

Pathfinding in the central nervous system and periphery by identified embryonic *Drosophila* motor axons

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Summary

We have studied the pattern of axon outgrowth from the identified embryonic *Drosophila* motoneurons, RP1, RP3, RP4 and RP5, from the onset of axonogenesis to the time of arborization over target muscles. Lucifer Yellow was intracellularly injected into each of these neurons to obtain a detailed description of the morphology of their growth cones and of the pathways that they follow. We have divided the sequence of axon growth from these neurons into five major phases. In the first phase, the growth cone of each RP axon grows medially along its contralateral homologue along the anterior commissure. Each RP axon follows a separate path across the midline in the anterior commissure. After crossing the ventral midline, the axons wrap around specific contralateral RP somata. In the second phase, each axon grows posteriorly and dorsally down the contralateral longitudinal connective, fasciculating with the other RP axons. In the third phase, the axons

turn into the intersegmental nerve *via* the anterior nerve root, then cross over to the segmental nerve, before contacting the external surfaces of intermediate muscles 15/16. They do not fasciculate with the pioneering aCC and RP2 axons at this time. In the fourth phase, the axons advance laterally across the ventral muscle group. During this phase, each axon extends processes over a number of inappropriate muscles as well as contacting its correct, target muscle. In the final phase, the processes to inappropriate muscles are withdrawn, generating the mature pattern of motor axon projections. There is no consistent, clear difference between the RP motoneurons in the relative timing of axon outgrowth.

Key words: *Drosophila* embryo, motoneurons, neural development, axon guidance, insect embryo, growth cones.

Introduction

We would like to understand how the specific pattern of connections between motoneurons and muscles is established during the course of embryonic development. The advanced genetic techniques available in the fruitfly *Drosophila melanogaster* (Rubin, 1988; Thomas and Crews, 1990) make it an attractive model organism for investigating the molecular basis of this developmental phenomenon. However, there are considerable technical difficulties associated with working at the cellular level on an embryo of the small scale of *Drosophila*. Consequently, only a limited amount of information is available about the cellular processes underlying the establishment of specific neuromuscular connections in this insect. This is in contrast to our detailed view of motor axon growth in the much larger locust embryo (Ball *et al.* 1985; Whittington, 1989; Myers *et al.* 1990). This lack of information at the cellular level has hampered the interpretation of genetic/molecular studies of neuromuscular specificity.

In reports of previous studies of the *Drosophila* embryo (Thomas *et al.* 1984; Canal and Ferrus, 1987;

Jacobs and Goodman, 1989b), the choices of pathway made by a subset of identified motoneurons within the central nervous system (CNS) have been described. However, earlier descriptions of motor axon growth in the periphery (Hartenstein, 1988; Johansen *et al.* 1989) have been based on immunohistochemical techniques that stain groups of axons and do not reliably reveal the somata of these motoneurons. Thus a description of the complete sequence of axon growth from individual, identified motoneurons, from the earliest stages of axon outgrowth to arrival at the target muscle, is lacking. Detailed information of this type is needed to pinpoint the nature and degree of specificity of cues that guide the axons to their targets as well as the cellular mechanisms that generate the mature pattern of connections. Furthermore, this level of resolution would greatly assist interpretation of data such as the cellular localization of gene products or the pattern of defects seen in developmental mutants.

We have recently characterized the motoneuron population in abdominal segments A3 to A7 of the *Drosophila* embryo and have determined the muscle targets of those motoneurons that can be reliably

recognized from their soma positions (Sink and Whittington, 1991). In the present study, we have used intracellular dye injection to determine the cellular contacts made by the growth cones of a subset of these motoneurons, neurons RP1, RP3, RP4 and RP5, from the onset of axon outgrowth to the time of arborization over their muscle targets. We have pinpointed a number of neuronal and non-neuronal cells that are likely to be involved in the guidance of these motor axons at various phases of their growth. Furthermore, we have revealed that the final specific pattern of neuromuscular connections is attained in part by the initial extension of processes over both target and non-target muscles, followed by the retraction of inappropriate processes. A similar developmental sequence has recently been described for motoneurons in the locust embryo (Whittington, 1989; Myers *et al.* 1990).

Materials and methods

Wild-type *Drosophila melanogaster* (Oregon-R) embryos from our laboratory colony were used in this study. The embryos were collected from yeast-pasted food jars, chemically dechorionated by agitation in a 25% commercial bleach solution, and staged according to the morphological criteria of Campos-Ortega and Hartenstein (1985). They were dissected on a clean glass slide under *Drosophila* saline (6.5 g NaCl, 0.14 g KCl, 0.20 g NaHCO₃, 0.12 g CaCl₂, 0.01 g NaH₂PO₄ in 1 l of distilled water) held in a Vaseline dam. The anterior end of the egg was cut off and the embryo was squeezed from the vitelline membrane. The embryo was dissected longitudinally along the dorsal midline with a sharpened tungsten needle, the digestive system removed and the bodywall gently flattened onto the slide.

For intracellular dye injections, the RP motoneuron somata were identified in unfixed embryos using Zeiss Nomarski optics and a Leitz water immersion objective. Somata were penetrated with 30–60 M Ω microelectrodes filled with a 5% Lucifer Yellow (LY) solution. The LY was iontophoretically injected with a 0.2 nA DC hyperpolarizing current for 20 s.

Anti-LY immunohistochemistry was used to stain LY-injected neurons with a non-fading reaction product that could be visualized in transmitted light under Nomarski optics. Injected embryos were fixed in 4% paraformaldehyde in Millonig's buffer, washed in phosphate-buffered saline (PBS), and incubated overnight at 4°C in anti-LY antibody (prepared in our laboratory, according to the procedure of Taghert *et al.* 1982) diluted 1:500 in PBS/0.4% Triton X-100/0.25% bovine serum albumin (PBT). Embryos were then washed in PBS, and incubated overnight at 4°C in horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Amersham) diluted 1:250 in PBT. The embryos were then washed in PBS, incubated for 60 min in 0.5% diaminobenzidine in PBS, and reacted with 0.006% hydrogen peroxide until the injected neurons were stained dark brown. Following a final wash in PBS, the embryos were cleared and mounted in 100% glycerol.

Preparations were examined on a Zeiss photomicroscope with Nomarski optics using a Zeiss Planapo 100 \times or 63 \times oil immersion objective. Stained neurons were drawn with the aid of a camera-lucida and photographed with Tech Pan film.

Results

Muscle arborization of neurons RP1, RP3, RP4 and RP5 in the stage 16 embryo

We have previously described (Sink and Whittington, 1991) the pattern of arborizations of neurons RP1, RP3, RP4 and RP5 (hereafter called the RP neurons) on abdominal muscles in stage 16 embryos, a stage near the end of embryogenesis (hatching takes place at stage 17). The axons of RP1 and RP4 arborize along the medial face of muscle 13; the axon arborization of RP3 runs anteriorly and posteriorly along the border between muscles 6 and 7; while the axon of RP5 arborizes on muscles 13, 14, 15 and 16 (Fig. 1). For the purposes of this study, we have taken these to be the mature arborization patterns for each of these neurons. The somata of each of these neurons can be reliably recognized under Nomarski optics throughout axonogenesis. They lie in stereotypic positions with respect to the scaffold of axons formed by the transverse commissures and the longitudinal connectives in each segment. RP1 and RP4 lie dorsal to this axon scaffold, RP3 at middle levels of the scaffold and RP5 ventral to the scaffold.

Fig. 2 is a schematic map of the region of abdominal musculature encountered by these neurons as they grow to their target muscles. Our description of the muscle pattern accords with that provided by Crossley (1978), except that we recognize a new muscle, lying just lateral and slightly external to muscle 14. This muscle appears in Crossley's figures but is unnumbered. A recent study (Bate, 1990) used the number 14.2 for this muscle and renumbered muscle 14 as 14.1. We prefer to use number 30 for this new muscle as Bate's nomenclature may imply that there is a special relationship between this muscle and muscle 14.

General comments

The following description of the patterns of axon growth from the RP neurons is based on a total of 58 fills of RP1 in 51 embryos, 79 of RP3 in 57 embryos, 67 of RP4 in 49 embryos, and 37 of RP5 in 32 embryos. These observations were spread across the developmental period under investigation and were confined to neurons in abdominal segments A3–A7. Within an individual embryo at a given stage of development, any one of these motoneurons showed a similar morphology across these segments. There was no indication of a consistent anteroposterior sequence in axon outgrowth from segmentally homologous neurons within segments A3–A7.

The developmental sequence of axon growth from motoneurons RP1, RP3, RP4 and RP5

For the purposes of this study we have divided the sequence of axon growth from the RP motoneurons into five phases. Jacobs and Goodman (1989b) have described the pattern of axon growth for RP1 and RP3 from axonogenesis up to entry of the axon into the peripheral nerve. Our observations, obtained by intracellular LY injections, agree closely with their

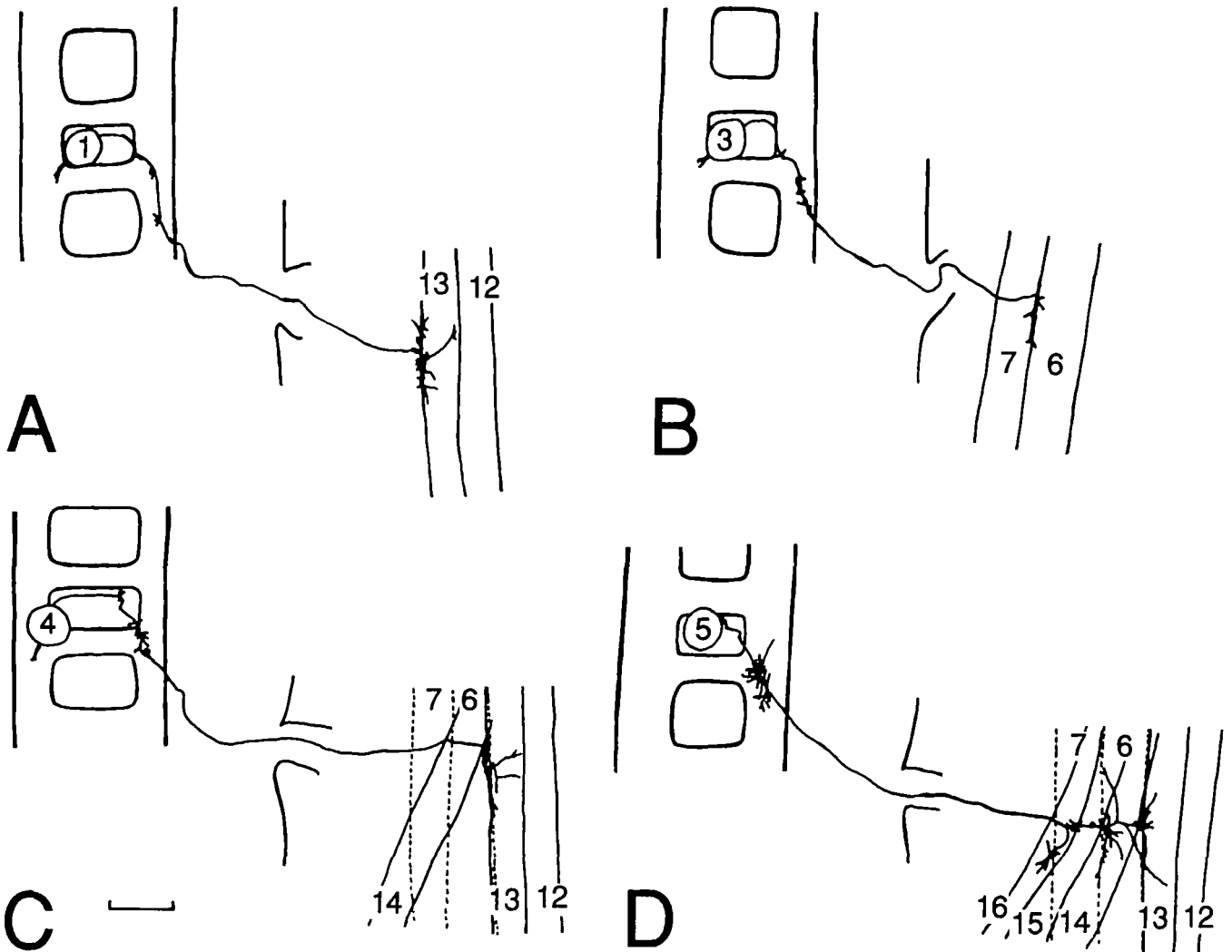


Fig. 1. Camera-lucida drawings showing arborizations of RP motorneurons over target muscles in stage 16 embryos (A) RP1 contacting muscle 13, (B) RP3 contacting muscles 6 and 7, (C) RP4 contacting muscle 13, (D) RP5 contacting muscles 13, 14, 15 and 16. Scale bar=10 μ m.

data, drawn from reconstructions of electron micrographs.

Growth across the midline

Axonogenesis begins in stage 12 for each of the RP neurons and follows a similar pattern in each case. A lamellipodial veil approximately 5 μ m long extends from the soma towards the ganglion midline. Short filopodia (1–1.5 μ m) are present along the lateral edges of the veil while one to two longer filopodia (up to 5 μ m) are at the leading edge (Fig. 3A).

Slightly later, the veil thins into an axon and extends medially, crossing the embryonic midline in the process. Dual fills in the same segment show that each of the RP axons fasciculates with the axon of its contralateral homologue at this time (Fig. 3B). Each RP axon pair runs in the anterior commissure, separated from the others along both the dorsoventral and the anteroposterior planes; the axons of RP1 and RP4 lie dorsal to those of RP3 and RP5. Growth of the

RP axons appears to be largely synchronous at this stage; none is obviously leading the others. This finding is based upon fills of two different RP motorneurons in the same hemisegment in the same individual (Fig. 3C). Fills of homologous neurons in different segments in the same individual reveal small differences in the extent of axon outgrowth, but these differences are not consistent and there is no apparent gradient along the anteroposterior axis (data not shown).

During stage 13, the growth cone of the RP1 motorneuron wraps around the anterior and lateral surface of its contralateral homologue and at the same time the somata move medially to take up their final positions inside the rectangular grid formed by the anterior and posterior commissures and the longitudinal connectives (Fig. 3D). The axons of RP3, RP4 and RP5 show a similar behaviour. However, while RP3's axon wraps around its contralateral homologous soma (Fig. 6A), the axon of RP5 appears to associate with the contralateral RP3 soma and not with the contralateral

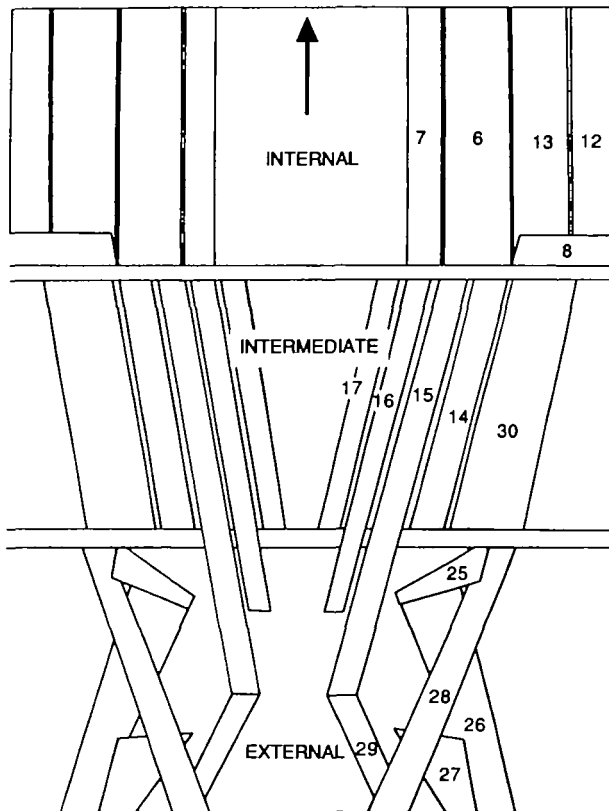


Fig. 2. Schematic diagram of the region of musculature encountered by the RP neurons, showing the muscles in three adjacent abdominal segments at internal, intermediate and external levels. External is closest to the cuticle. The arrow runs along the ventral midline and points anteriorly.

RP5 soma. Filopodia from RP4's growth cone contact the contralateral RP1 soma as well the contralateral RP4 soma. In five fills of RP1 (Fig. 3E) and four of RP3, the growth cones of these neurons also extended processes along the medial and posterior surfaces of their contralateral somata. Such branches are apparently withdrawn soon thereafter as they were never seen in later stage embryos.

Growth down the contralateral longitudinal connective

Contact with a contralateral RP soma brings the growth cone of each of the RP motorneurons within filopodial grasp of the contralateral longitudinal connective (CLC), which has been previously pioneered by the axons of neurons pCC, MP1, vMP2 and dMP2 (Jacobs and Goodman, 1989b); the longest filopodia span the width of the CLC at this stage. During stage 14, the axon of each of the RP neurons extends posteriorly down the CLC along a route that is oblique and sometimes not straight (Fig. 4A,B). The previously separate RP axons fasciculate within the CLC (Fig. 4A) and lie dorsally within this tract. Dual fills reveal that the RP axons have extended approximately the same distance posteriorly, although in different embryos there are variable differences in the extent of growth of these axons (Fig. 4A).

Growth to the ventral muscle group

In stage 14, the RP axons diverge from the CLC, turning laterally into and growing along the dorsal surface of the anterior root of the intersegmental nerve (ISN; Bate, 1982), which has been previously pioneered by the axon of neuron aCC. Typically, several long (up to 5 μm) filopodia are directed towards the anterior root of the ISN as the growth cone turns (Fig. 4B). While the growth cone is within the nerve root, filopodia extend across its whole width. All of the RP axons show the same behaviour at this stage.

The axons of motorneurons aCC and RP2 extend into the periphery along the anterior and posterior roots of the ISN, respectively, before the axons of the other RP neurons (Jacobs and Goodman, 1989b). This makes the aCC and RP2 axons candidates for guiding the RP axons by a process of selective axon fasciculation. To test this hypothesis, we performed dual fills of the RP neurons and aCC or RP2. We observed that the RP axons do not fasciculate with aCC or RP2 within the ISN.

Once outside the ganglion, the RP axons cross over from the ISN to the segmental nerve (SN; Bate, 1982) (Figs 4C, 6B). The RP axons remain fasciculated during this stage. Shortly thereafter, early in stage 15, the RP axons extend processes that contact the external surface of the ventral muscles 15 and 16 (Fig. 4D).

At about this time, each of the RP somata sends out a collateral branch that extends into the ipsilateral longitudinal connective (Fig. 4D). This branch is consistently present later in embryonic and larval development, attaining a length of up to 5 μm in late embryonic stages. Dendritic branches also start to appear within the neuropile contralateral to the soma (Fig. 4D).

Growth over the ventral muscle group

Having reached muscles 15/16, the RP axons diverge from the main SN nerve into SN branch b (SNb; Johansen *et al.* 1989) and contact the internal surface of muscle 28. RP5 also sends a process into nerve SN branch d (SNd; Johansen *et al.* 1989). Lateral to muscle 28, the RP axons grow between the most internal and its adjacent, intermediate muscle sheet (Fig. 2). While growing over the ventral muscles, the RP growth cones become more expansive and send processes over the surface of the muscles that they contact (Figs 4D, 5, 6C). Some of these processes may be axonal branches rather than filopodia, since they appear to be thicker than typical filopodia, are of non-uniform width and are sometimes branched. The pattern of muscles contacted by a RP neuron in any given embryo is variable (illustrated by RP3, Fig. 5). However, examination of a number of embryos suggests that each of the RP axons is able to arborize over all or most of the following muscles; the internal surface of muscles 12, 13, 14, 15, 28 and 30 and the external surface of muscles 6 and 7.

The pattern of initial axon arborization on a given muscle by an individual motorneuron is both variable and more widespread than at the end of embryogenesis, as shown, for example, by the arborization of RP3 over

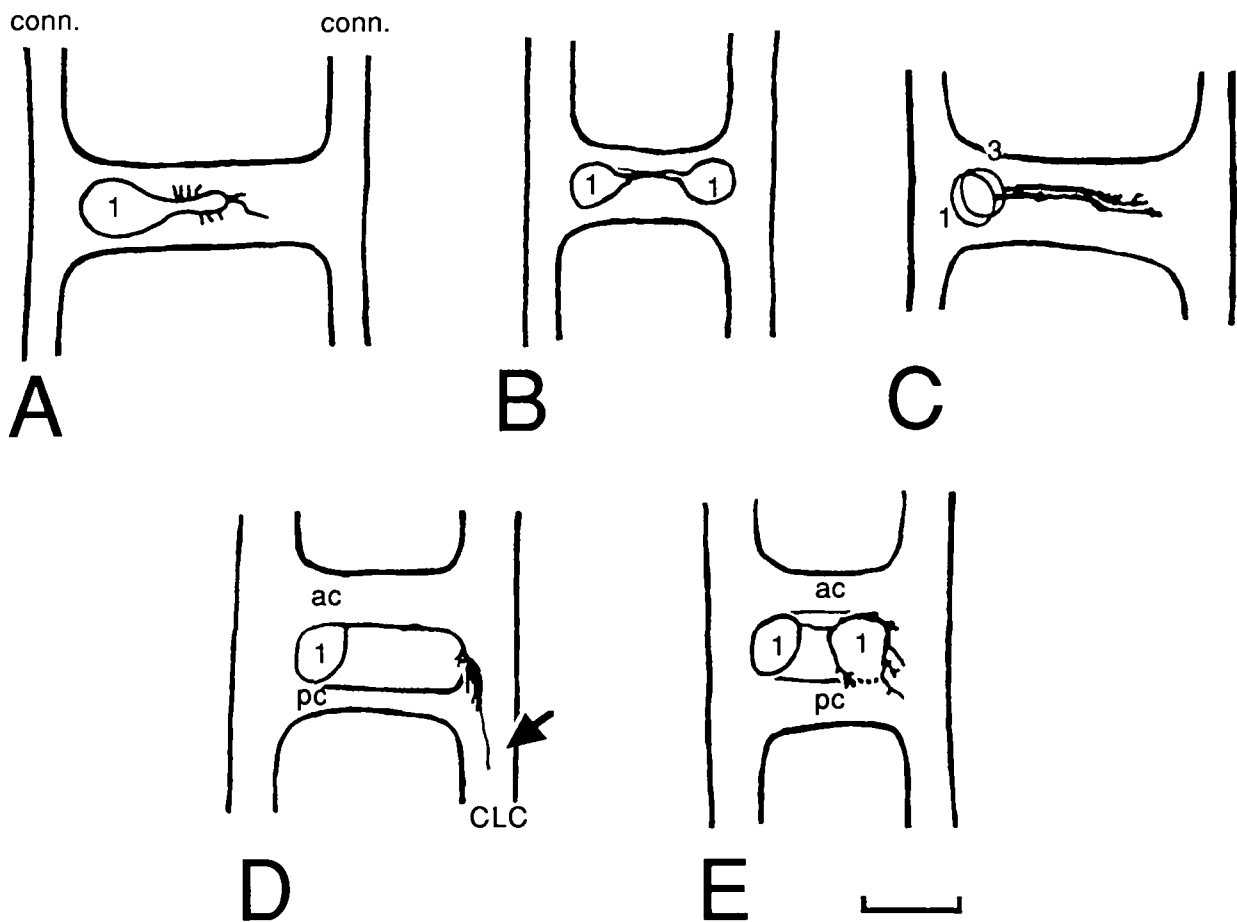


Fig. 3. Camera-lucida drawings of RP motorneurons during growth across the midline and into the CLC. (A) Extension of a lamellipodium by RP1 towards the ganglion midline. (B) Fasciculation of a pair of RP1 axons in the anterior commissure. (C) Dual fill of RP1/3 showing separate pathways taken by these axons in the anterior commissure. (D) RP1 axon entering the CLC. Arrow indicates a long filopodium at the leading edge of the growth cone. (E) Branches of RP1 axon wrapping around both the medial and lateral sides of the contralateral RP1 soma. ac, anterior commissure; pc, posterior commissure; conn., connectives; CLC, contralateral longitudinal connective. Scale bar=10 μm .

muscles 6/7 in Fig. 5. Whereas in stage 16 the arborization of this neuron is confined to the adjoining edges of muscles 6 and 7, during stage 15 it is often found spanning the complete mediolateral extent of these muscles.

These growth trajectories bring the RP axons into the vicinity of their target muscles, 13 (RP1 and RP4), 6/7 (RP3) and 13, 14, 15 and 16 (RP 5), over which they form the stereotypic arborization patterns evident at late embryonic stages (Sink and Whittington, 1991). In some cases, RP axons continue to grow beyond their target muscles and arborize over more distal muscles after having apparently contacted their target muscle (e.g. RP3 sometimes extends processes to muscles 12/13, Fig. 5, RP1, RP4 and RP5 to muscle 12.). However, they were never observed to extend distal to muscle 12.

Retraction of inappropriate axonal branches

During the period late stage 15, the RP motorneurons have arborizations over both their target muscles and the non-target muscles, which they had contacted en

route to their targets. Late in stage 15 and early in stage 16, the processes in contact with inappropriate muscles are withdrawn, leaving the mature arborization patterns (Fig. 1).

Discussion

This is the first study in the *Drosophila* embryo to have examined the cellular contacts made by the growth cones of identified motorneurons through the whole period of their growth, from the onset of axonogenesis to the time of arborization over muscle targets. Our observations of the behaviour of the RP axons provide insights into: (a) the cellular identity of putative guidance cues for these axons; (b) the degree of specificity of these cues with respect to different RP axons; and (c) the cellular mechanisms that underlie the generation of the stereotypic axonal morphologies of mature RP motorneurons. We discuss these issues below with respect to the five phases of RP motor axon growth that we define in this study.

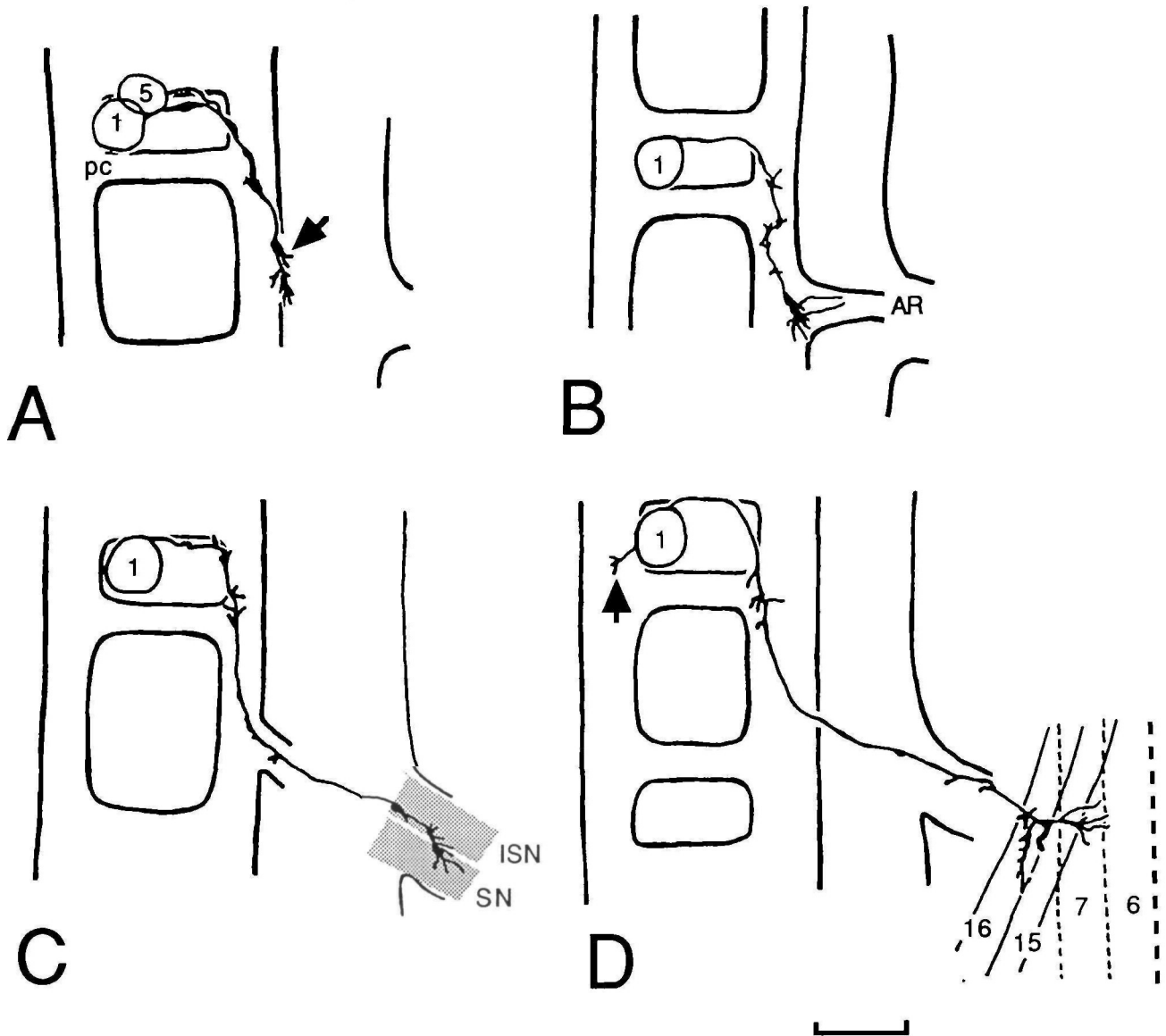


Fig. 4. Camera-lucida drawings of RP motorneurons during axon growth into the anterior root of the ISN; along the ISN, and onto oblique intermediate muscles. (A) Dual fill of RP1 and RP5 showing fasciculation of their axons within the CLC. RP5's growth cone (arrow) is just behind RP1's. (B) RP1 axon turning into the anterior root (AR) of the ISN. Note long filopodia in the direction of the turn. (C) RP1 axon crossing from the ISN to the SN. The subdivision of the peripheral nerve into the ISN and the SN fascicles is indicated by the dots. (D) Branching of RP1 axon on the external face of the intermediate, ventral muscles 15 and 16. A short dendritic branch has extended from the soma into the ipsilateral connective (arrow) at this time. AR, anterior root of the ISN; ISN, intersegmental nerve; SN, segmental nerve. Scale bar = 10 μm .

The earliest stage of axon outgrowth involved the formation of a lamellipodium on the medial side of the RP somata. Lefcort and Bentley (1989) have presented evidence that, in the case of the pioneer neurons in the grasshopper limb bud, the orientation of the mitotic spindle during the final cell division prior to the formation of these neurons determines the orientation of axon emergence. Whether such intrinsic factors or some external cue determines the direction in which the lamellipodium is formed in the RP motorneurons awaits further investigation.

After this early phase, the RP axons appear to use a variety of external cellular structures as guidance cues.

The first cellular feature with which the RP axons associate is the axon of their contralateral homologue. Our observations, together with those of Jacobs and Goodman (1989*b*) on RP1 and RP3, strongly suggest that this behaviour is due to specific self-self recognition, since the RP growth cones are within filopodial reach of all the RP axons, and grow out at the same time, yet fasciculate selectively with their homologous partners.

In the case of RP1, RP3 and RP4, this apparent self-self recognition continues as their growth cones wrap around their contralaterally homologous somata. However, the growth cone of RP5 changes its growth

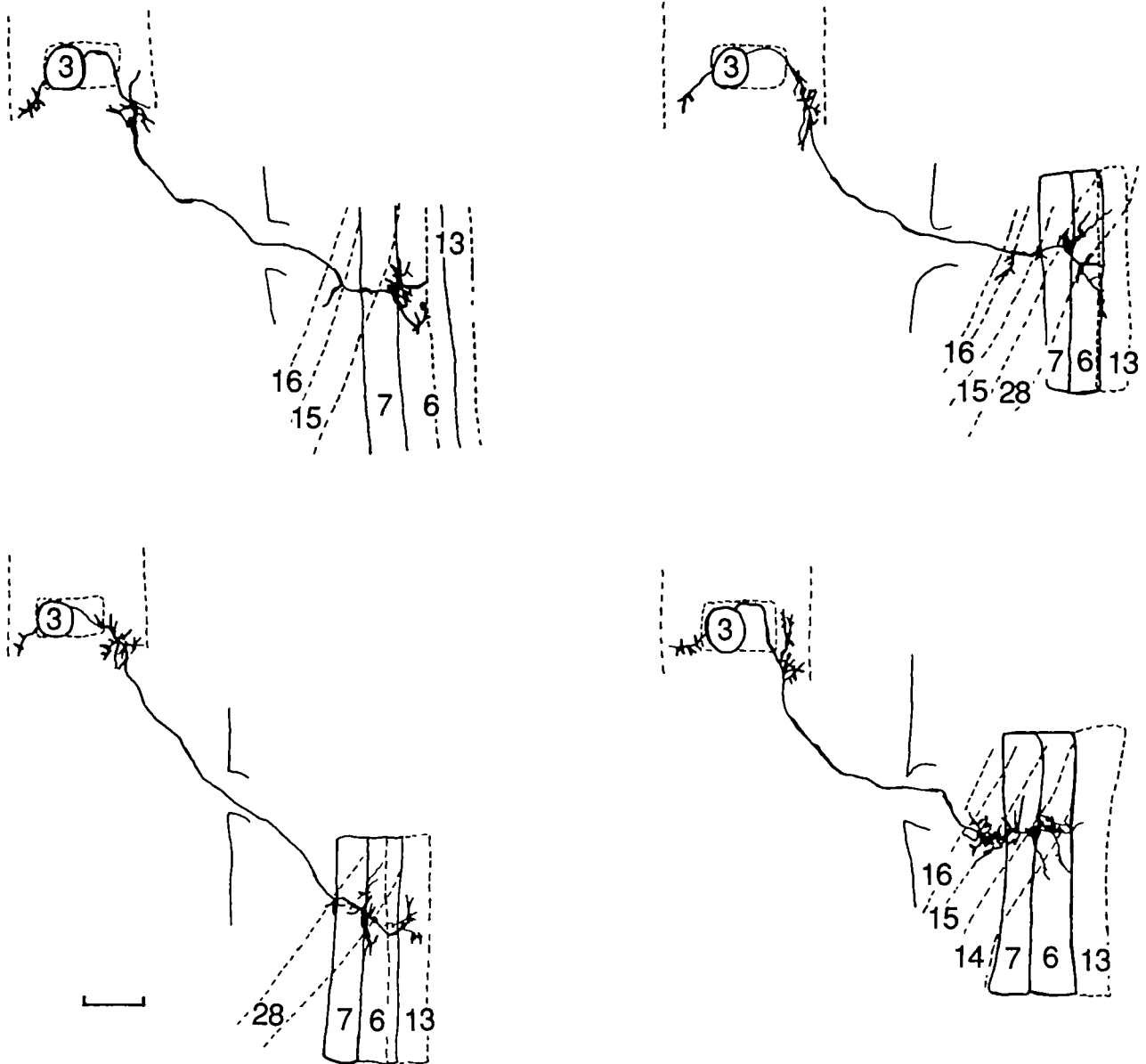


Fig. 5. Camera-lucida drawings of RP3 in 4 different embryos at a stage when the growth cone is contacting the target muscle 13. Note the variability in the pattern of muscles contacted and in the arborization patterns on individual muscles. Scale bar=10 μ m.

substratum at this point, appearing to associate with the contralateral RP3 soma, rather than with its homologue. This suggests that different markers are present on the RP cells which are selectively recognised by the different RP growth cones. Molecules that are selectively expressed on subsets of embryonic *Drosophila* CNS neurons have been identified (fasciclin I, Zinn *et al.* 1988; fasciclin III, Patel *et al.* 1987); however, the effects of deleting such molecules have not been examined at the single cell level.

Contact with contralateral RP somata brings the axons of the RP neurons within filopodial reach of the CLC, down which they subsequently grow, before diverging into the anterior root of the ISN and advancing to the edge of the CNS. Other axons precede

the RP axons into the CLC and ISN (Jacobs and Goodman, 1989b) and are possible candidates for guidance of the RP axons in these phases of their growth. However, dual fills show that two of these pioneers, aCC and RP2, are not followed by the RP axons. The RP axons may be guided by a trail of glial cells located on the dorsal side of the CNS, which has been suggested as the cue guiding the posteriorly directed growth of the pioneering aCC axon (Jacobs and Goodman, 1989b). The trajectory of the RP axons along the CLC is frequently not straight, and may be the result of a preferential adhesion to the adjoining edges of these glial cells. Studies in both vertebrates (Rakic, 1971; Alvarez-Buylla and Nottebohm, 1988) and invertebrates (Bastiani and Goodman, 1986; Carr

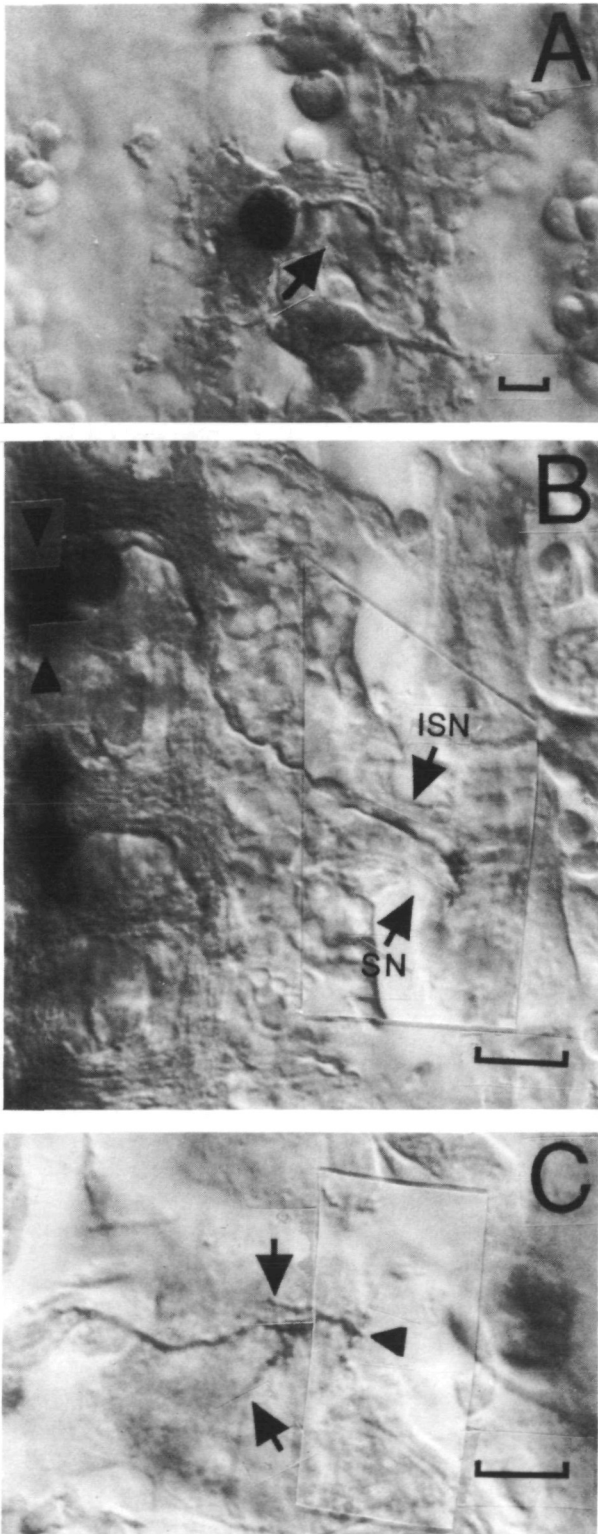


Fig. 6. Photomicrographs of RP neurons at different stages of development. (A) RP3 axon entering the CLC. The axon has grown along the anterior and lateral surface of the contralateral RP3 cell (arrow). Scale bar=5 μ m. (B) RP1 axon diverging from the ISN into the SN outside the ganglion. Arrowheads indicate inadvertently filled glial cells. Scale bar=10 μ m. (C) RP4 axon at target muscle 13 (arrowhead) showing processes in contact with non-target muscles 14 and 15 (arrows). Scale bar=10 μ m.

and Taghert, 1988; Jacobs and Goodman, 1989a) show that glial cells can delineate pathways for both cell and axon migration. Molecules specifically assisting neuronal cell adhesion (Ng-CAMs) have been identified in vertebrates (Grumet *et al.* 1984; Grumet and Edelman, 1988). In *Drosophila*, a Ng-CAM, neuroglian, has been identified (Bieber *et al.* 1989) but its functional role is unclear.

The RP axons encounter a choice point at the edge of the ganglion, leaving the ISN and crossing over to the SN, before exiting the segmental nerve onto the external surfaces of ventral muscles 15 and 16. In other cases, insect motor axons are guided into the periphery by sensory axons (Hartenstein, 1988) or pioneer motor axons (Whittington, 1989). Our observations, together with the results of the anti-HRP study by Johansen *et al.* (1989), suggest that the RP axons are not preceded onto the ventral muscle group by either sensory axons or other motor axons. Therefore, the RP axons apparently do not exit the segmental nerve by tracking other axons.

Glial cells known to be present along the proximal region of the segmental nerve (Fredieu and Mahowald, 1989) may act as guideposts, providing the cue for the RP axons to leave the nerve. The CAM neuroglian is expressed on both glia and dorsal neurons in the *Drosophila* embryo (Bieber *et al.* 1989), and could serve a role in guiding the RP axons from the nerve.

Alternatively, the RP axons may be exiting the segmental nerve in response to a chemoattractant released by the ventral muscles. Insect motor axons still exit the ganglion, albeit by different nerves, in the absence of the target muscles (Whittington *et al.* 1982; Whittington and Seifert, 1982; Nässel *et al.* 1986). Similarly, we have found that the RP3 axon still exits the segmental nerve in the absence of target muscles (Sink and Whittington, in preparation). This dismisses a role for a diffusible, target-derived neurotropic factor in guiding axons from the ganglion, but it is still possible that the RP axons are guided from the nerve by such a cue from more proximal muscles.

Once in contact with the ventral muscle group, the RP growth cones became more expansive, and extend distally over the surfaces of these muscles. The pathways followed by the RP axons across the ventral muscles are not delineated by any obvious physical structures. It is likely, therefore, that the pathways are based on preferential adhesion by the RP growth cones for substrata expressed on the surface of the muscles. Preferential growth cone adhesion has been identified as a mode of pathfinding both *in vitro* (Letourneau, 1975) and *in vivo* (Berlot and Goodman, 1984; Caudy and Bentley, 1986). The extension of processes by each individual RP motorneuron over many of the muscles in the ventral group indicates the broad distribution of adhesive substrata. The same molecular markers may therefore be used to guide the different RP motoraxons to their targets. This strategy of pathway delineation reduces the number of molecular markers that are needed to obtain a stereotypic pattern of axonal arborization over closely situated muscles. Similarly the fasciculation of RP axons during growth down the CLC

and into the ISN may reflect the usage of a common set of guidance cues by these neurons.

In summary, our results indicate that the RP axons use a variety of guidance cues as they grow towards their muscle targets, including guidepost cells, selectively labelled fascicles and adhesive substrata. We have recently embarked on a series of ablation experiments to test axon utilisation of individual guidance cues. These experiments will form the basis for a search for the molecules on these cues that mediate axon guidance. Several cell adhesion molecules (CAMs) have already been identified in *Drosophila* (fasciclin III, Patel *et al.* 1987; neuroglian, Bieber *et al.* 1989 and the gene product of *l(2)gl*, Klämbt *et al.* 1989) and implicated in axon fasciculation. In mutants for these genes, the CNS tracts appear relatively normal. One interpretation of this result is that axon fasciculation at the tract level involves the use of more than one CAM. Alternatively, these mutants may possess subtle defects in axon behaviour that might only be revealed by examination at the level of identified axons, with a thorough background knowledge of normal axon behaviour such as is provided in the present study.

Our observations indicate that two cellular mechanisms underlie the generation of the stereotypic axonal morphologies of mature RP motorneurons, namely (a) reliable axon steering along appropriate pathways and (b) more diffuse initial axon branching followed by retraction of inappropriate processes.

After crossing the ventral midline, processes of the RP growth cones were rarely found on the posterior and medial sides of the contralateral RP somata. This may result from a directed, preferential growth of processes from the outset along the anterior and lateral sides of the RP somata, due to an asymmetrical distribution of molecular markers on the surfaces of these cells. Alternatively, the growth cone may initially wrap around the entire cell, but those processes on the anterior and lateral sides of the cell may contact the CLC before those on the posterior and medial sides, due to their physical proximity to that pathway. As a result, the former branches may outcompete the latter ones for a limited maintenance factor and be retained at their expense. If retraction took place quickly, branches on the medial and posterior sides would be seen less frequently than those on the lateral and anterior sides.

When growing down the CLC and into the ISN, the RP motor axons appear to take a well-defined growth trajectory from the outset, with little evidence of branching outside their mature, appropriate pathways.

Once in contact with the ventral muscle group, the RP growth cones extend processes over non-target muscles. The inappropriate processes are retracted during stage 15–16 and are rarely observed in older stage 16 embryos. Studies of motor axon outgrowth in the embryos of locusts (Myers *et al.* 1990) and leeches (Kuwada, 1984; Baptista and Macagno, 1988) have shown that inappropriate axon branching consistently occurs during axon outgrowth by specific embryonic motorneurons. This is contrary to the commonly held

view that motor axon outgrowth is highly specific and error-free (Ball *et al.* 1985; Tosney and Landmesser, 1985; Eisen *et al.* 1986; Myers *et al.* 1986).

Inappropriate branches in the locust embryo rarely contact a non-target muscle (Myers *et al.* 1990), whereas inappropriate processes in the *Drosophila* embryo extend over non-target muscles. We have yet to determine if these processes form synapses with the non-target muscles. Functional connections with non-target muscles have been observed for target-deprived locust motorneurons (Whittington, 1985), demonstrating that insect motorneurons do possess the capacity to innervate muscles other than their normal targets.

Retraction of inappropriate processes may be the result of contact with the target muscle and the subsequent stabilisation of the appropriate branches at the expense of the inappropriate processes. Baptista and Macagno (1988) found that inappropriate branches in the leech are retained in the absence of normal muscle targets. This suggests that a signal is derived from the target muscle that stabilises the contacting branch, and causes the retraction of inappropriate branches. Alternatively, competition may occur between appropriate and inappropriate axon arborisations for innervation sites on the muscles. The results of our muscle ablation experiments argue for the first of these alternatives (Sink and Whittington, in preparation).

In several cases, RP motor axons grew beyond their target muscle(s) to contact more distal muscles, even after they had apparently contacted their target muscle. This observation provides evidence against the hypothesis that contact with the target muscle immediately leads to cessation of axon growth, a conclusion that is consistent with a recent study of axon growth by the S interneuron in the leech embryo (McGlade-McCulloch and Muller, 1989). However, it does not exclude the hypothesis that target muscle contact may stop axon growth following a delay.

In relation to the establishment of axon arborization patterns within the target muscle, we have observed that the initial arborizations of RP motor axons are both variable and more widespread than at late stages of embryogenesis. This finding appears to conflict with the claim by Johansen *et al.* (1989) that *Drosophila* motorneurons establish their stereotyped arborizations without significant process pruning, a conclusion based upon a comparison of arborizations in stage 16 embryos with those in stage 17 embryos and larvae (Fig. 11, Johansen *et al.* 1989). However, our observations show that the RP motor axons have virtually attained their mature morphologies by stage 16; more widespread branching patterns are only seen during stage 15.

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