Early ablation of target muscles modulates the arborisation pattern of an identified embryonic Drosophila motor axon

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Summary

The Drosophila RP3 motor axon establishes a stereotypic arborisation along the adjoining edges of muscles 6 and 7 by the end of embryogenesis. The present study has examined the role of the target muscles in determining this axonal arborisation pattern. Target muscles were surgically ablated prior to the arrival of the RP3 axon. Following further development of the embryo in culture medium, the morphology of target-deprived RP3 motor axons was assayed by intracellular injection with the dye Lucifer Yellow. Axonal arborisations were formed on a variety of non-target muscles when muscles 6 and 7 were removed and these contacts were maintained into stage 16. The pattern of axonal arborisations over non-target muscles varied between preparations in terms of the number of muscles contacted, and the distribution of arborisations on individual muscles. Following removal of muscle 6, the RP3 motor axon frequently contacted muscle 7, and axonal arborisations were present along the distal edge of the muscle. In the absence of muscle 7, the RP3 axon often did not contact muscle 6 and when muscle 6 was contacted, the arborisation of RP3 was poorly developed. Axonal processes were retained on non-target muscles when only one target muscle was present. Therefore, the establishment of a stereotypic arborisation by the RP3 motor axon is apparently dependent on growth cone contact with both target muscles.

Key words: Drosophila embryo, Drosophila motoneuron, neuromuscular specificity, muscle ablation, insect embryo.

Introduction

The characteristic axon morphologies attained by the end of embryogenesis are the culmination of growth cone responses to pathway options and final targets. Indications of the underlying cellular mechanisms for axon pathfinding and target recognition have come from observing the behaviour of single neurons in the relatively simple insect nervous system. Studies in the locust embryo in particular have identified candidate axon guidance mechanisms within the central nervous system (Goodman et al. 1984; Raper et al. 1983a,b,c) and periphery (Bate, 1976; Bentley and Keshishian, 1982; Berlot and Goodman, 1984; Myers et al. 1990; Meier and Reichert, 1990; Whittington, 1989), and tested the role of several cellular cues with ablation experiments (Bentley and Caudy, 1983; Raper et al. 1983a,b,c; Bastiani and Goodman, 1986; Bastiani et al. 1986; du Lac et al. 1986; Whittington et al. 1982; Whittington and Seifert, 1982; Whittington and Seifert, 1984). Despite this wealth of information on axon guidance at the cellular level, the relatively intractable genetics of the locust has constrained the analysis of the molecular basis of axon pathfinding and target recognition.

In contrast, the embryo of the fruit fly Drosophila melanogaster is particularly attractive for genetic and molecular studies (Thomas and Crews, 1990). Recent studies have identified the cellular contacts made by embryonic Drosophila motor axon growth cones that may be important for defining axon trajectories and establishing arborisation patterns (Hartenstein, 1988; Johansen et al. 1989a; Sink and Whittington, 1991b). We are taking these descriptive cellular studies a step further with ablation experiments, which are designed to pinpoint the growth cone contacts that are critical for the development of stereotypic motor axon morphologies. The results from ablation experiments may also indicate the specificity of marker molecule(s) distribution along axon pathways and on target muscles.

During normal development, the RP3 axon in the Drosophila embryo (Sink and Whittington, 1991b), like leg-innervating motorneurons in the locust embryo (Myers et al. 1990), extends processes over non-target muscles before ultimately establishing a stereotypic arborisation over the target muscles. These observations raise the question of whether a signal from the target muscles causes the axon to retract inappropriate processes and stabilise the processes contacting the target muscles. In the present study, we have examined the role of the target muscles in the establishment of the embryonic Drosophila RP3 motor axon's stereotypic arborisation pattern (Johansen et al. 1989a; Sink and...
Whittington, 1991a,b). Muscles were surgically ablated prior to the arrival of the axon, and following further development in embryo culture medium, axon behaviour was assayed by intracellular dye injection. Ablation of both target muscles resulted in variable arborisation patterns over non-target muscles. Results from the ablation of single target muscles indicate that RP3 growth cone contact with both target muscles prior to stage 16 is necessary for (a) the establishment of a consistent arborisation pattern by the middle of stage 16, and (b) the complete retraction of processes in contact with non-target muscles.

Materials and methods

Stock
Oregon-R wild-type embryos of Drosophila melanogaster were used in this study. Eggs were chemically dechorionated by agitation in a 25% commercial bleach solution. Embryos were examined under a dissecting microscope, and staged according to the morphological criteria of Campos-Ortega and Hartenstein (1985).

Dissections
Embryo dissections and culturing were carried out in modified M3 medium (Shields and Sang, 1978). In our version of M3 medium, equivalent quantities of l-alanine were substituted for the a- and b-alanine, choline-Cl was substituted for choline-HCl, and aspartic acid was omitted. Foetal calf serum (FCS) was omitted from the dissection solution to facilitate adhesion of the embryo to the slide.

Embryos were dissected on poly-L-lysine (Sigma)-coated glass slides under sterile FCS-free M3 medium held in a Vaseline dam. The anterior end of the egg was cut open, and the embryo was squeezed from the vitelline membrane. Embryos were dissected longitudinally along the dorsal midline using an electrolytically sharpened tungsten needle. The bodywall was gently flattened onto the slide, and the musculature encountered by the RP3 axon during outgrowth. In some cultured embryos, the RP3 axon had not contacted muscles 6 and 7 in control segments, and appeared to retain this arborization through larval development (Johansen et al. 1989a,b; Budnik et al. 1990) (Fig. 1A).

Immunochemistry
For anti-LY immunohistochemical processing, preparations were fixed in 4% paraformaldehyde in Millonig's buffer for 15–20 min, washed in phosphate-buffered saline (PBS), and incubated overnight in a rabbit anti-LY antibody (raised in our laboratory) diluted 1:500 in PBS/0.4% Triton X-100/0.25% bovine serum albumin (PBT). The next day the preparations were washed in PBS and incubated for 24 h in HRP-conjugated goat anti-rabbit IgG antibody (Amersham) diluted 1:250 in PBT. Preparations were then washed in PBS, incubated for 1 h in 0.5% diaminobenzidine in PBS, and reacted with hydrogen peroxide to give a stable reaction product in the injected neurons. Following a final wash in PBS, the embryos were cleared and mounted in 100% glycerol.

All preparations were examined on a Zeiss photomicroscope, equipped with Nomarski optics, using a Zeiss Planapo 100× oil immersion objective. Injected neurons were drawn with the aid of a camera lucida.

Results

General comments
In normal, unoperated embryos the RP3 motor axon in abdominal segments A3–A7 has formed a stereotypic arborisation along the adjoining edges of muscles 6 and 7 by mid stage 16 (Sink and Whitington, 1991a,b), and appears to retain this arborization through larval development (Johansen et al. 1989a,b; Whitington, 1991a,b). Fig. 2 shows schematically the region of muscle 7 by mid stage 16 in normal, untreated, homologous abdominal segment was also intracellularly injected with LY.

Ablation of muscles 6 and 7
Muscles 6 and 7, the targets for motoneuron RP3, are not contacted by RP3 growth cones in untreated, homologous abdominal segments. In some cultured embryos, RP3 axon arborisations to muscles other than 6 and 7 were only seen in unmanipulated segments when the musculature and/or the CNS was grossly abnormal in appearance. Such embryos were rejected.
located in the most internal muscle layer. This location makes it possible to remove surgically these two muscles without causing obvious disruption to other muscles in the region. Muscle ablations were performed at early stage 15, at which time RP3's motor axon has just exited the CNS (Sink and Whitington, 1991b) and has, therefore, not yet contacted muscles 6/7. The following observations are based on 21 manipulations (in 20 embryos) in which both muscles 6 and 7 were removed in an abdominal segment, (generally A4–A6).

In the absence of both target muscles, the RP3 axon arborised on a number of muscles in the ventral muscle group (summarised in Table 1). Axonal arborisation patterns differed between embryos in terms of the number of muscles contacted, and the identity of the muscles contacted (Fig. 3). In eight preparations, the target-deprived RP3 axon extended beyond the region where the target muscles are normally situated (Fig. 3B,C,E,F). In two preparations, the RP3 axon grew anteriorly, and extended processes along and across the segmental boundary (Fig. 3A,D). In one preparation, the RP3 axon fasciculated with the intersegmental nerve, which extends to more distal muscles.

Intramuscular arborisations of target deprived RP3 axons differed between embryos. For example, the axonal arborisations present on muscle 14, the most frequently contacted non-target muscle (Table 1), varied in both number, and extent of muscle surface contacted. In some cases (Fig. 3B), the axon has processes only along the distal edge of muscle 14 whereas in others (Figs 3C,D and F) the processes spread across the entire internal surface of the muscle. (We define distal as being further removed from the CNS and internal as being furthest removed from the epidermis).

**Ablation of muscle 6**

In the absence of muscle 6, the RP3 axon grew into the periphery, and in 11 out of 14 cases (in 14 embryos) contacted muscle 7 (Fig. 4A,C,D,E,F). The pattern of axonal arborisations in contact with muscle 7 varied between embryos but in all cases was restricted to the distal third of the internal face of the muscle. In one embryo, a process was extended for approximately 10 μm anteriorly along the distal edge of muscle 7 (Fig. 4D). In other embryos, the arborisation of RP3 was less extensive (Fig. 4E,F).

Although most (78%) of RP3 axons in the treated
Table 1. The frequency of contact of body wall muscles by the RP3 motorneuron after ablation of one or both of its target muscles M6 and M7

<table>
<thead>
<tr>
<th>Muscle ablated</th>
<th>Muscle contacted</th>
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<tr>
<td></td>
<td>6</td>
<td>7</td>
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<tr>
<td>Both M6 and M7 (n=21)</td>
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<tr>
<td>M6 only (n=14)</td>
<td>–</td>
<td>11</td>
</tr>
<tr>
<td>M7 only (n=9)</td>
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Fig. 3. Camera lucida drawings of RP3 motor axons in embryos after removal of both muscles 6 and 7 in the same hemisegment, followed by a 5 h culture period. In A the axon has extended a process anteriorly along the external surface of muscle 15, then distally along the segmental boundary (SB). In D a branch has extended anteriorly along muscle 14, then crossed the segmental boundary. Axons in B, C, D, E and F have extended distally beyond the region normally occupied by muscles 6 and 7. CNS, central nervous system; AXON, RP3 axon. Scale bar=10 μm.

Fig. 4. Camera lucida drawings of RP3 axons in segments where muscle 6 had been removed. Arrowheads indicate the axonal arborisations that contact muscle 7. Scale bar=10 μm.

Segments contacted muscle 7, in all cases the axon maintained some processes in contact with non-target muscles (Table 1). The most commonly contacted non-target muscle was muscle 14 (11/14 preparations).

The identity and number of non-target muscles contacted differed between embryos, as did the pattern of intramuscular arborisations on non-target muscles (e.g. compare muscle 15 in Fig. 4B and 4C). In one preparation, the RP3 axon crossed the anterior segmental boundary.

Ablation of muscle 7
In the absence of muscle 7, less than 50% (4/9) of the RP3 axons contacted muscle 6. Axonal contact with muscle 6, when it occurred, was minimal and was generally confined to the proximal edge of the muscle.
Fig. 5. Camera lucida drawings of RP3 axons in segments where muscle 7 had been removed. In A and F, the axon failed to contact muscle 6. In C the axon has crossed the anterior segmental boundary and terminated on muscles 6 and 7 in the next segment. Arrowheads indicate the axonal arborisations that contact muscle 6. Scale bar=10 μm.

(Fig. 5B,C,D,E). Arborisations over non-target muscles varied in both the number and the region of the muscle contacted.

Although the RP3 axon contacted muscle 6 in four preparations, the axon still retained processes on several non-target muscles in the ventral muscle group (Table 1). The most frequently contacted non-target muscles were muscles 28 and 14.

In one preparation, an RP3 axon had extended a process across the anterior segment boundary, and this terminated along the adjoining edges of muscles 6 and 7 in the next segment (Fig. 5C). In four preparations, the RP3 axons extended distally beyond muscle 6, and contacted muscle 13 (Fig. 5B), and in two preparations, muscle 12 (Fig. 5B).

Discussion

In the present study, the target muscles of the RP3 motoneuron were ablated prior to the arrival of the motor axon. Examination of the behaviour of target-deprived RP3 axons provides insights into the role of target muscles in (a) directing axon growth into the periphery; (b) the establishment of specific connections with the target muscles, including the retraction of processes contacting non-target muscles and; (c) the formation of a stereotypic intramuscular arborisation pattern.

Axon growth into the periphery

During normal development, the RP3 motor axon, once in the periphery, diverges from the segmental nerve onto the external surfaces of the ventral oblique muscles 16 and 15 (Johansen et al. 1989a; Sink and Whitington, 1991b). In the absence of both target muscles, the RP3 motor axon still diverged from the segmental nerve in this region, indicating that the axon is not guided from the nerve by chemotropic cues diffusioning from the target muscle.

In a similar experiment in the locust embryo, ablation of the 133a muscle pioneer (MP) cell prior to the arrival of the Df motor axon, resulted in the Df axon failing to branch from the main leg nerve (Ball et al. 1985). While the response of this motoneuron to ablation of its target is different to that seen for the RP3 neuron in Drosophila, it does not necessarily argue for the existence of diffusible chemotropic cues from the target MP cell, since the Df axon is within filopodial reach of the MP cell before it leaves the main leg nerve.

Establishment of specific connections with the target muscles and retraction of inappropriate branches

During normal development, motor axons in both locust and Drosophila embryos send processes in aberrant directions (Myers et al. 1990; Sink and Whitington, 1991b), pointing to the importance of selective axon retraction in generating specific neuro-muscular connections. At least two different mechanisms could underlie this process. Under the first mechanism, motor axon contact with the target muscle causes a withdrawal of branches of that same neuron which are in contact with non-target muscles. According to the second mechanism, withdrawal of a branch on a non-target muscle is a result of a competitive interaction between that branch and axonal branches arising from the appropriate motoneuron for that muscle. If only the first mechanism is in operation, a motoneuron should fail to withdraw branches to inappropriate muscles if prevented from contacting its target muscle.

In the presence of both its target muscles, the Drosophila RP3 motor axon attains a stereotypic axon arborisation pattern by the middle of embryonic stage 16 (Sink and Whitington, 1991a,b). At the end of the culturing period, the embryo appears to be well into stage 16, as judged by the morphology of RP axons in unoperated segments. When both target muscles are
identified as axon guiding mechanisms (Letourneau, 1975; Bonhoeffer and Huf, 1982; Hammarback et al., 1988) and in vivo (Berlot and Goodman, 1983; Caudy and Bentley, 1986). In the Drosophila embryo, an adhesive substratum that permits RP3 axon extension may be confined to this region of musculature. Alternatively, a substance may be present on the membranes distal to muscle 12 which inhibits advancement of the RP3 growth cone. Axon inhibition has been demonstrated in vitro for chick temporal retinal axons. These axons grow on membranes from the anterior tectum (Walter et al., 1987a), but actively avoid growing on membranes from the posterior tectum (Walter et al., 1987b).

In the absence of muscle 6, the RP3 motor axon frequently contacted muscle 7 (78%), whereas far fewer RP3 axons contacted muscle 6 after muscle 7 had been removed (<50%). A possible explanation of this result is that contact with muscles proximal to muscle 7 (e.g. muscles 16, 15 and 14) is generally sufficient to guide the axon of RP3 to that muscle, but that muscle 7 is required to guide the axon as far as muscle 6. Alternatively, muscle 7 may interact directly with muscle 6 to prepare it for innervation by RP3. In the absence of this interaction, RP3 may not be able to reliably contact or maintain an arborisation on muscle 6.

**Intramuscular axonal branching**

In normal embryos, RP3 forms a stereotypic arborisation along the distal and proximal faces of muscles 7 and 6, respectively. The arborisations present along muscle 7 in the absence of muscle 6 were variable in extent, but tended to be confined to the distal edge of the muscle, as in normal development. In addition, when RP3 made contact with muscle 6 in the absence of muscle 7, processes were confined to the proximal edge of the muscle 6, although the extent of contact never approached that seen in normal development. These observations speak against the hypothesis that the localisation of RP3's arborisation along the adjoining faces of muscles 6 and 7 depends upon maintained contact between those muscles.

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**References**


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