

## Nerve-muscle interactions during flight muscle development in *Drosophila*

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### SUMMARY

During *Drosophila* pupal metamorphosis, the motoneurons and muscles differentiate synchronously, providing an opportunity for extensive intercellular regulation during synapse formation. We examined the existence of such interactions by developmentally delaying or permanently eliminating synaptic partners during the formation of indirect flight muscles. When we experimentally delayed muscle development, we found that although adult-specific primary motoneuron branching still occurred, the higher order (synaptic) branching was suspended until the delayed muscle fibers reached a favourable developmental state. In reciprocal experiments we found that denervation caused a decrease in the myoblast pool. Furthermore, the formation of certain muscle fibers (dorsoventral muscles)

was specifically blocked. Exceptions were the adult muscles that use larval muscle fibers as myoblast fusion targets (dorsal longitudinal muscles). However, when these muscles were experimentally compelled to develop without their larval precursors, they showed an absolute dependence on the motoneurons for their formation. These data show that the size of the myoblast pool and early events in fiber formation depend on the presence of the nerve, and that, conversely, peripheral arbor development and synaptogenesis is closely synchronized with the developmental state of the muscle.

Key words: *Drosophila*, Indirect flight muscles, Motoneurons, Metamorphosis, Myoblast

### INTRODUCTION

The formation of a neuromuscular synapse results from a series of coordinated interactions between the motoneuron and the muscle. A major interest in understanding synapse development is to define the nature of the orthograde and retrograde signaling involved. The skeletal muscle synapse of vertebrates has been well studied towards understanding the nature of nerve-muscle interactions during neuromuscular junction formation (Hall and Sanes, 1993). These studies have shown that differentiation of the synaptic partners may initially be independent, but intercellular communication between the two cell-types is essential for the initiation, formation and maintenance of the neuromuscular synapse. Some of the prominent effects of the nerve on muscle differentiation, include the clustering of ACh receptors at synaptic sites, fiber-type specification and the insertion of synaptic membrane specific proteins (reviewed by Hall and Sanes, 1993). In contrast, the target muscle releases neurotrophins for nerve survival (Funakoshi et al., 1995), and affects neurotransmitter release (Xie and Poo, 1986). Interactions between muscle and its motoneuron continue through the life of an animal, and those that take place during initial formation are often recapitulated during nerve/muscle injury (Hall and Sanes, 1993).

The fruit fly *Drosophila* offers the use of a variety of genetic, molecular and cellular approaches to study the formation of neuromuscular junctions (NMJ) (Goodman and Doe, 1993;

Keshishian et al., 1996). Two sets of NMJs are formed during its life cycle: an embryonic set that is used predominantly during the larval stages, and an adult set that is used for specialized adult motor functions. A comparative study of their development is likely to bring to light common themes and pinpoint those genes that are redeployed and others that are novel to the adult system. While development of the two sets of muscles is similar in many ways, there are also major differences (Fernandes and Keshishian, 1995). For example, the muscle pattern in the embryo develops prior to arrival of the motoneurons (Johansen et al., 1989) but during adult development, the muscle and nerve branching patterns develop simultaneously (Fernandes and VijayRaghavan, 1993). Thus, during adult development there exists an opportunity for one synaptic partner to influence the patterning of the other, in a manner similar to vertebrate skeletal muscle.

Another interesting feature of the adult neuromuscular system is that larval motoneurons are respecified during metamorphosis to innervate newly developing adult muscles (Truman et al., 1993). In the periphery the motoneurons withdraw larval synapses and subsequently elaborate adult specific branches over the newly generated muscle fibers (Currie and Bate, 1991; Fernandes and VijayRaghavan, 1993). In the CNS, larval arbors are pruned back, and adult specific dendritic fields form (Truman et al., 1993). The respecified neurons make novel connections to drive an entirely different circuitry for adult specific behaviors (Kent and Levine, 1988). Studies in *Manduca* have shown that interactions with the

target muscle can modulate arbor development in the CNS (Kent and Levine, 1993).

We chose to study nerve-muscle interactions during synaptogenesis in the indirect flight muscles (IFMs) of *Drosophila*. The development of the neuromuscular pattern of these muscles is schematically depicted in Fig. 1. We have previously demonstrated that one muscle group that makes up the IFMs, the dorsal longitudinal muscles (DLMs), can be transiently delayed by 4-6 hours in its development by ablating the larval muscles that are used as scaffolds for myogenesis (Fernandes and Keshishian, 1996). Examining the development of innervation under these conditions allowed us to analyze the effects of target deprivation on motoneuronal outgrowth and differentiation. Our results show that although the target muscle fiber is not required for the formation of adult specific primary neuronal branches, it does exert an effect on the appearance of second order branches. It is therefore likely that surface features of the target fiber are important in the establishment of nerve branches.

In a reciprocal experiment, we cut the nerve that innervates the IFMs (Fig. 1) using a microbeam laser, and examined how the nerve influences development of the two muscles that make up the IFMs: the DLMs that use larval scaffolds, and the dorso-ventral muscles (DVMs) which develop de novo (Fernandes et al., 1991). We find that the nerve is involved in maintaining the size of the myoblast pool, as cutting the nerve resulted in considerably smaller muscle fibers. This is directly correlated with a decrease in the myoblast population. While denervation did not affect the initial distribution of myoblasts in the regions of myogenesis, nor the segregation of myoblasts into distinct muscle forming groups, it reduces the myoblast population. A striking result was that in the case of the DVMs, myoblast fusion was initiated but not sustained and muscle fiber formation was blocked, showing its dependence on the nerve. When the DLMs were forced to develop without their larval scaffolds, they showed an absolute dependence on the nerve for muscle fiber formation, a behavior similar to that of the DVMs. Our results suggest a trophic role for the nerve during myoblast proliferation and an additional role in DVM patterning.

## MATERIALS AND METHODS

### Fly strains

Laser ablations were carried out in transformant lines containing the Myosin Heavy Chain (MHC)-lacZ or the IFM specific Actin (88F)-lacZ. While the MHC-lacZ line was useful to visualize stages before the onset of IFM development, the Actin-lacZ was used to detect the presence of developing IFM fibers.

### Ablations

Ablations were performed in third instar larvae. The animals were anesthetised with diethyl ether and mounted in a drop of saline. The preparation was imaged using Nomarski optics and was observed on a video monitor. Laser ablations were performed as described by Cash et al. (1992) using a pulsed dye laser (VSL 337; Laser Sciences, Inc., Newton, MA USA). Coumarin 440 was used as the laser dye, yielding laser peak pulse energies of 30  $\mu$ J (3 nseconds pulse duration). The laser pulses were focussed on to the desired muscle or nerve using a 63 $\times$  planapochromatic objective (NA 1.4) on a microscope with enhanced Nomarski video imaging (Cash et al., 1992). Between two to four 1-second-long bursts of laser pulses were fired (15 Hz firing frequency) until the muscle or the nerve was severed.

### MF 9 ablations

Three persistent larval muscles in the mesothorax, MF9, 10 and 19' serve as scaffolds for DLMs a+b, c+d, and e+f respectively. The most dorsal of these, MF-9 was ablated. We have previously shown that as a result of ablating MF9, development of the resulting DLM fibers was delayed and fiber number was altered (Farrell et al., 1996; Fernandes et al., 1996). Development of innervation to these fibers was studied using nerve specific markers. The unoperated contralateral hemisegment served as an internal control.

### Nerve ablations

The mesothoracic nerve was cut at three distinct locations (see Fig. 1A): (1) close to the CNS to completely denervate the segment, (2) at the level of the ISN to specifically denervate the DLMs and (3) further in the periphery, (between MFs 10 and 19') to allow regeneration to take place. The ablations were performed in late second instars to early third instar larvae.

After the manipulation, larvae were transferred to food vials and allowed to recover. After the larvae pupated, they were removed at the desired ages, dissected, fixed and processed for antibody staining or for histochemical staining.

## Dye-fills and immunohistochemistry

### Dye-fills

Lucifer yellow (5% w/v in distilled water) was iontophoresed with either a continuous or a 0.5 Hz, 0.1 nA current to dye-fill MN5, the motoneuron that innervates DLMs a and b, the most dorsal pair of DLM fibers that are derived from MF-9. The dye-fills were done in dissected pupal preparations in physiological saline (Cash et al., 1992). Dye fills were initially checked by fluorescence, and were processed with antisera to Lucifer yellow with peroxidase cytochemistry.

### Antibodies

Nerves were visualized using polyclonal antibodies raised in goat (Jackson Labs, 1:250). Myoblasts were visualized using rabbit antibodies to Twist (1:500, gift from Maria Leptin). Antibody staining was carried out as described by Farrell et al. (1996). Polyclonal anti-Lucifer yellow antibodies (1:100) were a gift from Paul Taghert. For double staining, tissues were first processed for X-gal staining and subsequently incubated with antibodies. X-gal staining protocol was as described by Farrell et al. (1996).

### Quantitative analysis

Individual DLM fiber lengths and widths were measured using a video-based micrometry program. Between five and seven measurements were averaged for each fiber. These numbers were used for estimating muscle surface areas.

Extent of myoblast distribution around the DLM fibers was estimated by obtaining video images of the cells, and tracing a perimeter around the population visualized by anti-Twist staining. Each area estimate was an average of five to seven samples.

## RESULTS

### Role of the target muscle in the development of innervation

When MF9, the larval muscle from which DLMs a and b develop, is ablated in third instar larvae, DLM fibers still develop 70% of the time, and express the appropriate Actin isoform (Fernandes and Keshishian, 1996). These later-arising muscle fibers differ from their control counterparts in several ways. They are thinner than their normally developing counterparts, and the

number of fibers is variable (Farrell et al., 1996; Fernandes and Keshishian, 1996). They develop *de novo*, a mode of myogenesis used by the DVMs and almost all other muscles in the pupa. Development of the *de novo* DLMs is delayed by 2-6 hours. We have exploited this experimentally induced delay to test the effect of transient target loss on motoneuron development.

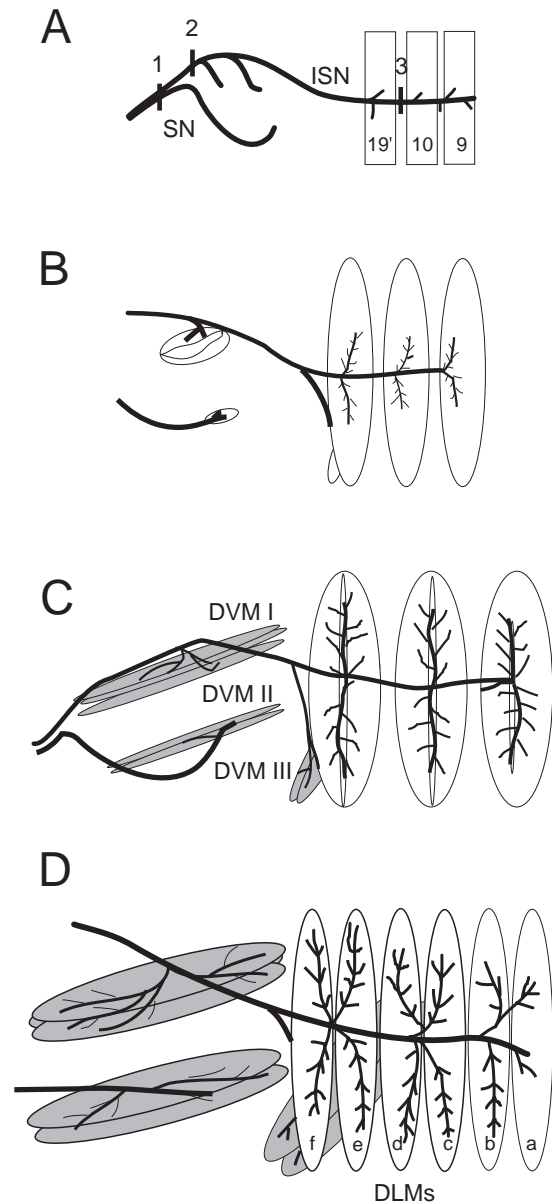
### Development of innervation in control hemisegments

The unoperated contralateral hemisegment served as an internal control for all experiments. Development of DLM innervation in these control hemisegments occurred on schedule as previously described (Fig. 1; see also Fernandes and VijayRaghavan, 1993). By 8 hours APF when three persistent larval muscles are still prominent, the neuromuscular junctions have largely been withdrawn. The larval nerve (ISN) maintains contact with the muscles, usually along their proximal edge (Figs 1A, 2A). By 12 hours APF, the larval muscles elongate, with two prominent nerve branches oriented along the A/P axis (Fig. 1B). The larval muscles subsequently split as myoblasts fuse into them, an event which is very prominent by 16-18 hours APF (Figs 1C, 2C). At this time, the primary branches extend along the surface of the muscles and sprout higher order (2°) branches which are presumably synaptic. By 24 hours APF, splitting is complete and the adult complement of six DLM fibers is present with a characteristic branch pattern that prefigures that of the adult (Figs 1D, 2E).

### Transient muscle loss delays motoneuronal differentiation

The distal end of the larval nerve remains otherwise intact in hemisegments where MF9 was previously ablated in larvae (compare Fig. 2A and B of 8 hour APF pupae). This occurs because the nerve innervates at least two other muscles in the vicinity of the ablated MF9, and contact with these muscles probably ensures retention of the distal ends. The onset of 1° branch outgrowth takes place on schedule despite the absence of a target muscle (Fig. 2D). However, we find that at least one additional branch is present, as compared to controls ( $n=11$ ). Usually, the dorsal most pair of developing DLM fibers is contacted by three 1° branches (Fig. 2C). In the operated hemisegment shown in Fig. 2D, four 1° branches can be seen. Moreover, in contrast to control hemisegments, the branches are not present in an ordered fashion. As a result of larval muscle ablation, DLMs appear *de novo* with a 2- to 6-hour delay and there is a corresponding delay in the elaboration of 2° nerve branches. The 1° branches can remain in the periphery without higher order branching as late as 24 hours APF (Fig. 2C-F).

The appearance of 'de novo' DLMs is preceded by the formation of small, Actin 88F expressing pre-fibers (Fernandes and Keshishian, 1996). However, these are not preferentially contacted by the 1° branches (Fig. 2D). As pre-fibers mature into larger fibers, 2° branches elaborate over the muscle surface (Fig. 2H). By 36 hours APF, when muscles have reached about 1/3 their adult size, all the *de novo* fibers are innervated (Table 1). In every case examined, there were fewer higher order branches in the experimental animals than in controls. Our results suggest that the generation of 2° branches is a response of the innervating motoneuron to cues coming from the developing muscle.



**Fig. 1.** Neuromuscular development of the indirect flight muscles (IFMs) during the first 24 hours of pupal development. The IFMs consist of the dorsal longitudinal muscles (DLMs, a-f) and the dorso-ventral muscles (DVMs, I, II, III). (A) 8 hours APF. Three persistent larval muscles (9, 10, 19') give rise to the DLMs. The larval nerves (intersegmental nerve, ISN, innervating dorsal targets and the segmental nerve, SN innervating ventral and lateral targets) have retracted their larval neuromuscular junctions. 1, 2 and 3 indicate regions of nerve cuts that resulted in complete, partial and transient denervation respectively (see Methods). (B) 12 hours APF. The larval muscles flatten and elongate, and adult specific nerve outgrowth is seen. In the region of the DVMs smaller outgrowths are noticeable. (C) 16 hours APF. The larval muscles split as myoblasts fuse with them to begin formation of the six DLM fibers. Simultaneously, the nerve also undergoes reorganization. Higher-order nerve branches arise at this time. This is the earliest time that DVM fibers can be seen. (D) 24 hours APF. The adult neuromuscular pattern is formed. The DLMs (a-f) and DVM III are innervated by the posterior dorsal mesothoracic nerve (PDMN), which arises from the restructuring of the ISN (Fernandes and VijayRaghavan, 1993). DVM I and II are innervated by the ADMN and the mesothoracic accessory nerve respectively (Sun and Wyman, 1996).

### MN5 retains its target specificity

We wanted to determine if the correct motoneuron is the source of innervation to the de novo DLM fibers, or whether other motoneurons are involved. DLMs a and b are normally co-innervated by a single motoneuron, MN5, while the rest of the DLM fibers are each innervated by single motoneurons (Fig. 3F; Coggshall, 1978; Ikeda and Koenig, 1988). The adult motoneurons are thought to be remodelled larval motoneurons (reviewed by Truman et al., 1993). This is primarily based on observations in a related insect *Manduca*, where intracellular dye fills have revealed both central as well as peripheral reorganization of persistent larval motoneurons. Given that in our case, nerve processes maintain minimal contact with the larval muscles subsequent to withdrawal of neuromuscular junctions, it is likely that MN5 is a persistent larval motoneuron. Alternatively, it could be contacting a muscle target for the first time.

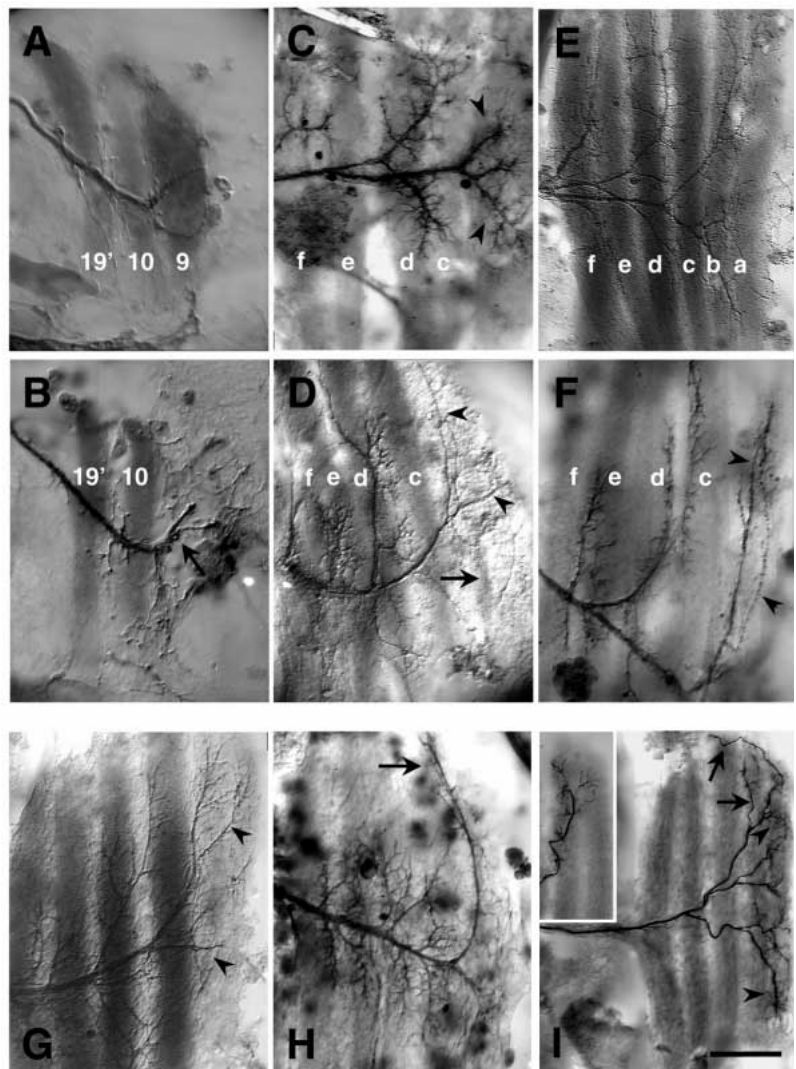
Lucifer yellow dye fills of MN5 in an unoperated animal at 24 hours APF reveals arborizations on DLMs a and b (inset in Fig. 2I), which are identical to those seen with anti-HRP (also see Fig. 2E,H). This confirms that the arbors on DLMs a and b, as revealed with HRP are normally those of MN5. Dye fills of target deprived MN5s at 24 hours APF reveal that

the motoneuron favourably directs its peripheral projections to the de novo DLM fibers (Fig. 2I). However, inappropriate branches are directed to the neighbouring DLM fibers at a low frequency (Table 1). Since such endings are rarely observed in controls, we believe that they are a response to target deprivation, a behavior also seen in studies in the *Drosophila* embryo (Cash et al., 1992). We observe these inappropriate endings as late as 36 hours APF (Table 1). Thus, although MN5 reserves most of its arbor for its target muscles, it is capable of contacting neighboring muscle fibers and making stable, incorrect connections with them following transient target loss.

### Muscle target alterations lead to precocious central arbor development of motoneurons

Apart from observing MN5's behaviour in the periphery we also examined the development of its central arbors. In addition to undergoing a change in morphology in the periphery, respecification of motoneurons also involves a reorganization of dendritic morphology to accommodate novel CNS connections (Truman and Reiss, 1988). The loss of larval arbors is followed by the appearance of adult-specific dendrites. We observed that in normal animals, dendritic

**Fig. 2.** Effect of target deprivation on the development of innervation to the DLMs. A,C,E are controls, at 8 hours, 16 hours and 24 hours respectively; B,D,F are the corresponding operated hemisegments. G-I show more examples of operated hemisegments at 24 hours APF. Nerves in A-G are labelled with anti-HRP. Muscles are visualized by X-gal staining of animals bearing MHC-lacZ (A,B) or 88FActin-lacZ (C-G) constructs. (A) 8 hours APF, control. The larval nerve maintains minimal contact with the persistent larval muscles that later begin splitting. (B) 8 hours APF, operated. The distal end of the nerve (arrow) remains unaffected in the absence of MF 9. (C) 16 hours APF, control. The nerve has elaborated primary and secondary branches (arrowheads) on the developing muscle fibers. (D) 16 hours APF, operated. While adult-specific outgrowths are observed (arrowheads), higher order branches are rarely seen in the region of DLMs a and b. Muscle prefibers, if present (arrow), are not preferentially innervated. Elaboration of nerve processes over the normally developing DLMs (c-f) takes place on schedule. (E) 24 hours APF, control. The 6 DLMs have formed and the nerve has elaborated branches over much of the muscle surface. (F) 24 hours APF, operated. Nerve processes on the de novo developing DLMs show undifferentiated endings (arrowheads), as compared to those on neighboring DLM fibers (c-f). (G) 24 hours APF, operated. Nerve endings on the de novo DLMs have higher-order branches (arrowheads), similar to those on neighbouring DLM fibers. Notice that the de novo fibers are expressing lower levels of Actin-lacZ as compared to DLMs c-f. (H) 24 hours APF, operated. A nerve branch that would normally innervate DLMs a/b makes an inappropriate ending on DLM c (arrow). (I) 24 hours APF, operated. Intracellular dye-fill of MN5 using Lucifer yellow reveals higher order branches (arrowheads) on the single de novo DLM. In addition, at least two inappropriate branches (arrows) are seen. Inset shows, at a higher magnification, DLMs a and b from a control hemisegment which are innervated by one of the three branches of MN5 (using anti-HRP). Bar, 32  $\mu$ m in F and inset in I; 50  $\mu$ m in the rest of the panels.



**Table 1. Effects of delaying DLM development on the maturation of the innervating motoneuron, MN5\***

Age (APF)	% MN5s with secondary branches <sup>†</sup>		% MN5s with inappropriate projections <sup>‡</sup>	
	Normal	Operated	Normal	Operated
14-18h (n=11)	100	0	0	36
20-26h (n=23)	100	26	0	39
>32h (n=14)	100	100	0	36

\*DLM development was delayed by ablating the larval precursor fiber in early third instar. The effects on MN5 development were determined by anti-HRP labeling.

<sup>†</sup>Secondary branches are higher order branches that sprout from the primary nerve branches. The formation of these branches is delayed as a result of target deprivation.

<sup>‡</sup>Inappropriate projections are primary branches of MN5 that contact neighboring DLM fibers (usually DLM c/d). Such branches are only seen in situations of muscle target deprivation.

arbors first appear around 24 hours APF (Fig. 3C). At this time the six DLM fibers have formed from their larval scaffolds, and 2° branches are present on the muscles. In target-deprived animals, we found that there was a precocious development of dendrites equivalent to 4-6 hours of development (Fig. 3B,D,F; Table 2). Similar behaviour is observed in other systems including leech (Jellies and Kopp, 1995; see Discussion). In our target-deprived or altered hemisegments, CNS dendrites were seen as early as 18 hours APF. The morphology bears a close resemblance to later wild-type stages, and hence is likely to reflect adult specific dendritic outgrowth.

### Role of the nerve during IFM myogenesis

To study the role of innervation on IFM myogenesis, the nerve was laser ablated at three distinct locations (see Fig. 1) during the early third larval instar. In the first set of experiments, the nerve was cut near the CNS to completely denervate the hemisegment. These denervations allowed us to examine the effects on both DLM and DVM myogenesis. In a second set of experiments, the nerve branch that projects to the DLMs was cut at a more distal point, where it enters the ventral muscle region. This was done to specifically affect DLM development, while allowing DVM development to take place normally. In a third set of experiments, nerve transections were performed in the periphery, and involved cutting the nerve between MFs 19' and 10, so that MF10 and the more distal MF9 were denervated. These manipulations allowed the peripheral tips to regenerate and therefore test the effect of delayed innervation on myogenesis.

### Denervation results in reduced myoblast distribution and smaller DLM fibers

In the first set of experiments the nerve was cut near the root (at position '1' of Fig. 1A), in flies bearing the MHC-lacZ or the 88F Actin-lacZ constructs. In denervated hemisegments the loss of myofibrillar organization within the persistent larval muscles (as detected by a loss in birefringence; data not shown), and their elongation (Fig. 4A,B) took place normally, indicating that these processes are nerve-independent. The subsequent muscle splitting also occurred in the absence of the nerve, but its onset was delayed by approximately 2-4 hours. This was consistent with the delayed appearance of DLM fibers. We also observed that muscle fiber size was significantly reduced (A).

Using the Actin 88F-lacZ line we observed that in denervated animals there was a 2-3 hour delay in the expression of the lacZ marker in the DLM fibers. The intensity of X-gal staining was lower in denervated fibers than controls (Fig. 4C,D). In order to quantify the effects of denervation, areas of denervated DLMs were measured during the formation of the six fibers from their larval templates (16-20 hours APF) and at later time points after the six fibers were distinguishable (24 hours APF, 36 hours APF; Fig. 5A). Between 16-24 hours APF, the denervated fibers were reduced to 50% of the control fiber areas. Their relative size decreased to 36% of control by 36 hours APF. Since myoblast fusion is largely completed by 26 hours APF (Fernandes et al., 1991), it is likely that the decrease observed at later stages is due to the secondary loss of already developed fibers. This was confirmed by our observations that the entire complement of DLMs was not always present (data not shown). This suggests that the smaller fibers may not be stable enough to proceed through the rest of pupal development.

We hypothesized that the slower pace of DLM development, the reduced fiber area and the lowered intensity of Actin-lacZ staining might reflect a reduced myoblast pool. At 12 hours APF, no differences are observed in the distribution of myoblasts (observed with anti-Twist) between control and denervated hemisegments (Figs 4B, 5A,B). Thus, prior to the initiation of fusion, the nerve has little or no effect on myoblast pool size. This continues to be the case even during 14-16 hours APF, when differences in muscle size/areas begin to be noticeable. Between 20 and 24 hours APF, when the six DLM fibers are formed, myoblast distribution in the denervated hemisegments is clearly reduced compared to the unoperated contralateral hemisegments. (Figs 4C,D, 5B). Although we have not directly tested for myoblast proliferation, our observations of reduced myoblast distribution in denervated hemisegments may reflect an involvement of the nerve in

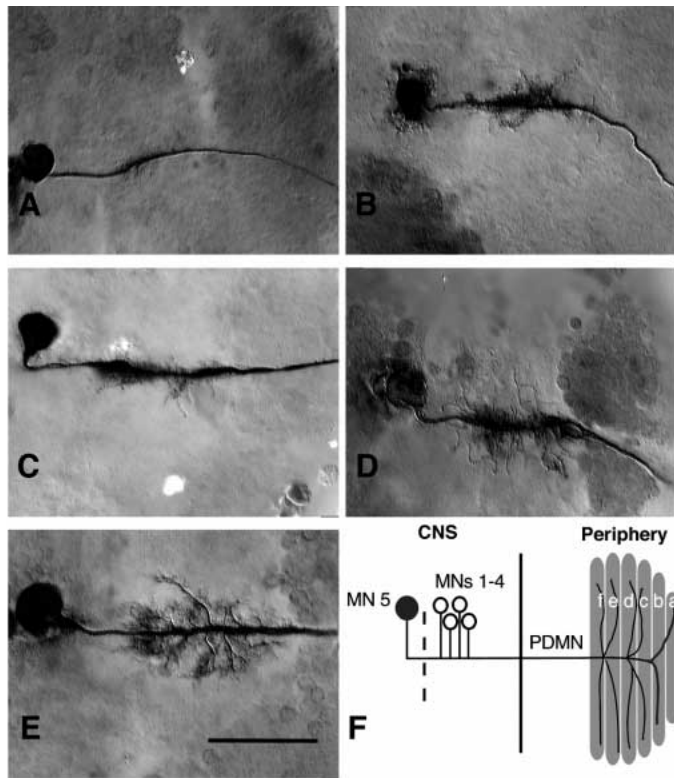
**Table 2. MN5's dendrites arise precociously when the development of the target DLM fibres is delayed\***

Muscle	Developmental status <sup>†</sup>	Age (APF)	% CNS dendrites <sup>‡</sup>	
			Control	Operated
Muscle splitting	Elaboration of motoneuron branching	16-20h	0 (n=10)	100 (n=5)
6 DLMs present	Principal adult branches established	22-24h	36 (n=11)	100 (n=3)
DLMs grow in size	Addition of higher order processes	26-30h	43 (n=14)	100 (n=5)

\*MN5's central arborizations were examined using Lucifer yellow dye fills.

<sup>†</sup>Developmental status refers to the normal status of MN5 and its muscle targets in unoperated wild-type animals.

<sup>‡</sup>The fraction of animals with dendritic processes emerging from the primary neurite of MN5 within the ganglion.

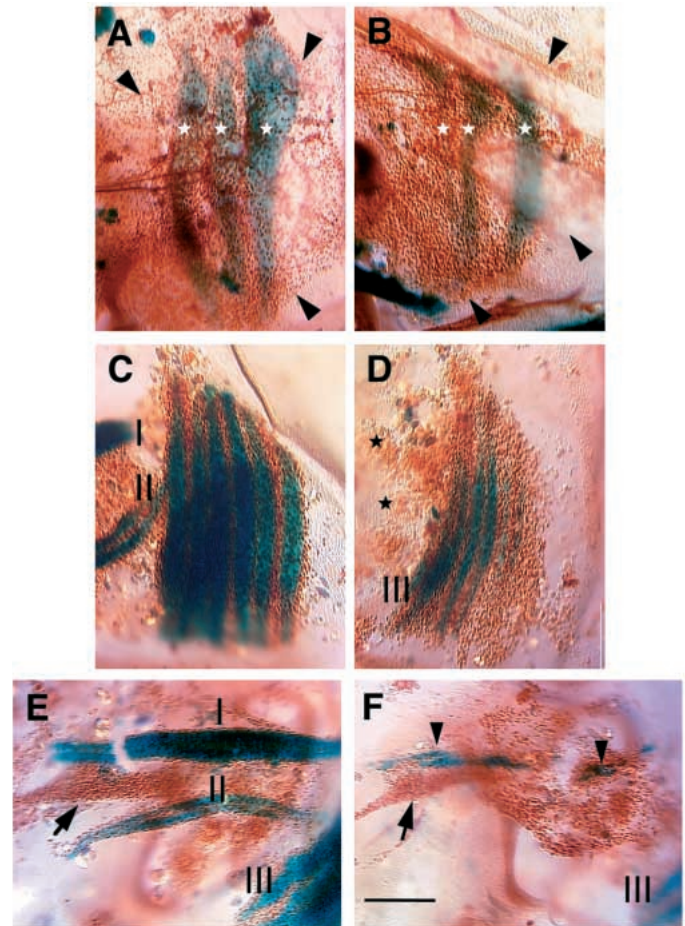


**Fig. 3.** MN5 dye-fills showing the effect of the muscle target on the development of CNS dendritic arbors. (A,C,E) controls at 18, 24, and 48 hours APF respectively. (A) 18 hours APF. The first signs of a dendritic arbor are seen along the axon. (C) 24 hours APF. The arbor becomes prominent. At this time, 6 DLMs have already formed in the periphery. (E) 48 hours APF. The dendritic field further increases. (B,D) Target-deprived hemisegments at 18 (B) and 24 hours APF (D) respectively. Precocious development of arbors is seen in every case examined. The arbors are more extensive than those seen in controls (A and C). (F) DLMs a and b are innervated by a single contralateral motoneuron (MN5), while DLMs c-f are each innervated by a single motoneuron (MNs 1-4). Bar, 50  $\mu$ m.

mitogenesis (see Discussion). Furthermore, since the initial myoblast distribution around the persistent larval muscles is unaffected, the nerve is likely to exert its influence once fusions are underway (see Discussion). Our experiments do not rule out the possibilities that the reduced myoblast pool is due to increased cell death or emigration of myoblasts. Apart from the above mentioned effects on DLM development, we also observed that nerve cuts had a more severe effect on the DVMs (discussed in a later section).

#### Effect of selective nerve cuts on DLM development

In a second set of manipulations one of the two nerve branches (the ISN, which innervates dorsal targets) was cut at a point where it enters the bodywall musculature (cut at position '2' of Fig. 1A). Nerves cut at this location should spare the DVMs. As expected, the DVMs were unaffected (Fig. 6;  $n=30$ ). In all the cases examined ( $n=25$ ), we found that DLM development was considerably retarded, which is similar to the situation when cuts were made at the nerve root. At 18 hours APF, when muscle splitting is well underway in the control hemisegment (Fig. 6A), the muscles in the denervated hemisegment



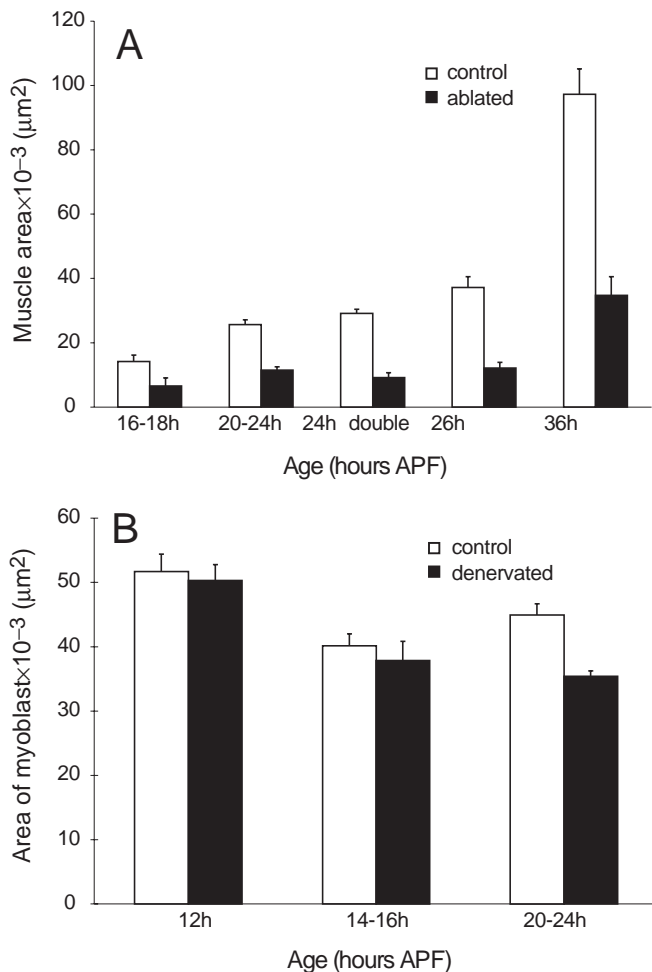
**Fig. 4.** Nerve influence on the myoblast population that gives rise to the DLMs and DVMs. Nerves are labelled with anti-HRP, muscles using MHC-lacZ (A,B) or 88FActin-lacZ (C-F) and myoblasts using anti-TWIST. (A) 12 hours APF, control and (B) 12 hours APF, nerve cut. Myoblast distribution in denervated hemisegments is identical to controls. Arrowheads demarcate the extent of myoblasts in the DLM region. These have also been labelled with HRP showing the nerve in A and a sensory nerve in B. The larval muscles are indicated with a star. The apparent shorter length of muscles in B is due to a fold in the preparation at the anterior end. (C) 24 hours APF, control and (D) 24 hours APF, nerve cut. Differences in myoblast distribution and fiber size are obvious at this stage. The DLM fibers are clearly thinner in the nerve cut hemisegment. DVMs I and II are often missing in denervated segments (stars). (E) 24 hours APF, control and (F) 24 hours APF, nerve cut. (E) Well developed DVMs I and II are absent in denervated hemisegments. Arrowheads indicate pre-fiber-like structures in the region of DVM I. The myoblast population forming the jump muscle (arrow) is smaller in denervated hemisegments as compared to controls. Bar, 50  $\mu$ m.

resemble the normal 12 hour stage. At 24 hours APF, it is not uncommon to find denervated muscles still in the splitting stage (Fig. 6D). The total DLM areas in nerve-cut hemisegments at 24 hours APF (Fig. 6C,D) showed a 50% reduction ( $n=13$ ,  $P<0.008$ ) when compared to control hemisegments.

#### Transient denervation delays DLM myogenesis

When muscle fibers 9 and 10 are peripherally denervated (cut at

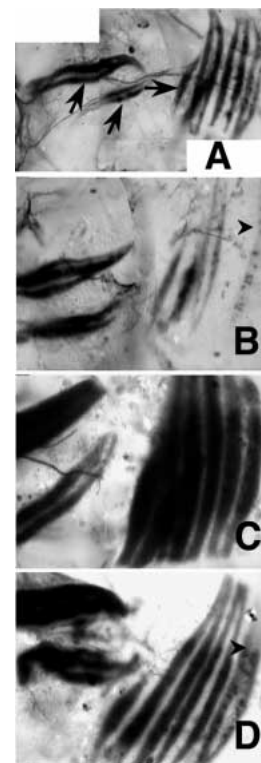
position '3' of Fig. 1), the onset of nerve regeneration is seen around 12 hours APF ( $n=22$ ). This is the time that 1° nerve branches extend over the muscle surface in control animals (Figs 1B, 7A). Since MF10 is closer to the nerve-cut, it is contacted by the nerve branches earlier than MF9 (59%). In a small number of cases, both fibers received contacts (14%). In the rest, neither fibers received nerve branches (27%; Fig. 7B). Nerve branches to MF 19' which is not denervated, are bushier than on the control side (Fig. 7A,B). Four hours later, when muscle splitting is underway (each persistent muscle gives rise to two DLM fibers), MF10 always develops in advance of MF9 (Fig. 7C), and is probably due to the fact that MF10 is reinnervated before MF9. Between 20 and 24 hours APF ( $n=21$ ), DLMs a-d are innervated (67%). At 32-38 hours APF ( $n=9$ ), after myoblast fusion is complete, the number of innervated DLMs increases (78%). In all the reinnervated animals, the pattern of innervation is different from the regular branching pattern seen in controls (compare Fig. 7C with 2E). Our results indicate that the delayed development of innervation does not deter formation of the normal complement of DLM fibers.



**Fig. 5.** Muscle areas and myoblast distribution compared in nerve-cut and control hemisegments. (A) Muscle areas of denervated DLMs compared with contralateral controls. Also included is a comparison of muscle areas in the double (nerve and muscle) ablation. (B) Myoblast distribution around the DLMs in denervated and unoperated controls.

### Innervation is essential for DVM formation

In experiments where the entire mesothoracic nerve was cut close to the CNS (Fig. 1), we observed that while DLM development was retarded (described previously), DVM development was severely affected. This indicates that unlike the DLMs, DVM development has an absolute requirement for innervation and reveals a basis for the difference in modes of development of the DLMs and DVMs. We find that although myoblasts are present in the region of the DVMs, and aggregates of myoblasts in the appropriate regions are seen, full length fibers do not usually develop. In many animals, we see traces of X-gal staining (Fig. 4E,F), indicating initial myoblast fusion events. Like the DLMs, there is a delay in the onset of fiber formation. A case in point is DVMII. At 16-18 hours APF, when DVMs I and III are present to varying extents, myoblast fusions in the region of DVM II as detected by Actin-lacZ staining, are undetectable. At 20-24 hours APF, we begin to see fusions in the region of DVM II, but these are not sustained to give rise to muscle fibers. At 36 hours APF, DVM II fibers are never observed.



**Fig. 6.** DLMs can be selectively denervated without affecting development of the DVMs. Muscles visualized using 88FActin-lacZ. (A) 18 hours APF, control. The six DLMs are still forming. Arrows indicate the three DVM fibers. (B) 18 hours APF, nerve cut. DLMs can be selectively denervated leaving innervation to the DVMs intact. As a result, DLMs are retarded in their development. Arrowhead indicates MF9, which has not yet begun splitting. (C) 24 hours APF, control. The full complement of IFMs. (D) 24 hours APF, Nerve-cut. In hemisegments where DLMs are selectively denervated, DLM fibers develop, but are smaller in size than the control counterparts in the opposite hemisegment. The dorsal most DLM fibers (arrowhead) are still in the process of splitting. The DVM fibers are unaffected. Bar, 150 µm (A) and 100 µm (B-D).

**Table 3. Distribution of DVM fibers in denervated hemisegments\***

Age (APF)	I, II and III (pres)	I, II and III (abs)	I and II (abs) III (pres)	II (abs) I and III (var)	II (abs) I and III (pres)	II (var) I and III (pres)
16-18h (n=18)	0%	33%	39%	17%	11%	0%
20-24h (n=43)	0%	14%	63%	2%	9%	23%
36h (n=17)	6%	0%	88%	0%	6%	0%

\*Several categories of experimental results were obtained. These are indicated in the columns, referring to the presence (pres), absence (abs), or variability (var) of the three DVM bundles, referred to as I, II, and III (see Fig. 1). Variable refers to very small, flimsy fibers. Denervation affects the DVMs in the following order: DVM I>DVM II>DVM III. DVM III develops underneath the DLM fibers.

The DVM primordia also appear smaller than the controls, as does the jump muscle (Fig. 4E,F). The occurrence of DVMs in denervated conditions was quantified and is shown in Table 3. The three DVM fibers are affected to varying degrees by denervation, with DVM II being the most affected. The order of severity is: DVMII>DVMI>DVMIII. For example, at 16-18 hours APF, the probability of finding DVM I or DVM II is 27%. However, by 36 hours APF, DVM I can be seen 6% of the time. At this stage, the only DVM to be present in a majority of the cases (88%) is DVM III. Taken together, the data suggest that in a majority of cases the progression of fusion, to give rise to fibers of DVMs I and II, is not sustained.

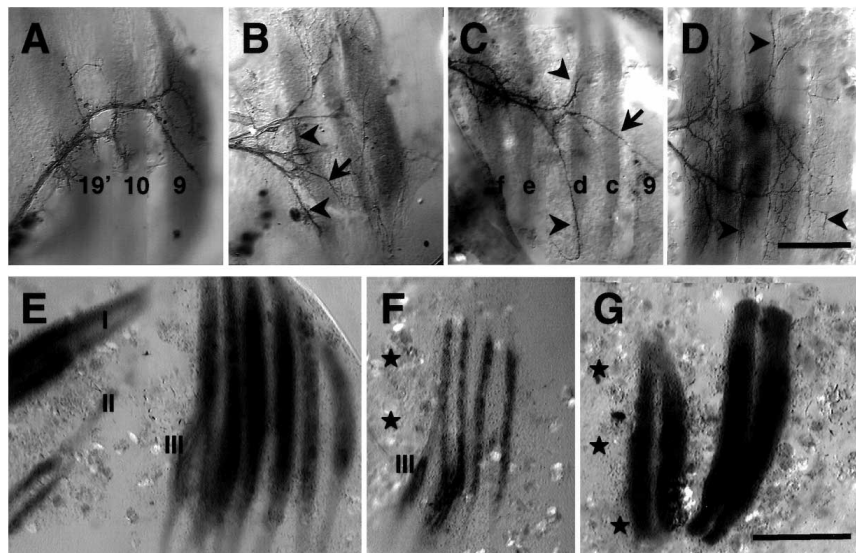
### Nerve cuts combined with muscle ablation

The key difference between the two modes of myogenesis in the IFMs is that the DLMs develop using persistent larval muscle scaffolds, whereas the DVMs develop de novo (Fernandes et al., 1991). We have previously shown that DLM fibers develop even when the larval scaffolds are ablated (Farrell et al., 1996; Fernandes and Keshishian, 1996; this study). Under these conditions, DLM myogenesis uses a developmental mode in essence similar to that of the DVMs (Fernandes and Keshishian, 1996). In the present study, we find that combining denervations with MF9 ablations results in a complete failure of DLM (a and b) development ( $n=14$ ). Although myoblast fusion is initiated, we never observe mature fibers (Fig. 7F,G). At 20-24 hours APF the DLMs consists solely of fibers c-f, and occupy an area which is smaller (32%) than observed for denervations alone (45%; fibers a-f).

### DISCUSSION

We investigated nerve-muscle interactions during pupal development of the Indirect Flight Muscles (IFMs) of *Drosophila*. Our findings demonstrate that as a result of target muscle loss, 2° nerve branches, which probably prefigure synaptic contacts, fail to develop. However, these 2° branches develop

when a replacement target muscle (de novo DLM) forms. Thus, unlike 1° branches, the formation of 2° branches awaits appropriate fiber formation. Furthermore, since the de novo DLMs are innervated by the native motoneuron, our studies demonstrate the existence of fiber specific connectivity in the IFM neuromuscular system. Upon denervation, the myoblast pool is reduced, and the DLM fibers are thinner. This indicates an important role of the nerve in regulating myoblast number. In contrast to the DLMs, the DVMs are more dependent on the nerve, since denervation severely affects DVM development. When the DLMs are forced to develop de novo (similar to the DVMs) their development becomes nerve dependent. Thus the nerve plays an additional role in fiber formation of a specific



**Fig. 7.** Further examples of the effects of denervation. Muscles visualized using MHC-lacZ (A,B) and 88FActin-lacZ (C-G). Nerves are labelled with anti-HRP. (A-D) Peripheral denervations. (A) 12 hours APF, *control*. Three persistent larval muscles with the first signs of nerve outgrowth. (B) 12 hours APF, Nerve-cut. Peripheral denervations that result in MFs 9 and 10 being denervated. MF 19' is innervated (arrowheads) and receives many more higher order branches than in the control. Arrow points to a regenerating branch to MF 10. MF 9 is not innervated at this time. (C) 16 hours APF, Nerve-cut. Regenerating branches on DLMs c and d (arrowheads) and to MF9 (arrow). MF9 has not begun splitting in this case. (D) 26 hours APF, Nerve-cut. Regenerating nerve branches (arrowheads) have innervated DLMs a-d. The branching pattern of the nerve is different from that seen in controls (compare with 2E). (E-G) Effects of denervation under conditions of MF9 ablation. (E) 20 hours APF, *control*. The full complement of the DLM and DVM fibers. (F) 20 hours APF, *operated*. 4 DLMs (c-f) and DVM III are present. Stars indicate the position of DVMs I and II. (G) 36 hours APF, *operated*. 4 DLM fibers (c-f) are present. All DVM fibers are absent (asterisks) in this animal. Bars: in D (A-D) 50  $\mu$ m; in G (E-G) 100  $\mu$ m.



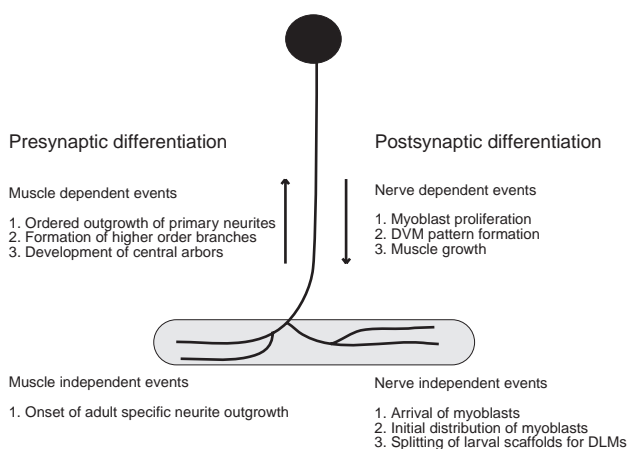
group of muscles, the DVMs. The studies we describe, have allowed us to dissect neuromuscular development of the IFMs into nerve- and muscle-dependent steps (Fig. 8).

### Role of the muscle fiber in the differentiation of the motoneuron

#### Peripheral branch development

The onset of adult-specific 1° branches is unaffected by target ablation. This suggests that 1° branch development is independent of the state of the target muscle fiber. Similar conclusions have also been drawn from studies in *Manduca* (Truman and Reiss, 1995). It is believed that ecdysteroids act directly on the cell body of the motoneuron to direct the peripheral outgrowth response (Prugh et al., 1992). Nevertheless, under conditions of target deprivation we observe an increased number of 1° branches and the pattern of outgrowth is not ordered as in controls suggesting that muscle-derived cues play regulatory or mechanical roles during the process. More striking is the observation that the subsequent development of 2° branches is stalled until a muscle fiber develops. This strongly argues for the involvement of retrograde cues in the formation of higher order nerve branches. A role for such cues during synaptogenesis has been indicated from embryonic studies in *Drosophila* (Prokop et al., 1996).

Similar conclusions were reached from studies in *C. elegans* (Plunkett et al., 1996). While the elimination of specific dorsal and ventral muscles led to normal morphological development of the innervating DD motoneurons, the subsequent development of synaptic varicosities did not occur. Our results in *Drosophila* indicate a specific instructive role for the target DLMs a and b for MN5s higher order branching. The adjacent fibers are permissive for the elaboration of higher order processes by MN5, as dye-fills revealed inappropriate contacts on the neighbouring DLMc. This is a common response to



**Fig. 8.** Orthograde and retrograde signalling events during neuromuscular development of the IFMs. This figure summarizes our key observations concerning the nerve-dependent and -independent events during adult myogenesis, as well as the influence of a muscle target on the development of motoneuron arbors. Development in this system depends on a complex interaction between pre- and postsynaptic partners, presumably through as yet unidentified signalling mechanisms.

denervation, and has also been observed in embryonic target deprivation studies (Cash et al., 1992). However, unlike the embryonic experiments, in the pupa a de novo muscle target arises after a brief delay. Under these conditions, the deprived motoneuron eventually makes endings on its normal muscle target, even when there is only one fiber instead of the normal two. Taken together, these results indicate that although the muscle surface is permissive for motoneuronal contacts, the appropriate motoneuron will reserve most of its endings (which presumably lead to synapse formation) for the correct muscle fiber targets.

#### Arbor development in the CNS

Studies in *Manduca* have established that persistent larval motoneurons are respecified during metamorphosis to innervate the adult muscles (Levine and Truman, 1985; Kent and Levine, 1988; Weeks and Truman, 1986). This involves withdrawal of larval branches both in the periphery and in the CNS, the subsequent outgrowth of new arbors, and their incorporation into novel adult neural circuits. Although the onset of dendritic remodeling is known to be target-independent, cues from the target are involved in shaping the growth of the CNS dendritic arbor (Kent and Levine, 1993). In our case, we show that target deprivation causes precocious development of CNS dendrites. Such behaviour has also been observed in leech, where ablating the heart muscle causes sprouting in the heart excitor (HE) motoneurons that normally innervate these muscles. An effect is also seen in the periphery, where neurites wander and make inappropriate connections (Jellies and Kopp, 1995). A different kind of regulation of central neurogenesis is seen in the case of the leech reproductive duct motoneurons. By ablating the peripheral targets, the genesis of several central neurons is affected, and is thought to be due to inductive signals which are retrogradely transported to the hemiganglion via specific central neurons (Becker et al., 1996). Thus, a peripheral target can influence the development of both peripheral as well as central arbors, and the processes are likely to involve retrograde signals.

### Role of the motoneuron in the differentiation of the muscle fiber

The role of the nerve during adult muscle development in *Drosophila* has been a long-standing question, since the observations of Lawrence and Johnston (1986) on the development of the male specific muscle (MSM), of the fifth abdominal segment. Clonal analysis indicated that the identity and formation of the MSM was determined by the innervating nerve (Lawrence and Johnston, 1986). Subsequently, it was shown that denervation of the appropriate hemisegment prior to metamorphosis, prevented formation of the MSM as well as the expression of a characteristic Actin isoform in fibers that developed in the region (Currie and Bate, 1995). These studies also showed that the somatic body wall muscles differentiated normally, although they were thinner than the controls. Similar results were obtained when the myoblast pool was reduced by using hydroxyurea to block dividing cells (Taylor and Knittel, 1995). The MSM is larger than the neighbouring body wall muscles, and its formation is believed to be more sensitive to fluctuations in the myoblast pool. It has been proposed that the product of the *fruitless* gene is involved in recruiting myoblasts into the developing MSM, and that this process may be nerve

dependent (Taylor and Knittel, 1995). By examining the role of the nerve during the early stages of myoblast proliferation and fusion, our experiments further suggest that the size of the myoblast pool may be influenced by the presence of the nerve, and that different muscle subsets may have an additional requirement for the nerve in establishing muscle pattern.

### Motoneuron influences on DLM myogenesis

One of the first observable effects of denervation is a delay in the appearance of DLM fibers. Prior to the initiation of fusion, the initial population of myoblasts appears normal. Thus the delay in fiber formation is likely due to a delay in the onset of fusion. Previous studies have suggested that a communication between the persistent larval muscles and the overlying myoblasts is involved in the onset and progression of fusion (Fernandes and Keshishian, 1996). Taken together with the present study, it thus appears that both the muscle and the nerve are involved.

As fusion progresses, it is evident that the DLM fibers are thinner in size, a condition that persists through later stages. A delay in fiber formation was also observed in studies where persistent larval muscles were ablated (Fernandes and Keshishian, 1996; this study). However, in contrast to our denervation study, the total muscle volumes in those studies remained constant (Farrell et al., 1996; Fernandes and Keshishian, 1996) presumably due to an intact myoblast pool. The formation of reduced DLM fibers in denervated conditions is therefore consistent with a delay in the onset of fusion as well as a reduced myoblast population. These results suggest a regulation between fusion and myoblast pool size. An initial myoblast pool is likely to be depleted as fusion gets underway. We propose that during normal muscle splitting, when fusion is at its peak, the myoblast pool needs to be maintained at a certain threshold level. It is conceivable that nerve-derived factors are involved in replenishing the myoblast pool by regulating myoblast proliferation rates.

Support for proliferation inducing nerve-derived factor(s) comes from in vitro studies done in *Manduca*, which showed that myoblast proliferation is increased when myoblasts are cultured in the presence of neurons (Luedeman and Levine, 1996). The same study showed that a contact-mediated or short-range signal was responsible for the observed increase in proliferation. Furthermore, studies of leg muscle development in *Manduca* have shown that accumulation and proliferation of myoblasts is compromised in denervated conditions (Consoulas and Levine, 1997), although the nerve-derived factors have not yet been identified. Reductions in fiber size brought about by denervation have also been observed in the *Drosophila* abdomen (Currie and Bate, 1996) as well as in butterfly (Neusch, 1985). A related situation exists in the *Drosophila* eye. During retinal development, the ingrowing retinal axons trigger a final round of division of the laminar precursor cells (Selleck and Steller, 1991). Recently, this effect has been shown to be mediated via the Hedgehog protein, which is transported down the axons (Huang and Kunes, 1996).

### Motoneuron influences on DVM patterning

We observed that while the DLMs develop, albeit more slowly, in the absence of a nerve, DVM myogenesis is abolished. The difference in nerve requirement between DLM and DVM development is yet another difference in the two related muscle

groups that has become apparent. There is a genetic basis for these differences as mutations exist that differentially affect the two muscles. For example, viable mutations at the *stripe* locus affect DLM development while sparing the DVMs (Costello and Wyman, 1986). In contrast, DVM development is specifically affected in mutations at the *rbp* complementation group of *Broad-Complex (BR-C)*, a locus regulated by 20-OH ecdysone, leaving the DLMs largely unaffected (Sandstrom et al., 1997). Both genes are involved in the formation of attachment sites, which is a later event in the formation of the adult muscles. Thus, other loci remain to be identified that might control the nerve influences on myogenesis.

The most striking difference between these two muscle groups is that they develop in different ways. Early during pupation, myoblasts segregate around the persistent larval muscles and fuse with them to give rise to the DLM fibers. In the DVM region, they form aggregates where fusion is initiated to give rise to the adult fibers. Denervation does not completely abolish the initial fusion in the DVM myoblasts, but prevents the progression to formation of a mature fiber. This effect is mimicked when the DLMs are forced to develop de novo by ablating their larval scaffolds. Under these conditions, if the hemisegment is also denervated, the DLMs do not develop. We also noted that in addition to the DVMs, the jump muscle had a reduced number of myoblasts. We do not yet know if the leg muscles and the direct flight muscles depend on innervation for their development in a manner similar to the DVMs. Our observations nevertheless suggest that nerve dependence during muscle patterning could be a common feature of de novo myogenesis. It is also consistent with a hypothesis put forward in an earlier study, that the use of larval scaffolds in DLM development is a superimposition on a common mode of de novo development (Fernandes and Keshishian, 1996).

### A working model

The early formation of the DLMs and the DVMs (during the first 24 hours of pupal development) can be divided into two stages (Fig. 8): (1) an initial nerve-independent stage, during which a pool of myoblasts is generated, and the persistent larval muscles are prepared for the initiation of fusion; (2) a second nerve-dependent phase when the size of the myoblast pool increases. The differences in formation of the DLMs and the DVMs following denervation is evident during this stage. We propose that the role of the nerve in myoblast proliferation is to establish a critical level of myoblasts during fusion. In the case of the DLMs, a lower level can sustain fiber formation because the myoblasts fuse with the larval scaffolds. The formation of the DVMs, which are not prefigured by larval muscles, is more sensitive to a myoblast pool falling below threshold. In this case there is an absolute requirement for the nerve, so as to ensure the initiation and progression of fusion to generate mature fibers. By virtue of the persistent larval muscles, the DLMs therefore can bypass a requirement for the nerve, but fiber size is compromised because the larval muscles are not sufficient to sustain myoblast proliferation and maintain the myoblast pool. The delay in DLM fiber formation (directly related to decreased fusion due to a decreased pool of myoblasts) suggests a link between fusion and the need to maintain a steady proliferation rate to replenish the myoblast pool.

The proposed requirement for a threshold level of myoblasts

to ensure continued fusion is supported by observations on the development of DVMIII. Among the three DVM fiber bundles, DVM III behaves differently from the other two fibers. It develops underneath the DLMs, and is the most frequently occurring DVM in denervated animals. It is possible that the proximity of DVM III to a larger source of myoblasts (the DLM pool) creates an above threshold pool for its myogenesis. The sustained fusion in this DVM could also reflect some trophic support from the DLMs developing in the vicinity. This raises the possibility of the muscle fibers also being a potential source of trophic support for myoblasts.

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