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Muscle development is independent of innervation during *Drosophila* embryogenesis

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SUMMARY

We have examined the role of innervation in directing embryonic myogenesis, using a mutant (*prospero*), which delays the pioneering of peripheral motor nerves of the *Drosophila* embryo. In the absence of motor nerves, myoblasts fuse normally to form syncytial myotubes, myotubes form normal attachments to the epidermis, and a larval musculature comparable to the wild-type pattern is generated and maintained. Likewise, the *twist*-expressing myoblasts that prefigure the adult musculature segregate normally in the absence of motor nerves, migrate to their final embryonic positions and continue to express *twist* until the end of embryonic development. In the absence of motor nerves, myotubes uncouple at the correct developmental stage to form single cells. Subsequently, uninnervated myotubes develop the mature electrical and contractile properties of larval muscles with a time course indistinguishable from normally innervated myotubes. We conclude that innervation plays no role in the patterning, morphogenesis, maintenance or physiological development of the somatic muscles in the *Drosophila* embryo.

Key words: *Drosophila*, myogenesis, innervation, ionic currents, invertebrate embryogenesis, *prospero*, *twist*

INTRODUCTION

Observations of neuromuscular development in *Drosophila* have shown that innervation is not involved in the early embryonic stages of muscle patterning of either the larval (Bate, 1990) or the adult (Bate et al., 1991) muscles. We have experimentally confirmed these observations and here extend the analyses to ask whether innervation plays a role in later myogenic events. For example, we wished to know if innervation plays a role in the late patterning and/or maintenance of both the larval muscles and the precursors of the adult muscles in the *Drosophila* embryo. Likewise, we wished to know if innervation may regulate the onset and progression of the larval muscle's physiological development in regards to the maturation of electrical properties and/or the contractile apparatus. Which aspects of myogenesis are intrinsic to myogenic cells and which depend on interaction with the innervating motor nerve? We have used a mutant, *prospero*, which delays peripheral motor innervation (Doe et al., 1991; Vaessin et al., 1991) to ask which, if any, of these stages of myogenesis are directed or regulated by innervation in the *Drosophila* embryo.

The earliest events of myogenesis occur prior to the pioneering of the peripheral motor nerves from the central nervous system (Johansen et al., 1989; Bate, 1990). The expression of nuclear proteins in small subsets of myoblasts occurs prior to germ band retraction (Dohrmann et al., 1990; Michelson et al., 1990; Bourgouin et al., 1992) and the initial myoblast fusion events that prefigure the larval muscle pattern begin with the onset of germ band retraction (Bate, 1990), yet pioneering of the motor nerves begins only after the completion of germ band retraction (Johansen et al., 1989; Broadie and Bate, 1993a). Hence, it is clear from timing alone that innervation is not involved in the initial crystallization of the larval muscle pattern (Bate, 1990). Likewise, innervation can not be required to initiate myoblast fusion; indeed, some of the smaller muscles (e.g. muscle no. 29) have completed fusion prior to neural contact (Bate, 1990). In contrast, peripheral motor nerves are closely associated with the developing myotubes for many hours prior to the establishment of the mature muscle pattern, while most muscles are still fusing with myoblasts and forming attachments to the epidermis (Johansen et al., 1989; Bate, 1990). Therefore, we wished to know whether innervation plays any role in late muscle patterning or formation of specific attachment sites. Furthermore, we wished to know if innervation plays a role in directing muscle morphogenesis and/or maintaining the differentiated muscle fibers.

In addition to the larval muscles, the prepattern of the adult musculature is established in the *Drosophila* embryo. This adult muscle prepattern is manifest as a pattern of persisting myoblasts which express the gene *twist* in the late embryo (Bate et al., 1991). These persistent myoblasts divide in the larva and pupa to produce *twist*-expressing cell groups fated to form specific subsets of adult muscle fibers (Currie and Bate, 1991; Broadie and Bate, 1991). In normal development, these persistent *twist*-expressing myoblasts

segregate from the much larger population of *twist*-expressing cells that produce the larval muscles and other mesodermally derived tissues. The segregation of persisting myoblasts begins prior to neural contact and so cannot be regulated by innervation (Bate et al., 1991). However, the segregating myoblasts quickly become closely associated with the developing nerves, an association that continues from mid-embryonic through to pupal stages when the adult muscles form (Bate et al., 1991; Currie and Bate, 1991). Therefore, we wished to know whether the motor nerves are required for the final segregation of the adult myoblasts, the persistent expression of the *twist* gene in these cells and/or the migration of the adult myoblasts in the embryo and later stages (Bate et al., 1991; Currie and Bate, 1991).

The first indication of the acquisition of muscle properties in the embryonic myotubes is the uncoupling of these cells to form single cell units (Broadie and Bate, 1993a). Immediately after uncoupling, the myotubes begin to develop the electrical and contractile properties of mature larval muscles (Broadie and Bate, 1993b). In normal development, myotube uncoupling coincides with the initial neuromuscular contact, physiological development begins immediately thereafter and the maturation of electrical and contractile properties occurs with constant neuromuscular interaction (Broadie and Bate, 1993a,b). We wished to know whether the acquisition of muscle properties may be signalled and/or subsequently regulated by the innervating motor nerve.

In this study, we combine morphological and physiological techniques to examine and compare all stages of myogenesis in normal and aneural (*prospero*) *Drosophila* embryos. Within the resolution of these techniques, we can detect no significant differences between muscles developing in the presence or absence of motor innervation. We conclude that myogenesis occurs independent of innervation at all stages of development in the *Drosophila* embryo.

MATERIALS AND METHODS

Fly stocks

The wild-type *Drosophilamelanogaster* strain Oregon-R was used as controls. *ru h th st* p^P *pros*^{14} *red e/ TM3 Sb* p^P *e* flies were outcrossed to wild type and their Sb^+ progeny crossed to generate the *pros*/+ stock used in these studies. Homozygous *prospero* (*pros*) mutants were distinguished by a grossly aberrant CNS evident in the dissected living embryo or stained whole embryos.

Preparation

Breeding flies were maintained on apple juice agar plates at 25°C and encouraged to lay eggs overnight. Wild-type and mutant (*pros/pros*) embryos were selected and staged by morphological criteria and dissected as reported earlier (Broadie and Bate, 1993a). All development times are reported in hours *a*fter *e*gg *l*aying (AEL) at 25°C and are displayed as decimals. Under these conditions, embryogenesis lasts 21±1 hours.

Immunocytochemistry

Several antibodies against components of the developing neuromusculature were used to stain both whole and dissected embryos: (1) anti-horseradish peroxidase antibody (anti-HRP; Cappell), which recognizes a neuron-specific cell surface antigen (Jan and Jan, 1982), (2) anti-*twist* antibody, which recognizes a nuclear protein expressed specifically in adult muscle precursors in the late embryo (Bate et al., 1991) and (3) anti-myosin heavy chain antibody (anti-MHC), which recognizes a major component of the contractile machinery in developing larval muscles. Staining procedures for whole and dissected embryos have been reported earlier (anti-HRP (Broadie and Bate, 1993a); anti-*twist* (Bate et al., 1991); anti-MHC (Drysdale et al., 1993)). Briefly, dissected embryos were stained as follows: embryos were dissected flat on polylysine-coated coverslips in normal saline, fixed for 15 minutes in 4% paraformaldeheyde, washed in phosphate-buffered saline + 0.3% triton X-100 (PBT), and blocked for 1 hour in 2% goat serum in PBT. The preparations were incubated in preabsorbed primary antibody (1:500 in PBT) for 1 hour at room temperature (anti-HRP) or overnight at 4°C (anti-*twist*, anti-MHC) with gentle agitation. The preparations were again washed in PBT, incubated with the appropriate biotinylated secondary antibody (Vectastain; 1:200 dilution in PBT, agitation, 1 hour at room temperature), washed in PBT and incubated with a commercial avidin-peroxidase complex (ABC kit; Vectastain) as directed for 30 minutes. The specimens were reacted with DAB, cleared in xylene and mounted for observation.

Intracellular dye-fills

Individual myotubes were injected with dye as reported earlier (Broadie and Bate, 1993a). Briefly; living myotubes were viewed with Nomarski optics and iontophoretically injected with a fluorescent dye under epifluorescence; a solution of 2% N-(2-aminoethyl) biotinamide hydrochloride (Neurobiotin (FW 322.47); Vector Laboratories) and 2% Lucifer Yellow. Lucifer Yellow was injected with small (nanoamps) hyperpolarizing current pulses under direct observation; to load Neurobiotin, the polarity of the current was reversed after confirming cell identity with the Lucifer Yellow. After injection, embryos were fixed for 1 hour in 4% paraformaldehyde, washed with PBT and incubated with a commercial avidin-peroxidase complex (ABC Elite kit; Vectastain) for 30 minutes. The specimens were then reacted with DAB, cleared in xylene and mounted to create a permanent preparation.

Electrophysiology: whole-cell patch-clamp techniques

For physiological experiments, the preparation was placed in a small perspex recording chamber and viewed in transmitted light with a compound microscope (Micro Instruments Ltd) fitted with differential interference contrast (Nomarski) optics and a 40× water-immersion lens. Whole-cell recordings were made at room temperature (18-22°C) with patch pipettes pulled from borosilicate glass (fiber filled) with tips fire-polished to final resistances of 5-10 Megohms. Whole-cell recordings were achieved using standard patch-clamp techniques as reported earlier (Broadie and Bate, 1993a,b). Signals were amplified using an Axopatch-1D (Axon Instruments) patch-clamp amplifier and filtered with an 8-pole Bessel filter at 2 kHz. Data were analyzed using PCLAMP 5.51 software (Axon Instruments).

Embryonic muscles required no treatment prior to patch-clamping and a tight seal (>10 gigohms) was achieved with slight suction. However, a muscle sheath covers the larval muscles and is first apparent at the time of hatching (20-21 hours AEL); this sheath was removed by incubation in collagenase (collagenase IV (Sigma); 1 mg/ml, 1 minute at room temperature) prior to patch-clamping.

All physiological recordings were performed in normal fly salines. The bath consisted of (in millimoles per liter): 135 NaCl, 5 KCl, 4 $MgCl_2$, 2 $CaCl_2$, 5 TES, 36 sucrose. The intracellular solution (in millimoles per liter): 120 KCl, 20 KOH, 4 $MgCl_2$, 5 TES, 5 EGTA, 0.25 $CaCl_2$, 4 ATP, 4 GTP, 36 sucrose. The pH of all solutions was buffered at 7.15.

To study the inward calcium current (I_{Ca}), CsCl was substituted for KCl in the intracellular patch pipette. Intracellular K^+ was

replaced with Cs^+ via perfusion in whole-cell configuration for at least 5 minutes. In this configuration, no outward K^+ currents could be recorded in a Ca^{2+}-free bath (Broadie and Bate, 1993b). Thus, the inward I_{Ca} can be recorded in the absence of all outward potassium currents. 20 mM $CaCl_2$ was used in the extracellular bath to amplify calcium currents.

Motor nerve stimulation

Embryonic motor nerves were stimulated while recording from patch-clamped muscles as reported earlier (Broadie and Bate, 1993a). Briefly; a small segment (0.5-1 μm) of the intact motor nerve was drawn into a pipette with gentle suction to form a tight seal. Stimulation was applied with a Farnell Pulse Generating System and current responses recorded from the patch-clamped myotube as described above. The shock artifact was decreased with the use of an isolated virtual ground.

RESULTS

A mutation in the *prospero* gene delays development of the peripheral motor nerves in the *Drosophila* embryo

The *prospero* (*pros*) gene is transcribed in the neuroblasts of the central nervous system (CNS) and sensory peripheral nervous system (PNS) during early neurogenesis (Doe et al., 1991; Vaessin et al., 1991) and encodes a nuclear protein expressed in neuronal ganglion mother cells (GMCs; Matsukaki et al., 1992). The *pros* gene is not expressed in the developing somatic musculature or elsewhere (Matsukaki et al., 1992). Mutations in *pros* alter neuronal fates and result in a morphologically abnormal CNS and sensory nervous system (Doe et al., 1991; Vaessin et al., 1991). It has been reported previously that mutations in *pros* prevent the early pioneering of the peripheral motor nerves (Vaessin et al., 1991).

Each abdominal hemisegment (A2-A7) of the *Drosophila* embryo has 30 syncytial muscle fibers innervated by two peripheral nerves; the anterior intersegmental nerve (ISN), which innervates the dorsal muscles, and the posterior segmental nerve (SN), which innervates the ventral muscles (Fig. 1; Johansen et al., 1989). In normal development, both nerves are pioneered by specific 'pioneer neurons' whose axons exit the CNS just after germ band retraction; first the ISN at 8.75-9.25 hours AEL, then the SN at 9.25-9.75 hours AEL (Broadie and Bate, 1993a; Fig. 1A). We have confirmed earlier reports that this initial pioneering of the peripheral nerves fails to occur in homozygous *pros* mutants (Fig. 1B). Furthermore, there is no indication of motor axons exiting the CNS for several hours (9-13 hours AEL) following the normal initiation of the peripheral motor nerves (Fig. 1C,D). At these early stages, the mutant phenotype appears nearly complete; peripheral motor nerves are observed in only a small subset of segments (<10%).

By 16 hours AEL, the motor nerves of wild-type embryos are fully established and robust neuromuscular junctions (NMJs) have formed on the somatic muscles (Figs 1C,E, 2). *pros* mutants at this stage have formed an extensive, albeit abnormal, sensory nervous system but still lack peripheral motor nerves (Fig. 1D). However, the mutant phenotype is less comprehensive at this late development stage; though most segments remain uninnnervated at 16 hours AEL, in some segments motor axons have exited the CNS and have begun to extend on to the muscles. By the end of embryogenesis (24 hours AEL), most segments manifest some degree of motor innervation and only a small subset (10-15%) of segments remain completely uninnervated (data not shown). From these observations, we conclude that the phenotype of *pros*/*pros* mutations is usually to delay greatly, rather than prevent, the outgrowth of motor axons from the CNS. For this reason, the state of innervation during late embryonic development was always independently assayed in the following analysis.

Peripheral motor nerves not required for larval muscle patterning

The development of the larval muscles begins with myoblast fusion events at the beginning of germ band retraction (7.5 hours AEL; Bate, 1990). Though the initial fusions occur in ventral myoblasts overlying the CNS, fusions among lateral and dorsal myoblasts occur during germ band retraction (7.5-8.5 hours AEL) in regions removed from possible neural contact (Bate, 1990). Thus, the initial fusion events, which produce the muscle pioneers that prefigure the larval muscle pattern, occur independently of innervation. After germ band retraction (8.5-9 hours AEL), motor axons exit the CNS (ISN at 8.75-9.25 hours AEL, SN at 9.25-9.75 hours AEL) and are present among the somatic myoblasts during subsequent myoblast fusion and the formation of epidermal muscle attachment sites (Fig. 1A). We used the *pros* mutation to remove the peripheral motor axons during these stages to ask what role, if any, do the motor nerves play in the patterning and maintenance of the larval muscles.

In normal development, the mature larval muscle pattern has been generated by 13 hours AEL (Bate, 1990); that is, myoblast fusions are complete and the myotubes have formed their mature attachments to the overlying epidermis (Fig. 3A). In segments lacking all motor innervation, a muscle pattern indistinguishable from the wild type is also formed by 13 hours AEL (Fig. 3B). We observed no differences in muscle numbers, size or attachments in aneural segments compared to normally innervated segments. Once formed, this muscle pattern is maintained normally through the end of embryogenesis (Fig. 3C,D). We conclude that innervation is not required for the formation or maintenance of larval muscles in the *Drosophila* embryo.

In *pros* mutants, mild muscle pattern aberrations are observed at a higher frequency than in wild type. These aberrations occur either as a small number of missing muscle fibers or patterning abnormalities in small regions of a segment (data not shown). Muscle defects occur primarily in abdominal segments A4/A5 but are observed at a low frequency in other segments. Therefore, though it is clear that motor innervation is normally not required to establish or maintain the muscle pattern, we cannot exclude the possibility that innervation plays some non-essential role in muscle formation or maintenance. Alternatively, the *pros* mutation may be affecting other neural tissues, either in the CNS or the periphery, which play a non-essential role in muscle patterning. Since *pros* is not expressed in the muscles or their precursors (Doe et al., 1991), it is unlikely that a mutation in *pros* is affecting muscle development directly.

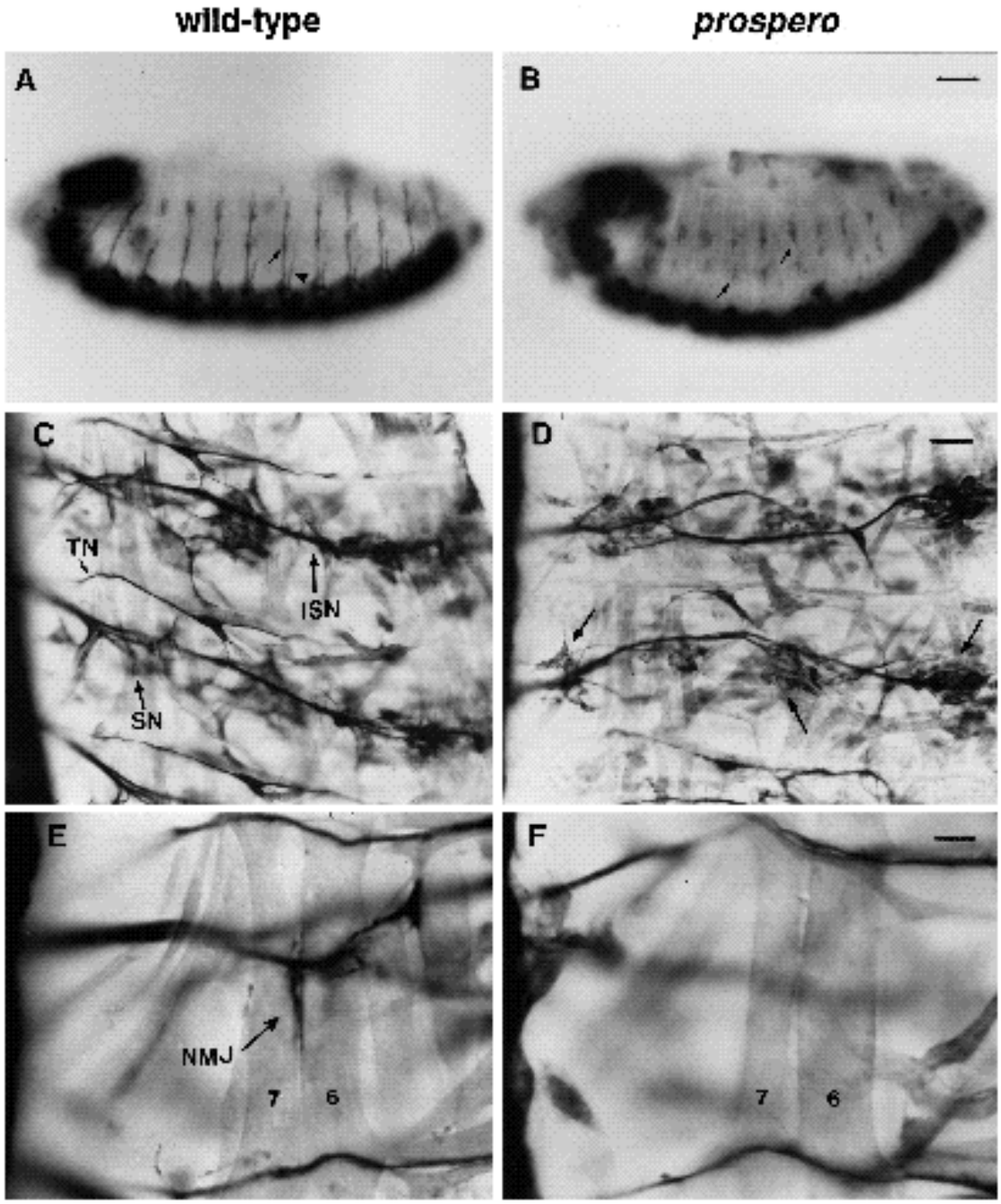

Fig. 1. A mutation in the *prospero* gene eliminates the peripheral motor nerves of the *Drosophila* embryo. (A,B) Intact embryos stained with anti-HRP antibody to reveal the nervous system at 10-11 hours AEL. Anterior is to the left, dorsal at the top. (A) In wild-type embryos, both the intersegmental nerve (ISN; arrow) and the segmental nerve (SN; arrowhead) have exited the ventral CNS. The peripheral sensory neurons are also differentiating and sending axons toward the CNS. At 10 hours AEL, the efferent motor axons and afferent sensory axons have just met and are beginning to fasciculate to form the mature peripheral nerves. (B) In *prospero* mutant embryos, both the ISN and SN fail to exit the CNS. However, the sensory neurons differentiate and send afferent axons (arrow) towards the CNS. The morphology of the CNS is grossly abnormal. (C-F) Dissected embryos stained with anti-HRP antibody at 16 hours AEL. The ventral CNS is left, anterior at the top. (C) In wild-type embryos, the peripheral nerves have attained their mature morphology; the ISN innervates dorsal muscles and carries sensory neurons from the dorsal regions, the SN innervates ventrally. The transverse nerve (TN) demarcates segmental boundaries; two hemisegments are shown. (D) In *prospero* mutant embryos, the ventral, lateral and dorsal sensory neurons (arrows) send axons into the CNS, but no motor axons are present in the periphery. Hence, only the afferent sensory axons are present in the peripheral nerves. These nerves elicit no muscle contraction when stimulated (see Fig. 2). (E) A higher magnification view of the ventral segmental nerve in one hemisegment. A branch of the SN innervates muscle 6 (NMJ), the focus of all our physiology studies. (F) In *prospero* mutants, the SN is absent and muscle 6 receives no innervation. Scales 50 μm (A,B), 10 μm (C,D), 5 μm (E,F).

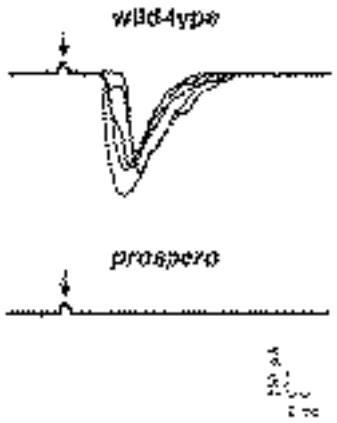

Fig. 2. A mutation in the *prospero* gene eliminates muscle innervation in the *Drosophila* embryo. These traces show current recordings in muscle 6 at 16 hours AEL. The peripheral nerve was stimulated with a suction electrode (arrow) where it exits the CNS and the synaptic current measured in the muscle voltage-clamped at −60 mV; 5 superimposed responses are shown. In wild-type embryos, a robust synaptic current is recorded when the peripheral nerve is stimulated. In *prospero* mutant embryos, no synaptic response is recorded upon nerve stimulation (n=9). Thus, by both morphological (Fig. 1) and physiological criteria, the *prospero* mutation eliminates motor innervation.

Peripheral motor nerves not required to establish or maintain the embryonic prepattern of the adult musculature

The *twist* gene is expressed in mesodermal cells prior to gastrulation and serves as a marker for undifferentiated mesodermal cells during later embryonic development (Bate et al., 1991). As mesodermal cells differentiate into embryonic tissues, they stop expressing *twist* and begin to express genes appropriate to their differentiated function (Bate et al., 1991). In the late embryo (>13 hours AEL), most mesodermal cells have differentiated into embryonic tissues and only a small subset continue to express *twist*; six cells per hemisegment (one ventral, two lateral and three dorsal) among the larval muscles, cells associated with the imaginal discs in the thorax and a larger number of cells associated with the heart and gut (Fig. 4A; Bate et al., 1991). These cells are the precursors of the adult muscles; they continue to express *twist* through to larval stages, divide to form *twist*-expressing groups in the larva and pupa, and cells of each group fuse to form specific sets of muscle fibers in the adult fly (Currie and Bate, 1991; Broadie and Bate, 1991). In normal development, these persistent *twist*-expressing cells become closely associated with peripheral motor nerves as they segregate from the adjacent embryonic cells and remain associated with these nerves through their later development

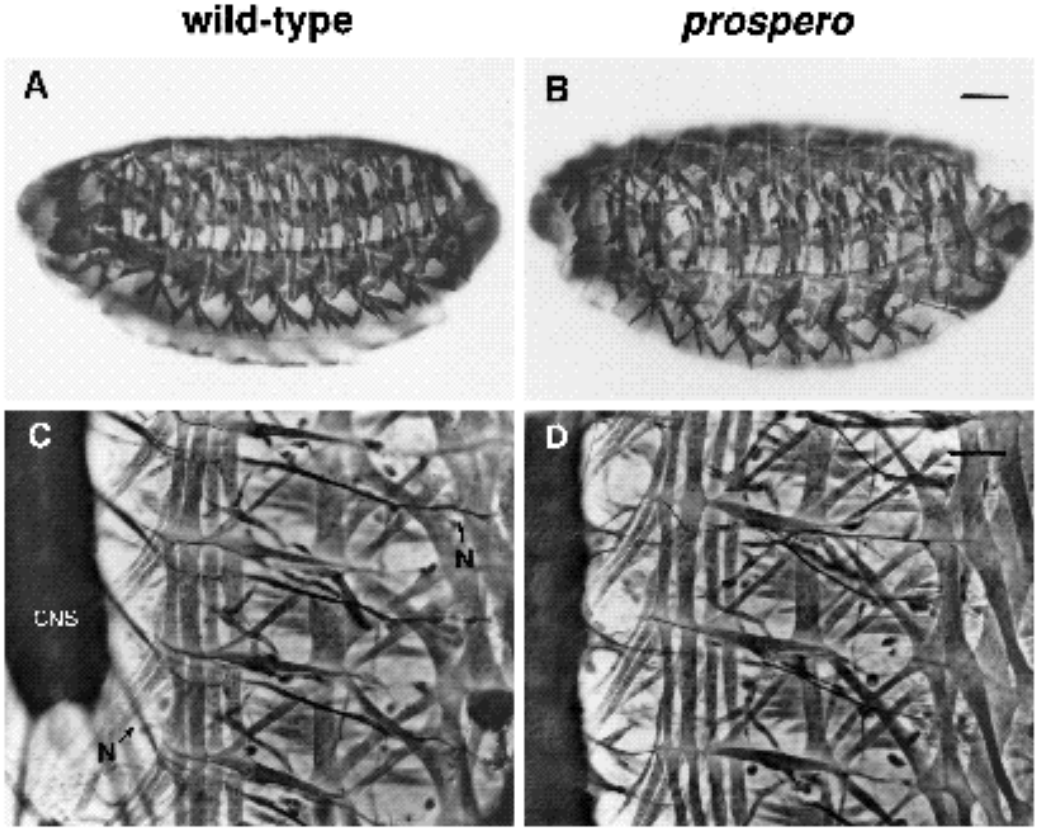

Fig. 3. The normal larval muscle pattern is generated and maintained in *prospero* mutant embryos lacking peripheral motor nerves. (A,B) Intact embryos stained with anti-MHC antibody to reveal the larval muscle pattern at 13-14 hours AEL. Anterior is to the left, dorsal at the top. (A) In wild-type embryos, the final muscle pattern is complete at 13 hours AEL. Each hemisegment contains 30 syncytial muscles, each with a specific size and epidermal attachment sites. (B) In *prospero* mutant embryos, the muscles develop with a time course similar to wild type and the mature larval muscle pattern is indistinguishable from wild type. (C,D) Dissected embryos double stained with anti-MHC antibody, to reveal the muscles, and anti-HRP antibody, to reveal the nervous system, at 16 hours AEL. Each panel shows 3 hemisegments; ventral is to the left, anterior at the top. (C) In wild-type embryos, the CNS has compacted anteriorly and peripheral nerves (N) extend posteriorly from the CNS to innervate each hemisegment. (D) In *prospero* mutant embryos, the aneural phenotype is not completely penetrant in the late embryo. As development proceeds, an increasing frequency of motor axons exit the CNS and innervate muscles in the periphery. However, in segments lacking motor innervation (shown here) the larval muscle pattern is maintained through the end of embryogenesis. The morphology of individual aneural muscles is indistinguishable from wild type. Notice that the CNS remains elongated and fails to compact as in the wild-type embryo. Scale 50 μm (A,B), 20 μm (C,D).

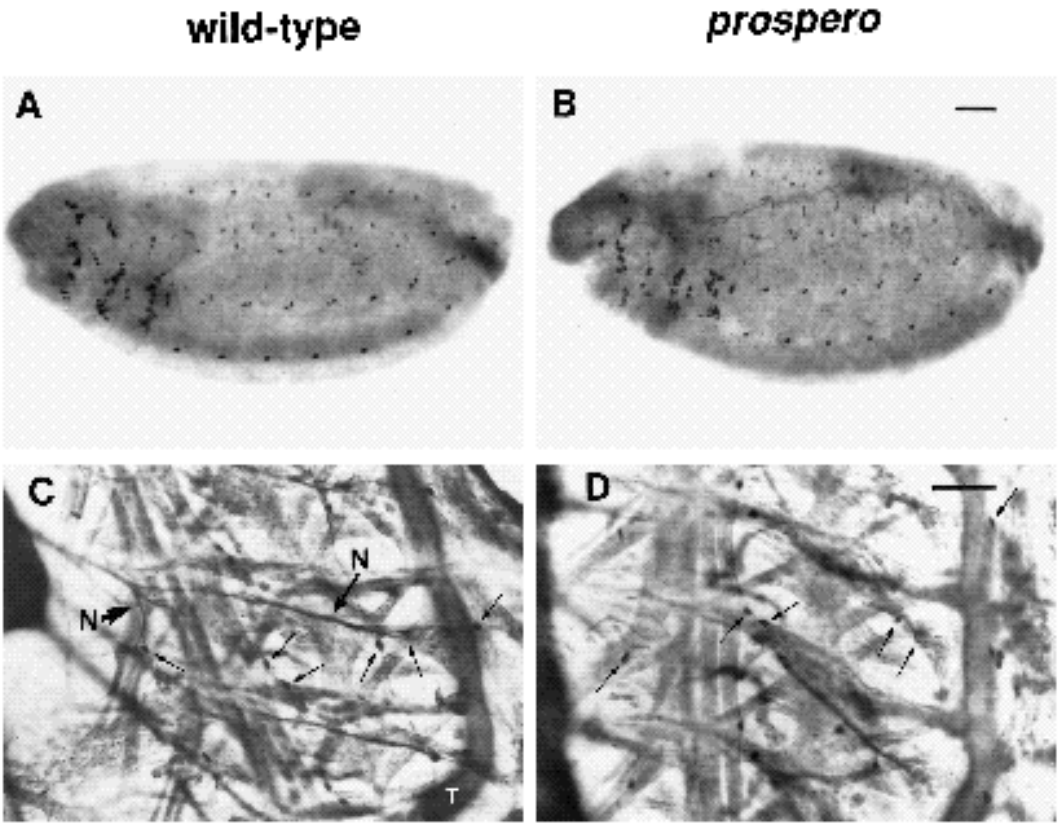

Fig. 4. The prepattern of the adult musculature is generated and and maintained in *prospero* mutant embryos lacking peripheral motor nerves. (A,B) Intact embryos stained with anti-*twist* antibody to reveal the adult myoblasts at 13-14 hours AEL. Anterior is to the left, dorsal is at the top. (A) In wild-type embryos, the adult myoblasts that prefigure the adult muscle pattern are identified by persistent expression of the *twist* gene. A simple pattern of 1 ventral cell, 2 lateral cells and 3 dorsal cells is present in each abdominal hemisegment; a larger number of *twist*-expressing adult myoblasts are associated with the imaginal discs in the thorax, the alimary muscle of the heart and the gut. (B) In *prospero* mutant embryos, the persisting *twist*-expressing cells segregate normally and establish a pattern of putative adult myoblasts indistinguishable from wild type. (C,D) Dissected embryos at 18 hours AEL double stained with anti-*twist* antibody and anti-HRP antibody to reveal the peripheral nerves. The ventral CNS is to the left, the dorsal tracheal trunk (T) to the right and anterior is at the top. (C) In wild-type embryos, the CNS has compacted anteriorly and peripheral nerves (N) extend posteriorly from the CNS to innervate each hemisegment. Persistent *twist*-expressing adult myoblasts (arrows; 1 ventral, 2 lateral, 3 dorsal) are closely associated with branches of the peripheral motor nerves (N). (D) In *prospero* mutant embryos, the aneural phenotype is not completely penetrant in the late embryo. As development proceeds, an increasing frequency of motor axons exit the CNS and innervate muscles in the periphery. However, in segments lacking motor innervation (shown here) the adult myoblast pattern (arrows) is maintained through the end of embryogenesis and these cells continue to express the *twist* gene. In the absence of motor nerves, the adult myoblasts usually become associated with the persisting sensory nerves but also exist without any neural association. Scale 50 μm (A,B), 20 μm (C,D).

(Bate et al., 1991; Currie and Bate, 1991). We used the *pros* mutant to ask if the peripheral motor nerves are required for the segregation or maintenance of the adult muscle precursors in the *Drosophila* embryo.

After germ band retraction (8.5-9 hours AEL), when the peripheral motor nerves begin to extend among the myoblasts, most mesodermal cells have differentiated and stopped expressing *twist* (Bate et al., 1991). From the remaining pool, a small number become closely associated with the developing nerves and maintain *twist* expression as the others differentiate and so signal their commitment to become adult muscle precursors. The simplest pattern occurs in the abdomen where only six cells per hemisegment (one ventral, a pair lateral and three dorsally) maintain expression into the late embryo (>13 hours AEL; Fig. 4A). In *pros* mutants lacking peripheral motor nerves, these persistent *twist*-expressing cells segregate with a time course indistinguishable from wild type. In the late embryo (>13 hours AEL), the pattern of *twist*-expressing cells is comparable between innervated and aneural segments (Fig. 4B). Moreover, in the absence of motor innervation, *twist* expression is maintained in these cells at least through the end of embryogenesis (21 hours AEL) and they successfully migrate to their final embryonic locations (Fig. 4C,D). We conclude that motor innervation plays no role in establishing or maintaining the adult muscle prepattern in the *Drosophila* embryo.

These experiments completely remove peripheral motor innervation, but elements of the peripheral sensory system are still present, albeit often abnormally patterned (Fig. 1). However, in previous studies (Bate et al., 1991), we have assayed the patterning of the *twist*-expressing adult myoblasts in *daughterless* (*da*) mutant embryos where virtually all the peripheral nervous system is removed. In *da* mutants lacking sensory nerves, the *twist*-expressing myoblasts are patterned in an essentially wild-type array (Bate et al., 1991). Therefore, sensory nerves, like motor nerves, are not required for the correct patterning of the *twist*-expressing myoblasts in the embryo. We cannot at present exclude the possibility that motor and sensory nerves may act as redundant adhesion and migration pathways for the *twist*-expressing myoblasts, because in the above exper-

iments one or the other is always present. We note, however, that in *pros* mutants both lacking motor nerves and with misrouted sensory nerves, the *twist*-expressing myoblasts do not appear to be incorrectly patterned by the misrouted sensory nerves. This observation suggests that patterning of the *twist*-expressing myoblasts may be independent of neural guidance and may rely instead on the closely adjacent tracheal system or other cues.

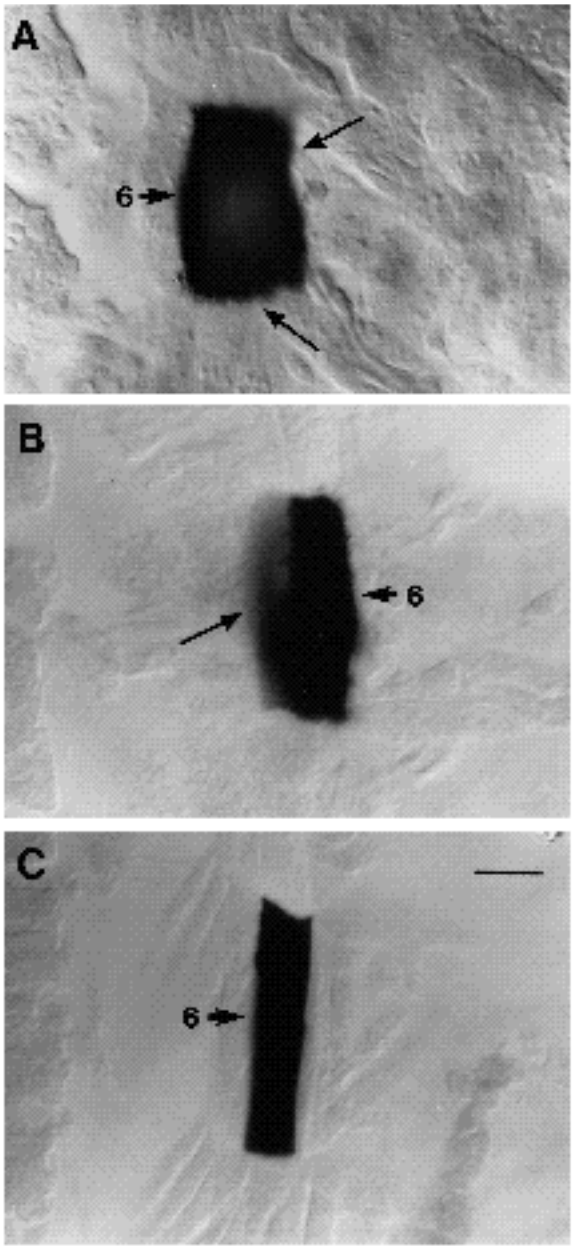

Fig. 5. Aneural muscles uncouple at the correct developmental stage in *prospero* mutant embryos. In *prospero* mutant embryos, muscle 6 was intracellularly injected with a small dye (Neurobiotin, FW 322.47) for 5 minutes and the extent of dye-coupling with adjacent muscles assayed (see Methods). (A) At 12 hours AEL, muscle 6 is dye-coupled to several adjacent muscles (arrows), but the extent of coupling appears variable (*n*=6/6 coupled). (B) At 12.5 hours AEL, muscle 6 remains coupled to adjacent myotubes (arrow), but the number of coupled muscles and the extent of coupling has decreased (*n*=11/13 coupled). (C) Soon after dorsal closure (13.25 hours AEL), all dye injected into muscle 6 is retained (*n*=8/8 uncoupled); uncoupling occurs abruptly between 12.75 and 13.25 hours AEL. Both the extent of muscle dye-coupling and the time-course of uncoupling are indistinguishable between innervated and aneural muscle 6. Scale (all) 10 μm.

Innervation not required for muscle uncoupling

Developing embryonic myotubes are electrically and dye-coupled to adjacent myotubes (Johansen et al., 1989). During normal development, myotubes abruptly uncouple at 12.75-13.25 hours AEL immediately after the initial neuromuscular contact (12.5-13 hours AEL; Broadie and Bate, 1993a). Previous studies have suggested the possibility that the motor nerve may be providing the signal for myotube uncoupling at this precise developmental stage (Broadie and Bate, 1993a). We have used the *prospero* mutant to ask what role, if any, the innervating motor nerve plays in myotube uncoupling.

When a small dye (Neurobiotin, F.W. 322.47) is injected into muscle 6 prior to neuromuscular contact with its innervating motor neuron (12.5 hours AEL), the dye transfers to several adjacent myotubes (Broadie and Bate, 1993a); though the exact number and identity of dye-coupled myotubes appears variable. However, when the dye is injected into muscle 6 immediately following neuromuscular contact (13.25 hours AEL) the dye is retained in the injected cell (Broadie and Bate, 1993a). In *pros* mutants lacking motor nerves, muscle 6 is similarly coupled to adjacent myotubes at 12.5 hours AEL (*n*=11/13; Fig. 5). Moreover, in aneural segments, muscle 6 is similarly uncoupled at 13.25 hours AEL (*n*=8/8; Fig. 5). We can distinguish no differences in the extent of myotube coupling nor the time course of uncoupling between innervated and aneural muscles. We conclude that the signal for myotube uncoupling does not come from the motor nerve and the temporal correlation between uncoupling and neuromuscular contact is coincidental.

Muscle contraction in aneural embryos

Prior to uncoupling, the embryonic myotubes are quiescent and will not contract when electrically stimulated (Broadie and Bate, 1993b). Though the myotubes express myosin heavy chain and actin during this stage, there is no evidence for the formation of the muscle's contractile apparatus as assayed with polarized light (Broadie and Bate, 1993b). Soon after uncoupling (13.5 hours AEL), the embryonic myotubes begin to contract when stimulated and endogenous contractions begin soon thereafter (13.75-14 hours AEL). Over the next hours (14-16 hours AEL), the myotube's contractile apparatus is rapidly assembled, resulting in a pronounced birefingence in polarized light and vigorous contraction when stimulated (Broadie and Bate, 1993b). Later development (>16 hours AEL) is characterized by the occurrence of frequent peristaltic muscle movements reminiscent of the larval locomotory movements. The development of the contractile apparatus correlates closely with the development of the neuromuscular junction (Broadie and Bate, 1993a,b); initial contraction apparatus assembly occurs soon after the initial neuromuscular contact, endogenous contractions begin with the onset of synaptic communication and subsequent muscle matura-

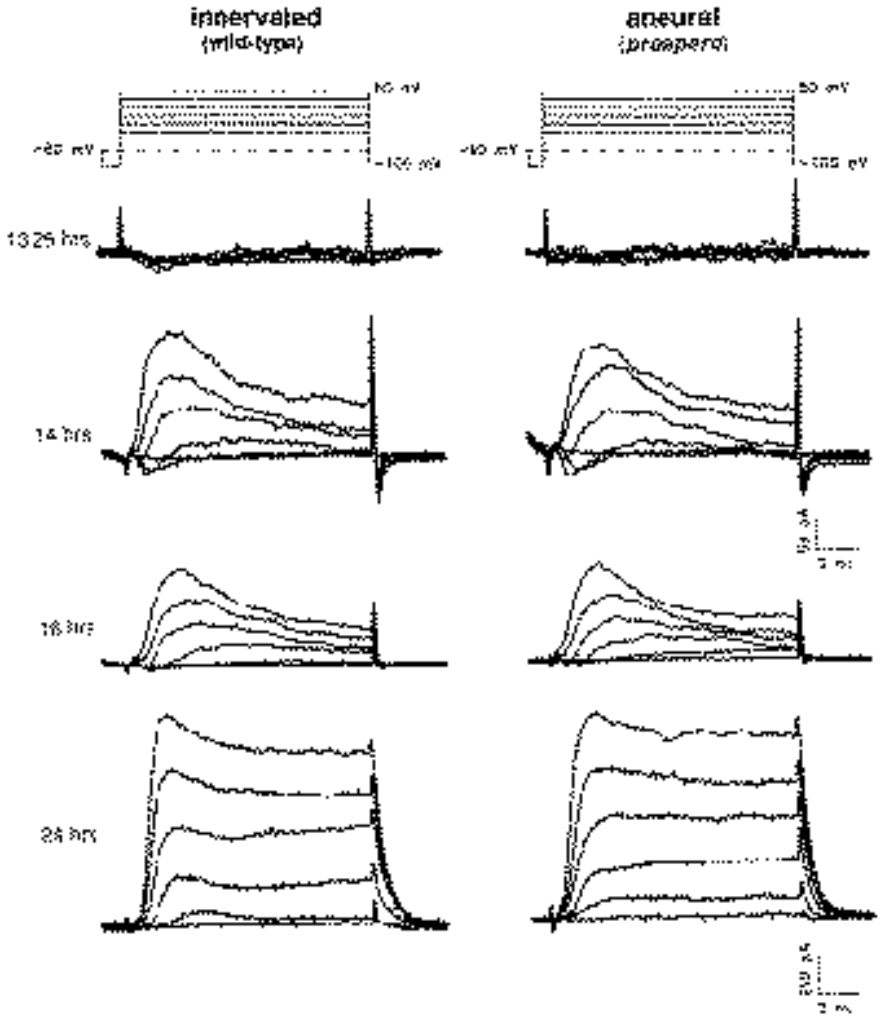

Fig. 6. The muscle ionic currents appear and develop normally in *prospero* mutant embryos lacking muscle innervation. Muscle 6 was patch-clamped in whole-cell configuration and voltage-clamped at −60 mV (see Methods). After a 2 second prepulse at −100 mV (to remove inactivation), the muscle was voltage-clamped from −20 to +80 mV in 20 mV increments. Five traces were averaged to generate the currents displayed here. In wild-type embryos, the voltage-gated whole-cell current develops from no response soon after muscle uncoupling (13.25 hours AEL) to a peak whole-cell current of several hundred picoamps (−100 to +80 mV) soon after hatching (24 hours AEL). Most of the whole-cell current is present as outward potassium currents, both fast and delayed, with the inward calcium current nearly obscured. In *prospero* mutants, aneural muscle generates a whole-cell current at the same developmental stage as innervated muscle and the current matures with a time course indistinguishable from wild type. The amplitude and kinetics of both the fast, inactivating potassium current and the delayed, non-inactivating potassium current are the same in both innervated and aneural muscle at all stages of development. The inward calcium current is considered in Fig. 7. In all cases, the state of innervation was independently assayed using either morphological (anti-HRP antibody staining; Fig. 1) or physiological (nerve stimulation; Fig. 2) tests. At least five embryos were examined for each genotype at each time point.

tion occurs with constant neuromuscular interaction. We used the *pros* mutant to ask what role, if any, the motor nerve plays in the development of the muscle's contractile apparatus and contractile activity.

There is no detectable difference in the progression of myosin expression in aneural and innervated muscles (data not shown). Likewise, the birefringent muscle contractile apparatus develops with a similar time course in innervated and aneural muscle. A muscle that has never received innervation develops the capacity to contract with the same time course as an innervated muscle and, in the mature embryo (21 hours AEL), will contract vigorously when electrically stimulated. We conclude that innervation plays no detectable role in the maturation or maintenance of a muscle's contractile abilities.

In mature wild-type embryos, injection of a toxin that blocks synaptic transmission prevents most isolated endogenous muscle contractions and all peristaltic locomotory movements (Broadie and Bate, 1993a). This result suggests that most muscle contraction in the embryo is neurally evoked. Similarly, in aneural *pros* mutants, there is a reduced incidence of endogenous muscle contractions and no evidence of peristaltic movements during the stages when muscular activity normally develops (13-16 hours AEL; Broadie and Bate, 1993a,b). The incidence of muscular activity in *pros* mutants increases towards the end of embryogenesis, consistent with the delayed motor nerve outgrowth and delayed establishment of functional neuromuscular junctions (Broadie and Bate, 1993c). Though peristaltic locomotory movements are observed in mature *pros* embryos, both their frequency and coordination remain dramatically reduced relative to wild type. These observation suggests that most, but not all, muscle activity is neurally evoked and that coordinated embryonic movement requires coordinated neural input.

Innervation does not initiate or regulate the maturation of muscle electrical properties

Prior to myotube uncoupling, the only detectable whole cell current present in the embryonic myotubes is a small, inward calcium current (I_{Ca}; Broadie and Bate, 1993b). Immediately after uncoupling (13.5 hours AEL), the other ionic currents present in the mature muscle begin to appear and develop with current-specific time courses. In addition to I_{Ca}, there are four outward potassium currents; two voltage-gated, the fast I_A and delayed I_K, and two calcium-gated, the fast I_{CF} and delayed I_{CS}. These five currents combine to generate the complex whole-cell current that characterizes the muscle

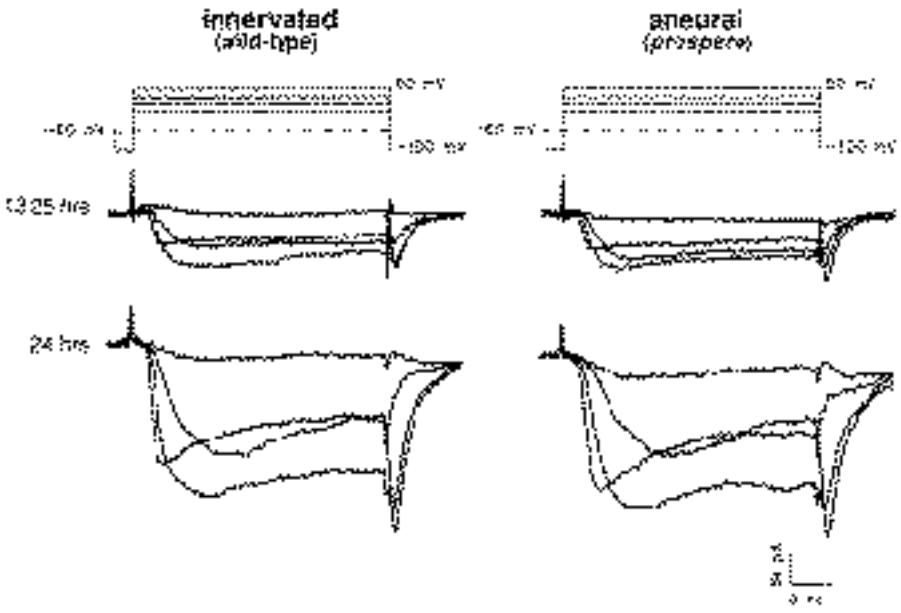

Fig. 7. The inward calcium current, which mediates muscle contraction, appears and matures normally in *prospero* mutant embryos lacking muscle innervation. Muscle 6 was patch-clamped in whole-cell configuration and voltage-clamped at −60 mV (see Methods). Cs^+ was substituted for K^+ in the patch pipette and the intracellular K^+ was allowed to diffuse away for 5 minutes prior to experimentation. This recording configuration abolishes the outward K^+ currents and allows the measurement of the inward calcium current in isolation (see Methods); 20 mM Ca^{++} was used in the external bath to increase current amplitudes. After Cs^+ perfusion, the muscle was given a 2 second prepulse at −100 mV and then voltage-clamped from 0 to +60 mV in 20 mV increments. Five traces were averaged to generate the currents displayed here. In wild-type embryos, a significant inward calcium current is present immediately after myotube uncoupling (13.25 hours AEL). This current develops continuously through the end of embryogenesis, approximately tripling its amplitude by the first instar (24 hours AEL). In *prospero* mutants, aneural muscle also contains a inward calcium current after uncoupling (13.25 hours AEL), which matures during development with a similar time course as wild type. Thus, the inward calcium current, like the outward potassium currents, is not induced or regulated by innervation. In all cases, the state of innervation was independently assayed using either morphological (anti-HRP antibody staining; Fig. 1) or physiological (nerve stimulation; Fig. 2) tests. At least five embryos were examined for each genotype at each time point.

response to a change in membrane potential. In earlier work (Broadie and Bate, 1993b), we have examined the embryonic development of the whole-cell current in muscle 6 and followed the maturation of its constituent currents. We observed that the whole-cell current appeared soon after the initial neuromuscular contact and matured rapidly during the initial phase of NMJ formation. Using the *pros* mutant to remove the innervating motor nerve, we here ask what role, if any, the motor nerve plays in the appearance and maturation of the muscle's electrical properties. In particular, we examine the maturation of the whole-cell voltage-gated currents and the inward calcium current responsible for muscle contraction in both innervated and aneural muscle.

Immediately after the myotubes uncouple (13.25 hours AEL), there is no measurable whole-cell current in myotube 6 in response to a depolarizing voltage step (Fig. 6). Yet by 14 hours AEL, a whole-cell current has developed composed of an inward current and both a rapid and delayed outward current (Fig. 6). The amplitude of the whole-cell current rapidly increases over the next several hours and increases at a slower pace through the end of embryogenesis (Fig. 6). The whole-cell voltage-gated current in aneural myotube 6 develops with an indistinguishable time course (Fig. 6); we could detect no significant differences in the appearance or maturation of the whole-cell current. Furthermore, we could detect no differences in the appearance or maturation of components of the whole-cell current: inward current and fast or delayed outward potassium currents. We conclude that the innervating motor neuron plays no regulative role in the development of the myotubes whole-cell electrical properties during embryonic development.

In whole-cell recordings, the small inward calcium current is usually completely masked by the large outward potassium currents, especially during late embryonic stages (Fig. 6). As a consequence, we examined the calcium current in isolation to ascertain whether the motor neuron plays a role in its appearance and regulation that is masked in the whole-cell current response. In normal development, the calcium current is the only current detectable immediately after the myotubes uncouple and develops with a constant, gradual rate through the end of embryogenesis (Fig. 7; Broadie and Bate, 1993b). In aneural muscle 6, the appearance and maturation of the calcium current was not significantly different from the wild type, innervated muscle (Fig. 7). Thus, the inward calcium current, like the outward potassium currents evident in the whole-cell recordings, develops independently of innervation.

We will not discuss the role of the presynaptic motor neuron in the development of the NMJ in this study, as it has been considered in detail elsewhere (Broadie and Bate, 1993c). We note only that the synaptic L-glutamate-gated current, unlike the voltage- and calcium-gated currents shown here, depends on interaction with the motor nerve to develop normally. Hence, though other muscle properties develop independently of innervation, the NMJ is a specialized aspect of muscle development that depends, at least in part, on direction from the presynaptic motor neuron (Broadie and Bate, 1993c).

DISCUSSION

The role of innervation in directing embryonic myogenesis in insects, and other invertebrate species, has received very

little attention. As far as we know, the sole study addressing this question in insects was performed by Haget (1965) on the embryo of *Leptinotarsa decemlineata*. He manually denervated the legs of the embryo during the fourth day of embryonic development when the leg anlagen contain thin syncitial muscles surrounded by undifferentiated myoblasts. Following denervation, muscles of typical size and histological structure developed normally. Therefore, from the time of operation, muscle development appeared to be independent of innervation.

In the present study, we have examined the requirement of innervation for the embryonic development of both the larval muscles and the adult muscle precursors in the embryo of *Drosophila melanogaster*. We have used a mutant in the *prospero* gene, expressed in neuronal precursors but not in the muscles or their precursors (Doe et al., 1991; Vaessin et al., 1991), which prevents the pioneering of the motor nerves from the earliest stages and allows us to examine non-invasively aneural muscle development. We have extended Haget's study (1965) by examining physiological as well as morphological aspects of aneural muscle development. We find that motor innervation plays no detectable role in establishing or maintaining the somatic muscles in the *Drosophila* embryo; in aneural (*prospero*) embryos, the larval muscles develop and are patterned normally, the adult muscle prepattern develops normally, and the larval muscles develop the electrical and contractile properties of mature larval muscles. We conclude that embryonic myogenesis, at least within the resolution of this study, is independent of innervation at all stages of development in the *Drosophila* embryo.

In contrast to the embryo, the role of innervation in directing postembryonic muscle development has been the subject of intense study for several decades in several insect species (reviewed in Nüesch, 1985). As in the embryo, postembryonic muscle anlagen differentiate histologically to form functional adult muscle fibers in the absence of all motor innervation. These denervated muscles attach to the epidermis and form normal muscle patterns containing distinct muscle types (Nüesch, 1985). However, unlike embryonic myogenesis, the development and maintenance of these adult muscles depends critically on innervation at several levels. First, early denervation suppresses mitosis in postembryonic myoblasts and so decreases the growth of the muscle precursor pool prior to differentiation (reviewed in Nüesch, 1985; Currie, 1991). Therefore, even though denervated myoblasts differentiate at the correct time to form normal muscle types, these muscles are variably decreased in size and always contain fewer nuclei. Secondly, in one case innervation has been shown to determine a muscle's differentiated fate. Lawrence and Johnston (1986) have shown that the genetic identity of the nervous system, but not the muscle or epidermis, determines the formation of the specialized male-specific muscle in the adult *Drosophila* abdomen. Finally, once differentiated, muscle is maintained only if it is innervated; denervated muscle variably decreases in size and often degenerates completely (reviewed in Nüesch, 1985). Thus, innervation directs postembryonic muscle development during anlagen growth, specification of at least some differentiated fates and maintenance of the differentiated muscle. It appears, therefore, that at least some of the mechanisms of muscle development and maintenance may be fundamentally different in embryonic and pupal insect development.

A similar developmental sequence has been described during the development of somatic muscles in several vertebrate systems (reviewed in Miller and Stockdale, 1987; reviewed in Miller, 1992). As in the *Drosophila* embryo, distinct types of muscle fibers form in both chicken (Miller and Stockdale, 1987) and rat (Condon et al., 1990a) embryos that are denervated or paralyzed. In both systems, the normal muscle fiber types are present and distributed in their characteristic intramuscular positions in the absence of motor innervation. Therefore, the early embryonic formation and differentiation of muscle appears largely innervation-independent in vertebrates as in *Drosophila*. In contrast, late fetal and postembryonic myogenesis is critically dependent on innervation in both chicken (Crow and Stockdale, 1986) and rat (Condon et al., 1990b) development. Interaction with the motor nerve is required to maintain and amplify the muscle patterns established in the embryo. Thus, the late development and maintenance of muscle is innervation-dependent, at least in part, in vertebrates as in *Drosophila*.

In all studied systems, vertebrate and invertebrate, embryonic and postembryonic, myogenesis shares a conserved central feature; myoblasts that have never received innervation differentiate into functional multinucleate muscles with distinct identities, which form intricate patterned arrays. Therefore, the primary myogenic developmental pathway is completely independent of direct neural control. We are left with the question of how the nature and time course of muscle development is regulated. In insects, the two likely possibilities are that myogenesis is autonomously controlled within the mesoderm and/or that myogenesis is regulated by ectodermally derived tissues, through local inductive signals (epidermis) or long-distance diffusible signals (nervous system). Experiments in at least one insect species (Williams and Caveney, 1980a,b) have shown that the muscle insertion sites are coded into the epidermal pattern and it appears, therefore, that the generation of muscle patterns is controlled, at least in part, by the epidermis. Likewise, in the *Drosophila* embryo, the formation of the specialized neuromuscular synapse is directed, at least in part, by the motor neuron (Broadie and Bate, 1993c). However, both these interactions involve the specialized association between muscle and another tissue, and may not typify strictly myogenic pathways. The cellular mechanisms regulating muscle patterning and differentiation remain to be elucidated.

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