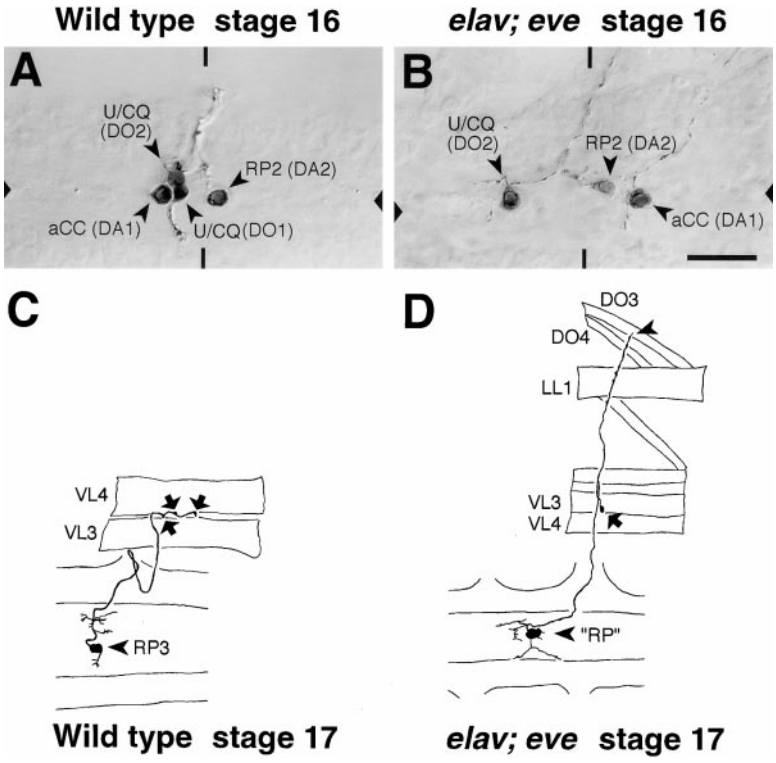

Figure 4. Eve Is Not Involved in the Specification of Target Muscles

(A and B) Photoconverted preparations of DiI retrograde labelings of late stage 16 wild-type (A) and *elav*-GAL4; UAS-*eve* (B) embryos. (A) Wild type aCC, RP2, and the two medial U/CQ neurons that innervate the most dorsal muscles DA1–2 [1 and 2] and DO1–2 [9 and 10]. (B) In *elav*-GAL4; UAS-*eve* embryos, the most dorsal muscles are correctly innervated: the anterior hemisegment shows RP2 and a U/CQ neuron (innervating muscles DA2 [2] and DO2 [10], respectively), the next posterior hemisegment shows aCC, which innervates muscle DA1 [1]. Triangles indicate the ventral midline, and segment borders are demarcated by vertical bars. Although the morphology of the motorneurons in *elav*-GAL4; UAS-*eve* embryos differs from wild type, they are still characteristically identifiable.

(C and D) Tracings of late stage 17 wild-type (C) and *elav*-GAL4; UAS-*eve* (D) embryos where the motoneurons innervating muscles VL3–4 [6 and 7] were retrogradely labeled. In the wild type, muscles VL3–4 [6 and 7] are innervated in the cleft between the two muscles by the RP3 motorneuron. The RP3 cell body lies medially and dorsally, and its axon projects contralaterally. The broad arrows point to varicosities (putative synaptic release sites) on muscles VL3–4 [6 and 7]. (D) *elav*-GAL4; UAS-*eve* embryo where an RP-like neuron was retrogradely labeled from the cleft between muscles VL3–4 [6 and 7]. In the periphery, this RP-like motorneuron projects into the dorsal ISN and terminates within the ISN near muscles DO3–4 [11 and 19] (arrowhead). No obvious varicosities have formed dorsally. A second axonal branch has subsequently formed, which reaches back onto muscles VL3–4 [6 and 7] forming at least one varicosity in the cleft between the muscles (broad arrows). Anterior is left and dorsal is up.

Scale bar: (A) and (B) = 15 μm; (C) and (D) = 20 μm.

Discussion

We have used the neuromuscular system of the *Drosophila* embryo as a model system in which to study the diversification of neurons that lies behind individual patterns of axonal growth. Specifically, we have focused on one of the main nerve trunks, the intersegmental nerve (ISN). ISN motor axons that innervate ventral targets defasciculate ventrally at the ISNb/d branch, while axons targeted to dorsal and dorsolateral regions of the muscle field remain fasciculated with the main nerve trunk (Landgraf et al., 1997).

eve Regulates Motorneuronal Pathfinding but Not Target Recognition Properties

Previous work in *Drosophila* has shown that two of the dorsally projecting motorneurons express the homeobox gene *eve* and that *eve* is required for the normal projections of these neurons (Doe et al., 1988b). We have reexamined the expression of *eve* in the *Drosophila* neuromuscular system and shown that *eve* selectively labels all motorneurons that project to the most dorsal muscles. Furthermore, we have demonstrated that *eve* is necessary and sufficient to direct motor axons via the ISN into the dorsal muscle field. While *eve* directs motor axons via the ISN into the dorsal sector of the muscle field, the *Drosophila* homolog of vertebrate LIM homeobox genes *islet-1* and *-2* has recently been shown to channel axons via the ISNb/d branch into the ventral region of the muscle field (Thor and Thomas, 1997). Thus, together *eve* and *islet* specify dorsal and ventral destinations in the ISN, and dorsal growth does not appear to be simply a default pathway from which *islet* diverts axons into the ventral muscle field (Figure 6). Such a model where *islet* and *eve* act as antagonists is supported by the fact that their patterns of expression are nonoverlapping in the wild type and that ectopic *eve* suppresses *islet* expression in motorneurons.

We have demonstrated that, while *eve* regulates patterns of axonal growth in motorneurons, it does not interfere with mechanisms underlying the recognition and innervation of their muscle targets. Therefore, axon pathfinding and target recognition appear to be distinct processes that are regulated separately. Although not proven, the published data on *Drosophila islet* do not rule out the possibility that *islet* may similarly regulate axonal pathfinding but not target recognition.

eve Directs Axonal Growth by Regulating Interaxonal Adhesion

We have shown that *eve* directs motor axons into the dorsal muscle field by promoting adhesion to the ISN and that this can be antagonized in *elav*-GAL4; UAS-*eve* embryos by coexpressing the antiadhesive Beat protein (Fambrough and Goodman, 1996). The phenotype that we observe in *elav*-GAL4; UAS-*eve* embryos (fusion of the main nerve trunks and failure of secondary nerve branching) might also be caused by ectopic *eve*

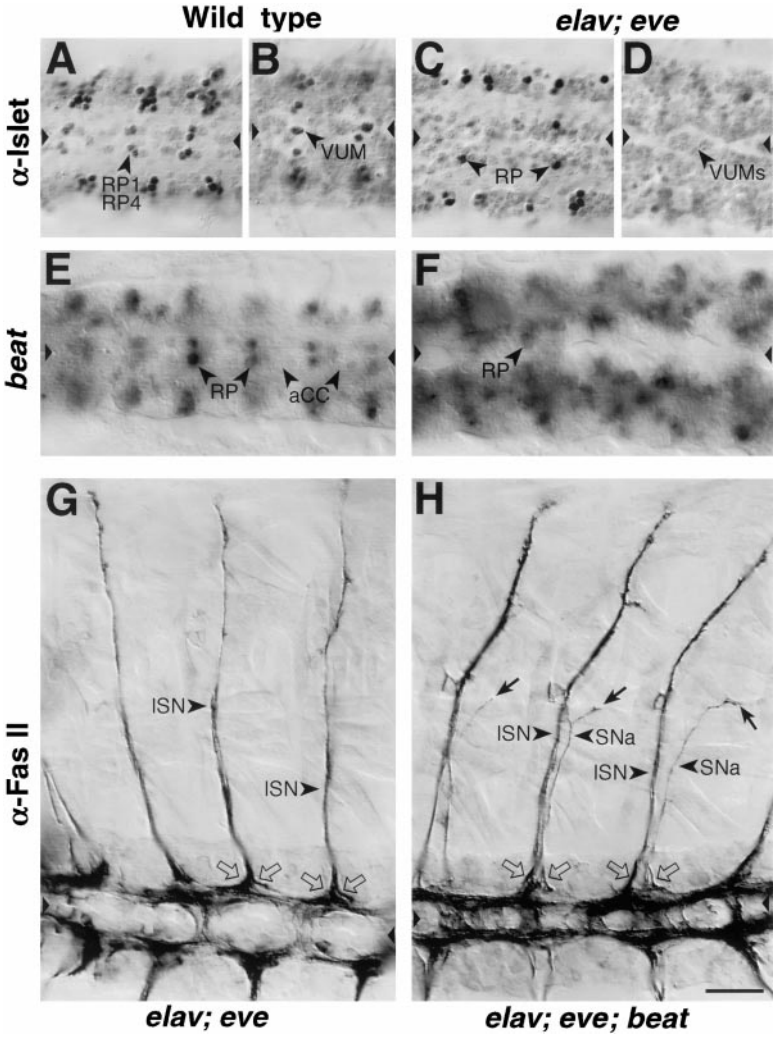

Figure 5. Eve Suppresses Islet and Regulates Axonal Adhesion

Islet expression pattern in the CNS of a late stage 16 wild type (A and B) and *elav*-GAL4; UAS-*eve* (C and D) embryo.

(A and C) Dorsal view: in the wild type (A), Islet is expressed in the RP1, 3, and 4 motorneurons medially; in *elav*-GAL4; UAS-*eve* embryos (C) Islet expression is reduced to a single dorsal and medial RP-like cell per hemisegment.

(B and D) Ventral view: in the wild type (B) Islet is expressed in a VUM motorneuron, but no Islet expression can be detected in the VUMs of *elav*-GAL4; UAS-*eve* embryos (D).

(E and F) *beat* in situ of early stage 16 wild type (E) and *elav*-GAL4; UAS-*eve* (F) CNS. (F) Ectopic Eve does not downregulate *beat* expression. The relative levels of *beat* (high levels in RP neurons and low levels in aCC) are unchanged in *elav*-GAL4; UAS-*eve* embryos (F).

(G and H) Anti-Fas II stainings of late stage 16 *elav*-GAL4; UAS-*eve* (G) and *elav*-GAL4; UAS-*eve*; UAS-*beat* (H) embryos. Coexpression of *beat* (H) partially rescues the phenotype of excessive fasciculation induced by ectopic Eve expression, decreasing the extent and frequency of ISN/SN fusion at the level of the nerve roots (open arrows) and facilitating defasciculation of SNa from the ISN (arrows indicate the distal end of SNa on its target muscles). Throughout, anterior is left and dorsal is up. The ventral midline is indicated by triangles.

Scale bar: (A)–(F), (I), and (J) = 20 μm; (G) and (H) = 50 μm.

interfering with peripheral signals for defasciculation. However, we think this unlikely as mutations in any one of several gene products that have been implicated as receptors for peripheral defasciculation cues (the receptor protein tyrosine phosphatases Dlar, DPTP69A, and DPTP99A [Desai et al., 1996; Krueger et al., 1996]) result only in minor defasciculation defects. In contrast, neuronal overexpression of a single CAM (Fas II) is sufficient to antagonize all secondary nerve branching (Lin and Goodman, 1994) in a similar fashion to what we observe in *elav*-GAL4; UAS-*eve* embryos. Furthermore, depending on the dose of ectopic Eve, ISN fusion with the SN can occur anywhere from within the neuropile to outside the CNS. Were *eve* to regulate signaling events that determine axonal pathway choice, then one would not expect this gradation of ISN/SN fusion phenotypes.

Interestingly, *eve* and *islet*, as well as the LIM homeobox gene *apterous*, are also expressed in subsets of interneurons (Lundgren et al., 1995; Schmidt et al., 1997; Thor and Thomas, 1997). Each of these subsets projects along a distinct common path, and for those expressing *islet* and *apterous*, the genes are not only required for the correct axonal projections but also for their fasciculation (Lundgren et al., 1995; Thor and Thomas, 1997). Thus, the adhesive properties of neurons, regulated by genes such as *eve* and *islet*, are likely to be central to their projection patterns and to the functional architecture of the CNS.

Evolution of the Neuromuscular System

There is an interesting correlation to be made between the expression of *islet* homologs in vertebrate and invertebrate motorneurons. However, while all vertebrate motorneurons express *islet-1* and/or *islet-2* (Tsuchida et al., 1994), only a subset of motorneurons express *islet* in *Drosophila* (Thor and Thomas, 1997). As we show here, another subset expresses *eve*, and there may well be further subsets expressing other genes that direct axons to different parts of the muscle field. For instance, the dorsolateral muscles DO3–5 [11, 19, and 20] and DT1 [18] are innervated by at least four intersegmental motorneurons that express neither *eve* nor *islet* (Figure 6; Landgraf et al., 1997; Thor and Thomas, 1997). Thus, there may be a third gene that defines the dorsolateral sector of the muscle field as the target area of these motorneurons. Interestingly though, we find that the axonal projections of the DO3–5 [11, 19, and 20] and DT1 [18] motorneurons are frequently affected by loss of Eve

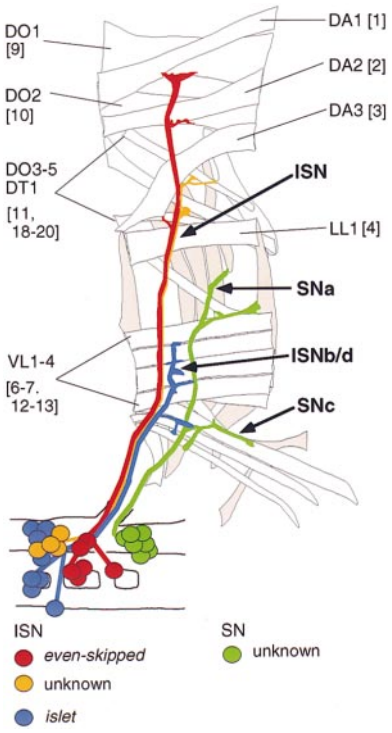

Figure 6. Genes that Govern Motorneuron Projections and Target Areas

Diagram of the body wall muscles (internal view) and their innervation of a late stage 16 abdominal half segment. The main nerve trunks are the ISN (red) and the SN (green). ISNb/d (blue) is the ventral branch of the ISN. Muscles innervated by the SN are shaded. *islet*-expressing motorneurons (blue) project via the ISNb/d to the ventral region of the muscle field. *eve*-expressing motorneurons (red) project via the ISN to the most dorsal (DA1–2 [1 and 2], DO1-2 [9 and 10]) and two dorsolateral muscles (DA3 [3] and LL1 [4]). A third set of intersegmental motorneurons (orange) projects to dorsolateral muscles DO3–5 [11, 19, and 20] and DT1 [18] but expresses neither *islet* nor *eve*. Motorneurons, which project through the SN (green), express neither *islet* nor *eve*, and it is likely that, for the SN motorneurons, as yet unknown genes might direct their axons either to the ventral or the lateral set of target muscles. Dorsal is up and anterior is left.

function. This suggests that these motorneurons rely on the axons of the Eve-expressing cells for pathfinding. In addition, motorneurons whose axons project through the SN express neither *eve* nor *islet*, and their growth patterns are likely to be regulated by other genes (Figure 6; Thor and Thomas, 1997).

Thor et al. (1997) have speculated that vertebrate motorneurons and their targets have all arisen from an original *islet*-expressing module, which in *Drosophila*, is still represented by the motorneurons innervating ventral muscles. Since many *Drosophila* motorneurons, including those that express *eve*, fall outside this *islet*-expressing unit, it seems likely that the insects at least have arisen from an ancestral form, with a much more diverse set of muscles and motorneurons. The muscle targets of the *eve*-expressing motorneurons in flies develop from a dorsal domain of the mesoderm that expresses the homeobox gene *tinman*, and this region also gives rise to the heart (Azpiazu and Frasch, 1993). In vertebrates, the *tinman*-expressing domain of the mesoderm also produces the heart, suggesting that there has been some conservation of the machinery that underlies the diversification of the mesoderm (for review see Bodmer and Venkatesh, 1998). However, this *tinman*-expressing region of the vertebrate mesoderm does not give rise to somatic muscles (Romer and Parsons, 1989), and it may be that it is this divergence in the fates of mesodermal cells in vertebrates and flies that accounts for the fact that there is a dorsally projecting set of motorneurons in *Drosophila*, which is not matched by a comparable set in vertebrates.

Interestingly, in the nematode *Caenorhabditis elegans*, a homolog of the *Drosophila eve* gene, *vab-7*, is also expressed in a set of motorneurons that go to dorsal targets, and it is required for their correct pathfinding (B. Esmaeili and J. Ahringer, personal communication). Thus, it appears that the function of *eve* in directing patterns of motorneuron growth is an ancient one.

Experimental Procedures

Fly Stocks

Muscles are named according to Bate (1993) and, in parentheses, Crossley (1978). Oregon-R flies were used as wild type. *elav-GAL4*, ftz_{ng}(neurogenic promoter)-GAL4, and UAS-*beat* were kindly provided by Corey Goodman (Lin and Goodman, 1994; Fambrough and Goodman, 1996). UAS-*even-skipped* on *TM6*/*P[rosy*$^{+}${*l(3)}]* was generously provided by Emma Dormand and Andrea Brand. UAS-*tau-lacZ* was kindly provided by Alicia Hidalgo and Andrea Brand (Hidalgo et al., 1995). All GAL4-UAS misexpression experiments were carried out at 29°C. *eve*^{ID19}/*CyO, ftz-lacZ* kindly provided by Chris Doe is a temperature-sensitive allele of the *even-skipped* (*eve*) gene, where protein function is abolished by shifts from the the permissive temperature (18°C) to the restrictive temperature (29°C; Doe et al., 1988b; Frasch et al., 1988). We balanced the *eve*^{ID19} allele over *CyO, wg-lacZ*, which gives strong epidermal β-galactosidase expression throughout embryogenesis. To remove Eve protein function, staged embryos (stages according to Campos-Ortega and Hartenstein, 1985) were shifted to the restrictive temperature by transferring them into a 29°C incubator on moist, prewarmed blotting paper. Unless stated otherwise, eggs were then kept continuously at the restrictive temperature until late stage 16/early stage 17. Homozygous *eveID19* embryos were unambiguously identified by lack of β-galactosidase expression and muscle defects in abdominal segments A_2 and A_6. Segments A_3–A_5 are wild type at the permissive temperature, and only these were used for analysis of the mutant phenotype (Doe et al., 1988b). We find that independent of the genetic background, nerve branch morphology is consistently altered by such temperature shifts, though the pattern of axonal projections and their innervation remains normal.

Immunohistochemical Methods

Immunocytochemical staining of embryos was carried out following standard techniques for whole mounts (Rushton et al., 1995) and flat preparations (Broadie and Bate, 1993). Whole mounts were mounted in capillaries (Hilgenberg) and could thus be viewed from all sides. Mouse anti-Fasciclin II (MAb 1D4; Van Vactor et al., 1993), used at a dilution of 1:20, and Mouse anti-Fasciclin III (MAb 2D5; Patel et al., 1987), used at a dilution of 1:4, were generous gifts from Corey Goodman. Mouse anti-Islet (MAb 3A4), a generous gift from Tom Jessell (Tsuchida et al., 1994), was used at a dilution of 1:20. Mouse anti-Connectin (kindly provided by Rob White [Meadows et al., 1994]) was used at a dilution of 1:20. Mouse anti-Eve (courtesy of Nipam Patel [Patel et al., 1992]) was used at dilutions of 1:50 to 1:200. Rabbit anti-Synaptotagmin (kindly provided by Hugo Bellen and Troy Littleton [Littleton et al., 1993]) was used at a dilution of 1:1000. Rabbit anti-Myosin Heavy Chain (a gift from Dan Kiehart [Kiehart and Fegali, 1986]) was used at a dilution of 1:500. Rabbit anti-β-galactosidase (Cappel) was used at a dilution of 1:5000. *beat*

mRNA was kindly provided by Corey Goodman, and in situs were carried out following standard procedures.

DiI Labeling and Photoconversions

DiI labelings and photoconversions were carried out as previously described in Landgraf et al. (1997).

Electron Microscopy

Ultrastructural analyses were carried out as described previously (Prokop et al., 1996, 1998). In brief, embryos were injected with 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2), the injected specimens were cut open at their tips with a razor blade splinter, postfixed for 30–60 min in 2.5% glutaraldehyde in 0.05 M phosphate buffer, briefly washed in 0.05 M phosphate buffer, fixed for 1 hr in aqueous 1% osmium solution, briefly washed in dH_2O, treated en bloc with an aqueous 2% solution of uranyl acetate for 30 min, dehydrated, and transferred to Araldite. Serial sections of 30–50 nm (silvergray) thickness were transferred to formvar-covered carbon-coated slot grids (Galay and Nilsson, 1966), poststained with lead citrate for 5–10 min, and examined on a Jeol 200CX. Transverse serial thin sections were taken about 10–15 μm behind the anterior border of the denticle belts, which can be visualized in semithin sections with the light microscope.

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