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Polyneuronal innervation of an adult and embryonic lobster muscle

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SUMMARY

Motor innervation of the deep extensor muscle in the abdomen of lobsters (*Homarus americanus*) was compared in adults and embryos using electrophysiological techniques. There is widespread innervation of the adult muscle by the common excitor and inhibitor axons and regionally restricted or private innervation by three more excitor axons. In the embryo the earliest sign of functional innervation revealed a single inhibitory and two to three excitatory axons thus denoting simultaneous innervation by the full complement of axons. In corroboration, serial-section electron microscopy revealed several axon profiles invading the embryonic deep extensor muscles and giving rise to well-defined neuromuscular synapses with presynaptic dense bars. Innervation patterns to homologous regions of the embryonic and adult muscles were similar, consisting of a few large inhibitory synapses and many small excitatory ones. Consequently the adult pattern of polyneuronal innervation occurs simultaneously and *in toto* during embryonic development.

INTRODUCTION

Each abdominal muscle in tailed decapods is innervated by as many as five excitatory axons and a single inhibitory axon (Kennedy & Takeda, 1965*a*, *b*; Parnas & Atwood, 1966). The inhibitor and at least one of the excitors innervates all of the muscle fibres within a single hemisegment. The remaining excitors have a more restricted distribution, innervating the muscle fibres confined to a small region of the hemisegment. Under these circumstances innervation of an abdominal muscle might occur initially by the common excitor axons with the regionally restricted ones following later i.e. a sequential form of innervation. Alternatively, the full complement of motoneurons may innervate the muscle simultaneously.

In one of the first studies of the abdominal muscles of embryonic lobsters, Lang

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(1977) revealed the presence of excitatory and inhibitory neuromuscular synapses in electron micrographs of the abdominal deep extensor muscle. Subsequently, Cole & Lang (1980) confirmed the functional nature of these synapses by recording both excitatory (EJP) and inhibitory (IJP) junctional potentials in muscle fibres of embryos. Indeed they showed that the embryonic deep extensor muscles possess excitatory synapses that have phasic properties similar to those found in the adult. This finding was confined to the single excitatory axon which they found to be the predominant pattern of innervation in this muscle. Occasionally they detected innervation by an additional excitatory axon, but not by three or more, as is the case in some regions of the adult muscle for crayfish and rock lobsters (Parnas & Atwood, 1966). On the basis of these findings they suggested that polyneuronal innervation of the embryonic deep extensor muscle occurs by the gradual recruitment of axons rather than by their concurrent establishment of synaptic contacts.

In order to test these alternative hypotheses we have compared the innervation pattern of the deep abdominal extensor muscles in the adult and embryo using electrophysiological techniques and electron microscopy. Our findings suggest that polyneuronal innervation of this muscle occurs simultaneously rather than sequentially. Moreover, the pattern established initially during development is similar to the pattern found in the adult.

MATERIAL AND METHODS

Adult

Adult lobsters (*Homarus americanus*) weighing approximately 500 g were trapped in the local waters of the Cape Cod Bay or purchased from local suppliers and held in running sea water at the Marine Biological Laboratory, Woods Hole, Massachusetts. A preparation was made by isolating the abdomen from the rest of the animal and carefully removing the superficial and deep flexor muscles and the gut along the length of the abdomen in order to expose the deep extensor muscles. During this dissection a 3–5 mm length of the nerve root to the extensor muscles was left intact. Innervation of the deep extensor muscle was determined by stimulating the nerve root and recording the resultant muscle junctional potentials following the methods of previous workers (Abbott & Parnas, 1965; Parnas & Atwood, 1966). These electrophysiological experiments were done on both left and right sides of the second to the fifth segment.

In order to examine the fine structure of the innervation, the deep extensor muscles were exposed and superfused with fixative. During the first buffer wash small segments of the muscle were cut from the medial and lateral bundles of the second and third segments and were processed separately. Techniques for fixing, embedding, and sectioning the tissue were conventional to our laboratory (Govind & Pearce, 1982). Selected areas of the muscle showing innervation were serially sectioned and measurements of various morphological features were made from micrographs with a final magnification of $\times 27\,000$.

Embryo

Egg-bearing females were obtained from the Massachusetts State lobster hatchery on Martha's Vineyard and maintained in running sea water. Eggs at different stages covering the period of embryonic development were examined. The most visible sign of embryonic development is the pigmented eye spot. As eye spot enlargement is linearly related to the development of the abdomen (Perkins, 1972; Cole & Lang, 1980), it was used as a diagnostic tool. In practice the long and short axis of the eye spot were averaged to give an eye index which served as the index of embryonic developmental stage.

For electrophysiological recordings, the embryo was dissected minimally to expose the deep abdominal extensor muscles together with their nerve roots on one side of the abdomen by methods described previously (Cole & Lang, 1980; Stephens & Govind, 1981). Electrical stimulation of brief (0·1 msec) pulses was delivered via fine platinum wire electrodes to the second nerve root and the resulting junctional potentials recorded *via* KCl-filled electrodes inserted into the muscle fibres. The display and recording of signals was by conventional methods.

For electron microscopy, the embryonic abdomens were isolated from the thorax and telson and prepared, such that segments two to four were intact. Cross sections of the entire abdomen were obtained and viewed on single-slot grids. This enabled us to identify unequivocally the various groups of flexor and extensor muscles and ensure that we were viewing the muscle of our choice. Measurements of morphological features were made from serial micrographs with a final magnification of × 27 000.

RESULTS

1) *Adult innervation*

a) *Electrophysiology*

The deep abdominal extensor muscle consists of a medial (DEAM) portion and lateral (DEAL) portion shown diagrammatically in Figure 1. The DEAM is divided into anterior (DEAM1) and posterior (DEAM2) parts; within each part the

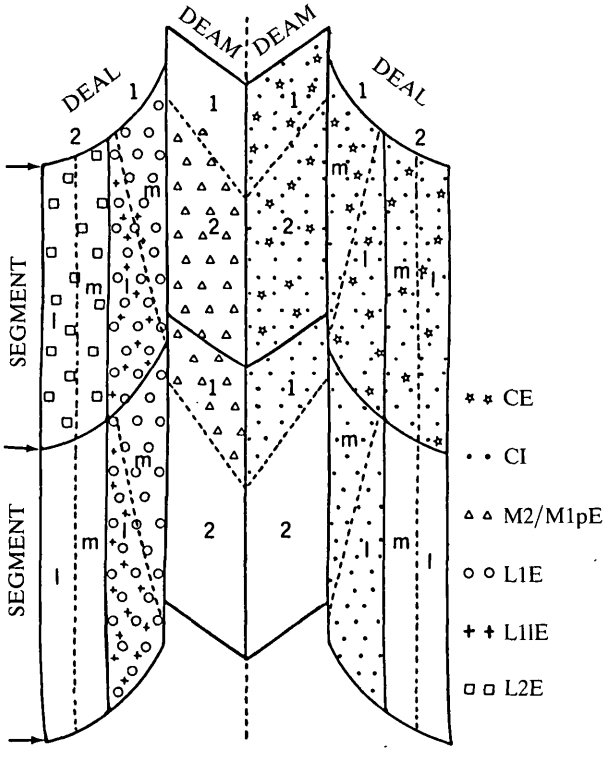

Fig. 1. Polyneuronal innervation of the deep abdominal extensor muscles in an adult lobster showing the distribution of the common excitor (CE), and common inhibitor (CI) axons (right hemisegments) and the four private excitor axons (M2/M1pE, L1E, L1lE, L2E) (left hemisegment) in their own segment (upper) and in the next posterior segment (lower).

Table 1. *Innervation patterns of the common excitor (CE), common inhibitor (CI) and of four specific exictors (M2/M1pE, L1E, L1lE, L2E) to the medial (DEAM) and lateral (DEAL) components of the deep abdominal extensor muscles in adult lobsters.*

	Motor neurons		
Muscle	own segment	next posterior segment	total number
DEAM 1	CE CI	M2/M1pE CI	2E 2I
DEAM 2	CE CI M2/M1pE		2E 1I
DEAL 1m	CE CI L1E	L1E CI	3E 2I
DEAL 1l	CE CI L1E L1le	L1E L1lE CI	5E 2I
DEAL 2m	CE CI L2E		2E 1I
DEAL 2l	CE CI L2E		2E 1I

muscle fibres run helically with a pitch of 1·5 turns per hemisegment. The DEAL on the other hand is divided into adjacent sections, DEAL 1 and DEAL 2, each of which in turn is subdivided into medial (m) and lateral (l) parts. Only the DEAL 1 fibres are helical with a pitch of 0·5 turns per hemisegment; the DEAL 2 fibres run parallel to the rostrocaudal axis. The motor innervation to these different parts of the extensor muscle is summarized in Figure 1 and Table 1, and described below.

A single excitor, referred to as the common excitor (CE), goes to all parts of the muscle in its own hemisegment. It exerts a powerful influence as a single stimulus will invariably evoke an action potential (AP) in all parts of the muscle except in DEAL 2 where it evokes an excitatory junctional potential (EJP) and occasionally an AP (Fig. 2). Whereas a short bout of repetitive stimulation results in synaptic facilitation, continued stimulation of the CE at 1 Hz or at 10 Hz results in synaptic fatigue.

The remaining four excitors have innervation confined to restricted parts of the extensor muscle and are referred to as private axons as compared to the common axon which has a widespread distribution. We have named these private axons according to their distribution which is shown graphically in Fig. 1. All of the private

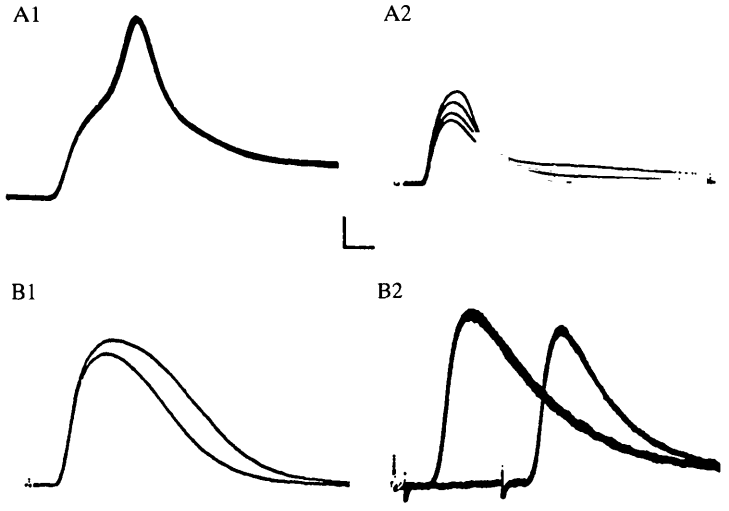

Fig. 2. Representative recordings showing innervation of the adult deep extensor muscle by the common excitor (A) and by the common inhibitor (B) axons. In fresh preparations a single stimulus to CE evokes an EJP which develops into an AP (A1) while in less-fresh preparations, EJPs show facilitation with repetitive stimulation (A2). Effect of CI stimulation is to reduce the EJP evoked by CE (B1) and by L2E (B2) excitor axons. Calibration vertical, 10mV in A, 5mV in B., horizontal, 5 msec.

axons except L2E provide innervation to the next posterior segment. The strength of the synaptic input provided by these axons declines from the midline outwards as judged by their ability to produce an AP from an EJP. Thus, while an AP is evoked by a single stimulus to M2/M1pE, repetitive stimulation at 1 Hz of L1E is required. However repetitive 1 Hz stimulation only occasionally results in an AP when applied to L1E and rarely when applied to L2E. With all four excitors, the EJPs facilitate with repetitive stimulation, though no attempts were made to measure the degree of facilitation.

A single common inhibitor (CI) innervates all portions of the extensor muscle in its own segment and limited portions in the next posterior segment. Inhibitory junctional potentials (IJPs) were rarely seen but since their effects on EJPs were easily demonstrated (Fig. 2) it was possible to determine the distribution of the CI as being widespread (Fig. 1, Table 1).

In summary, fibres of the adult deep extensor muscle are innervated by a single inhibitory and between two and five excitatory axons (Table 1).

b) *Electron microscopy*

The fine structure of polyneuronal innervation was examined in the DEAM of adult lobsters. Since fibres of the DEAM receive both excitatory and inhibitory axons (Table 1), it was necessary to distinguish between them in the electron micrographs. This was done on the basis of the differences in shape of the synaptic vesicles of the two axons following aldehyde fixation (Uchizono, 1967; Atwood,

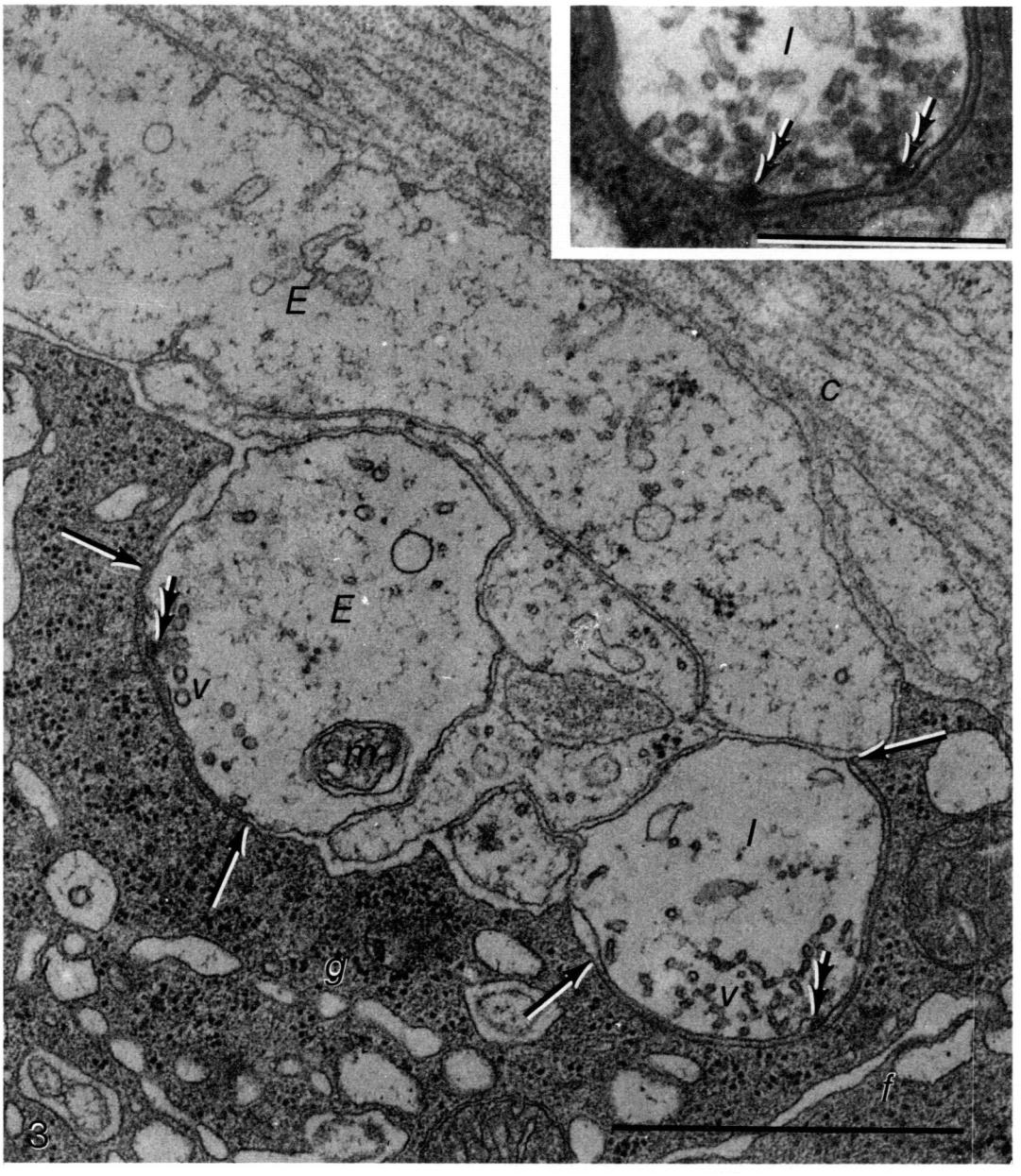

Fig. 3. An innervation site consisting of two excitatory (*E*) nerve terminals from different excitatory axons, and one inhibitory (*I*) nerve terminal to the adult DEAM muscle. Round and ellipsoid synaptic vesicles (*v*) which respectively characterize *E* and *I* terminals, cluster around presynaptic dense bars (double arrows). The insert shows an omega-shaped profile occurring adjacent to the dense bars (double arrow) in an inhibitory terminal. Synapses are indicated between arrows; *f*, myofibril; *g*, granular sarcoplasm of muscle; *m*, mitochondria; *c*, connective tissue. Scale bar, 1 μm main figure; 0·5 μm insert.

Lang & Morin, 1972; Tisdale & Nakajima, 1976); vesicles of the excitatory terminal were spherical and those of the inhibitory terminal were ellipsoid (Fig. 3). Since the only inhibitory innervation DEAM 2 receives is via the CI axon of its own segment, the neuromuscular inhibitory terminals with ellipsoid-shaped vesicles belong to this axon. The excitatory terminals, however, cannot be assigned to specific axons since DEAM 2 receives excitatory innervation from two axons.

Qualitative features of the innervation to the DEAM were characteristic of those found in other crustacean muscles (reviewed by Atwood, 1976). Branches of the motor axon gave rise to nerve terminals that contained mitochondria and synaptic vesicles, and made synaptic contacts with the granular sarcoplasm-containing region of the muscle fibre (Fig. 3). Synaptic vesicles were clustered around presynaptic dense bars and occasionally omega-shaped figures were adjacent to these bars, suggesting exocytosis of vesicles (Fig. 3) and implicating these bars as active sites of transmitter release (cf. Govind & Chiang, 1979).

An examination of the DEAM in five separate lobsters revealed a typical pattern of innervation consisting of a single inhibitory terminal situated close to either one or two excitatory terminals (Fig. 3). In the latter case, the terminals remained as separate entities in sections taken serially and at periodic intervals for distances of

Table 2. *Comparison of morphological features of excitatory (E) and inhibitory (I) innervation to the deep extensor muscles in adult and embryonic lobsters using serial section electron microscopy.*

	Adult		Embryo	
	E	I	E	I
Muscle length serially sectioned (μm)	13·773	13·773	16·810	16·810
Terminal length serially sectioned (μm)	23·039	18·523	53·155	33·815
Synapses:				
Total number	17	3	49	12
Total surface area (μm^2)	12·927	30·198	6·045	6·797
Mean surface area (μm^2)	0·760	10·065	0·123	0·566
(X ± S.E.M.)	± 0·529	± 0·852	± 0·007	± 0·098
	$P<0·01$		$P<0·01$	
Presynaptic dense bars				
Total number	14	25	51	16
Number per synapse	0·82	8·33	1·04	1·33
% of synapses with no dense bar	47	0	6	17
% of synapses with 1 dense bar	19	0	84	50
% of synapses with 2 dense bars	18	0	10	25
% of synapses with 3 and more bars	6	100	0	0
Total length (μm)	1·439	3·175	7·635	6·965
Mean length (μm)	0·102	0·127	0·150	0·435
(X ± S.E.M.)	± 0·043	± 0·054	± 0·010	± 0·124
	ns		$P<0·01$	

10 to 50 μm and were therefore regarded as belonging to separate axons. Synapses of the excitatory terminal were relatively small compared to those of the inhibitory terminal, and this size difference was validated with serial-section electron microscopy. For this procedure we examined, in two separate lobsters, innervation sites for approximately 7 μm each, giving a combined total of approximately 14 μm of muscle fibre length that was analysed for its innervation (Table 2). In this length there were only three inhibitory synapses as compared to 17 excitatory ones. The inhibitory synapses were more than ten-fold larger in surface area than the excitatory synapses. Not only were the inhibitory synapses larger, but they possessed more dense bars than their excitatory counterparts. The dense bars were of similar length in both types of terminals. In terms of these morphological criteria, the area of inhibitory innervation is considerably larger, than the area of excitatory innervation to the adult DEAM muscle.

2) *Embryonic innervation*

a) *Electrophysiology*

The innervation of the deep extensor muscles was determined in embryos with an eye index ranging from 90 to 520 μm. Early embryonic development was examined in several stages of approximately 10 μm increments from 90 to 150 μm eye index with five to seven embryos examined at each stage. In these early embryos functional innervation was not present judging from the fact that no junctional potentials were recorded either spontaneously or following stimulation of the nerve roots. In later embryos with eye indices between 160 to 520 μm encompassing nine size categories functional innervation was present. The transition from the non-innervated to the innervated condition in the abdominal extensor muscles therefore occurred in embryos with an eye index between 150 to 160 μm respectively which represents approximately 48 h of development at ambient water temperatures of 25–28 °C. The innervation consisted of both excitatory (EJP) and inhibitory (IJP) junctional potentials (Fig. 4A, B). The IJPs were usually hyperpolarizing in sign, but occasionally showed a reversal to become depolarizing during the course of an experiment. A single IJP was found in individual muscle fibres suggesting the presence of a single inhibitor axon. This confirmed previous findings on this system by Cole & Lang (1980) and Stephens & Govind (1981).

The EJPs from an individual fibre, on the other hand, showed two to three distinct increments in amplitude when the stimulus intensity was increased (Fig. 4C, D). This suggested innervation of individual muscle fibres by two to three excitatory axons. Very rarely did we find a single class of EJPs in a muscle fibre. Such dual and triple excitatory innervation was present in embryos with eye indices between 160 to 520 μm; in other words, in all embryos in which innervation was detected. In contrast to our previous findings of a single excitatory axon in these embryonic muscles (Stephens & Govind, 1981; Cole & Lang, 1980), our present results demonstrate that right from the beginning of functional innervation muscle fibres receive input from two to three excitatory and a single inhibitory axon.

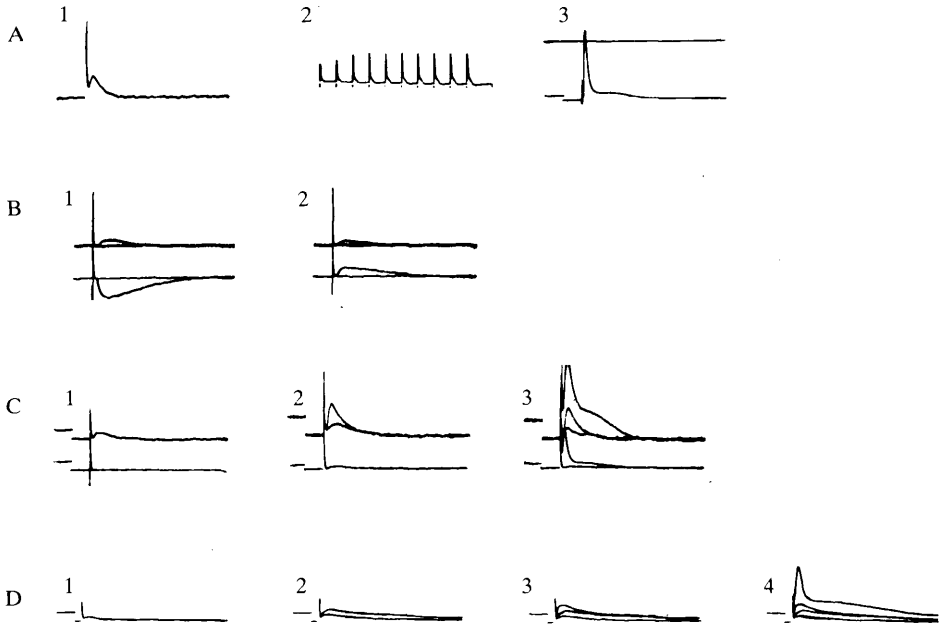

Fig. 4. Innervation of the deep extensor muscle in embryonic lobsters revealed by stimulation of the second nerve root in minimally dissected preparations. (A) representative features of excitatory innervation showing unitary EJP (A1), facilitation of EJPs (A2) and a regenerative response (A3) in three separate embryos with eye indices of 450, 490, and 520 μm. (B) inhibitory innervation indicated by an hyperpolarizing IJP (B1, lower trace) which becomes depolarizing in sign (B2) during the course of an experiment in an embryo with an eye index of 410 μm. For comparison an EJP (upper trace) is shown in the same fibre. (C) dual (lower trace) and triple (upper trace) innervation of two separate fibres revealed by increasing stimulus intensity in three steps (C1 to C3) in an embryo with an eye index of 310 μm. (D) single inhibitory axon innervation indicated by an IJP which is depolarizing in sign (D1) and triple excitatory axon innervation indicated by three distinct sizes of EJPs (D2, D3, D4) in a single muscle fibre of an embryo with an eye index of 160 μm. Calibration: vertical, 10 mV: horizontal, 10 msec for D, 20 msec for C, B, A1, A3, 200 msec for A2.

Other features of excitatory innervation include a small to moderate amount of facilitation of the EJPs with repetitive stimulations and regenerative responses arising from the EJP (Fig. 4A). The regenerative responses varied in amplitude and occasionally overshot zero.

In summary, embryonic innervation of fibres of the deep abdominal extensor muscles consist of a lone inhibitory and two to three excitatory axons.

b) *Electron microscopy*

In early embryos, with eye indices between 90 to 150 μm, profiles of axons or neuromuscular terminals were not found in the deep extensor muscles. This is in keeping with the electrophysiological data which revealed a lack of innervation in these early stages. In later embryos, with eye indices greater than 150μm, innervation

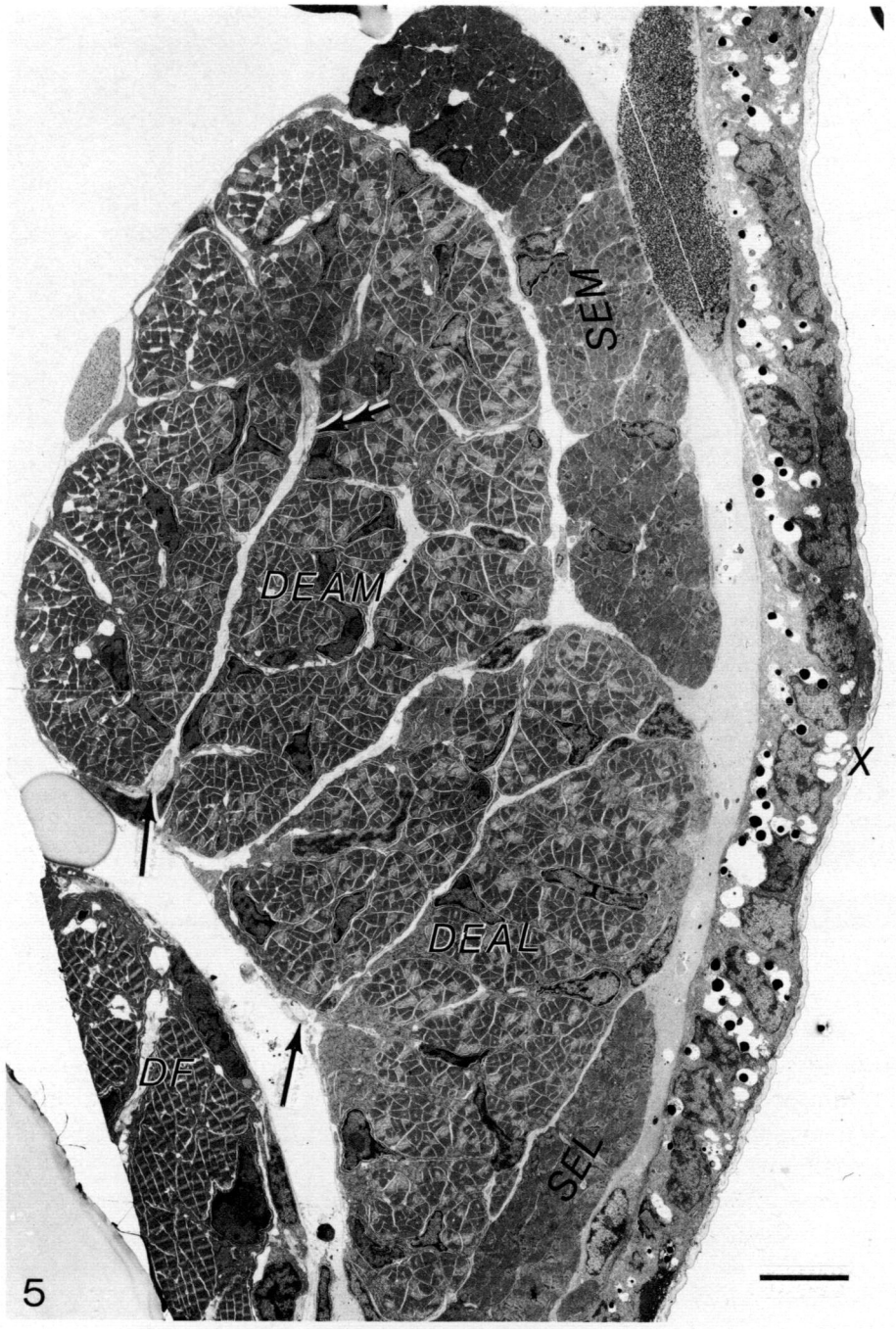

Fig. 5. Subdivision into medial and lateral components of the superficial (*SEM, SEL*) and deep (*DEAM, DEAL*) extensor abdominal muscles in an embryonic lobster. Site of innervation analysed in this report within the DEAM is shown by a double arrow while axon profiles along the periphery of the deep extensors are shown by single arrows. *DF*, deep flexor muscle; *X*, exoskeleton. Scale bar, 1 μm.

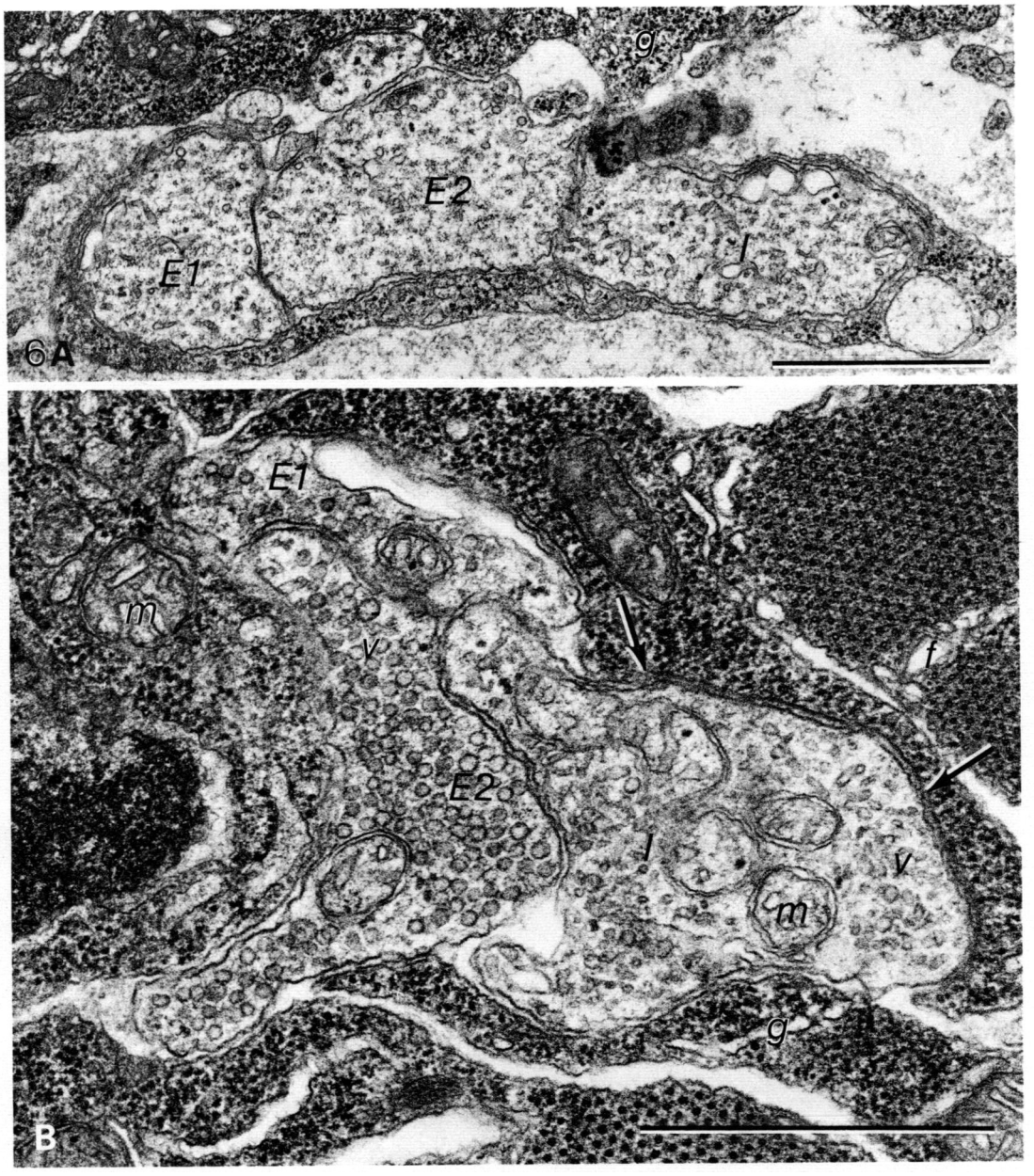

Fig. 6. An inhibitory (*I*) and excitatory (*E1, E2*) motor axon profiles from separate axons, (A) travel together to the embryonic DEAM muscle and (B) give rise to neuromuscular terminals which are characterized by clear synaptic vesicles (*v*) and a synapse (between arrows). *f*, myofibril; *m*, mitochondria; *g*, muscle granular sarcoplasm. Scale bar, 1 μm.

consisted of a few discrete pockets scattered over the cross section to the DEAM (Fig. 5). An embryo with an eye index of 350 μm was examined with serial-section electron microscopy and yielded most of the data described in this report. In this

case the innervation site consisted of three (Fig. 6A) and occasionally four axon profiles which were followed in both directions i.e. towards their origin and their destination. The axons were followed centrally for a long-enough distance to be certain that they were separate axons, not branches of a single axon.

In the direction of their destination the axons gave rise to neuromuscular terminals (Fig. 6B) with well-defined synapses, presynaptic dense bars and synaptic vesicles. As in the case of the adult muscle, in the embryonic muscle also, there is a close juxtaposition of a single inhibitory terminal with one or two excitatory terminals, and the inhibitory synapses appear to be larger than their excitatory counterparts. However, unlike the adult condition, in the embryo the neuromuscular terminals appear to be more densely populated with synaptic vesicles (cf. Figs 3 and 6B). That this is due to fixation procedure is unlikely as both adult and embryonic tissue were subjected to the same fixation procedure.

The quantitative analysis in Table 2 shows the relationship between excitatory and inhibitory innervation in the embryo to be similar to that in the adult in some respects. For instance, inhibitory synapses are far fewer and considerably larger than excitatory ones. While the great majority of excitatory synapses possessed a single dense bar, many of the inhibitory synapses had two and some even three dense bars. Consequently the larger inhibitory synapses tended to support more dense bars than the smaller excitatory ones as was also the case in the adult. Unlike the adult condition however, in the embryo the inhibitory dense bars were significantly greater in length than their excitatory counterparts.

DISCUSSION

In terms of the total innervation to the embryonic lobster deep extensor muscle in each segment, there are as many as three excitatory and a single inhibitory neuron innervating each muscle fibre. Since the same degree of polyneuronal innervation is characteristic of adult muscle fibres it suggests, at least in the embryonic stages we examined, that muscle fibres were functionally innervated by the adult complement of axons.

It has been previously suggested that the innervation to these muscles is sequential with a single axon forming the initial early contacts and acting as a pioneer axon to the later following axons (Cole & Lang, 1980). In the present study of several early embryonic stages with a 10 μm difference in size of eye index representing approximately 48 h of development, the muscle fibres are innervated simultaneously by the full complement of motor axons and not sequentially. This does not preclude the possibility that one (or more) of the axons act as a pioneer to establish the pathway in a manner similar to that found in insect nervous systems (Goodman, 1982). We would not have seen such sequential innervation if it occurred within a 48 h time span which was the minimum period separating the developmental stages that we examined.

The way in which the common and private excitor axons define their fields of

innervation appears to differ during ontogeny. Early in development the CE axon innervates the deep extensor muscle in its own hemisegment and in those on either side (Stephens & Govind, 1981). This widespread innervation by the CE axon to three segments is seen in embryonic, larval and early juvenile lobsters but is lost later, and the innervation becomes restricted to a single segment in late juveniles and adults. The peripheral innervation field of the CE axon is therefore defined by synapse elimination during development. The private excitor axons, on the other hand, do not appear to distribute widely to non-target areas since stimulation in one segment did not evoke responses in adjacent segments. Rather they appear to zero in on their correct targets at the time of first innervation. What cues direct these axons to their specific targets while allowing the CE axon to spread out widely and cross segment boundaries remains an intriguing mystery. The results, however, illustrate two separate mechanisms for defining the peripheral fields of motor neurons: one in which an overproduction of synapses occurred and the other in which only the requisite number are produced. The co-existence of both mechanisms emphasizes the fact that the formation of neural connections is guided not by a single force but possibly by several forces which interact to produce the final pattern of connections.

The other finding of interest was the close resemblance between the embryo and the adult both in the fine structure and pattern of neural connections. Apart from the fact that the embryonic synapses were smaller in size than the adult ones, they were otherwise similar in form and in having presynaptic dense bars. They also resemble each other in the fact that there are fewer inhibitory than excitatory synapses and that the former are considerably larger than the latter in both embryonic and adult DEAM muscles. The significance of this almost equivalent amounts of both types of innervation to the DEAM is unclear, though one possibility is that the inhibitory innervation may serve to eliminate any residual tension in the extensor muscles. In this way the antagonistic deep flexor muscles which are responsible for the rapid tail flips can exert their full effect (Kennedy & Takeda, 1965*a*). Whatever may be their role, our data reveals that an almost adult-like pattern of neuromuscular connections to the DEAM are laid down in full, well in advance of hatching. In contrast, the neuromuscular connections to the lobster limb muscle differentiates after hatching into the larval stages (King & Govind, 1980; Govind & Pearce, 1982). This somewhat precocious development of the neuromuscular connections to the abdominal muscles *vis-a-vis* the limb muscles in the embryonic lobsters is correlated with the fact that immediately upon hatching the larvae propel themselves by means of their abdomen and accessory limb appendages (Herrick, 1895; Neil, MacMillan, Robertson & Laverack, 1976).

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