THREE-DIMENSIONAL ULTRASTRUCTURE OF THE CRAYFISH NEUROMUSCULAR APPARATUS

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ABSTRACT

The synapse-bearing nerve terminals of the opener muscle of the crayfish *Procambarus* were reconstructed using electron micrographs of regions which had been serially sectioned. The branching patterns of the terminals of excitatory and inhibitory axons and the locations and sizes of neuromuscular and axo-axonal synapses were studied. Excitatory and inhibitory synapses could be distinguished not only on the basis of differences in synaptic vesicles, but also by a difference in density of pre- and postsynaptic membranes. Synapses of both axons usually had one or more sharply localized presynaptic "dense bodies" around which synaptic vesicles appeared to cluster. Some synapses did not have the dense bodies. These structures may be involved in the physiological activity of the synapse. Excitatory axon terminals had more synapses, and a larger percentage of terminal surface area devoted to synaptic contacts, than inhibitory axon terminals. However, the largest synapses of the inhibitory axon exceeded in surface area those of the excitatory axon. Both axons had many side branches coming from the main terminal; often, the side branches were joined to the main terminal by narrow necks. A greater percentage of surface area was devoted to synapses in side branches than in the main terminal. Only a small fraction of total surface area was devoted to axo-axonal synapses, but these were often located at narrow necks or constrictions of the excitatory axon. This arrangement would result in effective blockage of spike invasion of regions of the terminal distal to the synapse, and would allow relatively few synapses to exert a powerful effect on transmitter release from the excitatory axon. A hypothesis to account for the development of the neuromuscular apparatus is presented, in which it is suggested that production of new synapses is more important than enlargement of old ones as a mechanism for allowing the axon to adjust transmitter output to the functional needs of the muscle.

The innervation of the crayfish opener muscle consists of a singe excitatory (E) axon and a single inhibitory (I) axon, which have been extensively investigated by physiological methods and later by means of electron microscopy (for reviews, see 2–4). The system is of interest because it has some features in common with various neurons in the central nervous systems of both vertebrates and invertebrates. Detailed study of this accessible system may lead to principles which can be applied elsewhere.

Previous ultrastructural studies have allowed

identification of E and I synapses, and have shown the existence of axo-axonal synapses between E and I nerve terminals (7, 9, 10, 21). However, no attempt was made previously to provide a complete reconstruction of the neuromuscular apparatus through serial sectioning. Therefore, no exact statements could be made about the number, locations, and sizes of the synapses on the two axons, or about the general shape of the synapse-bearing terminal regions. In order to provide such information about ultrastructure, serial sectioning must be done; other methods such as scanning electron microscopy are not adequate for this purpose (21).

In the present study, we have reconstructed from serial sections a number of regions containing synaptic terminals, and have measured the sizes of synapses on the two axons. A more quantitative statement can now be made about the morphology of these representative regions of the crayfish neuromuscular apparatus.

MATERIALS AND METHODS

The animals used in this study were specimens of *Procambarus blandingii*, obtained from a dealer in Wisconsin. The opener muscle of the chelate first walking leg was chosen for the investigation. Several of these muscles were fixed, but most of the nerve terminals investigated in detail were chosen from a single muscle. Examination of other specimens showed that they were qualitatively similar to the one studied in detail.

Muscles were fixed according to the method of Peracchia and Mittler (25). Small pieces of the muscles, consisting of a few fibers and the attached innervation, were embedded in an Epon-Araldite mixture and sectioned with a diamond knife on an LKB Ultrotome III. Longitudinal sections were cut from several samples until regions showing nerve terminals were found. Serial sections of these regions were then cut and mounted on single-slot grids coated with Formvar. The use of single-slot grids was necessary for viewing the entire synaptic region, which often was quite extensive (see Fig. 1). The method for collecting the sections and aligning them on the single-slot grids was that developed by Moens (23) from the technique of Galey and Nilsson (17). A continuous record was kept of the sections as they were produced. Note was taken of the thickness of each section (as judged by the interference color) and of any sections that were missed. Most of the sections were 70–90 nm in thickness.

The grids were stained in saturated aqueous uranyl acetate for 40 min, and then in 0.4% lead citrate for 10 min (33). They were viewed with a Philips EM-200 electron microscope, and photographs were taken of the regions of interest.

Reasonably complete series of electron micrographs were obtained for four representative regions each 3–10 μm in total length. Several other regions were also followed but in less detail. From the micrographs, models

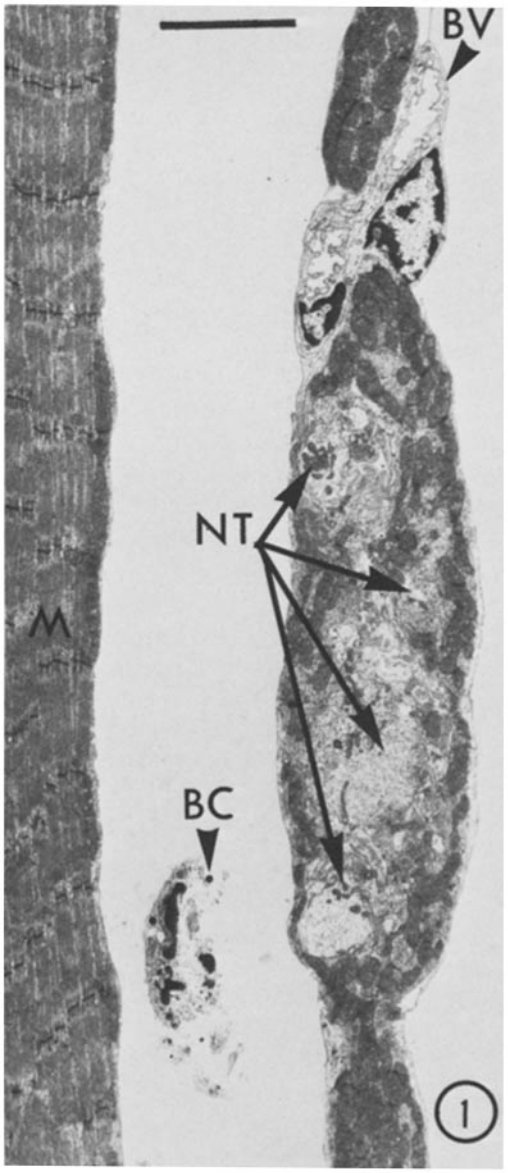

FIGURE 1 Low-power electron micrograph of synaptic region in the crayfish opener muscle on a single-slot grid. This region was located in a semi-isolated outgrowth of the muscle fiber which contained the nerve terminals (some indicated: *NT*), mitochondria, and granular sarcoplasm. The two parent axons each provide two large terminals and two small ones to this region. Other structures include: *BV*, blood vessel; *BC*, blood cell; *M*, muscle fiber. Scale mark, 5 μm.

of the terminal regions were reconstructed by cutting thin pieces of Styrofoam to the shape of each nerve terminal in each section. Synapses were marked on the Styrofoam pieces which were glued together in the correct orientation, using the micrographs as templates. The models provided a way to visualize the rather complex relationships of E and I nerve terminals.

Measurements of the dimensions of nerve terminals and synapses were made from the micrographs of each section to permit calculation of nerve terminal surface areas, synaptic contact areas, etc. For each synapse, the length of the synapse measured on the micrograph was multiplied by the thickness of the section; the values so obtained from each section in which the synapse appeared were added together to yield a value for contact area of the synapse. Nonsynaptic terminal areas were computed in the same way. The percentages of synaptic and nonsynaptic membrane for E and I nerve terminals could be calculated from these figures. Separate calculations were made for different branches of each axon, to see whether any variation was evident in different parts of the terminal apparatus.

RESULTS

E and I Synapses

Areas containing nerve terminals were identified with low-power electron microscopy (Fig. 1). The identity of the terminals (E or I) was established with high-power electron microscopy using the differences in morphology of synaptic vesicles described in previous studies (9, 10, 31). E terminals have more regular and statistically somewhat larger synaptic vesicles than I terminals, when fixed in glutaraldehyde and postfixed with osmium tetroxide.

Other differences between E and I terminals were apparent. In particular, synapses of E terminals generally had somewhat more densely stained pre- and postsynaptic membranes than those of I terminals (Figs. 2 and 3, or Figs. 4 and 10). Similar differences in staining reactions of synaptic contact regions have been described among vertebrate central synapses by Gray (18, 19). Differences in shape and in relative numbers of E and I synapses will be dealt with later.

At many E synapses, and a few I synapses, a thin sheet of electron-dense material was present in the synaptic cleft (Fig. 2). This material appeared to be attached by periodic dense projections to the postsynaptic membrane. At most I synapses, the dense material in the synaptic cleft was not seen (Fig. 3). However, a less prominent layer of material, with periodic interruptions, could sometimes be seen on the postsynaptic membrane. It is likely that the precise appearance of the dense material within the synaptic cleft, and whether or not it is firmly bound to the postsynaptic membrane, depends on conditions during fixation. Similar postsynaptic specializations have been observed at insect neuromuscular synapses (11, 26).

Presynaptic Dense Bodies

Densely staining presynaptic structures of several different forms have been observed in both vertebrate and invertebrate central nervous tissue (see Sandeman and Luff [27] for examples and further references), and at vertebrate neuromuscular junctions, where they have sometimes been referred to as "active zones" (12, 13). In serial sections of crayfish synapses, it was apparent that many synapses of both E and I types had one or more "dense bodies" associated with the presynaptic membrane around which synaptic vesicles were densely clustered. Two of these structures appear in Fig. 3. They did not have any apparent substructure which could be resolved in the material prepared for this study. The dense bodies were

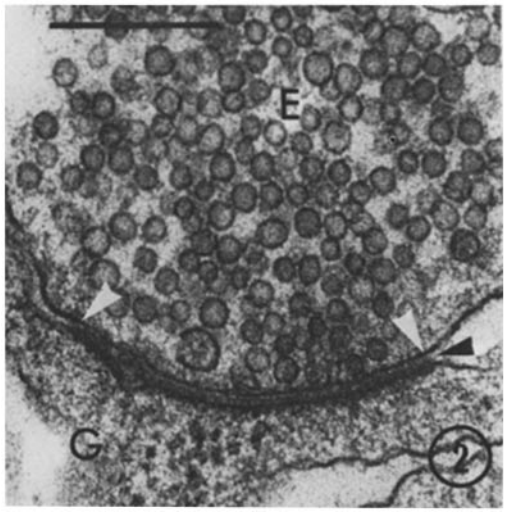

FIGURE 2 High-power view of a typical E neuromuscular synapse (between white arroheads), showing regular presynaptic vesicles, densely stained pre- and postsynaptic membranes, intervening dense material (black arrowhead). The synapse is considered to extend over regions in which densely stained pre- and postsynaptic membranes have a uniform separation of approximately 200 Å. *G*, granular sarcoplasm; *E*, excitatory terminal. Scale mark, 0.25 μm.

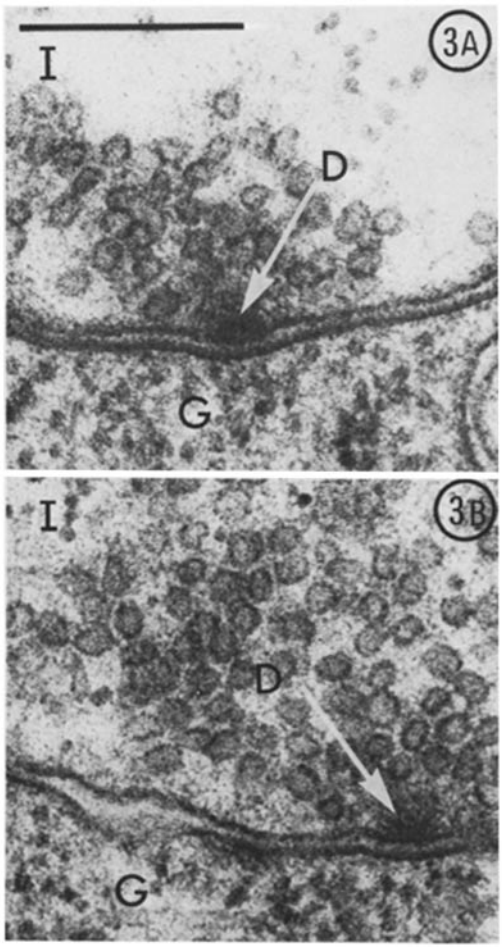

FIGURE 3 Two I neuromuscular synapses (Fig. 3 A and B) showing clusters of less regular inhibitory presynaptic vesicles near presynatpic dense bodies (*D*, white arrows). The pre- and postsynaptic membranes are less heavily stained than those of E terminals. *G*, granular sarcoplasm; *I*, I nerve terminals. Scale mark, 0.25 μm.

generally of hemispherical configuration, 300–600 Å in diameter, and seen usually in only one section of a series.

Synaptic vesicles were observed more densely clustered at the presynaptic dense bodies than elsewhere. In Fig. 4, serial sections through an I synapse are presented to show localization of two dense bodies along the synapse, with associated clustering of vesicles. Graphs of vesicle density along two other I synapses are presented in Fig. 5 to illustrate the characteristic increase in vesicle density near the dense bodies. One of these syn-

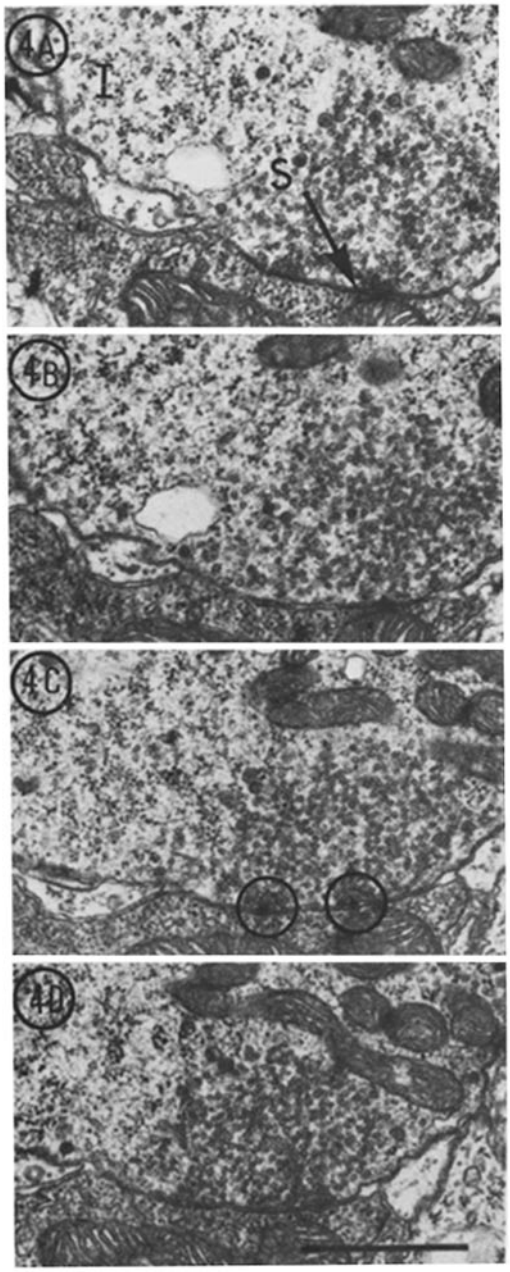

FIGURE 4 Several sections from a series taken through an I nerve terminal (*I*) with a neuromuscular synapse (*S*), to show locations of two presynaptic dense bodies (circled in Fig. 4 C), with clustering of the synaptic vesicles. Distance along the synapse from Fig. 4 A to 4 B, 0.3 μm; from Fig. 4 B to 4 C, 0.15 μm; from Fig. 4 C to 4 D, 0.22 μm. Scale mark, 1 μm.

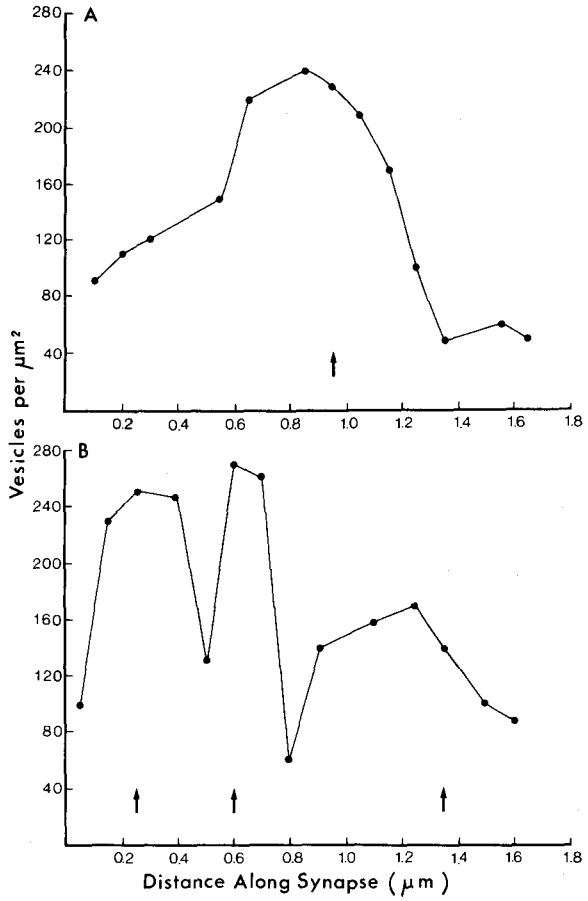

FIGURE 5 Graphs of synaptic vesicle density (within 0.25 μm of the synaptic membrane) measured in successive sections along the synapse, for two large I neuromuscular synapses. Positions of presynaptic dense bodies are indicated by arrows.

apses contained only one dense body, whereas the other had three, each associated with a peak in vesicle density.

Some synapses were found which did not have a dense body. In such synapses, a well-defined peak in vesicle density was not apparent. This is illustrated in Fig. 6, which shows vesicle density plots for two E synapses, one of which had a presynaptic dense body and the other of which did not. Only the former showed a peak in vesicle density along the synapse.

It was estimated that about 22% of E synapses (in a sample of 34) lacked the presynaptic dense bodies, compared to about 14% of I synapses (in a sample of 27). Synapses lacking the dense bodies were smaller than average in area of contact; few if any of the larger synapses lacked a dense body. Thus, among "synapses" showing the characteris-

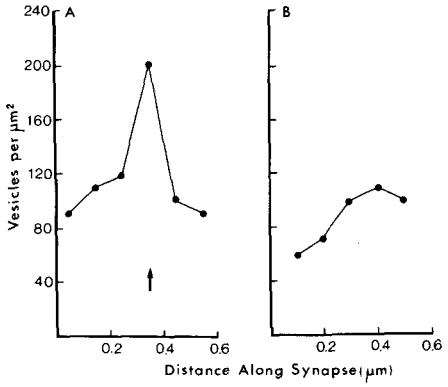

FIGURE 6 Graphs of synaptic vesicle density (within 0.25 μm of the synaptic membrane), measured in successive sections along the synapse, for two E neuromuscular synapses, one of which (Fig. 6 A) had a dense body, and the other of which (Fig. 6 B) did not.

tic increase in density of pre- and postsynaptic membranes, a distinction can be made between structures having one or more dense bodies, and those having none.

Among E synapses having one or more dense bodies, it was determined that approximately 60% ($^{16}/_{26}$) had one dense body and 40% ($^{11}/_{26}$) had two. Among I synapses, 52% ($^{13}/_{23}$) had one dense body, 26% ($^{6}/_{23}$) had two, 17% ($^{4}/_{23}$) had three, and 5% ($^{1}/_{23}$) had four. The results suggest a tendency for I synapses to posses more dense bodies than E synapses, although the distinction is not a sharp one.

Configuration of Nerve Terminals

Several nerve terminals usually appeared close together in synaptic regions. They, along with associated glial cells, were embedded in specialized regions of the muscle fiber containing granular sarcoplasm devoid of contractile filaments. Sometimes the specialized region formed a partly isolated outgrowth of the muscle fiber, as in Fig. 1.

Serial sections showed that the two parent axons (E and I) gave rise to all of the synaptic terminals of each region through complex branching. The branches of the two axons did not "follow" each other closely at this level, so that the "diplotomic" pattern observed with methylene blue (32) was not preserved. The reconstruction of Fig. 7, for example, shows the E axon dividing into three branches and the I axon into two.

An interesting feature of the branching of the I axon in Fig. 7 is the extreme narrowness of the neck connecting the side branch (I_2) with the main branch (I_1). This feature was commonly observed at branch points of both E and I axons. The necks were often 0.2 μm in diameter or less.

Both the main trunks of the axons and the side branches had synapses, but the side branches often had fewer glycogen granules and mitochondria, and a greater density of vesicles than the main trunk. The side branch (I_2) of the I axon (Fig. 7) shows this feature very well. It is jammed full of synaptic vesicles, whereas in the main trunk (I_1), the vesicles are limited mainly to the region of the synapse. Similarly, a branch of the E axon (E_1) is densely packed with vesicles. (A second branch, E_2, appears vacuolated and empty, perhaps due to an artifact of fixation.)

Figs. 8 and 9 show a reconstruction of another synaptic region made from a longer series of sections which illustrates additional features of the nerve terminals. Great diversity of branch size is apparent; for example, the thin nonsynaptic branch I_2 of Fig. 8 is many times smaller than the main trunk I_1. Some of the very small nonsynaptic side branches of both E and I axons contained microtubules, suggesting that they may have been points of growth for the axon. One such potential growth point (*EB*) can be seen in Fig. 9.

The narrow branch points mentioned previously are apparent in this reconstruction (e.g. E_3 and I_4 of Fig. 8). Moreover, some axonal branches showed periodic enlargements and narrowings even when side branches were not given off (Fig. 8, E_1; Fig. 9, *E*). The narrow necks were often very small and could easily have been overlooked in random sectioning. Often they contained a mitochondrion, which imparted a peculiar dense appearance to them (see Fig. 9, inset).

The E axon generally contributed more branches to a synaptic region than the I axon. However, the average size of I axon branches was greater, and the very largest branches seen were from the I axon.

Location of Axo-Axonal Synapses

Axo-axonal synapses were observed rather infrequently in this study, as in previous ones. One or two axo-axonal synapses could be found for each 3–4 μm of the terminal region sectioned, compared with 20 or more inhibitory neuromuscular synapses from the same terminals.

Many of the axo-axonal synapses were found at branch points or narrow necks of the E axon although some were also seen on enlarged regions of the E axon terminals. Illustrative examples are provided in Figs. 9 and 10. In Fig. 9, a small axo-axonal synapse is shown which involves the main trunk of the I axon and a narrow neck of the E axon. The synapse, although small, had a localized dense body and a cluster of vesicles at the presynaptic membrane. The synapse of Fig. 10 is much larger and occurs on a larger neck which joins one of the main trunks of the E axon (E_2-*i*) to a large side branch (E_2-*ii*). (Sections taken on either side of the region in the illustration showed that E_2-*ii* was a side branch of limited extent and that E_2-*i* was a main axon.) The synapse had two presynaptic dense bodies and many synaptic vesicles clustered near the presynaptic membrane. The contract area of this synapse was about 0.38 μm^2, but it did not occupy the whole of the apposed surface of the neck (Fig. 10).

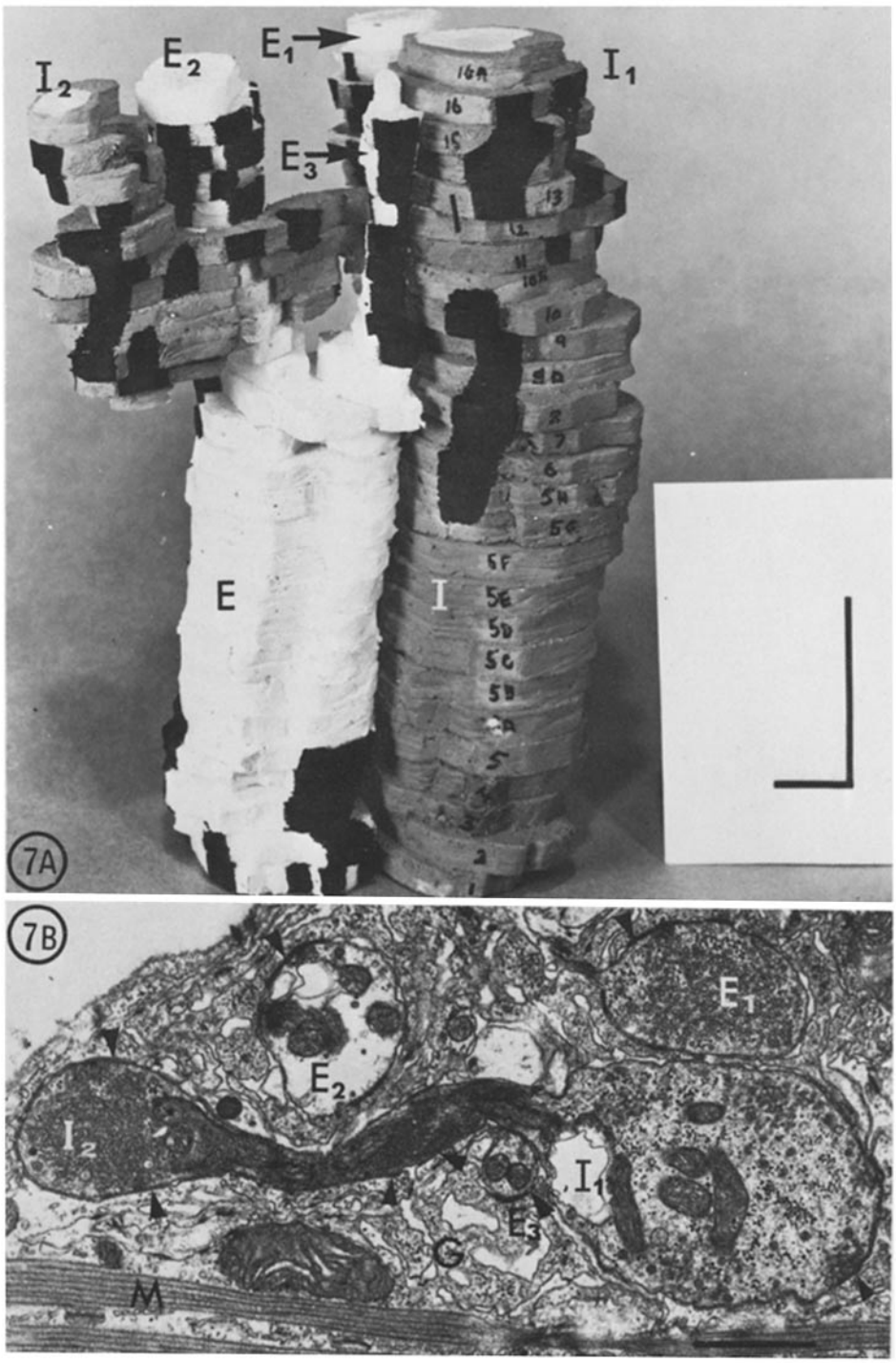

FIGURE 7 Fig. 7 A: Reconstruction of part of the terminal apparatus from serial sections. E (*E*) and I (*I*) axons both give rise to branches (E_1, E_2, E_3 and I_1, I_2). Synapses are painted black on the model. Scale marks: horizontal, 1 μm; vertical, 0.6 μm. Fig. 7 B: Electron micrograph from the series used for Fig. 7 A, to show a narrow side branch of the I axon giving rise to a secondary terminal (I_2). Neuromuscular synapses are indicated by arrows. The terminals are numbered as in the reconstruction. *M*, muscle fiber myofilaments; *G*, granular sarcoplasm. Scale mark, 1 μm.

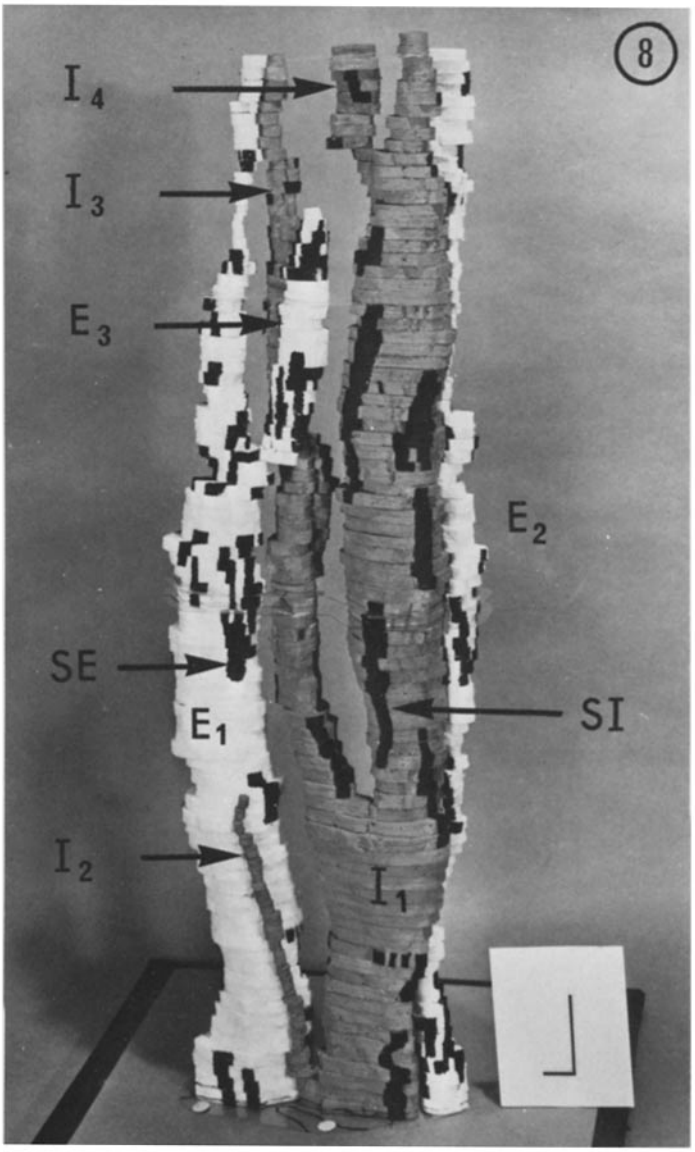

FIGURE 8 A more extensive reconstruction of another terminal region to show the forms of the axon terminals. E branches, E_1, E_2, and E_3; I branches, I_1, I_2, I_3, and I_4; representative E neuromuscular synapse, *SE;* representative I neuromuscular synapse, *SI*. Scale marks: horizontal, 1 μm; vertical, 0.6 μm.

These representative examples illustrate strategic placement of axo-axonal synapses at narrow necks and branch points of the E axon. In addition, a correlation between the size of the synapse and the size of the postsynaptic structure involved is apparent. The synapses located on narrow necks of diameter 0.2 μm were always small (less than 0.1 μm^2 in contact area). Those on larger necks were considerably greater in area (up to 0.4 μm^2). Furthermore, axo-axonal synapses usually involved the main trunk or a large branch of the I axon, and never occurred in our sample at branch points or narrow necks of the I axon.

Location and Sizes of Neuromuscular Synapses

Neuromuscular synapses were located on both the main trunk and the side branches of both E

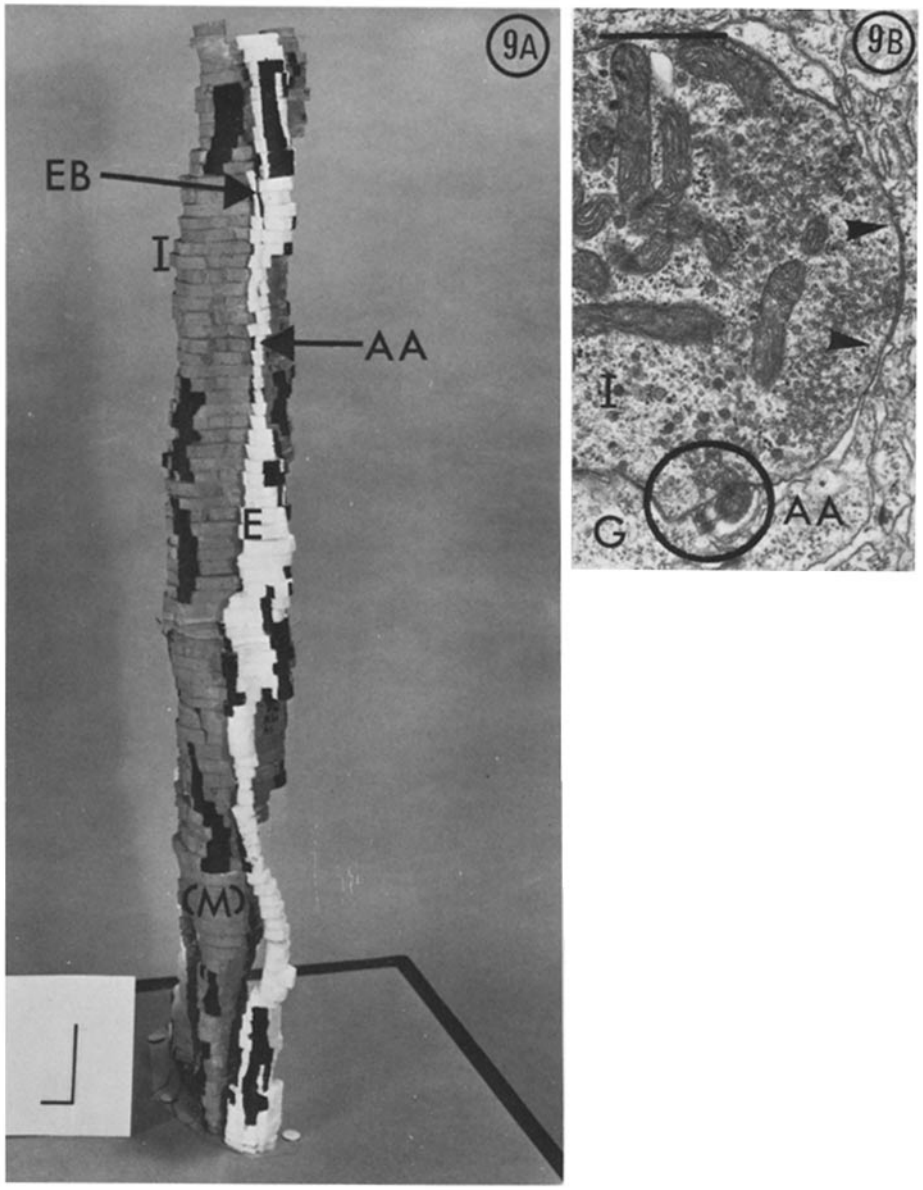

FIGURE 9 Fig. 9 A: Side view of the reconstruction of Fig. 8 to show the location of an axo-axonal synapse (*AA*), and the periodic expansion and construction of the E branch. A small "bud" or "sprout" of the E axon (*EB*) is indicated. *M*, sequence of five missing sections. Scale marks: horizontal, 1 μm; vertical, 0.6 μm. Fig. 9 B: Electron micrograph used for reconstruction, to show the axo-axonal synapse (*AA*) on the narrow neck of the E axon (circled). *I*, main trunk of the I axon; *G*, granular sarcoplasm; arrowheads, I neuromuscular synapse. Scale mark, 1 μm.

and I terminals. Small sprouts, such as I_2 in Fig. 8 and *EB* in Fig. 9, did not have synapses, but synapses did occur on some of the narrow necks and branch points (e.g. Fig. 7, I_2).

There was a wide range in size and shape of neuromuscular synapses. Many were roughly ovoid (Fig. 8, *SI*) whereas others were irregular or branched (Fig. 10, *BS*). E terminals showed a higher proportion of irregular or branched synapses than I terminals. In addition, E synapses

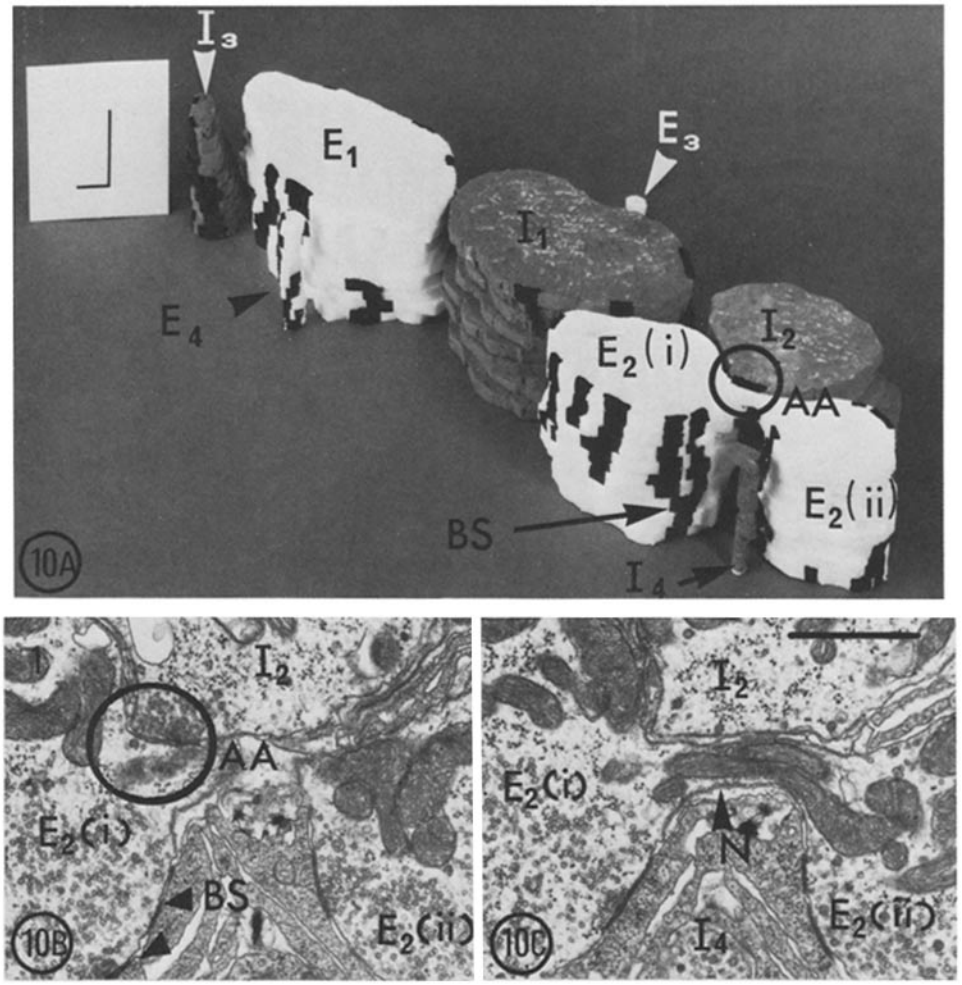

FIGURE 10 Fig. 10 A: Reconstruction of part of the terminal region shown in Fig. 1, to illustrate the location of an axo-axonal synapse (*AA*, circled) on a narrow neck joining two E terminals (E_2-*i* and E_2-*ii*). E_1, E_2, E_3, E_4, E terminals; I_1, I_2, I_3, I_4, I terminls; *BS*, branched synapse of the E axon. Scale marks: horizontal, 1 μm; vertical, 0.6 μm. Fig. 10 B: Electron micrograph from the series used for the model, showing the axo-axonal synapse (*AA*, circled) on the neck joining E_2-*i* and E_2-*ii*. The junction of the two E branches is not complete at this point. Fig. 10 C: Electron micrograph from the same series, just past the point at which the axo-axonal synapse disappeared. The neck (*N*) joining the two E branches is complete at this point. Terminals are numbered as in the reconstruction. Scale mark for Figs. 10 B and C, 1 μm.

were more numerous than I synapses in all synaptic regions studied.

A comparison of size distributions for E and I synapses whose contact areas could be accurately measured from serial sections is made in Fig. 11. The mean contact areas for synapses of E and I axons were, respectively, 0.389 $\mu m^2 \pm$ SE 0.028 (n = 52) and 0.455 $\mu m^2 \pm$ SE 0.070 (n = 31). The means were not significantly different, nor were the samples shown to be different by a Komolgorov-Smirnoff two-sample test. However, it is evident that some of the large I synapses exceed any of the E synapses in size. These large synapses occurred on the main trunks of the I axon, and had three or four presynaptic dense bodies.

An analysis of the total surface area devoted to synaptic contacts was made for three different synaptic regions (Tables I–III). One of the regions

(no. 1) was selected to include synapse-bearing axons near their entry into the muscle fiber; a second (no. 3) was taken close to the ultimate terminations of the axons; and a third (no. 2) was from an intermediate position. Some other regions were examined in less detail, and in general the observations accorded with those made in the three regions chosen for more detailed analysis.

Information for side branches and for the main axonal trunks was analyzed separately. Usually there was no difficulty in telling which structures were the main trunks, and which structures were offshoots from the main trunk. Sometimes, two large (>1 μm diameter) trunks were encountered for one of the axons in the same region (see Figs. 1 and 10); in such instances, both were considered as main trunks rather than side branches, because of their size. We did not determine the pattern of connectivity between two main trunks of the same axon since our series of sections did not extend far enough.

The results of the analysis of the E terminals of

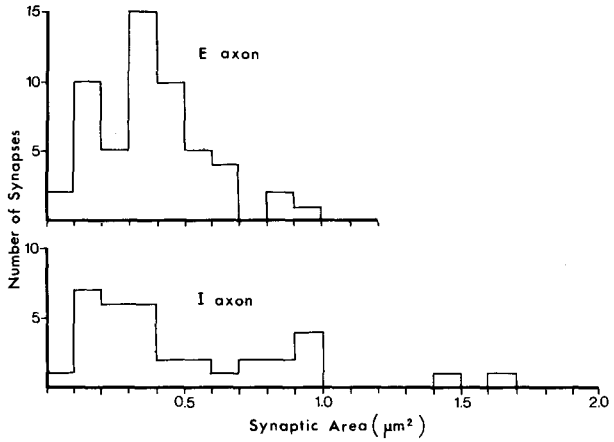

FIGURE 11 Histograms to show size distributions for computed contact areas of E and I synapses. Only the synapses for which a complete series of sections was obtained are included; many others were sampled less completely.

TABLE I

Synaptic Contact Areas for Branches of the E Axon in Three Terminal Regions

Terminal region	Branch	Length sectioned	Number of synapses	Total synaptic area	Surface area of branch	Synaptic area
		μm		μm^2	μm^2	%
1	Main branch A	3	13	4.91	36.43	13.4
	Main branch B	3	24	8.02	58.53	13.7
	Side branch A	3	8	3.51	18.84	18.6
	Side branch B	2	2	1.89	7.02	26.9
	Totals		47	18.33	120.82	15.2
2	Main branch	7	23	8.72	40.75	21.4
	Side branch A	7	14	3.96	19.41	20.4
	Side branch B	2	7	2.09	5.09	41.1
	Totals		44	14.77	65.52	22.5
3	Main branch	1.5	8	3.18	11.69	27.2
	Side branch A	1	5	0.96	3.49	27.1
	Side branch B	1.5	7	2.02	6.79	29.8
	Side branch C	1	2	0.30	1.57	18.9
	Totals		22	6.46	23.54	27.4

TABLE II

Synaptic Contact Areas for Branches of the I Axon in Three Terminal Regions

Terminal region	Branch	Length sectioned	Number of synapses	Total synaptic area	Surface area of branch	Synaptic area	Axo-axonal synapses	Area of axo-axonal synapses
		μm		μm^2	μm^2	%		%
1	Main branch A	3	8	2.66	33.50	7.9	1	1.1
	Main branch B	3	11	4.68	48.29	9.7	1	0.2
	Side branch A	1.5	3	1.06	6.07	17.5	0	0
	Side branch B	2	2	1.47	6.96	21.0	0	0
	Totals		24	9.87	94.83	10.4	2	0.5
2	Main branch	7	15	7.79	64.74	12.0	3	0.3
	Side branch A	5	9	3.10	18.91	16.4	1	0.2
	Side branch B	1	2	0.23	1.86	12.6	0	0
	Side branch C	1	2	0.43	2.54	16.7	0	0
	Totals		28	11.55	88.05	13.1	4	0.2
3	Main branch	2.5	6	2.25	18.46	12.2	1	0.2
	Side branch	1	5	1.27	4.73	26.9	0	0
	Totals		11	3.52	23.19	15.2	1	0.1

TABLE III

Percentages of Surface Area and Synapse Area Contributed by Main and Side Branches of E and I Axons in Three Terminal Regions

	Main branches				Side branches			
	Total surface area		Total synapse area		Total surface area		Total synapse area	
Terminal region	I	E	I	E	I	E	I	E
	%	%	%	%	%	%	%	%
1	86.3	78.6	74.0	70.4	13.7	21.4	26.0	29.6
2	73.5	62.2	67.5	59.1	26.5	37.8	32.5	40.9
3	79.6	49.7	63.8	49.2	20.4	50.3	36.2	50.8

the three regions are summarized in Table I, and of the I terminals, in Table II. Some overall comparisons between the two axons, and between main branches and side branches, are given in Table III.

Among the significant features which emerged from the analysis, the following are worth comment.

(*a*) In all three regions, there were more individual E synapses than I synapses; in fact, the ratio is close to 2:1. Also, the total contact area was higher for E synapses than for I synapses, even though in one region (no. 2), the total axonal surface area was higher for the I terminals, and in region no. 3 the total surface areas were equivalent.

(*b*) The percentage of surface area devoted to synaptic contacts was greater for E terminals than for I terminals. For both types of terminal, the percentages were lower in region no. 1 than in the other two regions.

(*c*) In most cases the percentage of surface area devoted to synaptic contacts was larger for side branches than for the main trunks, but the total amount of synaptic membrane was always higher for the main trunks.

(*d*) Side branches contributed a higher percentage of total synapse area than of total membrane area in all regions and for both axons (Table III). Thus, more synaptic contact area occurs per unit surface area on side branches than on main branches.

(*e*) The amount of membrane devoted to axo-axonal synapses was a very small fraction of the total available (Table II).

DISCUSSION

The measurements presented here indicate that E and I axons each have their own morphological personalities, even though their terminals grow closely together and are, to some extent, "matched" in their physiological properties (5).

In the opener muscle of the crayfish walking leg, the E and I synaptic potentials recorded with microelectrodes are small and show pronounced facilitation. Large, poorly facilitating potentials of the type seen in certain crab muscles (28) have not been recorded. Thus, the present study provides a picture of the situation in terminals of adult crayfish which generate small, facilitating synaptic potentials. Further work is necessary to compare these terminals in more detail with those of fatigue-sensitive phasic or "fast" axons and with those of tonic axons which generate large, poorly facilitating, postsynaptic potentials (28). Preliminary work has already revealed some morphological differences among physiologically different terminals (6, 28).

The catalog of differences between E and I axons in the crayfish opener muscle now includes differences in density of synaptic contact regions as well as differences in synaptic vesicles. Thus, the crayfish peripheral E and I synapses have several features in common with type 1 and type 2 vertebrate central synapses, which also are thought to subserve excitation and inhibition, respectively (1, 18, 19).

The dense bodies observed at crayfish peripheral synapses seem to be similar to other presynaptic structures observed in many vertebrate and invertebrate central synapses (27). The "dense projections" observed on presynaptic membranes with special staining techniques (see, for example, 1, 22) are not equivalent to the less frequent dense bodies considered here. The dense bodies observed here may be similar to the active zones of the vertebrate end plate (12, 13); however, the latter is an elongate structure and is more extensive than the small semispherical dense bodies of crayfish neuromuscular synapses.

The fact that only one to four of these dense bodies appeared per synapse, and that synaptic vesicles were often more densely clustered at these structures than elsewhere, suggests some physiological role for the dense bodies. They may be concerned in some way with release or re-uptake of transmitter, as is believed to be the case for the active zone of the frog neuromuscular junction (12, 13). Their presence may indicate a physiologically active synapse, and their absence a physiologically inactive one. If this hypothesis is correct, some of the synapses on the terminals studied here may be physiologically inactive, since they had no dense bodies. The synapses without dense bodies were usually smaller than average, and may have been recently formed, immature, or senescent. In any case, the present observations point to the possibility that not all morphologically defined "synapses" need also be physiologically active synapses. According to our hypothesis, the most physiologically active synapses may be those with the most dense bodies. As a corollary, some of the large I synapses may be the most active physiologically of those studied here, which would fit in with the observation of a higher quantal content of inhibitory than of excitatory transmission on individual crayfish muscle fibers (5).

The differences in size of the various branches of both E and I axons are striking. This feature renders inoperative any attempt (such as that of Hoyle and McNeill [20]) to identify different nerve terminals supplying a crustacean muscle purely on the basis of relative size.

Side branches differed from the main axonal trunks in having (usually) a higher percentage of membrane devoted to synaptic contacts, a greater density of vesicles, and fewer mitochondria and glycogen granules. These differences suggest not only different stages in development, but possible differences in physiological responsiveness to stimulation as well. Possibly, rates of facilitation and fatigue may differ at the two locations. However, it would be difficult to show this by extracellular microelectrode recording because of the closeness of the various branches in a synaptic complex. In fact, it has been found that both E and I responses can be recorded at the same location (30).

The constrictions or necks at branch points and at various places along the axon terminals probably limit the passage of synaptic vesicles and other materials from one part of the axon to another. The constrictions were often small and usually plugged with a mitochondrion. Furthermore, the constrictions probably represent points of low

safety factor for spike propagation (cf. 24, 29). Whether they normally result in decremental spread of the action potential into the terminals as suggested by Dudel (14–16) is difficult to judge on present evidence (8). The fact that axo-axonal synapses are often located at such constrictions of the E axon strongly suggests that the electrical potential past the point of constriction must change during presynaptic inhibition. Due to the short-circuiting effect of the I synapse, the potential past the point of constriction very likely becomes decremental. Thus, E synapses stationed distal to the point of constriction would experience a potential change less than normal, and hence release less transmitter. Any synapses located proximal to the constriction would experience little change in potential and would release transmitter in the normal fashion. At present, we cannot given an accurate estimate of the percentage of total synapses in this category. It is worth noting that presynaptic inhibition is never strong enough to eliminate *all* transmitter output from the E axon, and that the persistent output may be from synapses proximal to points of constriction which are relatively unaffected by presynaptic inhibition.

A further point of interest in connection with the axo-axonal synapses is that, although they are located at points of *low* safety factor for the E axon, they occur at points of *high* safety factor for the I axon. The functional importance of this situation is obvious; but the developmental mechanism responsible for it presents an interesting problem.

The occurrence of side branches and nonsynaptic "growth points" in the crayfish neuromuscular apparatus suggests a general hypothesis for growth and maturation of the axon terminals. Side branches may arise initially from small microtubule-containing "buds" which, at some stage in the animal's life, elongate into small branches (e.g. I_2 in Fig. 8). Synapses then start to develop and enlarge, producing a relatively high ratio of synaptic to nonsynaptic membrane. Not all of the synapses are physiologically active. The rate of production and growth of synapses soon slows down, but the branch continues to enlarge, so that the percentage of synaptic membrane gradually decreases. Most synapses do not continue to enlarge greatly after they have matured, but new ones may form, permitting the output of transmitter from the axon to keep pace with the growth of the muscle fiber. Growth and synapse formation occurs more actively near the ends of the terminals than more proximally, so the percentage of synaptic membrane is higher near the distal ends of the terminals.

The hypothesis accounts for the observations made in this muscle so far, and suggests that formation of new synapses, rather than enlargement of old ones, is the more important mechanism in adjusting output of transmitter to the growth and needs of the muscle. This idea is supported by the fact that E synapses from all regions examined did not differ significantly in mean size. However, the largest synapses for the I axon were found in the most proximal region of the terminal (region no. 1). Also, the occurrence of a "tail" of large I synapses and the less frequent occurrence of I synapses suggest that synaptic enlargement may be emphasized more, and production of new synapses less, in I axons than in E axons. The ability to enlarge existing synapses would be functionally important in allowing the I axon to retain control over an E axon branch point which, though initially very small, may in time grow larger.

The hypothesis can be tested further by examining material from very young crayfish in which nerve branches and synapses are being formed.

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