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From growth cone to synapse: the life history of the RP3 motor neuron

Kendal Broadie[1], Helen Sink[2], David Van Vactor[2], Douglas Fambrough[2], Paul M. Whitington[3], Michael Bate[1] and Corey S. Goodman[2]

[1]Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK
[2]Howard Hughes Medical Institute, Department of Molecular and Cell Biology, Life Science Addition Room 519, University of California, Berkeley, Berkeley, California 94720, USA
[3]Department of Zoology, University of New England, Armidale N.S.W. 2351, Australia

SUMMARY

In *Drosophila*, the ability to analyze the development of individually identified neurons with a variety of imaging and biophysical techniques can be complemented by sophisticated genetics and molecular biology. This powerful combination is allowing the development and function of single neurons and their synaptic connections to be unraveled at an unparalleled level of resolution. In this article, we focus on a single, identified motoneuron — RP3 — arguably the best understood neuron in the fruitfly. Many events in the life history of RP3 are well characterized, including cell migration, axon outgrowth and pathfinding within the central nervous system, pathfinding in the periphery to its appropriate muscle target domain, the specific recognition of its muscle targets, the events of synapse formation and maturation, and its mature function in the locomotion of the fly larva. Genetic analysis has revealed mutations in a number of different genes which affect specific aspects of RP3 development from axon outgrowth to synapse formation.

Key words: *Drosophila* embryonic development, RP3 neurogenesis, motor neuron synaptogenesis

INTRODUCTION

Invertebrate neurobiologists have worked with identified neurons for decades. Indeed, one of the most compelling reasons for working with "simpler" nervous systems is the consistency with which individual neurons can be uniquely identified from animal to animal as distinct and stereotyped elements of relatively simple and well defined neural networks. Embryologists too have taken advantage of the notion of identified cells, having worked with specific progenitor cells for a century or more. Over the past decade, these two approaches, the embryological and the neurobiological, have come together, with neurons being identified early in their development in embryonic insect nervous systems and the origin of these same neurons being linked, by means of cell lineage analysis, to specific divisions of identified progenitor cells, called neuroblasts. In principle, the complete developmental pathway from neuroblast to neuron to synapse can be defined, and the mechanisms controlling these events analyzed by a wide range of surgical, biochemical and molecular genetic manipulations.

The developmental analysis of identified neurons began more than a decade ago in the large embryo of the grasshopper (e.g., Bate, 1976a,b; Goodman and Spitzer, 1979; Goodman and Bate, 1981; Bate and Grunewald, 1981; Goodman et al., 1982; Bentley and Keshishian, 1982; Raper et al., 1983; Bentley and Caudy, 1983; Raper et al., 1984; Ball et al., 1985), an excellent system for cellular approaches including cell lineage analysis, intracellular dye injections, and a variety of different experimental manipulations.

Fortunately, the common blue-print that underlies the diversity of insect nervous systems has meant that the homologues of cells and cell-cell interactions identified first in the grasshopper embryo could, almost without exception, be found in the embryo of *Drosophila* (e.g., Thomas et al., 1984; Goodman et al., 1984; Jacobs and Goodman, 1989; reviewed by Goodman and Doe, 1993). Moreover, the constant burgeoning of technical advances – from monoclonal antibodies, to enhancer trap lines, to reporter gene constructs – has provided powerful cell-specific probes that have enhanced our ability to study identified neurons, their processes, and their interactions in the developing nervous system of *Drosophila*. It is also possible in *Drosophila* to use the larva to study the physiology of neuromuscular synapses (Jan and Jan, 1976a,b), and the embryo to study the physiology of these same developing synapses as they form and mature (Broadie and Bate, 1993a-c). Thus, a complete range of genetic, molecular, embryological and physiological techniques can be brought to bear on the construction of a neural network, from migration and process outgrowth to synapse formation and maturation.

This analysis is furthest along for an identified motor neuron called RP3, although here too, some gaps still remain in our knowledge. In this article, we review what is known about the development of RP3 and its synapse with muscles 6 and 7 in *Drosophila*. The ability to study individual motor neurons and their muscle targets has greatly aided the

analysis of the function of genes and molecules involved in specific aspects of neuromuscular development and function.

IDENTIFICATION OF RP MOTOR NEURONS

At present, nearly 20 of the approx. 200 embryonic neurons on each side of each CNS thoracic and abdominal neuromere can be readily recognized from embryo to embryo and uniquely identified based on their cell body location and axon trajectory (reviewed by Goodman and Doe, 1993). Most of these cells are early-born neurons with cell bodies near the inner (dorsal) surface of the developing CNS. Thus, the cell bodies are accessible in dissected preparations and bracketed by a scaffold of dorsal axon tracts that provide a grid of spatial reference points for uniquely identifying these cells.

From this population of identified cells, the RP neurons, a cluster of five prominent motor neurons (RP1-5), are amongst the best characterized (Thomas et al., 1984; Jacobs and Goodman, 1989; Sink and Whitington, 1991a,b; Halpern et al., 1991; Goodman and Doe, 1993; Van Vactor et al., 1993). Finding memorable names for identified neurons is not always easy, and in this instance the acronym *RP*, picked late one night over a decade ago by M. B. and C. S. G., stands for *Raw Prawn*, an Australian phrase aptly describing the delicate and innocent appearance of two cells — RP1 and RP2 — as they were first discovered in the embryo of the moth *Manduca sexta* and then by homology in the grasshopper and fruitfly (Thomas et al., 1984). RP3, RP4, and RP5 were subsequently identified in *Drosophila* (Sink and Whitington, 1991a).

The RP neurons form a cluster of five somata lying in or just dorsal to the plane of the 'CNS ladder' (Fig. 1A), a region bounded by the anterior and posterior commissures and the lateral borders of the longitudinal connectives. At maturity, all of the RP neurons send their axons out of the intersegmental nerve (ISN) into the periphery to innervate identified muscle targets. However, whereas four of these neurons (RP1, RP3, RP4, and RP5) extend their axons across the midline of the CNS and then out the next-posterior contralateral ISN, RP2 extends its axon out the next-anterior ipsilateral ISN. In the periphery, the axons of RP1, RP3, RP4, and RP5 switch to the segmental nerve (SN) and then to branch b of the segmental nerve (SNb) to innervate ventral domain muscles, whereas RP2 continues to extend in the ISN and innervates a dorsal domain muscle (Fig. 1).

Apparently, the RPs do not form a single lineage group, but rather are descended from different neuronal precursor cells, called neuroblasts (NBs). For example, RP2 is known to arise from NB 4-2 (Doe, 1992), whereas the other RPs appear to be generated from one or more distinct NBs (C. Q. Doe, personal communication; N. H. Patel, personal communication). The precise lineage of RP3 is not yet known, although this should now be possible due to recent advances in cell lineage techniques (Udolph et al., 1993). Although most of our knowledge about RP3 begins shortly after its birth at the onset of its cell-specific migration, it should be possible in the future to study the life history of this neuron from birth to mature function.

RP3 MIGRATION AND AXON GUIDANCE TOWARDS THE MIDLINE

Active cell migrations appear to play only a limited role in determining cell positions in the developing fly CNS (Goodman and Doe, 1993). Most neurons are born and differentiate without migrating and are only passively displaced internally during successive rounds of NB division. Nevertheless, some specific migrations are known to occur for both neurons and glia. Of particular interest here, RP3 and RP1 migrate during early neurogenesis to take up their characteristic medial dorsal positions in the CNS (Patel et al., 1987; Jacobs and Goodman, 1989). RP3 is born in a lateral position, many cell diameters away from its final position near the ventral midline. It begins to migrate towards the midline at about 9 hours of development (stage 12) just before extending its first axonal growth cone (Patel et al., 1987; Jacobs and Goodman, 1989) and continues migration as axonogenesis proceeds (9.5 hours; stage 13). At present, nothing is known about the mechanisms controlling this cell-specific migration.

The RP neurons begin to display distinct cell surface properties about the time RP3 migrates toward its medial position and commences axonogenesis. The motor neurons RP1, RP3, RP4, and RP5 express the homophilic cell adhesion molecule (CAM) fasciclin III (Fas III; Patel et al., 1987; Snow et al., 1989) on their cell bodies and begin extending axons around 9 hours of development (Patel et al., 1987; Halpern et al., 1991; H. Sink, unpublished results). Several of the same neurons (e.g., RP1 and RP3) also express the homophilic CAM Fasciclin I (Fas I) on both their cell bodies and axons (Zinn et al., 1988; McAllister et al., 1991). A different, but overlapping subset of RP neurons express the homophilic CAM connectin (Nose et al., 1992; Meadows et al., unpublished data) during the same developmental period. In particular, RP3 expresses Fas III, Fas I, and Connectin on its cell body and axon during its medial migration and the onset of axonogenesis. Mutations in any one of the genes that encode these proteins does not affect early neurogenesis and, in particular, RP3 migration and axon outgrowth are normal in single mutants (Elkins et al., 1990; A. Nose et al., unpublished results). These observations suggest that if these CAMs do play a significant role in directing cell-specific migrations and axon outgrowth in the early CNS, then it is their combinatorial and overlapping expression that controls these events. Indeed, a double mutation in the *fasI* gene and in the *abelson* (*abl*) gene, which encodes a cytoplasmic tyrosine kinase, blocks RP1 and RP3 axon extension across the CNS midline (Elkins et al., 1990). In the absence of crossing the midline, in the *fasI; abl* double mutant, the RP1 and RP3 axons instead extend on their own side of the CNS and in many but not all cases, project out the ipsilateral ISN. The extension of the RP1 and RP3 growth cones across the CNS midline is also blocked in mutations in the *commissureless* gene (Seeger et al., 1993), as described below.

RP3 PATHFINDING AND TARGET RECOGNITION

RP3 develops a prominent contralateral axon, which

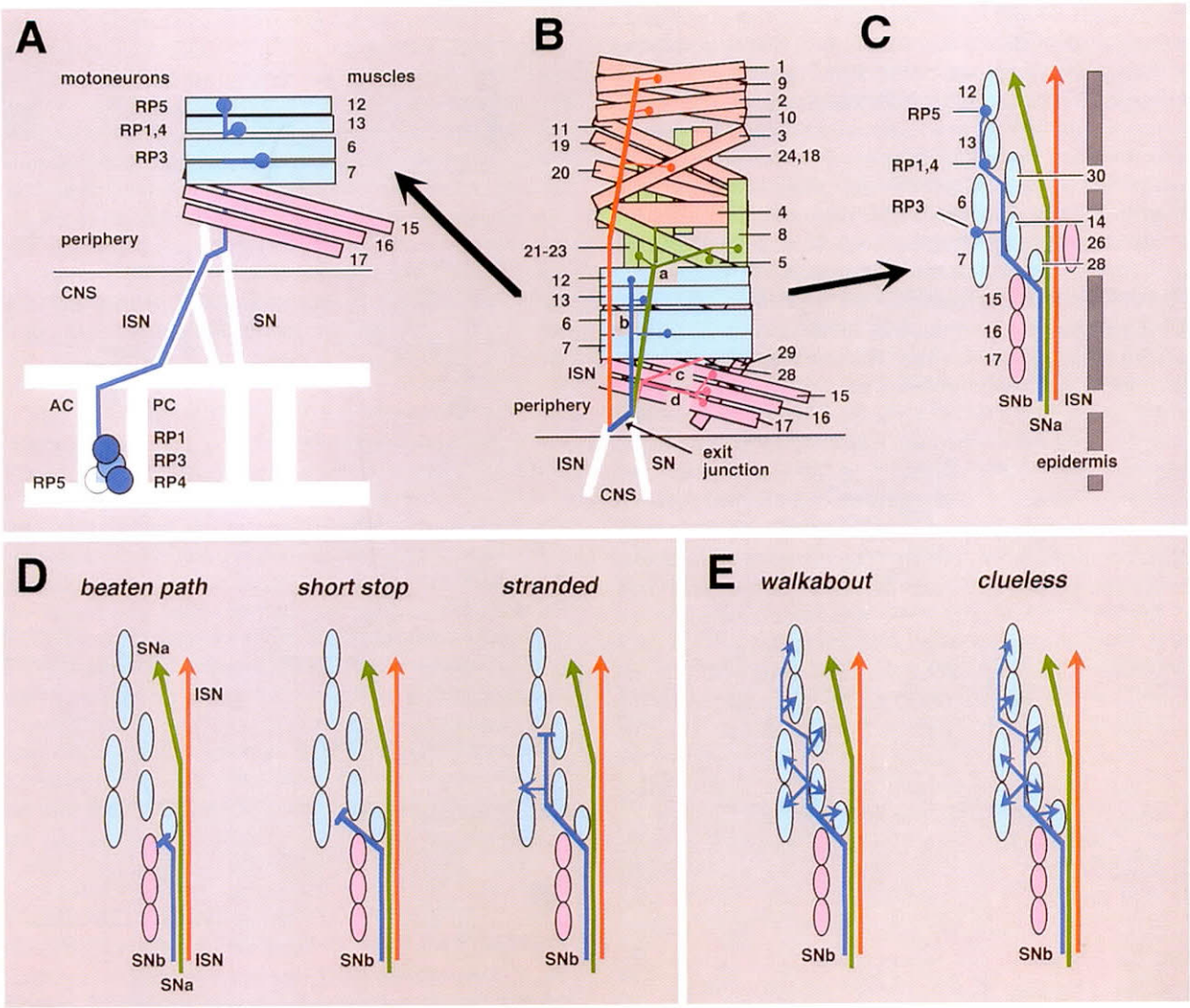

Fig. 1. Motoneuron projections in wild-type and mutant *Drosophila* embryos. (A) The location of the cell bodies of the RP neurons (RP1, RP3, RP4 and RP5) in the CNS, and the common trajectory of their axons as they exit the CNS in the ISN (intersegmental nerve), cross over to the SNb (segmental nerve b) at the exit junction, and then extend to contact the ventrolateral muscles (in blue). Once they reach their common target domain, these identified motoneurons make specific synaptic contacts with particular muscle fibers such that RP3 innervates muscles 6 and 7, RP1 and RP4 innervate muscle 13, and RP5 innervates muscle 12. AC, anterior commissure; PC, posterior commissure. (B) The embryonic motoneuron branches and muscles in an abdominal hemisegment (A2-A7) are shown schematically. Each major nerve branch is shown in a different color as it emerges from the exit junction outside of the CNS, with its muscle target field shown in a corresponding but lighter shade. The ISN (in red) projects to dorsal muscles 1, 2, 3, 4, 9, 10, 11, 18, 19, and 20. The SNa (in green) projects to lateral muscles 5, 8, 21, 22, 23, and 24. The SNb (in blue) projects to ventrolateral muscles 6, 7, 12, 13, 14, 28 and 30 (14 and 30 are hidden beneath the internal muscles; see Fig. 2). Branches SNc and SNd (in purple) both innervate the ventral muscles 15, 16, 17, 26, 27, and 29. Although SNa, SNb, and the ISN actually grow beneath the ventral muscles, the trajectory of each nerve branch has been brought to the foreground for clarity. This pattern corresponds to embryonic stage late 16 and 17. The periphery is represented as a flattened fillet preparation, with ventral CNS at the bottom and the dorsal midline at the top. Anterior is to the left. (C) The trajectory of the SNb and the specific pattern of target muscle innervation of the identified motor neurons (RP3, RP1, RP4 and RP5) are shown in schematic cross section. The SNb splits away from the ISN and SNa at its entry to the ventral muscle field near muscle 28. (D,E) The development of mutant SNb motor projections. (D) The location where SNb growth cones stop in *beaten path* mutants approximately corresponds to the first major choice point in SNb pathfinding where growth cones make extensive contacts with the surfaces of muscles 28 and 14 as they split away from the ISN and SNa. *short stop* mutant growth cones stop just beyond this first choice point at contacts with muscle 14 where the SNb must shift trajectory to grow between the ventral longitudinal muscles (internal) and the more external muscles 14 and 30. The location where SNb growth cones stop in *stranded* mutants corresponds to the second major choice point in SNb pathfinding at muscle 30 where RP motor neurons 1, 4 and 5 must shift to a pathway internal to muscles 13 and 12. (E) SNb growth cones in *walkabout* and *clueless* mutants extend into the ventral domain, but fail to correctly recognize the appropriate target muscles, instead arborizing over a range of adjacent ventral muscle surfaces. Although not shown, these mutant axons also can be found crossing segment boundaries, and occasionally following a pathway along the internal or external surfaces of the ventral muscle mass. (Adapted from Van Vactor et al., 1993).

projects across the CNS midline and then out the ISN to innervate its specific muscle targets. RP3 first generates an axon during its medial migration at 9.5 hours of development (stage 13) (Patel et al., 1987). The axon projects within the anterior commissure to join the contralateral ISN (stage 14) (Jacobs and Goodman, 1989).

Jacobs and Goodman (1989) described the pattern of RP3 axon outgrowth in the CNS from reconstructions of serial electron micrographs. Subsequently, several groups (Halpern et al., 1991; Sink and Whitington, 1991b; Broadie and Bate, 1993a) described RP3 axon pathfinding within the CNS and into the periphery to its target muscles using intracellular injection of the fluorescent dye, Lucifer Yellow (LY), and with antibodies specific for small subsets of motor axons (i.e. Fas III expression on RP3). Sink and Whitington (1991b) divided the sequence of axonogenesis for the RP1, RP3, RP4, and RP5 motor neurons into five phases. Thus, although axonogenesis for all four neurons is very similar up to the time they contact and make their synaptic connections on specific ventral muscles, the actual sequence shown in Fig. 2, and described below, is specifically for RP3 (H. Sink and P. M. Whitington, unpublished results). First, during its soma migration, the growth cone of RP3 extends medially along the axon of its contralateral homologue in the anterior commissure to contact and wrap around the contralateral RP3 cell body (stage 12; Fig. 2A,B). Second, the RP3 axon grows posteriorly in the dorsal contralateral longitudinal connective, fasciculating with other RP axons within the dorsal RP tract (stage 13; Fig. 2C). Third, the RP3 axon leaves the longitudinal tract, turns laterally, and enters the ISN via the anterior ISN nerve root previously pioneered by the aCC growth cone (Fig. 2D). Once outside the CNS, the RP3 axon crosses over to the SN at the exit junction (Fig. 2E), before contacting the surfaces of the ventral muscles (stage 14). Fourth, having reached muscles 15 and 16, RP3 and the other RP axons diverges from the main SN into the SNb (Johansen et al., 1989) to contact the internal face of muscle 28 (Fig. 2F). This location where RP3 and other RP axons leave the main SN to form the SNb has been termed a choice point to denote that several alternate paths are available and that different growth cones make divergent choices when confronted with this choice (Van Vactor et al., 1993). Lateral to muscle 28, RP3's axon grows between the most internal and intermediate of the three muscle layers (Bate, 1990), advancing laterally across the ventral muscles (stage 15). During this period, RP3 axonal processes ramify over a restricted ventral muscle field (see below), contacting both target and non-target muscles. Fifth, RP3's inappropriate processes are withdrawn to generate its mature projection pattern onto its target muscles (Fig. 2G), the two ventral muscles 6 and 7 (stage 16).

In addition to its primary axon, RP3 develops additional processes within the CNS. As RP3's motor axon is ramifying within the ventral musculature (stage 15), the soma extends a collateral process in the ipsilateral longitudinal connective (Sink and Whitington, 1991b). Dendritic branches develop within the dorsal neuropile contralateral to the soma (Fig. 2E-G).

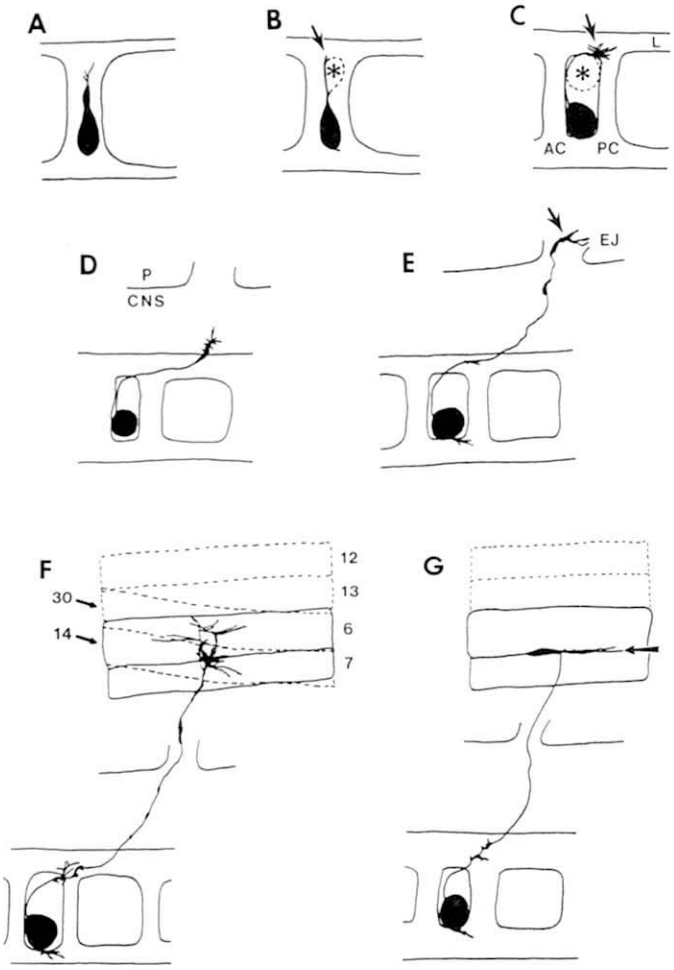

Fig. 2. Development of RP3 motoneuron from axonogenesis to synapse formation. Camera-lucida drawings of RP3 motoneuron as revealed by intracellular injection of Lucifer Yellow dye into the cell body in dissected embryo fillets. (A) Stage early 13: initial RP3 growth cone extends across the midline as the cell body finishes its migration towards the midline. (B) Stage mid-13: extension of RP3 growth cone (arrow) around the cell body of its contralateral homologue (asterisk). (C) Stage late 13: after extending around cell body of contralateral homologue (asterisk), RP3 growth cone (arrow) turns posteriorly towards the intersegmental nerve root. (D) stage early 14: RP3 growth cone turns laterally to begin heading out the intersegmental nerve (ISN) root. (E) Stage late 14: just after the RP3 growth cone (arrow) exits the CNS in the ISN, it enters the exit junction (EJ) region where it leaves the ISN as a distinct fascicle with other RP axons forming the segmental nerve b (SNb). (F) Stage late 15: expanded RP3 growth cone explores the surface of ventrolateral muscles 14, 30, 7 and 6 prior to retracting exuberant filopodia and selecting its specific target muscles 7 and 6. (G) Stage late 16: the RP3 growth cone has transformed into a presynaptic ending (arrow) in the cleft between muscles 7 and 6 (H. Sink and P.M. Whitington, unpublished results).

TARGET ABLATIONS LEAD TO SPECIFIC DEFECTS IN RP3 PATHFINDING AND TARGETING

Several studies (Sink and Whitington, 1991c; Chiba et al., 1993; Keshishian et al., 1993) have examined RP3 axono-

genesis in the absence of its target muscles (6 and 7). RP3's muscle targets have been deleted using genetic, experimental and laser ablation techniques. In the absence of target muscles 6/7, RP3 still diverges from SNb into the correct ventral muscle field, arguing against the release of chemoattractants by these specific target muscles (although such chemoattractants might be released by the more general ventral domain of muscles and thus by the remaining ventral muscles). Without its normal targets, RP3's axon ramifies over the normal field of ventral muscles and, within these muscles, apparently makes contacts at random and often innervates one or more incorrect targets (Fig. 3B). In the absence of only one of its targets (6 or 7), RP3 still can identify the remaining target, although not as reliably as when both targets are present. In the absence of adjacent non-target muscles, which RP3 normally encounters either en route to its target or following target contact, RP3 still contacts and recognizes its muscle 6/7 target.

These observations suggest that (1) RP3 leaves the SN to enter the ventral muscles using cues from sources other than its specific target muscles, and (2) that once in the ventral muscle region, RP3 receives particular cues from muscle 6/7 which enable it to identify them as it's targets. Moreover, contact between RP3 and its correct targets appears to play a decisive role in signaling the retraction of inappropriate processes. Thus, muscles 6 and 7 must possess unique characteristics that identify them as RP3's primary targets and these characteristics are maintained even if the number of ventral muscles, and therefore their relative positions, are altered.

CERTAIN MUTATIONS LEAD TO SPECIFIC DEFECTS IN RP3 PATHFINDING AND TARGETING

Seeger et al., (1993) conducted a near saturation screen for mutations that affect the pattern of commissural and longitudinal axons in the developing CNS. This screen identified several genes required for the construction of longitudinal connectives [e.g., *longitudinals lacking* (*lola*), *longitudinals gone* (*logo*)] and axon commissures [e.g., *commissureless* (*comm*) and *roundabout* (*robo*)]. These mutations identify genes that are required for the early stages of neuronal pathfinding and navigation within the developing CNS, including the navigation and path choices made by the RP3 growth cone. In particular, mutations in *comm* block the early navigation of the RP3 axon towards and across the CNS midline in the anterior commissure. In *comm* mutants, RP3's contralateral projection is blocked and instead an axon projects ipsilaterally, eventually leaving the CNS in the ipsilateral ISN, switching to the SNb, and innervating the ipsilateral homologue of its target muscles (6 and 7), a phenotype intriguingly similar to that of the *fasI; abl* double mutant mentioned above (Elkins et al., 1990). Thus, *comm* is one element in a genetic hierarchy that specifies progressive choice points in the navigation of the RP3 axon within the CNS.

Van Vactor et al., (1993) conducted a further genetic screen of the second chromosome (approx. 40% of the genome) in *Drosophila* for mutations affecting pathfinding outside the CNS and the development of neuromuscular connectivity (Fig. 1D,E). This screen identified several genes required for motor neuron pathfinding [e.g., *beaten path* (*beat*), *short stop* (*shot*) and *stranded* (*sand*)] and muscle target recognition [e.g., *clueless* (*clu*) and *walkabout* (*wako*)].

beat, *sand*, and *shot* are genes required for motor neurons to correctly navigate through specific choice points in both the ISN and SNb (Fig. 1D). In particular, the SNb defects observed in these mutants suggest that these three genes control discrete steps in RP motor neuron pathfinding. In *beat* mutants, RP axons (like RP3) fail to separate from the ISN and SNa to pioneer SNb. Thus, *beat* is required for the RPs to enter the ventral muscle domain. In *shot* mutants, RPs succeed in pioneering SNb and just enter the ventral muscle domain, but usually do not succeed in contacting distal target muscles. In *sand* mutants, RP axons get a little further and usually manage to navigate past muscles 28 and 14 before terminating. RP3 often reaches its target muscles, but more dorsally projecting RPs fail to reach their targets. Thus, *beat*, *shot*, and *sand* appear to define a hierarchy of genes that is required for proper pathfinding of the RP axons to leave the SN, enter the SNb, and then properly enter and explore the ventral muscles (Fig. 1D).

Mutations in two genes — *wako* and *clu* — prevent RP neurons from recognizing their correct target muscles. In these mutations, all the RP axons have access to and contact with their target muscles, but still fail to form appropriate neuromuscular synapses (Figs 1E, 4, 5). Unlike the pathfinding mutants *beat*, *shot*, and *sand*, mutations in *wako* and *clu* (in the alleles $wako^1$, $wako^2$, and clu^1) appear to affect specifically the axons in the SNb and a small portion of the motor axons in SNa, while other axon trajectories are normal (Van Vactor et al., 1993). In *wako* and *clu*, RP axons in SNb ramify widely over the ventral muscles, occasionally even crossing the segment boundary (Figs 4, 5), and appear, in the light microscope, to form neuromuscular contacts at random among these muscles. In particular, the normal RP3 target synaptic site on muscles 6 and 7 receives little or no innervation in these mutants and, instead, RP3 synapses at unpredictable locations on one or more adjacent ventral muscles (Fig. 5). However, as in wild-type development, RP3's axon is always restricted within a subset of the ventral muscles and will only form synapses with muscles within this restricted field. Thus, both *wako* and *clu* are genes that are required for the RP axons in SNb to recognize their correct target muscles once they have entered the ventral muscle domain.

The SNb is unique among the peripheral nerve branches because most of the motor axons in this nerve (i.e. RP1, RP3, RP4, and RP5), and their muscle targets (i.e. 28, 7, 6, 14, 13 and 12 — listed in order from the ventral midline), have been individually identified (Fig. 1A-C). This has allowed us to follow individual axon trajectories in various mutant backgrounds or experimental conditions. RP3, in particular, has been studied by intracellular LY injection in mutant (e.g. *wako*) genetic backgrounds (Fig. 5B). For example, in embryos mutant for *wako*, RP3 fails to recognize its target in the synaptic cleft between muscles 6 and 7, and instead continues to grow over and past its target and even cross the segment boundary (Van Vactor et al., 1993). This mutant phenotype is intriguingly reminiscent of

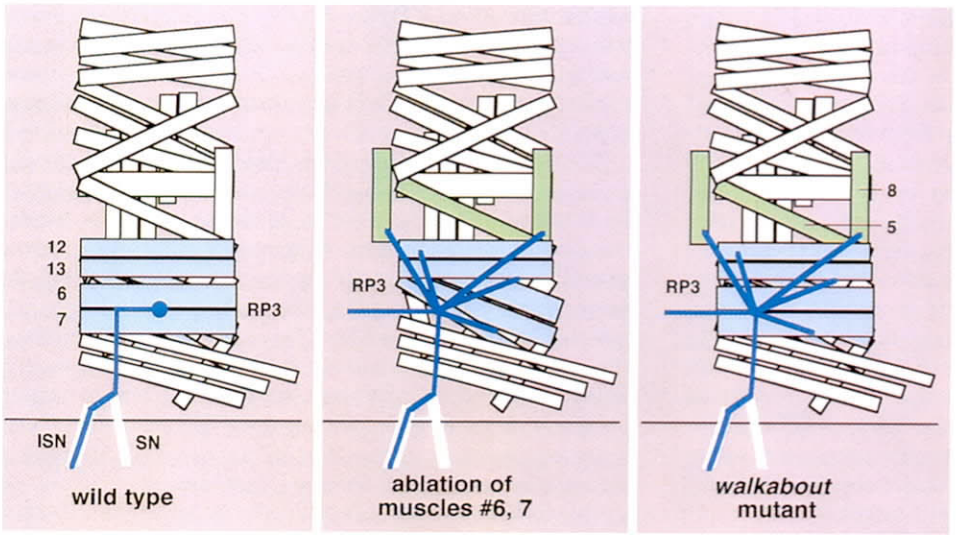

Fig. 3. Trajectory of RP3 in wild-type, target muscle ablation, and *walkabout* mutant embryos. (A) Wild-type innervation of RP3 in the cleft between muscles 6 and 7. (B) Summary of aberrant projections of wild-type RP3 axons in embryos in which muscles 6 and 7 had been surgically ablated prior to target exploration (based on intracellular injections of Lucifer Yellow dye) (summary of data from Sink and Whitington, 1991c). (C) *walkabout* RP3 growth cones fail to recognize their appropriate target muscles 6 and 7, and instead contact alternative surfaces within the ventrolateral target domain. Mutant axons often cross segment boundaries to interact with muscles in adjacent ventral muscle fields. The ventral target domain to which RP3 is restricted in *walkabout* mutants (Van Vactor et al., 1993) is identical to the muscles innervated by RP3 when its target muscles 6 and 7 are surgically ablated (as shown in B, Sink and Whitington, 1991c). In addition to the ventrolateral muscles normally innervated by SNb motor neurons (shown in blue), in either *walkabout* mutant embryos or muscle 6/7 ablations, RP3 appears to selectively contact muscles 5 and 8 (shown in green) which are normally innervated by the lateral branch of SNa. Interestingly, in addition to the motor neurons in the SNb, the only other motor neurons that appear to be abnormal in *walkabout* mutant embryos are the ones that normally innervate muscles 5 and 8. (Adapted from Van Vactor et al., 1993).

RP3 behavior when its muscle targets have been ablated (Fig. 3; Sink and Whitington, 1991C). In both cases, RP3's axon wanders over and arborizes, apparently at random, with neighboring muscles — but only in a highly restricted ventral muscle set. These observations have led to the idea of "muscle target domains" (Van Vactor et al., 1993), defined as a group of neighboring muscles sharing common signals that attract appropriate cohorts of motor neuron growth cones. In the case of RP3, the muscle domain includes the normal muscle targets of the SNb motor neurons as well as the more lateral muscles 5 and 8 (Fig. 3; Van Vactor et al., 1993). This concept suggests that (1) groups of motor neurons and their target muscles share a common identity, and (2) specific genes (e.g. *wako* and *clu*) operate in pathways that subdivide these common identity groups so that individuals can be distinguished and synaptically coupled according to unique identities (e.g., RP3 with muscles 6/7).

RP3 SYNAPSES ON MUSCLES 6 AND 7

The RP3 axon projects in SNb to innervate a pair of large, ventral, internal-longitudinal muscles [numbers 6 and 7 (Fig. 6); Crossley, 1978; Bate, 1990]. The first indication of synaptogenesis is the transient expression of the homophilic CAM Fas III (Snow et al., 1989) at the synaptic site along the medial lateral border between the two muscles — termed the synaptic cleft — starting about 12 hours of development (Halpern et al., 1991). Fas III is also strongly expressed on the RP3 growth cone prior to and during the early stages of neuromuscular contact (Fig. 7B; Halpern et al., 1991). In the absence of RP3, Fas III is still expressed on its target muscles at the correct synaptic site (Broadie and Bate, 1993c). These observations lead to the suggestion that differential adhesion mechanisms might be involved in either neuromuscular recognition or the recognition of specific synaptic sites on the muscle target.

However, arguing against these two hypotheses is the observation that spatially restricted Fas III expression is neither necessary nor sufficient to mediate synaptogenesis between RP3 and muscles 6/7. Null mutations in *fas III* are viable (Elkins et al., 1990) and do not appear to perturb the development of neuromuscular specificity (Keshishian et al., 1993). Moreover, mutations in *wako* or *clu* do not perturb normal Fas III expression and yet they block RP3's recog-

Fig. 4. SNb development in wild-type and *walkabout* mutant embryos. Both photographs show late stage 16 embryonic fillets stained with mAb 1D4, which recognizes the transmembrane forms of fasciclin II. In the wild-type in A, the two target muscles (7, 6) of RP3 are identified. In the *walkabout* mutant embryo shown in B, muscles 7 and 6 are also identified as reference points. In *walkabout* mutant embryos, the ventral muscles appear normal. (A) Ventral longitudinal muscles 7, 6, 13 and 12 are innervated in a highly stereotyped pattern in abdominal segments of wild-type embryos (A2-A7). The arrow shows the branch of SNb which innervates muscles 7 and 6 (motor neuron RP3). SNc (c) and SNd (d) can be seen clearly in this photograph, although the SNa (a) is out of focus. The ISN can also be seen as it passes beneath these ventral muscles. The lateral edge of the ventral CNS is shown below. (B) SNb growth cones, failing to recognize their correct targets in *walkabout* mutant embryos, often cross the segment boundary to search ventral longitudinal muscles of adjacent segments (arrow). Other types of abnormal SNb projections are seen in adjacent segments. (Adapted from Van Vactor et al., 1993).

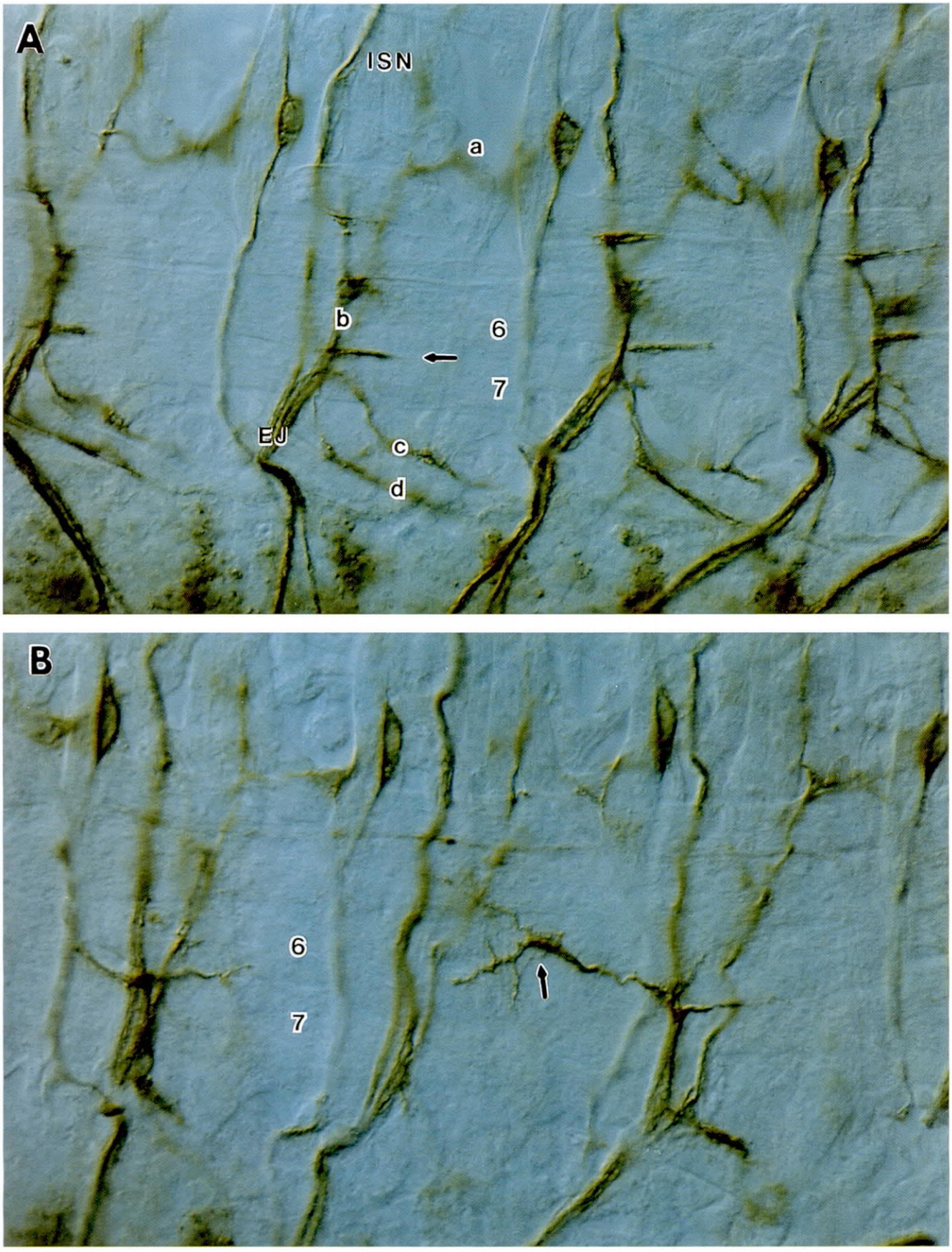

Fig. 4

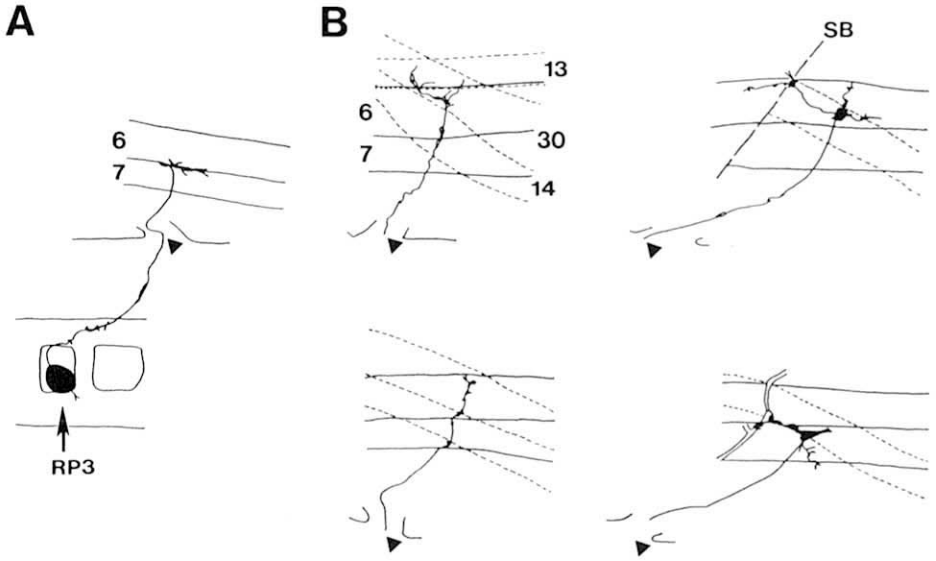

Fig. 5. RP3 axonal projections in *walkabout* mutant embryos. (A) The normal trajectory and muscle target contacts of the SNb motor neuron RP3 is shown in this camera lucida drawing from a wild-type, Lucifer Yellow (LY)-filled RP3 at stage 16. The RP3 cell body (in black) assumes a characteristic position and cell layer within the CNS neuropil scaffold. After crossing the midline, the RP3 axon extends towards the exit junction (marked with a triangle). Outside of the CNS, RP3 joins the SNb (not shown), and finally makes a stereotyped terminal arbor in the cleft between muscles 7 and 6. (B) Four examples of RP3 trajectories in *wako*^{1} homozygous mutants are shown. As in A, the triangles mark the RP3 exit point from the CNS. In each case, the surfaces of ventral muscles other than the targets of RP3 are drawn in dashed lines to show that abnormal contacts are now made with these adjacent muscles. In 7 out of 9 individually dye-filled, *wako*^{1} RP3 neurons, we found the same types of mistargeting phenotypes where RP3 could be seen extending beyond target muscles 7 and 6 to contact alternative muscles or to cross the segment boundary (SB). (Adapted from Van Vactor et al., 1993).

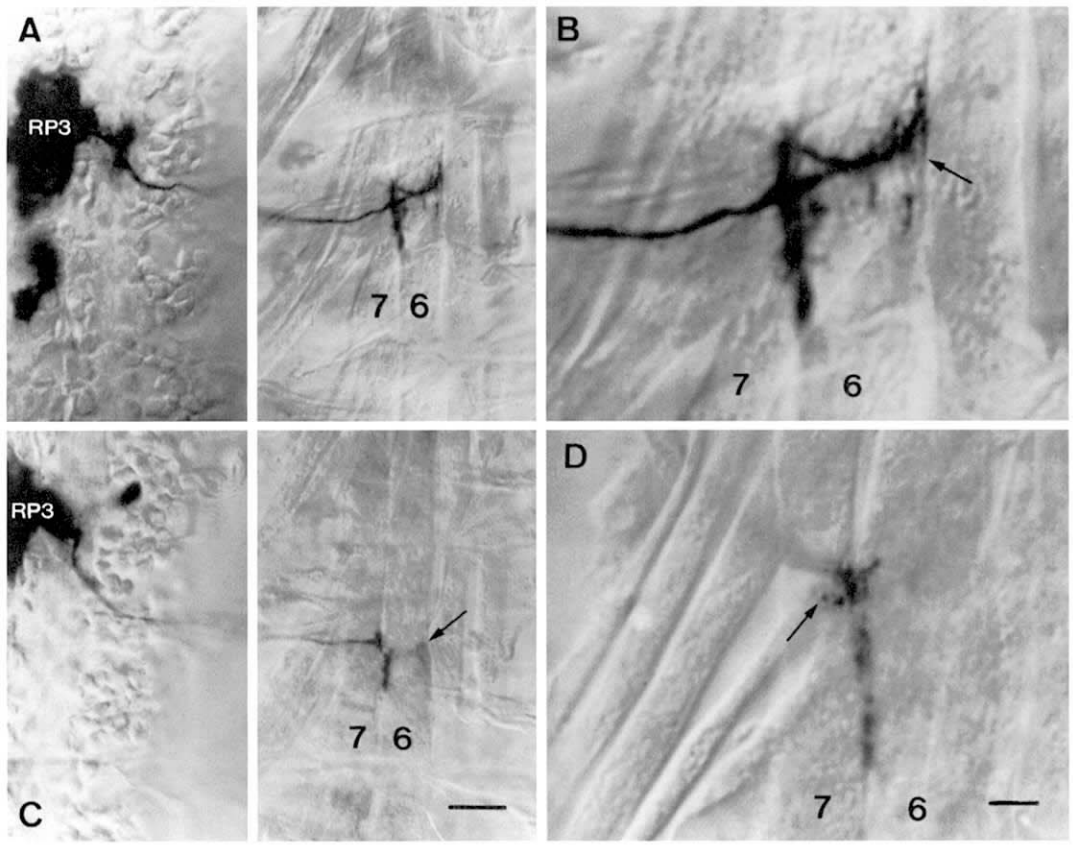

Fig. 6. The late stages of morphological synaptogenesis between RP3 and muscles 6 and 7. Staged embryos were dissected along the dorsal midline, a dye injected into the RP3 cell body and the preparation examined with Nomarski optics (Broadie and Bate, 1993a): the CNS is left and anterior is at the top. (A) In early stage 16 (13-14 hours), extensive filopodial exploration of the ventral muscles occurs. RP3's axon terminal takes on a complex morphology with extensive arborization in the cleft between muscles 6/7 and apparently variable production of incorrect side branches onto adjacent muscles. (B) Detail of the incorrect branches (arrow) shown in panel A. These side branches contact all the muscles in the ventral muscle domain without apparent pattern or restriction. (C) By mid-stage 16 (14.5 hours), most of RP3's incorrect branches have been retracted (arrow shows single remaining process). (D) By stage 17 (16 hours), all incorrect branches have been retracted and RP3 has established its mature projection onto muscles 6 and 7. The NMJ occupies the mature synaptic site in the anterior cleft between its two targets and contains numerous varicosities (arrow), or boutons, characteristic of presynaptic transmitter release sites. Scale bars; 10 μm (A,C), 3 μm (B,D).

nition of its target (Van Vactor et al., 1993). Thus, Fas III may work in some combinatorial way to establish or strengthen early synapses, or alternatively, it may not play a significant role in these events but rather may control some as yet undefined functions. Other proteins, such as Toll (Nose et al., 1992), are known to be expressed at the synaptic cleft of muscles 6/7 with a pattern similar to Fas III and may play a role in similar mechanisms.

Several studies (Johansen et al., 1989a; Halpern et al., 1991; Sink and Whitington, 1991b; Broadie and Bate, 1993a) have described the morphological differentiation of the neuromuscular junction (NMJ) between RP3 and muscles 6/7 using intracellular LY injections in RP3 (Fig. 6) and immunohistological observations at both the light and scanning electron microscope levels (Fig. 7). NMJ morphogenesis can be divided into six stages. First, morphological differentiation of the NMJ begins with three prominent RP3 growth cone processes in the synaptic cleft, two anterior and 1 posterior to the anterior lateral axon entry point (Fig. 7A; 12.5-13 hours). Second, during these initial stages of RP3 contact with its target, RP3 maintains exuberant processes over a variable range of muscles in the ventral muscle domain, often even extending beyond its target to contact muscles 30, 12, and 13. These processes are distinct from growth cone filopodia, in that the processes are both thicker and longer than filopodia, and perhaps should be termed 'axon branches' (Fig. 6A,B). Third, in early stage 16 (13-14 hours), RP3 refines its terminal arbor in the synaptic cleft between muscles 6 and 7. Inappropriate axonal branches to non-target muscles are retracted and the anterior process within the cleft is also retracted, leaving the mature projection of RP3 processes restricted to the synaptic cleft posterior to the axon entry point (Fig. 6C,D). Fourth, RP3 develops presynaptic specializations in the form of varicosities or boutons (presynaptic transmitter release sites) at 14-15 hours (Figs 6D, 7C). Fifth, a second motor neuron synapses at RP3's pre-established synaptic site (15-16 hours; Fig. 7B). Sixth, during stage 17, the NMJ develops its mature morphology (Figs 6D, 7C).

Broadie and Bate (1993a,b) described the accompanying physiological development of the NMJ between RP3 and muscle 6 with whole-cell patch-clamp techniques and divided synaptic differentiation into several progressive

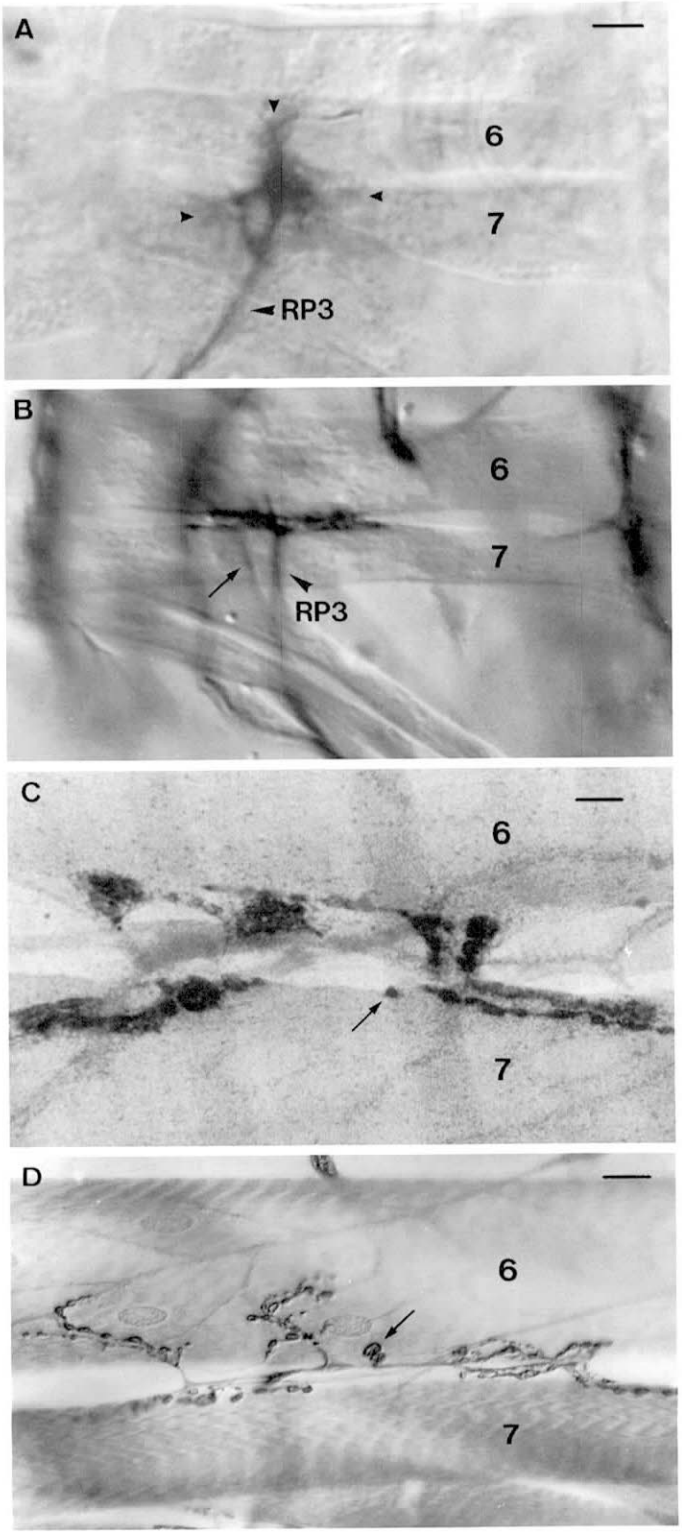

Fig. 7. The differentiation of the neuromuscular synapse between RP3 and muscles 6 and 7. (A) Dye injection into RP3's cell body reveals the axonal growth cone during its initial contact with target muscles 6 and 7 in late stage 15 (12.5-13 hours). RP3's growth cone contacts muscles 6/7 at a characteristic position in the anterior cleft between the muscles and prominent filopodia (arrowheads) explore both the cleft and the adjacent muscles. (B) Immunohistological staining of Fasciclin III (Fas III) reveals the double innervation of muscles 6/7 in stage 17 (16 hours). Fas III is expressed on RP3's axon, on the later axon (arrow), and on the muscle membrane at the shared synaptic site.
(C) Immunohistological scanning electron microscopy (SEM) against a neural cell-surface antigen (anti-horseradish peroxidase; Jan and Jan, 1982) reveals the NMJ between RP3 and muscles 6 and 7 in late stage 17 (18-19 hours). The NMJ has developed the rudiments of its mature morphology including restriction to the mature synaptic site, numerous boutons (arrow) and axonal bridges spanning the cleft between the two muscles.
(D) Immunohistological staining of the excitatory neurotransmitter, L-glutamate (Broadie and Bate, 1993a), reveals the mature NMJ between RP3 and muscles 6 and 7 in the wandering third instar larva. The NMJ is considerably elaborated during larval life and develops numerous boutons (arrow) and higher order synaptic branches on the muscle surfaces. Scale bars: 5 μm (A,B); 1 μm (C) and 20 μm (D).

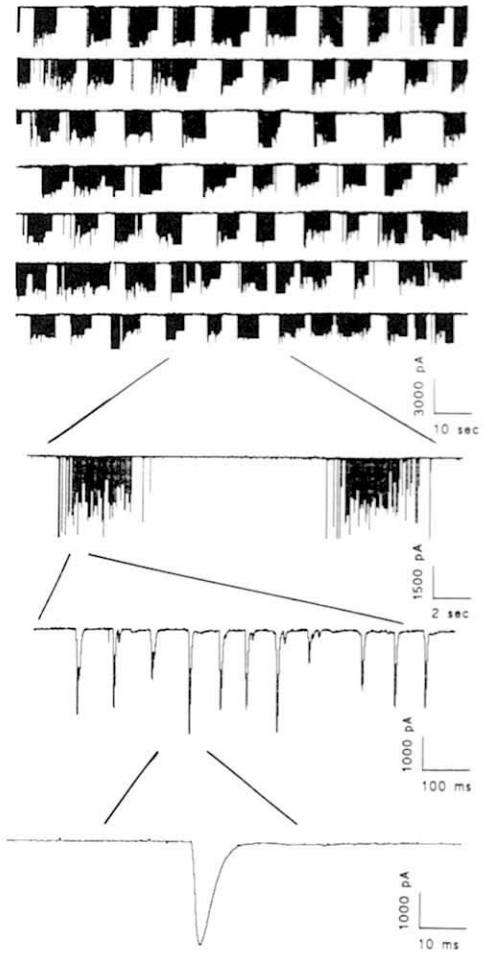

Fig. 8. Excitatory junction currents (EJCs) recorded at the NMJ between RP3 and muscle 6 in the newly hatched larva. The muscle was voltage-clamped at −60 mV in whole-cell configuration and endogenous synaptic EJCs recorded in normal saline (Broadie and Bate, 1993a). In the larva (24 hr), synaptic currents occur as EJC bursts with a regular periodicity. Each burst triggers a wave of peristaltic muscle contraction that occurs during locomotory movements. Between EJC bursts, synaptic activity is strongly suppressed and large EJCs (>100 pA) rarely occur.

steps. First, during the initial stages of RP3/muscle 6 contact (12.5-13 hours), muscle 6 is electrically and dye-coupled to several adjacent muscles in the ventral muscle domain. Second, muscle uncoupling (13 hours) heralds the onset of a rapid period of differentiation; the muscle membrane rapidly develops its mature electrical properties, the contractile apparatus becomes functional, and physiological receptors for the excitatory neurotransmitter, L-glutamate, are expressed in the muscle membrane. Third, glutamate receptors (gluRs) are initially homogeneously expressed in the muscle membrane (13-14 hours), but are localized to the synaptic cleft soon after the refining of the presynaptic arbor (14-16 hours AEL). Fourth, the NMJ becomes functional and RP3 activity begins to drive contractions in its target muscles (14-15 hours). Coordinated peristaltic muscle movements soon develop. Fifth, during stage 17 (16 hours onwards), the density of gluRs in the postsynaptic receptor field is dramatically increased. Sixth, by the end of embryogenesis, RP3 generates rhythmic bursts of synaptic excitatory currents that drive the periodic contraction of muscles 6 and 7 during locomotory movement (Fig. 8).

RP3 plays no discernible role in the differentiation of muscle properties in its target muscles (Broadie and Bate, 1993d); in the absence of RP3, muscle 6 develops normal muscle contractile and electrical properties. In contrast, RP3 does induce postsynaptic specializations in muscle 6 (Broadie and Bate, 1993c). In the absence of RP3, muscle 6 expresses functional gluRs but fails to localize these receptors to the synaptic domain. Furthermore, the upregulation of functional gluR expression associated with late synaptic development also fails to occur in the absence of RP3. Thus, RP3 provides signals to its muscle target that induce the construction of the postsynaptic receptor field.

POSTEMBRYONIC DEVELOPMENT AND FUNCTION OF RP3

At hatching, the morphology of the NMJ between RP3 and its targets, like other NMJs, is relatively rudimentary (Fig. 7C). During postembryonic development, the NMJ is refined with higher order synaptic branching and the elaboration of presynaptic boutons (Fig. 7D; Keshishian et al., 1993; Atwood et al., 1993; Jia et al., 1993). In addition, as the larva grows rapidly during its four day life, the synapse must also grow accordingly to accommodate the enlarging muscle. Thus, though the basic neuromuscular connectivity is established during embryogenesis, synaptic development continues well into postembryonic life. In the embryo, neural pathfinding and morphological synaptogenesis proceed through largely activity-independent mechanisms, such as selective adhesion. For example, RP3 develops a normal terminal arbor on muscles 6 and 7 in the complete absence of neural electrical activity (Keshishian et al., 1993; K. Broadie and M. Bate, unpublished observations). However, postembryonic synaptic development proceeds through distinct activity-dependent mechanisms. Larval NMJs, including that of RP3 on muscles 6 and 7, show use-dependent morphological plasticity (Budnik et al., 1990; Jia et al., 1993), such that increased electrical activity (e.g., in the hyperexcitable double mutant *eag shaker*) increases both the number of synaptic branches and boutons.

These NMJs show altered synaptic plasticity in memory mutants with a defective cyclic AMP (cAMP) cascade (Zhong et al., 1992). For example, *dunce* (*dnc*), which affects the cAMP-specific phosphodiesterase, increases the number of branches and boutons of RP3 on muscles 6/7. Likewise, *rutabaga* (*rut*), which affects adenylate cyclase, suppresses this *dnc*-mediated effect. Thus, the cAMP messenger cascade affects morphological development. Similarly, the cAMP cascade plays a role in both synaptic facilitation and potentiation between RP3 and muscle 6 (Zhong and Wu, 1991) thus, both activity-dependent and cAMP-dependent plasticity are important elements of synaptic development as they provide mechanisms whereby the animal can selectively strengthen or weaken neuromuscular connections based simply on their use.

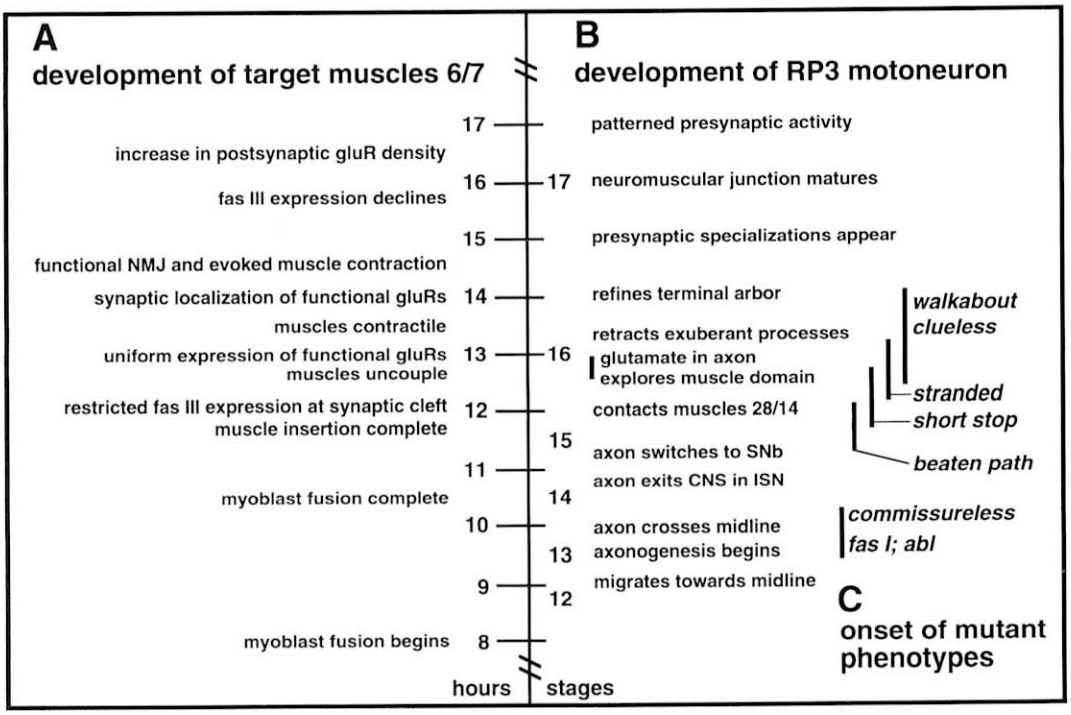

Fig. 9. Time line of the development of the RP3 motoneuron and its target muscles 6 and 7. The development of muscles 6 and 7 (A, left side) and RP3 motor neuron (B, right side) is summarized, showing the temporal sequence of notable events. RP3 development has been thoroughly studied from the beginning of axonogenesis to the physiological maturation of its synapse with muscles 7 and 6 (Patel et al., 1987; Johansen et al., 1989a, b; Halpern et al., 1991; Sink and Whitington, 1991a, b; Broadie and Bate, 1993a, b; Van Vactor et al., 1993). Further studies of neuromuscular development beyond stage 17 (including larval stages) are not covered in this timeline. (C) The range of time when defects arise in different mutants are indicated by black bars. The *commissureless* and *fas I/abl* mutant phenotypes are described by Seeger et al. (1993) and Elkins et al. (1990), respectively. The other mutants are described by Van Vactor et al. (1993). The approximate time after egglaying is shown relative to embryonic stage as described by Campos-Ortega and Hartenstein (1985). (Adapted from Broadie and Bate, 1993a, and Van Vactor et al., 1993).

Jan and Jan (1976a,b) first described the morphological and physiological characteristics of the mature larval NMJ between RP3 and muscles 6 and 7. In the larva, muscles 6 and 7 are among the largest longitudinal muscles and, working as a jointly innervated muscle unit, provide much of the force for the animal's normal locomotory movements. RP3 is an excitatory motor neuron, which elicits muscle contraction via the excitatory transmitter L-glutamate (Figs 7D, 8; Jan and Jan, 1976b; Johansen et al., 1989b). The larva moves with a series of regular peristaltic muscle movements. Movement is controlled in each muscle with a brief burst of high frequency excitatory junctional currents (EJCs) at the NMJ (Fig. 8). Periodic bursts of neural action potentials can be recorded in the peripheral motor nerves (Budnik et al., 1990) and, in the case of the NMJ between RP3 and muscle 6, this activity triggers a similar pattern of periodic EJC bursts at the neuromuscular synapse.

CONCLUSION

In this article, we summarize the life history of a single identified motor neuron — RP3 — and focus in particular on the events from its cell migration and axon outgrowth to the formation of its mature neuromuscular synapses (Fig. 9). The location of the RP3 cell body has made it possible to study its development throughout much of its embryonic and larval life. Likewise, the identity and development of RP3's synaptic targets — muscles 6 and 7 — have been described throughout their development (Fig. 9A). Finally, the excitatory neuromuscular synapse of RP3 onto muscles 6 and 7 has been examined both morphologically and physiologically throughout its development and during its mature function in the locomotion of the fly larva. This detailed characterization of a single neuromuscular unit has proven invaluable in the analysis of the function of molecules and genes implicated in neuromuscular development and function (Fig. 9C). In this review, we have briefly mentioned numerous genetic mutations, which have been isolated and/or analyzed using on gained from the resolution of working with individually identified neurons, muscles, and synapses. In the future, we hope to characterize these and other mutations and in so doing to gain an understanding of the molecular and genetic pathways which control the specificity, formation, maturation, and ongoing remodeling of identified synaptic connections in the fruitfly.

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