Growth Cone Choices of *Drosophila* Motoneurons in Response to Muscle Fiber Mismatch

Akira Chiba, Huey Hing, Sydney Cash,^a and Haig Keshishian

Department of Biology, Yale University, New Haven, Connecticut 06511

In *Drosophila* embryos, each motoneuron is accurately matched to one or more singly identifiable muscle fibers. In this article we altered the number and pattern of the embryonic muscle fibers using genetic, heat shock, and laser ablation methods to test whether motoneuron growth cones are able to recognize specific targets. The choices made by two motoneurons were assayed using both intracellular dye fills and immunocytochemistry. The motoneurons RP1 and RP3 have nearly identical central and peripheral axonal trajectories. However, RP3 innervates the two most ventral longitudinal muscle fibers, 7 and 6, while RP1 grows past these fibers to innervate only muscle fiber 13. In *rhomboid* mutants muscle fiber 7 does not develop. Despite the loss of one of its targets, RP3 faithfully innervated the remaining muscle fiber 6 in over 80% of the observed cases. Furthermore, neuron RP1 accurately innervated muscle fiber 13, although it traversed one fiber fewer to reach it. Laser ablation of muscle fiber 7 confirmed the target choices shown by the motoneurons. In *numb* mutants, multiple muscle fibers, including 7, 13, and 12, fail to develop. This allowed us to test whether fibers distal to the target are involved in muscle fiber recognition, possibly by halting the growth cone advance. In mutant embryos, RP3 innervated muscle fiber 6 at the same frequency regardless of the absence of the distal muscle fiber 13. By contrast, RP1, which had lost its target entirely, frequently failed to innervate any muscle fiber during the period examined. Finally, muscle fiber 13 can be duplicated in wild-type embryos by means of a brief heat pulse during myogenesis. Presented with two targets, RP1 innervated both fibers in each case examined, while RP3 synapsed with muscle fibers 7 and 6 normally. Neuron-specific antibodies revealed that the embryonic growth cone choices were not transient, but persisted into the larval neuromuscular projections. These results indicate that each motoneuron growth cone has a primary target preference, which is retained even when the numbers of the muscle fibers, and therefore their relative positions, are altered. We therefore suggest that synaptic recognition by *Drosophila* motoneuron

growth cones relies on unique features of the individual muscle fibers.

[Key words: cell recognition, *Drosophila*, growth cone, laser ablation, neuromuscular junction, numb, rhomboid, synaptic specificity, target mismatch]

One of the goals of developmental neurobiology is to understand how embryonic neurons select their synaptic partners. There is good evidence that the guidance cues used by growth cones often derive from the immediate cellular environment. Where individual choices were assessed using singly identified neurons, specific filopodial contacts have been found to play important roles in steering growth cones along their central and peripheral pathways (Palka et al., 1983; Raper et al., 1983; Bastiani et al., 1985; Grenningloh et al., 1991; Bentley and O'Connor, 1992; Bernhardt et al., 1992; Eisen and Pike, 1992). Dynamically expressed cell-surface molecules have been identified on both developing neurons and muscle fibers, and their role in regulating growth cone guidance and target recognition is a focus of extensive analyses in both vertebrate and invertebrate systems (Dodd and Jessell, 1988; Goodman et al., 1992; Landmesser, 1992; Schachner, 1992; Tosney, 1992).

Mismatch and translocation experiments have shown that vertebrate motoneurons have preferred muscle targets. In zebrafish embryos, each of the primary motoneurons becomes committed to its characteristic trajectory a few hours prior to the initiation of axon outgrowth. Also, primary motoneurons grow to the appropriate axial musculature despite translocation (Eisen, 1991; Eisen and Pike, 1992). In the chick embryo, translocation of motoneuron pools by spinal cord reversal or rearrangement of limb bud segments shows that motoneurons project accurately to their appropriate targets despite initially aberrant pathfinding (Lance-Jones and Landmesser, 1981; Whitelaw and Hollyday, 1983a,b; Landmesser, 1992).

The innervation of the *Drosophila* body wall is well suited for testing hypotheses about synaptic recognition during development. In this system, neuromuscular synapse formation can be examined *in vivo* with single-cell resolution. The embryonic and larval abdominal musculature consists of a simple, segmentally repeated array of 30 muscle fibers (Johansen et al., 1989a). Each multinucleated muscle fiber can be individually identified by virtue of its attachment sites, orientation, and shape (Crossley, 1978; Anderson et al., 1988; Johansen et al., 1989a,b; Bate, 1990), as well as by characteristic molecular expression patterns (Halpern et al., 1991; Harkins and Keshishian, 1991; Nose and Goodman, 1991; Halfon et al., 1992; Harkins et al., 1992; Nose et al., 1992). At least 30 motoneurons innervate the muscle fibers in each hemisegment (Sink and Whittington, 1991a), over a dozen of which have been uniquely identified (Halpern

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Correspondence should be addressed to Akira Chiba, Department of Biology, Yale University, P.O. Box 6666, New Haven, CT 06511.

^aPresent address: Columbia University School of Medicine, New York, NY 10032.

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et al., 1991; Sink and Whittington, 1991a; Cash et al., 1992; Keshishian et al., 1993).

During the last third of embryogenesis, the motoneurons exit the CNS in each hemisegment via two principal nerves (Johansen et al., 1989b). The resultant connectivity is accurate, with each motoneuron matched to one or more specific muscle fibers (Halpern et al., 1991; Sink and Whittington, 1991a,b; Cash et al., 1992). Furthermore, the behavior of growth cone target selection has been analyzed in detail for the *Drosophila* embryonic RP neurons (Halpern et al., 1991; Sink and Whittington, 1991b). These studies suggest that specific growth cone choices are responsible for the observed accuracy of synaptic connections.

The cell bodies of the RP motoneurons (Fig. 1) lie adjacent to each other in the dorsal CNS (Jacobs and Goodman, 1989; Halpern et al., 1991). The axons project through essentially the same cellular environment within the CNS and exit via a common nerve [segmental nerve branch b (SNb)] in the periphery. As the filopodia of efferent growth cones can sample widely in the body wall, it is likely that both neurons contact many of the same muscle fibers in the vicinity of their synaptic targets (Chiba et al., 1990; Halpern et al., 1991; Sink and Whittington, 1991b). However, despite the similarity of their trajectories, when the two neurons approach their respective targets they show distinct behaviors (Halpern et al., 1991; Sink and Whittington, 1991b). The RP3 growth cone advances between the external and internal muscle fiber layers, turning at the cleft between the two most proximal longitudinal muscle fibers 7 and 6 (Fig. 1*B*, arrow). There, the growth cone projects onto the fibers' internally facing surfaces. By contrast, the RP1 growth cone extends past muscle fibers 7 and 6 to innervate the internally facing surface of muscle fiber 13 (Fig. 1*B*).

In this article we have tested the hypothesis that motoneuron growth cones can distinguish between individual muscle fibers by challenging RP1 and RP3 with altered arrays of putative target cells. We altered both the number and pattern of the longitudinal muscle fibers in the embryo prior to their innervation. The resulting choices of the two RP growth cones were examined using both dye fills and immunocytochemistry. Here we describe three complementary situations: (1) the deletion of muscle fiber 7, one of the two normal targets of RP3; (2) the further deletion of muscle fiber 13, the sole normal target of RP1; and (3) the creation of an extra muscle fiber 13, duplicating RP1's target.

These mismatch experiments showed that the RP motoneurons projected to their normal targets despite alterations in the number and pattern of adjacent muscle fibers. We also found that, when their targets were either duplicated or halved in number, the innervating motoneurons accommodated these changes without significant targeting errors. Therefore, these results suggest that *Drosophila* motoneurons have preferred synaptic partners, and that target recognition depends on specific growth cone choices.

Some of the results in this article have been presented in abstract form (Chiba and Keshishian, 1991).

Materials and Methods

Fly strains. Wild-type Canton S (CS-5; Woodard et al., 1989) *Drosophila melanogaster* was used throughout the study. The mutants used were *rhomboid^{lac1}* (= *veinlet²*), generated by P element-insertional mutagenesis (Bier et al., 1989, 1990), and the *numb¹* allele, first isolated as *l(2)neo⁷* by Cooley et al. (1988) and subsequently characterized by Uemura et al. (1989).

Figure 1. A schematic representation of the RP1 and RP3 motoneurons and their target muscle fibers in *Drosophila* embryos. *A*, Dorsal view showing two abdominal CNS segments (light gray area) alongside the ventral longitudinal muscle fibers 7, 6, 13, and 12 (rectangles) in a right body-wall hemisegment. Anterior is to the top. The RP1 and RP3 trajectories and their synaptic targets are based on both previous studies (Jacobs and Goodman, 1989; Halpern et al., 1991; Sink and Whittington, 1991a) and this article. The cell bodies of both RP1 and RP3 lie within the space surrounded by the anterior and posterior commissures (ac and pc) and the right and left longitudinal connectives (lc). In this figure the contralateral RP1 and RP3 neurons are not shown. The axons cross the midline of the CNS within the anterior commissure. Turning posteriorly at the contralateral longitudinal connective, both axons extend posteriorly to exit the CNS via the intersegmental nerve tract (ISN). As they exit the CNS, both the RP1 and RP3 axons cross to the segmental nervebranch b (SNb). The RP3 axon innervates the first two ventral longitudinal muscle fibers 7 and 6, while RP1 extends distally to innervate the third ventral longitudinal muscle fiber 13. *B*, A transverse view showing the point where the two RP growth cones diverge to innervate their respective targets (arrow). The growth cone filopodia grow distally to contact multiple muscle fibers (Chiba et al., 1990; Halpern et al., 1991; Sink and Whittington, 1991b). The RP3 growth cone turns internally to innervate both 7 and 6, while the RP1 growth cone advances across the width of muscle fiber 6 to innervate muscle fiber 13 (Halpern et al., 1991). The RP axons extend between two layers of muscle fibers, the ventral longitudinal fibers, 7, 6, 13, and 12, and the ventral oblique fibers, 17, 16, 15, 14.2, and 14.1. Dorsal is to the top, and all these arrangements are symmetrical along the ventral midline of the animal.

Staging. Embryos were staged according to the morphological criteria of Campos-Ortega and Hartenstein (1985), with each stage being further subdivided by calibration to specific events during midgut development in the intact embryo (Halpern et al., 1991). Embryos were collected with 1 hr egg lays on yeast apple juice agar plates from population cages and incubated at 18°C. Late stage 16, when most of the dye fills in this study were performed, corresponds to an embryo with three midgut lobes and a highly convoluted hindgut, but before the appearance of air-filled trachea. At 25°C this would correspond to approximately 15 hr of embryogenesis.

Laser ablation. Single muscle fibers in the whole-mount stage 15 embryos were ablated by focused pulses from a dye laser operating at 440 nm, as described by Cash et al. (1992). The embryos were transferred to food plates and allowed to grow to the third larval stage, when they were dissected for analysis.

Muscle fiber duplications. Wild-type eggs were collected from population cages over a 30 min interval and were incubated at 18°C, except

Figure 2. Embryonic heat shock induces the duplication of muscle fiber 13. *A*, Heat shock duplications were most frequent in abdominal segments A3 and A4 ($n = 37$ duplications in 180 larvae). The duplications were generated by several heat shock protocols (see Materials and Methods). *B*, The frequency distribution of muscle fiber 13 duplication, sampled in segments A2–A6 (mean = 4.1%; $n = 98$ hemisegments in 10 larvae), is closely modeled by a Poisson distribution with the same mean value of 4.1%. The correspondence between the expected and observed frequency distributions suggests that each duplication is a random and independent event. *C*, The critical period for inducing muscle fiber 13 duplication falls within embryonic stages 14–15. A 2 hr heat shock (from 18°C to 31°C) was delivered at the onset of the embryonic stages indicated ($n = 98$ –127 hemisegments assayed in 10–13 larvae for each data set). The critical period overlaps with myogenesis and occurs before synapse formation (Johansen et al., 1989b; Bate, 1990; Halpern et al., 1991; Sink and Whittington, 1991b).

during the period of the heat shock. On the basis of the experiments described below, we adopted a standard heat shock protocol of a 2 hr 31°C pulse beginning at the onset of stage 15. In one series of experiments the same pulse was given at various times between stages 10 and 17 to determine the critical period for the muscle fiber duplication. In another set of experiments, a 10 min heat pulse(s) was given by transferring the eggs on mesh between water baths. Data were collected in the third instar larvae, except for those in which the RP ending morphology was analyzed at late stage 16 immediately following the heat shock.

Following standard heat shock treatment, a duplication of muscle fiber 13 was observed in 4.1% of the sampled third instar larval hemisegments ($n = 98$ hemisegments in 10 larvae). On average, a single duplication event occurred in every two or three abdominal body-wall preparations examined in this group. A similar rate of duplication was found for stage 16 embryos from the same treatment group ($n = 250$ hemisegments in 30 embryos).

Muscle fiber 13 was more likely to duplicate than other fibers. Muscle fibers 6 and 12 duplicated at approximately 1% frequency, while neither muscle fiber 7 nor any of the ventral oblique muscle fibers, 17, 16, 15, 14.2, and 14.1, whose positions are shown in Figure 1*B*, duplicated after heat shock ($n = 98$ hemisegments in 10 larvae). The rate of deletion for any of the muscle fibers was equally small (1% or less).

Within the abdominal segments A1–A7, the duplication of muscle fiber 13 occurred most frequently in the middle segments A3 and A4 (Fig. 2*A*). There was no left–right bias for the heat shock sensitivity (53% and 47%, respectively; $n = 30$ hemisegments in 19 larvae). The

frequency distribution for duplication events was closely modeled by a Poisson distribution (Fig. 2*B*). Thus, in a given animal, muscle fiber 13 duplications probably occur as independent events.

The critical period for muscle fiber duplication occurred during stages 14–15, as determined by testing successively later 2 hr heat pulses (Fig. 2*C*). The critical period for duplication of the other ventral muscle fibers occurred during the same period (data not presented here). The onset of the critical period overlapped with the period of embryonic muscle formation but preceded synaptogenesis on the ventral longitudinal muscle fibers examined in this study (Johansen et al., 1989b; Bate, 1990; Halpern et al., 1991). The heat-shocked animals survived to adulthood at a rate similar to that for untreated animals (78% and 85% for, respectively, heat shock and untreated controls; $n = 40$ each). Finally, we noted that temperature shifts rather than high-temperature incubation were responsible for the effect (Table 1). Also, a single 10 min pulse was effective for generating duplications. Therefore, sudden temperature change during a critical period (stages 14–15) is responsible for inducing muscle fiber duplication.

Immunocytochemistry. Antibody labeling was performed as described by Halpern et al. (1991). A serum antibody to HRP was used as a general label for neurons, as these antibodies recognize all neurons in *Drosophila* (Jan and Jan, 1982). Specific neurons and/or muscle fibers were labeled using antibodies to fasciclin III (Patel et al., 1987; Snow et al., 1989) and Toll (Hashimoto et al., 1991) and also with monoclonal antibody (mAb) 22C10 (Fujita et al., 1982). β -Galactosidase expression in *rhomboid^{lacI}* flies was determined using a mouse monoclonal antibody

Table 1. Muscle fiber 13 duplications induced by various heat shock protocols^a

	Constant ^b		Shifts ^c		Pulses ^d		
	18°C	31°C	Up	Down	2 hr	10 min	6 times 10 min
Duplication frequency	0.0%	0.0%	1.8%	2.0%	4.1%	2.8%	5.8%
Hemisegments (larvae) examined	109 (12)	120 (12)	109 (12)	99 (11)	98 (10)	108 (12)	104 (11)

^a Data from abdominal A2–A6 hemisegments, scored in third instar larvae.

^b Development was either at a constant 18°C for 8 d or a constant 31°C for 4 d. Note that development roughly doubles in speed from 18°C to 31°C.

^c Upward shifts were from 18°C to 31°C at 22 hr of embryogenesis (stage 15), followed by a constant 31°C for 4 d. Downward shifts were from 31°C to 18°C at 11 hr of embryonic development (stage 15) followed by a constant 18°C for 8 d.

^d Constant 18°C for 8 d, except for 31°C pulse(s) applied at 22 hr of embryogenesis. The treatments were either a single 2 hr pulse, a single 10 min pulse, or six 10 min pulses each spaced with 10 min intervals at 18°C.

Figure 3. Differential interference contrast images of the ventral longitudinal muscle fibers in right abdominal hemisegments of unfixed control and experimental *Drosophila* embryonic fillets, viewed at late stage 16. Anterior is to the top of each of these and all subsequent photographs and tracings. *A*, In a wild-type embryo, four ventral longitudinal muscle fibers, 7, 6, 13, and 12, are present at characteristic positions in each hemisegment. *B*, In the *rhomboid* mutant, only three of the four ventral longitudinal muscle fibers are present. *C*, The *numb* mutant had only one longitudinal muscle fiber per hemisegment. The ventral oblique muscle fibers are located directly beneath the single longitudinal muscle fiber provisionally identified as 6. *D*, Heat shock occasionally induced muscle fiber 13 duplication. The two muscle fibers 13M (medial) and 13L (lateral) insert at the normal muscle fiber 13 site (compare *A*). Scale bar, 20 μ m.

(Promega). The Lucifer yellow dye fills were also labeled in some preparations using a rabbit serum (Taghert et al., 1982). The primary antibodies were visualized using peroxidase-labeled avidin-biotin complexes (Vectastain Elite ABC Kit, Vector Laboratories). Muscle fiber nuclei were visualized by incubating with Hoechst 33258 at 0.2% dilution in PBS (Halpern et al., 1991).

Growth cone visualization in embryos. Unfixed stage 16 embryonic fillets were prepared to expose the CNS and body-wall musculature (Johansen et al., 1989b; Halpern et al., 1991). Single RP motoneurons were identified by their characteristic cell body positions within the CNS. The cell bodies were iontophoretically injected with Lucifer yellow in living preparations maintained in balanced insect saline, and the growth cones of the dye-filled RP motoneurons were visualized using enhanced digital microscopy (Halpern et al., 1991).

Results

The CNS organization and growth cone behavior of the RP motoneurons in wild-type embryos. Figure 1 illustrates the muscle fibers and motoneurons examined in this study, as they appear in wild-type abdominal hemisegments A2–A7. The four ventral longitudinal muscle fibers 7, 6, 13, and 12 are identified by their position, their epidermal insertion sites (Fig. 3*A*; Crossley, 1978; Anderson et al., 1988; Johansen et al., 1989a,b; Bate, 1990), and their characteristic molecular expression patterns. The muscle fibers 7 and 6 express the membrane proteins Toll (arrow, Fig. 4*D*; Halfon et al., 1992; Nose et al., 1992) and fasciclin III (arrowhead, Fig. 4*F*; Halpern et al., 1991) on the surfaces where

the muscle fibers contact each other. Fasciclin III, in addition, accumulates heavily at the insertion sites for these muscle fibers. Also, in wild-type muscle fibers there are characteristic numbers of nuclei (Bate, 1990; Budnik et al., 1990; see also Table 2). Each of the ventral longitudinal muscle fibers is innervated by motoneurons projecting from SNb (Fig. 4*B*; Johansen et al., 1989b).

The motoneurons RP1 and RP3 are individually identified by (1) the characteristic positions of their cell bodies in each abdominal segment of the CNS (Fig. 4*A*; Thomas et al., 1984; Patel et al., 1987; Jacobs and Goodman, 1989; Halpern et al., 1991; Sink and Whittington, 1991a), (2) their expression of the membrane protein fasciclin III (Fig. 4*E*; Patel et al., 1987; Halpern et al., 1991) and their immunoreactivity to mAb 22C10 (Fig. 4*C*; Grenningloh et al., 1991), and (3) their characteristic axonal trajectories within the CNS (Fig. 4*G*; Jacobs and Goodman, 1989; Halpern et al., 1991; Sink and Whittington, 1991a). As noted above, the motoneurons RP1 and RP3 exit the ganglion via the same nerve root and diverge in the vicinity of their targets to innervate separate muscle fibers (Fig. 1*B*). Dye fills show that in the wild-type embryo RP3 innervates both muscle fibers 7 and 6 with more or less equal-sized branches, while motoneuron RP1 innervates only muscle fiber 13 (Fig. 5*A,B*). The morphological development of the RP synapses, from their initial growth cone contacts into differentiated motor endings,

Figure 4. The CNS and ventral longitudinal muscle fibers of wild-type embryos. *A*, The ladder-like arrangement of the major axon tracts within the neuropil of a single CNS segment in a late stage 16 embryo, shown with anti-HRP immunocytochemistry. The motoneuron RP3 is in focus at this level of the ganglion. RP1 lies directly dorsal to RP3, and is out of the optical section (see also Fig. 1*A*). *B*, The motoneuronal projections (arrows) to the ventral longitudinal muscle fibers 7, 6, 13, and 12 via SNb revealed using anti-HRP labeling. *C*, mAb 22C10 labels a subset of neurons within the CNS of a stage 16 embryo, including both RP1 and RP3 (out of optical section), as well as their axons. The antibodies also labels motoneurons RP4, RP2, and aCC. *D*, The membrane protein Toll accumulates on muscle fibers 7 and 6 in stage 15, especially at the cleft (arrow), where the two muscle fibers touch each other. *E*, Fascilin III immunocytochemistry labels the cell bodies and axons of both RP1 and RP3 (out of optical section) in a stage 16 embryo. RP4 is also labeled. *F*, Fascilin III expression by a subset of the motoneuron growth cones in

Table 2. Number of nuclei (mean $\pm$ SEM) in ventral longitudinal muscle fibers^a

Muscle fiber	Wild-type ^b <i>n</i> = 114 (22)	<i>rhomboid</i> ^c <i>n</i> = 18 (4)	<i>numb</i> ^c <i>n</i> = 22 (5)	Heat shock ^d <i>n</i> = 29 (19)
7	8.27 $\pm$ 0.15	—	—	8.17 $\pm$ 0.34
6	13.40 $\pm$ 0.28	9.44 $\pm$ 0.32*	—	12.62 $\pm$ 0.61
13	9.68 $\pm$ 0.16	9.28 $\pm$ 0.25	9.91 $\pm$ 0.29*	9.00 $\pm$ 0.31 (13M)
			—	8.83 $\pm$ 0.36 (13L)
12	9.01 $\pm$ 0.14	10.28 $\pm$ 0.23*	—	8.86 $\pm$ 0.34

^a The nuclei were visualized by using nucleic acid labeling (see Materials and Methods) and data were collected in A2–A6 hemisegments of bodywalls in either third instar larvae (“Wild-type” and “Heat shock”) or late stage 16 embryos (“*rhomboid*” and “*numb*”). Number in parentheses is number of animals examined.

^b Similar values were obtained for wild-type embryos at late stage 16, as well as for heat-shocked larvae without muscle fiber duplications (data not shown).

^c In both *rhomboid* and *numb* mutants, the muscle fiber provisionally identified as 6 had significantly more nuclei than the wild-type muscle fiber 7 but fewer than expected for a wild-type muscle fiber 6.

^d With heat shock–induced duplication of muscle fiber 13, both 13M and 13L each had nuclear numbers very similar to that of single unduplicated muscle fiber 13s.

*, Significantly differs from the wild-type counterpart (by two-tailed *t* test at *p* < 0.01 level).

was examined in detail by Halpern et al. (1991) and Sink and Whittington (1991b). It was found that by late stage 16 the RP growth cones project to and differentiate at their respective targets. We have therefore examined RP targeting at this and later stages, and where possible, have also examined motor projections in mature larvae.

The rhomboid mutation deleted muscle fiber 7. The *rhomboid* gene is involved both early in dorsoventral patterning (Mayer and Nüsslein-Volhard, 1988) and later in CNS development (Klambt et al., 1990). The gene product is also required for developing sensory organs and muscle fibers (Bier et al., 1990). The homozygous *rhomboid* embryos have only three ventral longitudinal muscle fibers per abdominal hemisegment, instead of the usual four (Fig. 3B).

Using morphological criteria, including size, orientation, and location of attachment sites for each of the muscle fibers, we concluded that in the *rhomboid* mutant, muscle fiber 7 had failed to develop while muscle fibers 6, 13, and 12 remained intact. Consistent with this morphologically based identification, only the most proximal of the three longitudinal muscle fibers, provisionally assigned here as muscle fiber 6, was immunopositive for both Toll and fasciclin III (Fig. 6D,F). In *rhomboid* mutants, the fasciclin III and Toll labeling distributed uniformly over the muscle fiber surface, suggesting that their wild-type localization (to the 7–6 cleft; Fig. 4D,F) is due to contact between two muscle fibers expressing the proteins. The nuclear number of the fasciclin III–positive muscle fiber 6 in *rhomboid* mutants, however, did not correspond to the wild-type values for either muscle fiber 7 or 6 (Table 2). The *rhomboid* mutation also affected development of other muscle fibers in dorsal regions of the body wall, as well as the ventral oblique muscle fibers 14.1 and 14.2 (Bier et al., 1990).

Homozygous *rhomboid* mutants lack several of the midline glia in the CNS. This narrows the distance between the two longitudinal connectives (Klambt et al., 1990). Despite this

change, the RP motoneurons were observed at their characteristic location between the anterior and posterior commissures (Fig. 6A). As in the wild-type, the neurons in the *rhomboid* mutants were immunopositive for both 22C10 (Fig. 6C) and fasciclin III (Fig. 6E). Furthermore, the axon trajectories revealed both by the 22C10 immunocytochemistry and dye fills were normal (Fig. 6C,G), indicating that the behavior of the RP neurons within the CNS was minimally affected by the mutation. Anti-HRP labeling showed that the developing SNb, which normally contains efferents from the RPs, exited the CNS normally to innervate the ventral longitudinal muscle fibers (Fig. 6B).

RP3 innervated muscle fiber 6 when muscle fiber 7 is absent. We examined the growth cone choices of RP motoneurons in the *rhomboid* mutants using Lucifer yellow dye fills. In the absence of muscle fiber 7, motoneuron RP3 innervated the remaining muscle fiber 6 in 83% of embryos examined at late stage 16 (*n* = 12 dye fills in 12 embryos; Fig. 7A). We did not observe collateral branches from the motoneuron on adjacent muscle fibers. In the wild-type embryo, RP3 innervates both muscle fibers 7 and 6 in the cleft (Figs. 1, 5A; Halpern et al., 1991). However, when confronted by a single muscle fiber 6 in the *rhomboid* mutant, the RP3 axon innervated it from either the proximal or distal side (Fig. 7A, left and middle panels). When innervating from the distal side, the axon first extended beneath muscle fiber 6, turning inward between fibers 6 and 13 (Fig. 7A, middle panel).

In 17% of the dye fills examined, the RP3 growth cone failed to synapse with the remaining muscle fiber 6, and projected distally toward the dorsal longitudinal muscle fibers. It was not observed to differentiate into a motor ending on alternate muscle fibers during the period examined (Fig. 7A, right panel). However, as the mutant embryos do not develop beyond stage 17 (Bier et al., 1990), we cannot rule out the possibility that these RP3 projections would have made synapses at a later time.

←

SNb, including both RP1 (arrow) and RP3 in stage 16. Also labeled is the membrane contact between muscle fibers 7 and 6 (arrowhead). G, An intracellular Lucifer yellow fill of RP1 at stage 16 revealing its axon trajectory within the CNS. The vertical line indicates the midline of the CNS. H, A dye-filled RP1 growth cone projecting at an early stage of its contact with the edge of muscle fiber 13. It has grown past the cleft of muscle fibers 7 and 6 (out of optical section). Visualized using anti-Lucifer yellow immunocytochemistry. Abbreviations are as in Figure 1. Scale bar, 20 μ m.

Figure 5. Tracings of Lucifer yellow-filled RP motoneuron growth cones in late stage 16 wild-type embryos. The dotted lines correspond to the portion of the RP growth cone and axon underneath (i.e., external to) the muscle fiber shown. Schematic cross sections indicating the axon trajectories are shown underneath each figure. *A*, The RP3 growth cone innervated muscle fibers 7 and 6 along the cleft. *B*, The RP1 growth cone, by contrast, innervated the single muscle fiber 13 after extending beyond the cleft of muscle fibers 7 and 6, as depicted by the schematic cross-sectional view. Scale bar, 20 μ m.

RP1 innervated muscle fiber 13 normally despite the absence of muscle fiber 7. The growth cone choices of RP1 were equally informative. Normally the neuron innervates muscle fiber 13 by projecting beneath the first two longitudinal muscle fibers to reach its destination (Fig. 5*B*). In the mutant embryo, RP1 invariably innervated muscle fiber 13 despite traversing one

fiber fewer to reach it ($n = 13$ dye fills in 13 embryos; Fig. 7*B*). Furthermore, the ending morphology resembled the wild-type (Halpern et al., 1991; also compare Figs. 5*B*, 7*B*). Thus, the deletion of a muscle fiber proximal to its own target did not cause noticeable errors in the RP1 growth cone behavior.

Laser ablation phenocopied the loss of muscle fiber 7 in rhomboid mutants. The *rhomboid* mutation used in this study is an embryonic lethal and may also have pleiotropic effects. Therefore, we have also deleted muscle fiber 7 in stage 15 embryos by means of laser ablation, and examined the morphology of muscle fiber innervation in mature larvae. Nearly 70% of the muscle fiber 7-ablated embryos ($n = 24$) developed into the third instar. Each experimental animal had muscle fiber 7 ablated in one to three body-wall hemisegments.

Anti-HRP labeling of all the motor endings in the third instar larvae showed that the remaining muscle fiber 6 was innervated in 95% of the hemisegments examined ($n = 24$ hemisegments in 15 larvae; Fig. 8*B–D*). As was observed in the *rhomboid* embryos (Fig. 7*A*), the larval muscle fiber 6 in the laser-operated animals was innervated from either its proximal or distal sides, with the former more than twice as common as the latter (Fig. 8, *B* and *C*, respectively). Also, at low frequency (8%), muscle fiber 6 remained uninervated (Fig. 8*D*), consistent with the observation that in a subset of the *rhomboid* mutant embryos RP3 failed to innervate the remaining muscle fiber 6 (Fig. 7*A*, right panel). The larval muscle fiber 6 innervation had wild-type features, such as normal-sized synaptic boutons and branches confined to the muscle fiber edge (A. Chiba and H. Keshishian, unpublished observations). The motor ending morphology on neighboring muscle fibers, such as 13 and 12, was unaltered after the loss of muscle fiber 7 (Fig. 8*B–D*). These observations suggest that the RP growth cone choices in the *rhomboid* mutants were likely due specifically to the absence of muscle fiber 7, and that the basic pattern of innervation was determined by the initial growth cone behavior.

The numb mutation deleted multiple muscle fibers. The *numb* mutation disrupts the differentiation of peripheral sensory neurons (Uemura et al., 1989), and in addition results in the loss of several ventral longitudinal muscle fibers. In *numb* mutant embryos, only one (or occasionally two) of the ventral longitudinal muscle fibers develops (Fig. 3*C*). We have provisionally identified the single remaining cell as muscle fiber 6 on the basis of its size, position, and apodeme insertion sites. Furthermore, this muscle fiber was both Toll and fasciclin III immunopositive (Fig. 9*D,F*), consistent with either muscle fiber 7 or 6 identity. However, as was observed for the *rhomboid* mutation, the nuclear number did not correspond to either wild-type value for muscle fiber 7 or 6 (Table 2).

The organization of the CNS neuropil, as judged by anti-HRP, 22C10, and fasciclin III immunocytochemistry, appeared largely unaltered in the mutant embryos (Fig. 9*A,C,E*). Also, the apparently normal pathway selected by the RP axons within the CNS suggested that the cellular environment is relatively intact

Figure 6. The CNS and ventral longitudinal muscle fibers of *rhomboid* mutant embryos. The panels in this figure parallel those of Figure 4, where the corresponding wild-type features are shown. *A*, Anti-HRP immunocytochemistry in the CNS at late stage 16 showed that both individually identified neurons, including RP3 and RP1 (out of optical section), and the principal axon tracts of the neuropil have developed. The distances between both longitudinal connectives (*lc*) and between anterior and posterior commissures (*ac* and *pc*) were reduced in comparison to the wild-type (Fig. 4*A*; Bier et al., 1990; Klämbt et al., 1990). The CNS phenotype of *rhomboid^{lac1}* used here was less severely affected than that of *rhomboid^{Δ43}*, which Klämbt et al. (1990) characterized. *B*, In the periphery, the motoneuron growth cones, visualized by anti-HRP, innervated the ventral longitudinal muscle fibers 6, 13, and 12. The innervation of muscle fiber 6 in this example was on its distal side (arrow). *C*, mAb 22C10 revealed

virtually normal neuronal organization in the CNS at stage 16. *D*, Toll protein accumulated on the membrane surface of muscle fiber 6 at stage 15. The protein was more uniformly spread over the surface, instead of accumulating heavily at the edges as seen in the wild-type (Fig. 4*C*). *E*, Fasciclin III immunocytochemistry revealed a normal number of RP motoneurons. The cell bodies were located closer to the midline than in wild-type. *F*, In the periphery fasciclin III immunolabeling was confined to a single muscle fiber (noticeably darker than its neighbors), as well as to the cell's insertion sites. We provisionally identified this cell as muscle fiber 6. Note that in the absence of a muscle fiber 7 the fasciclin III redistributed uniformly on the muscle fiber (compare Fig. 4*E*). Muscle fiber 6 was innervated by the fasciclin III-positive motoneurons (presumably RP3, arrow) from its distal side in this example. *G* and *H*, A Lucifer yellow fill of RP1 shown both within the CNS (*G*), and in the periphery (*H*), where it innervated muscle fiber 13 normally (compare Fig. 4*H*). Scale bar, 20 μ m.

Figure 7. Tracings of dye-filled RP motoneuron growth cones in late stage 16 *rhomboid* mutant embryos. Schematic cross sections indicating the axon trajectories are shown underneath each figure. **A**, The RP3 growth cone in the absence of muscle fiber 7 innervated the remaining muscle fiber 6 using either of two routes (*left* and *middle* panels). The growth cone drawn in the *middle* panel is at a different level than the adjacent muscle fiber 13 (as shown in the cross-sectional view). Occasionally the RP3 motoneuron failed to innervate any muscle fiber and grew distal to muscle fiber 6 (*right* panel). **B**, The RP1 growth cone, by contrast, innervated muscle fiber 13 without error, despite the loss of muscle fiber 7. Scale bar, 20 μ m.

in *numb* mutant embryos (Fig. 9G). As was observed in the *rhomboid* mutants, the motoneurons exited the CNS through the correct nerve, with SNb projecting a subset of its axons to the single remaining ventral longitudinal muscle fiber (Fig. 9B).

The *numb* mutation allowed us to test whether the preference of RP3 for muscle fiber 6 depended on the presence of adjacent muscle fibers. For example, successful target selection might depend on a repulsion from muscle fiber 13, which would halt RP3's distal growth. Furthermore, in the *numb* mutation RP1 is faced with the complete loss of its muscle fiber target, allowing us to test whether the motoneuron responds by selecting alternate muscle fibers.

RP3's preference for muscle fiber 6 was independent of neighboring muscle fibers. The RP3 dye fills in the late stage 16 *numb* mutants showed that it innervated the remaining muscle fiber 6 at high frequency (Fig. 10A, left panel). However, the RP3 growth cone occasionally failed to innervate the muscle fiber (Fig. 10A, right panel), a feature also observed for *rhomboid* mutants. Nevertheless, the preference for muscle fiber 6 was essentially the same both for *rhomboid* mutants, where muscle fiber 7 was missing but where distal fibers 13 and 12 were intact (83%, $n = 12$ dye fills in 12 embryos), and for *numb* mutants,

where muscle fibers 7, 13, and 12 were absent (86%, $n = 7$ dye fills in 7 embryos; compare Figs. 7A, left panel, and 10A, left panel). This suggests that the presence or absence of neighboring nontarget muscle fibers had no deciding role in defining RP3 choices.

RP1 grew aberrantly in the absence of its target. In contrast to the RP3 growth cone, the RP1 growth cone generally did not innervate the remaining ventral longitudinal muscle fiber and instead grew toward the dorsal midline, well beyond the site of its normal destination (Fig. 10B, right panel). We did not detect synaptic contacts by the RP1 growth cone at the distal sites during the period of embryogenesis examined. RP1 grew past muscle fiber 6 in 87% of the cases ($n = 8$ dye fills in 8 embryos), even though it was the sole available ventral longitudinal muscle fiber. This suggests that the RP1 growth cone retained its relative muscle fiber preferences in the proximal part of its trajectory. The continued distal growth of RP1 suggests that contact with its normal target, muscle fiber 13, may be needed to halt its projection. However, we did observe one example of RP1 innervating muscle fiber 6 (Fig. 10B, left panel). Since the mutants die before reaching the first instar stage, we were unable to assess the long-term effect of target loss on RP1. However, it is possible

Figure 8. Mature larval innervation patterns following laser ablation of muscle fiber 7 (see Materials and Methods). Following ablation of muscle fiber 7 in whole-mount stage 15 embryos, the animals were allowed to develop to the third larval instar. The ending morphology was examined using anti-HRP immunocytochemistry. *A*, A wild-type control abdominal hemisegment with an intact muscle fiber 7. Muscle fibers 7 and 6 are innervated by branches largely restricted to the cleft region. On muscle fiber 13 the motor endings project posteriorly, while on 12 there is generally a multibranched bifurcation (Johansen et al., 1989a; Budnik et al., 1990). *B–D*, Following the embryonic ablation of muscle fiber 7, muscle fiber 6 was either innervated on the proximal side (arrow, *B*), innervated on the distal side (arrow, *C*), or left uninervated (*D*). This was consistent with the embryonic observations with the *rhomboid* mutants (Fig. 7). The innervation on the neighboring muscle fibers 13 and 12 appeared unaltered. Schematic cross sections (*b–d*) correspond to each of (*B–D*) and show the axon trajectory patterns. Scale bar, 50 μ m.

that RP1 would eventually innervate alternative muscle fiber(s), as has been shown for muscle fiber loss elsewhere in the body wall when examined in mature larvae (Cash et al., 1992).

Heat shock occasionally caused the duplication of muscle fiber

13. When wild-type embryos received a brief heat shock during mid-embryogenesis, duplication and deletion of the abdominal body-wall muscle fibers occurred at low frequency (Fig. 2). When incubated at either a constant high or low temperature, the

Figure 9. The CNS and ventral longitudinal muscle fibers of *numb* mutant embryos. The panels in this figure parallel those of Figure 4, where the corresponding wild-type features are shown. *A*, Anti-HRP immunocytochemistry showed virtually normal organization of the major axon tracts within the CNS at stage 16. Cell bodies of RP3 and RP1 (out of optical section) were found in the characteristic positions. *B*, In the periphery some growth cones innervated the remaining muscle fiber 6, in this example, from the proximal side (arrow). Other growth cones (out of focus) extended beneath the muscle fiber projecting distally. *C*, mAb 22C10 revealed virtually normal organization within the CNS of a stage 16 mutant embryo. *D*, Anti-Toll antibodies labeled the single longitudinal muscle fiber at stage 15, consistent with its identity being either muscle fiber 6 or 7 (here provisionally designated as 6). *E*, Antibodies to fasciclin III labeled a normal set of RP motoneurons, including RP1 and RP3 (out of optical section), within the CNS of stage 16 mutant embryo. *F*, As with the muscle fiber 6 in the *rhomboid* mutation (Fig. 6*F*), the remaining muscle fiber

muscle fiber duplications in the body wall were absent (Table 1). The most frequent effect was the duplication of muscle fiber 13, first evident prior to innervation by late stage 15 (Fig. 3D).

The duplicated muscle fibers, termed here 13M (medial) and 13L (lateral), were identified primarily by their attachment sites, position, orientation, and size (Fig. 3D). As the rate of muscle fiber duplication was low and its occurrence random (Fig. 2B; see Materials and Methods), the immediately anterior and posterior segments usually contained the normal number of muscle fibers and therefore provided unambiguous reference points for muscle attachment sites on the embryonic body wall. Also, within the same hemisegment the surrounding unduplicated muscle fibers served as consistent landmarks.

We found that each of the duplicated muscle fibers averaged approximately nine nuclei, similar to the number in control, unduplicated muscle fiber 13s (Table 2). In embryos each of the duplicated muscle fibers had essentially the same width as an unduplicated muscle fiber 13. Muscle fibers 7, 6, and 12 in the same hemisegment retained their normal number of nuclei despite the addition of an extra muscle fiber. Therefore, it is unlikely that the duplication resulted from splitting a fully developed muscle fiber 13, but rather is likely to involve events prior to the completion of myocyte fusion (Bate, 1990).

Anti-HRP antibodies showed that the principal axon tracts within the CNS of the heat-shocked embryos were indistinguishable from untreated embryos (Fig. 11A). The path followed by the RP neurons along the anterior commissure, longitudinal connective, and the intersegmental nerve tract was normal. The full complement of the dorsal RP motoneurons as well as neurons aCC and pCC were present in their characteristic locations and numbers, as judged by both differential interference contrast optics and immunocytochemistry using anti-HRP (Fig. 11A) and anti-fasciclin III (data not shown).

Muscle fiber duplication of 13 had no effect on RP3's innervation of fibers 7 and 6. RP3 normally innervates muscle fibers 7 and 6, which lie proximal to the duplication (Fig. 5A). Muscle fiber 13 duplication had no visible effect on the ability of RP3 to innervate muscle fibers 7 and 6 successfully (Fig. 12A), and did not lure collateral branches from the motoneuron ($n = 4$ dye fills in 4 embryos). We also performed double-dye fills to monitor the behavior of both RP3 and RP1 in a hemisegment with a duplication of muscle fiber 13 (Fig. 11C). The results suggest that the RP3 growth cone is insensitive to changes in the number and relative positions of nontarget muscle fibers, even when the fibers lie within its filopodial reach.

In addition, the differentiation of RP3's axon terminals was unaffected by the adjacent duplication of muscle fiber 13. In both the control and experimental situations, the RP3 growth cone turned internally at the cleft between muscle fibers 7 and 6 and from that location extended processes posteriorly along the cleft on the internally facing surfaces of the two muscle fibers (compare Figs. 5A, 12A). Although we did not examine growth cone behavior prior to the embryonic stage 16, the degree of the synaptic terminal maturation appeared morphologically indistinguishable between the control and experimental situations.

Figure 10. Tracings of dye-filled RP motoneuron growth cones in *numb* mutant embryos. Schematic cross sections indicating the axon trajectories are shown underneath each figure. **A**, The RP3 growth cone innervated the sole ventral longitudinal muscle fiber with high reliability. The frequency was similar to the previous case for the *rhomboid* mutants (86% vs 83%), where more distal muscle fibers, such as 13 and 12, were present (Fig. 7A). This suggests that the growth cone's target preference does not depend on neighboring muscle fibers, acting as a backstop. **B**, By contrast, the RP1 growth cone, having lost its normal muscle fiber 13 target, usually failed to innervate any muscle fiber during the normal period of synaptogenesis, and instead extended distally beyond the normal stopping point (see Fig. 5B). However, we observed a single case where the RP1 growth cone innervated muscle fiber 6 (left). Scale bar, 20 μ m.

This lack of effect on muscle fiber 7 and 6 ending anatomy was also observed in third instar larvae with duplicated muscle fiber 13s (see below).

RP1 innervated both muscle fiber 13s. We next examined the response of the RP1 growth cone to the duplication of its normal targets in the embryos. In all cases examined, RP1 projected to

in *numb* mutants inserted at sites heavily labeled for fasciclin III. The muscle also showed moderate fasciclin III immunoreactivity. **G**, A Lucifer yellow fill of a single RP1, after being processed with anti-Lucifer yellow immunocytochemistry, showed that the axon trajectory within the CNS was virtually intact. **H**, The growth cone of a dye-filled RP1 that failed to innervate any muscle fiber and projected past the normal stopping point (compare Fig. 4H). Abbreviations are as in Figure 1. Scale bar, 20 μ m.

Figure 11. The CNS and the ventral longitudinal muscle fibers of heat-shocked wild-type animals with duplication of muscle fiber 13. *A* and *B*, Neuron-specific anti-HRP immunocytochemistry of late stage 16 embryos showing the CNS (*A*) and motoneuron endings (*B*) at the ventral body wall of two hemisegments, the anterior one bearing a duplication of muscle fiber 13. Despite the extra muscle fiber, all adjacent muscle fibers have received normal innervation. Meanwhile, the duplicated muscle fiber 13s were both innervated (arrows). Abbreviations are as in Figure 1. *C*, In this serial double dye fill, first RP1 and then RP3 was injected with Lucifer yellow at the late embryonic stage 16. This demonstrated that the RP1 growth cone innervated both muscle fibers 13M and 13L (arrows), while the RP3 growth cone (without arrow) innervated muscle fibers 7 and 6 normally. *D* and *E*, Third instar larval body-wall hemisegments containing duplicated muscle fiber 13s were labeled with anti-HRP. Both the duplicated and unduplicated muscle fibers were innervated (arrows). While the neighboring unduplicated muscle fibers showed normal innervation, the innervation of the duplicated muscle fibers showed some variability (see Discussion). Scale bar: 20 μ m for *A–C*; 50 μ m for *D* and *E*.

both muscle fiber 13s with equal-sized branches ($n = 5$ dye fills in 5 embryos; Fig. 12*B*). This indicates that the RP1 motor ending, which is normally confined to a single fiber, can expand to innervate two muscle fibers when presented the duplicated postsynaptic partners.

Although RP1 innervated both muscle fiber 13s, the axon trajectory was variable. In some cases the RP1 axon turned internally at the cleft between muscle fibers 6 and 13M and projected over the surface of both duplicated muscle fibers (Fig. 12*B*, left panel). This trajectory resembled the normal path taken by RP1 when it innervated a single muscle fiber in control hemisegments (Fig. 5*B*). However, in nearly half the cases, the RP1 axon grew more distally, turning internally between the two muscle fiber 13s, and bifurcated at that site to innervate them both (Fig. 12*B*, right panel).

The innervation of the duplicated muscle fiber 13s was confined to the internally facing surfaces of the muscle fibers, as is found for most fibers in the *Drosophila* body wall (Johansen et al., 1989a,b; Fig. 12*B*). Once on this surface, the RP1 growth cone extended posterior branches along the proximal edges of the muscle fibers, similar to its behavior in controls (Fig. 5*B*). Thus, features of the growth cone behavior typical of RP1 remained intact when the motoneuron was challenged with a target duplication.

Larval neuromuscular endings on duplicated muscle fibers. Since the heat-shocked animals survived to adulthood (see Materials and Methods), we were able to examine the body-wall neuromuscular endings in mature larvae. The motor ending morphology in the third instar body wall, as revealed by anti-HRP immunocytochemistry, showed extensive similarities to the embryonic body wall (compare Figs. 11*D,E*, 12*B*).

Within the hemisegments where muscle fiber 13 was duplicated, both muscle fibers 13M and 13L in larvae were innervated in 93% of the observed cases ($n = 30$ hemisegments in 19 larvae), a rate similar to that found for embryos (100%). Furthermore, the pathway variations for the axons innervating the two muscle fiber 13s resembled those observed for the RP1 axon in the embryo (Fig. 12*B*). In general, the larval axons turned either internally at the cleft between muscle fibers 6 and 13M or, alternatively, projected between muscle fibers 13M and 13L at approximately equal rates (Fig. 11*D,E*; Chiba and Keshishian, unpublished observations). These observations of axonal projections onto duplicated larval muscle fibers, together with those of the laser ablations (Fig. 8), are consistent with the idea that synaptic organization is largely established by the embryonic growth cone choices (Johansen et al., 1989b; Halpern et al., 1991; Cash et al., 1992).

Discussion

The experiments in this article were designed to test whether *Drosophila* growth cones can distinguish between individual muscle fibers during synaptogenesis. We found that growth cones retained their target preferences even when the numbers and patterns of muscle fibers were altered (summarized in Fig. 13). The elimination or duplication of a muscle fiber did not lead to a cascade of targeting errors that rippled across the body wall. As long as a muscle fiber remained intact, innervation was accurately made by the appropriate motoneuron, despite alterations of the adjacent musculature. Similarly, doubling or halving of the number of muscle fibers led to an accommodating response by the innervating motoneuron, expanding or restricting its synaptic contacts appropriately. These observations suggest

Figure 12. Tracings of Lucifer yellow-filled RP motoneuron growth cones in the late stage 16 wild-type embryos, following heat shock-induced muscle fiber 13 duplication. Schematic cross sections indicating the axon trajectories are shown underneath each figure. *A*, An RP3 growth cone that has innervated muscle fibers 7 and 6 normally, despite the presence of an extra muscle fiber 13 (compare Fig. 5*A*). *B*, An RP1 growth cone that innervated both muscle fibers 13M and 13L. Axon trajectories varied when duplicated muscle fibers were innervated; sometimes the axon turned internally at the cleft between muscle fibers 6 and 13M, but in other cases it turned internally at the cleft between the two muscle fiber 13s. In every case, however, the innervation occurred on the internally facing surfaces of the muscle fibers. Although we observed no case where RP1 growth cone innervated only one of the two muscle fiber 13s, anti-HRP immunocytochemistry of mature larvae showed that this may occur as a low rate, (Chiba and Keshishian, unpublished observations). Overall, the close resemblance to the larval innervation patterns (Fig. 11*D,E*) suggests that the embryonic growth cone choices largely determine the mature ending morphology. Scale bar, 20 μm.

that motoneuron growth cones respond specifically to their muscle fiber targets and in this fashion sort out their contacts during the early phases of innervation.

In a recent study, Sink and Whittington (1991c) deleted muscle fibers in acutely cultured *Drosophila* embryonic fillets, examining the behavior of motoneuron RP3. They found that when either muscle fiber 7 or 6 was surgically extirpated with a micropipette, the RP3 growth cone often projected to the remaining muscle fiber. This is consistent with our own observations

Figure 13. Summary of the target mismatch experiments examined in this article. *A*, In the wild-type (control), individual motoneurons innervated specific muscle fibers, demonstrating precise connectivity. Motoneuron RP3 innervated the two proximal ventral longitudinal muscle fibers 7 and 6, while motoneuron RP1 innervated a single muscle fiber 13 lying immediately distal. Other motoneurons not discussed in this study innervate these same muscle fibers, as well as more distal muscle fiber 12. *B*, Muscle fiber 7, one of the two normal targets of RP3, was deleted by either the *rhomboid* mutation or by means of laser ablation. RP3 in the mutant embryos innervated the remaining muscle fiber 6 at high frequency. When it failed to do so, its axon extended distally without innervating any muscle fiber, when examined at the end of stage 16. By contrast, in the same mutants RP1 innervated muscle fiber 13 with high reliability, even though its growth cone crossed one muscle fiber fewer to reach its destination. *C*, The *numb* mutation alters the muscle fiber pattern more radically. Muscle fibers 7, 13, and 12 were absent, leaving muscle fiber 6 as the only ventral longitudinal muscle

using the *rhomboid* and *numb* mutants, and the laser ablation of muscle fiber 7 in intact embryos (Fig. 13). Sink and Whittington (1991c), however, noted a differential preference by RP3 for muscle fiber 7 over 6, a point not directly tested in our study. Furthermore, removal of both fibers led to RP3 projections onto a wide array of alternate muscle fibers. This is comparable to the behavior of RP1 in *numb* mutants described in this article, as well as the results of Cash et al. (1992), who characterized alternate choices for target deprived motoneurons. Overall, these studies show that growth cones are capable of recognizing single cells as targets despite alteration of the muscle fiber pattern, and that in the complete absence of their preferred targets, motoneurons can project to alternate destinations.

The mismatch experiments described in this article rule out several mechanisms for synaptic connectivity in *Drosophila*. For example, it is unlikely that the motoneurons select muscle fibers as a function of their relative order within the body wall. According to this scenario RP3 would innervate the first two longitudinal muscle fibers it encountered. In the cases where muscle fiber 7 was missing, as in the *rhomboid*, *numb*, and laser experiments (Fig. 13*B,C*), RP3 never innervated two fibers, but confined its projection to the single remaining muscle fiber 6. Competition for available muscle fibers is also unlikely as a mechanism for identifying synaptic partners, as we observed normal targeting despite situations where muscle fibers were either duplicated or halved in number.

It is also unlikely that motoneurons identify their targets solely by using nonmuscle cells as guidance cues in the body wall. Normally, motoneuron growth cones contact multiple cell types as they project to their muscle fiber targets (Wang and Keshishian, 1992). However, as they reach their destinations, the growth cone filopodia are usually in contact with multiple muscle fibers (Halpern et al., 1991; Sink and Whittington, 1991a,b; present study). This suggests that the final target selection depends on the ability to respond selectively to the multiple surfaces contacted. This hypothesis is supported by the behavior of RP1 in *numb* mutants (Fig. 13*C*). In the absence of its preferred target, the RP1 growth cone continued to project aberrantly toward the dorsal musculature. This suggests that contact with the preferred target is important in halting the growth cone advance.

Muscle fiber patterning in mutant embryos. Two mutations were used in this study that affect the differentiation of embry-

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fiber in each hemisegment. The RP3 growth cone, nevertheless, innervated the remaining muscle fiber 6 with high accuracy. Compared to the previous case where only muscle fiber 7 was absent (*B*), the target recognition was not degraded by adjacent muscle fiber loss. By contrast, RP1, having lost its normal target altogether, usually extended beyond its normal stopping point and failed to innervate any muscle fiber, as indicated by the arrow. However, it can innervate muscle fiber 6 at a low rate, demonstrating that the target preference by the motoneuron is not absolute. *D*, When muscle fiber 13 is duplicated (arrows) in heat-shocked embryos, the individual motoneurons retain specific target preferences. RP1 replicated its contacts to innervate the duplicated muscle fibers. The two proximal muscle fibers 7 and 6 remain correctly innervated by RP3, while the distal muscle fiber 12 had motor endings of normal morphology (see Fig. 11). This supports the hypothesis that the duplication did not affect the specificity of motoneurons innervating adjacent muscle fibers. These observations show that manipulations of muscle fiber pattern and number have relatively benign effects as long as the preferred muscle fiber target of a motoneuron remains intact in the periphery.

onic muscle fibers. The role for either the *rhomboid* or *numb* product in normal myogenesis remains unknown (Uemura et al., 1989; Bier et al., 1990). The β -galactosidase reporter associated with the *rhomboid^{lac1}* insertion is not expressed by body-wall muscle fibers, but rather by midline glia in the CNS and by body-wall epithelial cells, suggesting that the effects on the musculature of the *rhomboid* mutation are indirect (data not shown). In both mutants there is a single ventral longitudinal muscle fiber that expresses both Toll and fasciclin III, phenotypes typical of both muscle fibers 7 and 6. We have provisionally identified this cell as muscle fiber 6 on the basis of its insertion sites, orientation, and position. However, molecular probes that distinguish between muscle fibers 7 and 6 are required to confirm this identity, or to rule out that the muscle fiber is a composite of 7 and 6. Nevertheless, motoneurons RP1 and RP3 retained their relative target preferences to the putative muscle fiber 6. Furthermore, laser ablation of muscle fiber 7 in the embryo led to motor ending development that was consistent with the growth cone choices made in *rhomboid* and *numb* mutants.

Effects of target manipulation. We found that motoneuron growth cones sometimes projected aberrantly in the manipulated body walls. Variable axon trajectories occurred when the expected number of target muscle fibers was altered. When muscle fiber 7 was absent (as in the *rhomboid* mutant, and following laser ablation), the remaining muscle fiber 6 was innervated from either the proximal or distal side, while trajectories to adjacent unaltered muscle fibers were normal (Figs. 7, 8). A similar local effect was observed when muscle fiber 13 was duplicated, where multiple axon trajectories were followed (Figs. 11*D,E*; 12). These observations suggest that axonal guidance depends on local cues in the vicinity of the muscle fibers. However, correct synaptic choices were usually made independent of the pathway followed to reach the targets.

We also observed cases where the RP neurons failed to make synaptic connections during the period of embryogenesis examined. These errors occurred when motoneuron targets were either partially or completely removed (Figs. 7*A*; 10*A,D*). Ball et al. (1985) described a similar situation following the loss of single muscle pioneers in developing grasshopper limb buds. The coxal depressor motoneuron Df normally targets a single muscle pioneer, but following the ablation of that cell the motoneuron projects to distal regions in the limb bud. Complete removal of the limb bud in grasshopper embryos results in target-deprived motoneurons projecting nonspecifically to alternate body-wall muscles to make functional synapses (Whittington and Seifert, 1984; Whittington, 1985).

In a related study, third instar larvae were examined following the embryonic loss of the oblique muscle fiber 5 (Cash et al., 1992), which is normally innervated by a branch of nerve SNa. The displaced motor endings were located on either of two alternate muscle fibers, on sites approaching the posterior segment border, where SNa terminates. This and the above-mentioned studies in grasshopper suggest that following the elimination of a preferred target, insect motoneurons are capable of innervating alternate muscles. Normally, insect motoneurons project accurately to specific muscle targets during development. Furthermore, as shown in our study, the normal target is preferentially selected by the motoneuron following various alterations of the muscle fiber patterns. Therefore, it is likely that the target muscle fiber exerts an active influence on the exploring motoneuron growth cone, and that in the absence of this signal,

the motoneuron's default affinity for other muscle fibers is revealed.

We also note that neuromuscular innervation in *Drosophila* is robust and resistant to disruption. Muscle fiber alterations have effects largely limited to the innervating motoneurons, leaving the remainder of the peripheral innervation unaltered. We speculate that the existence of primary motoneuron target preferences confers stability to the motor system, reducing the chance of catastrophic innervation errors following changes to the patterning of the musculature.

Cellular recognition during synaptogenesis. The ability of growth cones to respond selectively to single cells is not unique to this system. Similar behavior has also been observed for the reticular pioneer axons of isogenic *Daphnia* (Lopresti et al., 1974), the axons projecting within the CNS of the nematode *Caenorhabditis elegans* (Walthall and Chalfie, 1988; Miller et al., 1992; White et al., 1992), the cercal sensory afferents in crickets (Chiba et al., 1988), and both peripheral and central pioneer axons of grasshopper and *Drosophila* embryos (Bentley and Caudy, 1983; Keshishian and Bentley, 1983; Palka et al., 1983; Bastiani et al., 1984, 1985; Raper et al., 1984; O'Connor et al., 1990; Grenningloh et al., 1991).

Various synaptic target duplication experiments in insects also support the hypothesis of single-cell recognition. In the moth *Manduca sexta*, when *de novo* muscle fibers arise ectopically in the mutant *octopod*, motoneurons respond by coinervating both the ectopic muscle fibers and their normal targets (Miles and Booker, 1991). In homeotically transformed adult *Drosophila* with mutations in the bithorax complex, the descending giant fiber duplicates its mesothoracic arborization in the transformed metathorax, suggesting that the growth cone responds positively to the replicated neuropil generated by homeosis, to innervate duplicated synaptic partners (Thomas and Wyman, 1984).

The active recognition of single cells by growth cones has also been observed in a variety of vertebrate embryos. In the spinal cord of the fish medaka, longitudinal axons specifically contact the segmentally repeated Rohon-Beard cells, although these cells do not serve as obligate guideposts (Kuwada, 1986). Also, the inputs made onto Mauthner cells in the brainstem are consistent with single-cell targeting (Kimmel et al., 1990; Leber and Model, 1991). Furthermore, growth cone selectivity is responsible for the primary muscle preferences of motoneurons observed in both zebrafish and chick embryos (Lance-Jones and Landmesser, 1981; Tosney and Landmesser, 1985; Eisen, 1991; Eisen and Pike, 1992; Landmesser, 1992; Pike et al., 1992).

Partially ablated muscles are correctly innervated in the embryonic chick limb (Whitelaw and Hollyday, 1983a,b; Tosney and Landmesser, 1984), indicating that local cues, possibly derived from the muscles, guide the appropriate motor inputs. Furthermore, Whitelaw and Hollyday (1983b) observed correct innervation following parallel muscle duplications. These observations indicate that chick motoneurons retain their target preferences despite mismatch, and are consistent with our own work in *Drosophila*. By contrast, when muscle masses were serially duplicated in separate segments, Whitelaw and Hollyday (1983b) found that the distal muscles were not innervated by the expected neurons. It is possible that the second, more distal muscle mass is too distant to be detected by the appropriate motoneurons in the embryonic chick limb, which normally make accurate and direct projections to their muscle destinations (Tosney and Landmesser, 1985). By contrast, in *Drosophila* the

experimentally duplicated muscle fibers in a hemisegment are each within the filopodial reach of the RP growth cones.

Molecular mechanisms for target recognition. In general, following experimental mismatch, neurons attempt to innervate their normal targets. This suggests that in both vertebrate and invertebrate systems growth cones have intrinsic synaptic preferences (Langley, 1895; Grinnell and Herrera, 1981; Lance-Jones and Landmesser, 1981; Purves et al., 1981; Landmesser, 1992). It is not known whether chemoaffinity is responsible for the accuracy of synaptic connectivity seen in *Drosophila*, but this is an attractive hypothesis. However, not all the phenomena of synaptic development can be explained by a model based solely on primary target preferences (Prestige and Willshaw, 1976), and recognition is often accompanied by refining mechanisms that may be activity dependent (Meyer, 1982; Schmidt and Edwards, 1983; Reh and Constantine-Paton, 1984; Cline and Constantine-Paton, 1989; Fraser and Perkel, 1990). The role of electrical activity in target refinement during *Drosophila* embryogenesis remains an open question, although changes in electrical activity can regulate the degree of branch arborization in larval motor endings (Budnik et al., 1990).

A mechanism based on growth cone recognition of specific cell-surface molecules has been demonstrated in the *Drosophila* CNS. In the developing nervous system, the expression of fasciclin II, a glycoprotein belonging to the immunoglobulin superfamily, is required for several axon fascicle recognition events (Grenningloh et al., 1991). It is likely that molecules with similar roles exist for peripheral guidance and synaptogenesis.

There is good evidence that *Drosophila* muscle fibers are molecularly heterogeneous, differentially expressing putative transcription factors (Dohrmann et al., 1990), as well as a variety of muscle fiber-specific membrane and cell-surface glycoproteins. For example, glycoproteins known to mediate cellular adhesion *in vitro* (fasciclins I and III; Goodman et al., 1992; Toll: Keith and Gay, 1990; connectin: Nose and Goodman, 1991; Nose et al., 1992) are expressed at specific synaptic contacts during embryogenesis, in some cases by both synaptic partners (Halpern et al., 1991; Nose and Goodman, 1991; Chiba et al., 1992; Halfon et al., 1992; Nose et al., 1992). In addition, enhancer detector lines have been found that reveal genetic loci expressed by specific subsets of muscle fibers (Harkins and Keshishian, 1991; Nose and Goodman, 1991; Hartenstein and Jan, 1992).

Furthermore, cell adhesion molecules may be modified by their degree of glycosylation, and the perturbation of glycosaminoglycans has been shown to disrupt pioneer neuron guidance in insects (Wang and Denburg, 1992). The recent report of polysialic acid (PSA) in *Drosophila* embryos (Roth et al., 1992) raises the possibility that the adhesivity of cell-surface molecules in that system may be regulated by PSA glycosylation, as occurs for NCAM in vertebrate embryos (Rutishauser et al., 1988). In the embryonic chick limb, the degree of PSA glycosylation of NCAM regulates the defasciculation of motor axons in specific regions of developing muscles, and also modulates the adhesivity of other cell adhesion proteins, such as L1 (Landmesser et al., 1990).

In conclusion, the target mismatch experiments described in this article indicate that motoneuron growth cones respond preferentially to specific muscle fibers during the early phases of synapse formation, and that the growth cones retain these preferences despite alterations in the pattern of the musculature. The observations are consistent with the hypothesis that indi-

vidual muscle fibers in the *Drosophila* embryonic body wall have unique features, which could be involved in target chemoaffinity. Identifying the molecular mechanisms that govern neuromuscular target preferences remains a major challenge. Functional tests involving either genetic disruption or the ectopic expression of candidate molecules will be required to test the existence of molecular recognition during synapse formation in this system.

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