CRUSTACEAN MOTOR NEURON CONNECTIONS TRACED BY BACKFILLING FOR ELECTRON MICROSCOPY

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Individual neurons can be marked by injection through a microelectrode of the fluorescent dye Procion yellow (17) or of cobalt chloride (13). These materials can also be used to study neurons which have peripheral connections by allowing the markers to move up the cut axons into the cell bodies and central processes (5, 11). This "backfilling" method is facilitated by application of electrical current through the cut ends of the axons (5).

So far, both Procion yellow and cobalt chloride have been more useful for studies carried out by light microscopy than for those requiring electron microscopy, although attempts have been made to trace Procion yellow-injected cells with the electron microscope (14). The microinjection of Procion brown instead of Procion yellow (2) and a modification of the cobalt chloride method (3) have made electron micrscope studies of injected neurons more feasible. In addition, the modified cobalt chloride method can be used to trace backfilled motor neurons in the central nervous system and to study their central connections with other neurons.

The present paper was written for three purposes: (*a*) to show that the modified cobalt chloride method can be combined with the simple backfilling technique to trace motor neurons into the neuropile; (*b*) to show that both light and electron microscopy can be employed on a specimen to trace the motor neurons; (*c*) to demonstrate the

utility of these simple techniques in the study of specific central synapses of crustacean motor neurons by electron microscopy.

MATERIALS AND METHODS

The motor neurons selected for study were those innervating the fast flexor muscles in segments 2, 3, and 4 of the abdomen of the crayfish *Procambarus clarki* Girard (6, 7). One of the segmental motor neurons, the "motor giant" fiber, forms electrically transmitting synaptic contacts with giant fibers of the nerve cord near the point of exit of the third root, and these electrical synapses have been examined by cobalt chloride injection (9), and in the electron microscope by several investigators (most recently by Krasne and Stirling [8], and Stirling [16]). The other neurons of the fast flexor muscles make their synapses with the giant fibers within the ganglion (15, 18) and have not previously been studied with the electron microscope.

Isolated ganglia were mounted in a partitioned dish containing van Harreveld's crayfish solution on one side of the partition. A branch of the third root containing several motor neurons of the fast flexor muscles was threaded through a thin slot in the partition and its cut end was placed in a pool of 0.1 M cobalt chloride. The nerve was sealed firmly into the slot with Vaseline to prevent mixing of the solutions on opposite sides of the partition. The preparation was left 5–6 h at 20°C, during which time cobalt moved up some of the axons into the intraganglionic portions of the neurons. Applied current was not used in the preparations reported on here.

The cobalt-treated preparation was carefully removed from the partitioned dish and rinsed for 15 min in fresh van Harreveld's solution to remove some of the interstitial cobalt chloride. It was then exposed to 0.03% H_2O_2 in van Harreveld's solution for 5–10 min, and afterwards to 0.5 mg/ml 3,3-diaminobenzidine tetrahydrochloride (DAB) freshly prepared in van Harreveld's solution and adjusted to pH 7.4–7.8 with NaOH. The fast flexor neurons, if successfully filled with cobalt, turned dark blue, usually within a few minutes, due to formation of a polymer (3). A much longer time for the reaction was required if pretreatment with H_2O_2 was omitted. The polymer-containing neurons could be seen clearly in the intact ganglion, especially after clearing in methyl benzoate (Fig. 1 A). As the labeled neurons were visible even without clearing, this procedure was omitted for electron microscope studies.

Specimens judged to contain successfully filled neurons were fixed for electron microscopy by the method of Peracchia and Mittler (12). The DAB polymer took up osmium and became electron-dense during the osmication phase of the fixation, which was often extended overnight. On occasion, fixation in buffered 2% OsO_4 without glutaraldehyde was tried; this treatment usually produced larger granules within the backfilled neurons, but generally poorer preservation of the tissue.

After osmication, specimens were dehydrated in ethanol and embedded in an Epon-Araldite mixture. The hardened blocks were serially sectioned at 5 μm and the sections were mounted sequentially on cover slips. The backfilled neurons contained granules visible under the light microscope after the 5-μm sections had been stained with toluidine blue (Fig. 1 B). Points of contact between the motor neurons and the giant axons, and fine branches of the motor neurons in the neuropile, were located in serial thick sections of the ganglion.

Thick sections containing points of interest were remounted and thin sections were cut from them for electron microscopy. An Epon stub with a flat surface was moistened with a drop of epoxy and pressed down over the section on the cover slip. After the epoxy had hardened, the cover slip was removed by application of dry ice (4). Serial thin sections of the region of interest could then be cut with a diamond knife and mounted on single slot grids by the method developed by Moens (10). The sections were viewed and photographed with a Philips EM-200 electron microscope.

RESULTS

Fast flexor motor neurons with cell bodies ipsilateral to the cobalt-treated third root were traced in the present study. Usually two or three of these neurons were filled but the appearance of the label within the neurons was sufficiently distinct to enable one to follow individual neurons. The inhibitory neuron (F-10 of Selverston and Remler [15]) and the motor giant cell (F-1 [15]) were not included in the present study.

Labeled neurons traced into the ganglion with light microscopy were viewed in regions of potential interest with low-power electron microscopy. They contained granules of various sizes and had a prominent darkening of the inner surface of the membrane. In addition, the neuroplasm often had a "bleached" appearance (Fig. 1 C). Frequently, two or more labeled neurons in the same preparation were quite different in appearance and could easily be distinguished in the electron micrographs. Probably the differences result from variations in cobalt uptake by the neurons.

Labeled neurons gave off branches of the cord giant fibers and to the neuropile; the former made apparent electrical synapses, while the latter received numerous chemical synapses (Fig. 1 C). Examples of commonly observed chemical synapses are shown in Fig. 2 A, B. The features described for crayfish central synapses previously by others (8) were present: dense material in the synaptic cleft; "fuzz" on the postsynaptic membrane; clustering of synaptic vesicles at the presyn-

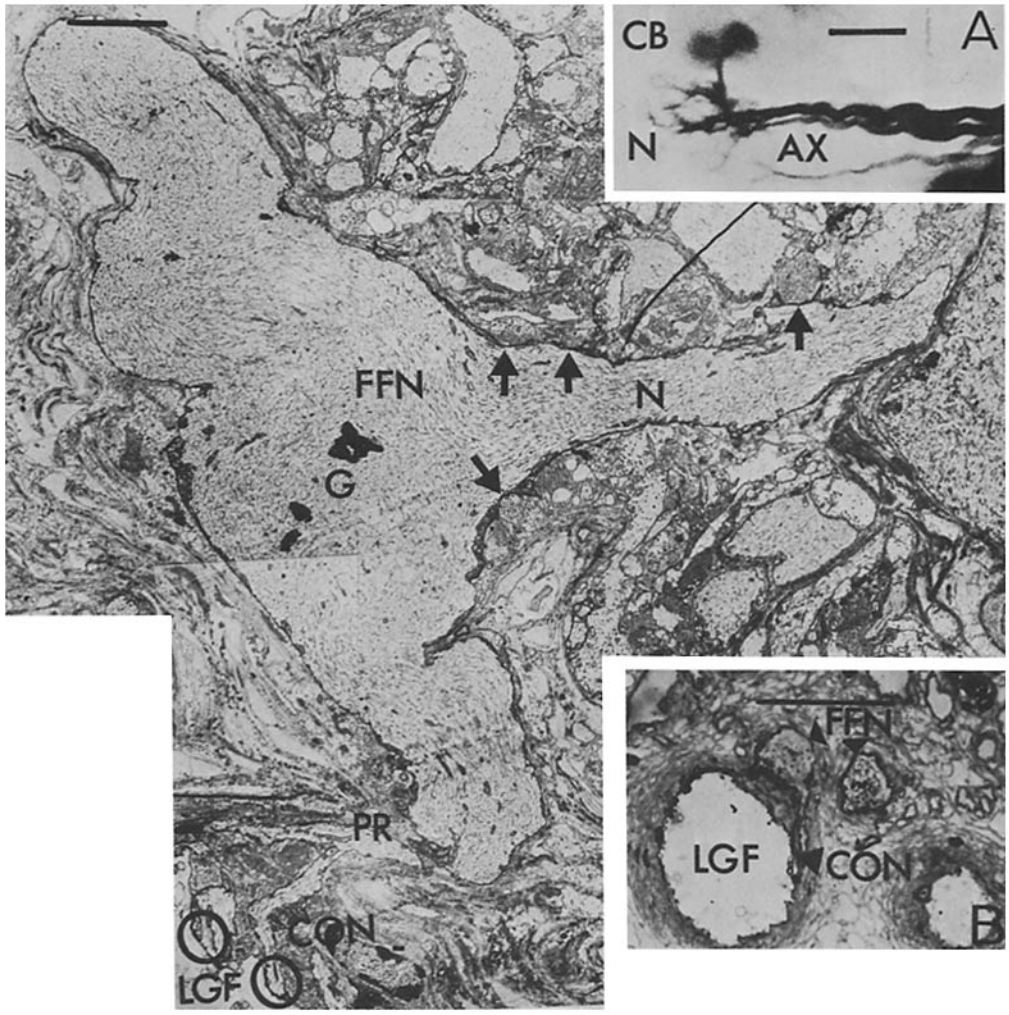

FIGURE 1 A Whole mount of a crayfish ganglion cleared in methyl benzoate to show backfilled motor axons containing the dark precipitate of diaminobenzidine. The cell bodies (*CB*), axons (*AX*), and neurites (*N*) of two fast flexor motor neurons can be seen. Scale mark, 250 μm.

FIGURE 1 B Light micrograph showing two labeled fast flexor motor neurons (*FFN*), one of which sends a process to form a contact (*CON*) with the lateral giant fiber (*LGF*). The labeled neurons contain granules which are visible in the light microscope. Scale mark, 75 μm.

FIGURE 1 C Low-power electron micrographs (montage) of a lightly labeled fast flexor motor neuron (*FFN*) in the region of dorsal ganglionic neuropile. The neuron contains granules (*G*) of the precipitate. A neurite (*N*) receives numerous synapses (arrows) from other neurons; they all appear to be of the chemical type. The neuron sends another process (*PR*) to form contacts (*CON*) with the lateral giant fiber (*LGF*). These contacts appear to be electrical synapses. Scale mark, 5 μm.

aptic membrane. The type of synapse illustrated had somewhat irregular synaptic vesicles, such as at crayfish inhibitory neuromuscular junctions (1). In addition, prominent clusters of dark granules (probably glycogen) appeared near the synapse. Another type of ending had a high proportion of dense-cored vesicles. The chemical synapses were most numerous on the branches and at junctions of

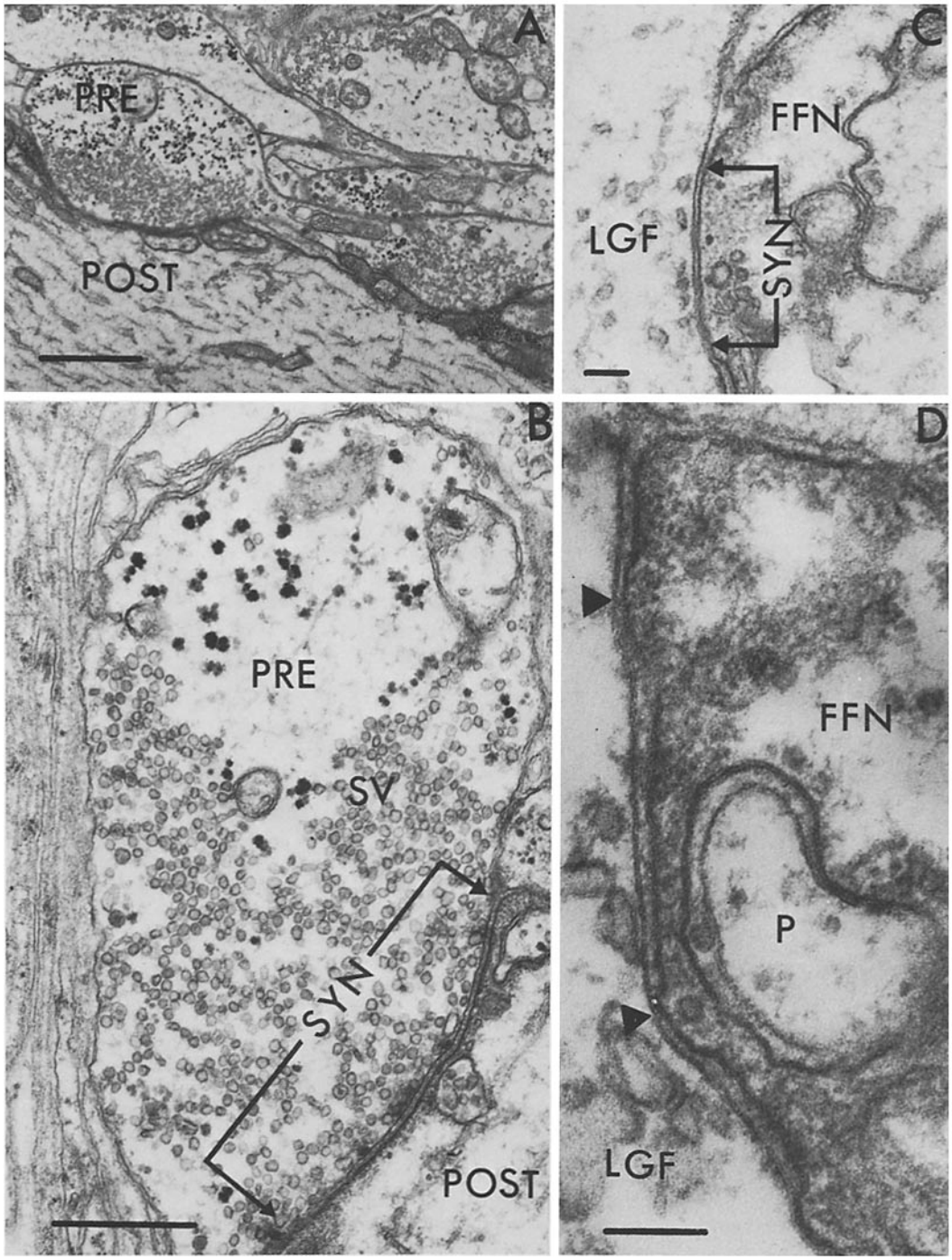

FIGURE 2 Details of chemical (A,B) and electrical (C,D) synapses on a fast flexor motor neuron (*FNN*). A. A presynaptic ending (*PRE*) contacts the motor neuron (*POST*) and forms two chemical synapses. Scale mark, 1 μm. B. Enlarged view of one type of chemical synapse on the same neuron, showing the region of contact (*SYN*), the somewhat irregular synaptic vesicles (*SV*), and nearby glycogen granules in the presynaptic terminal (*PRE*). A dense material is found at the synapse in the postsynaptic neuron (POST). Scale mark 0.5 μm. C. Contact between the fast flexor motor neuron (*FFN*) and the lateral giant fiber (*LGF*) showing the apparent electrical synapse (*SYN*). Only a very few vesicles occur in the LGF. Scale mark, $\frac{1}{8}$ μm. D. Enlarged view of a region of contact, showing regions of very close approach (arrows) between the membranes of the lateral giant fiber (*LGF*) and the fast flexor motor neuron (*FFN*). A peg (*P*) of the lateral giant fiber is enclosed within the flexor neuron and may provide additional regions of electrical contact. Scale mark, 0.1 μm.

branches with the main axon process; relatively few were found on the main process. The function of these chemical synapses is at present unknown.

Junctions of the labeled neurons with the giant fibers appeared to be electrical synapses (Fig. 2 C, D). Usually, several synapses were formed by each motor neuron with the giant fiber (Fig. 1 C). Only a few vesicles occurred near these synapses (Fig. 2 C), in contrast to the large number seen at the chemical synapses. The prevalence of vesicles was much less than at the electrically transmitting synapse between the motor giant axon and the lateral giant fiber (16). Pre- and postsynaptic membranes were generally separated by about 140 Å, but in places the separation was about 60 Å and less (Fig. 2 D). It is possible that the regions of close approach may represent gap junctions, but we have no definitive evidence on this point. Pegs of the presynaptic fiber were often embedded in the motor axon branch, providing for intimate association between the two neurons (Fig. 2 D).

DISCUSSION

The present study demonstrates the utility of the modified cobalt chloride method (3) in the study of the central connections of backfilled motor neurons. The Procion brown method (2) could probably also be used for this purpose, but we have found in preliminary trials that it is less satisfactory than the modified cobalt chloride method in this context. The advantages of the cobalt method which we have found include: (*a*) more rapid backfilling; (*b*) different appearances of different filled neurons in the same preparation; (*c*) longer osmication time, giving more contrast within the fixed tissue. The possible advantage of the Procion brown method is the greater contrast between the filled neuron and others, allowing easier tracing with light microscopy.

Because of the ease of combining light and electron microscope studies of the same section, this method permits serial reconstruction of neuron geometry in the light microscope and a subsequent search for synaptic contacts on specific branches in the electron microscope (manuscript in preparation). Neurophysiologists must know the exact location of synapses on neuronal dendrites to determine the potency of the inputs to the postsynaptic cell.

Different branches of the fast flexor neurons seem to be specialized for chemical and electrical transmission. The appearance of the junctions between the giant fibers and the motor neurons seems to rule out the possiblity of any substantial chemical component of transmission at this region, and confirms the electrical nature of transmission deduced from physiological observations (19, 20). However, since *all* of the contacts between the cord giant fibers and individual flexor motor neurons have not yet been examined, either morphologically or physiologically, the possibility of *some* chemical component in transmission cannot be rigidly excluded.

Although the nature of the chemical synapses that we have found is not yet known, Takeda and Kennedy (18) described both excitatory and inhibitory inputs to fast flexor motor neurons which are probably mediated by chemical synapses. We have observed at least two types of chemical synapse which could be involved in such pathways.

We thank Ms. Irene Kwan and Mr. Rudy Villadiego for invaluable technical assistance, and Mr. Rhanor Gillette for helpful discussions during the course of this work.

We received grant support from the National Research Council of Canada and from the Muscular Dystrophy Association of Canada.

Received for publication 22 March 1974, and in revised form 21 May 1974.

REFERENCES

1. ATWOOD, H. L., and W. A. MORIN. 1970. Neuromuscular and axo-axonal synapses of the crayfish opener muscle. *J. Ultrastruct. Res.* **32**:351–369.
2. CHRISTENSEN, B. N. 1973. Procion brown: an intracellular dye for light and electron microscopy. *Science (Wash. D.C.).* **182**:1255–1256.
3. GILLETTE, R., and B. POMERANZ. 1973. Neuron geometry and circuitry via the electron microscope: intracellular staining with osmiophilic polymer. *Science (Wash. D.C.).* **182**:1256–1257.
4. GRIMLEY, P. M. 1965. Selection for electron microscopy of specific areas in large epoxy tissue sections. *Stain Technol.* **40**:259–263.
5. ILES, J. F., and B. MULLONEY. 1971. Procion yellow staining of cockroach motor neurons without the use of microelectrodes. *Brain Res.* **30**:397–400.
6. KENNEDY, D., and K. TAKEDA. 1965. Reflex control of abdominal flexor muscle in the crayfish. I. The twitch system. *J. Exp. Biol.* **43**:211–227.
7. KENNEDY, D., and K. TAKEDA. 1965. Reflex control of abdominal flexor muscles in the crayfish. II. The tonic system. *J. Exp. Biol.* **43**:229–246.
8. KRASNE, F. B., and C. A. STIRLING. 1972. Synapses of crayfish abdominal ganglia with special attention to afferent and efferent connections of the lateral giant fibers. *Z. Zellforsch. Mikrosk. Anat.* **127**:526–544.

9. MITTENTHAL, J. E., and J. J. WINE. 1973. Connectivity patterns of crayfish giant interneurons: visualization of synaptic regions with cobalt dye. *Science* (*Wash. D.C.*). **179**:182–184.
10. MOENS, P. B. 1970. Serial sectioning in electron microscopy. *Proc. Can. Fed. Biol. Soc.* **13**:160.
11. PEARSON, K. C., and C. R. FOURTNER. 1973. Identification of the somata of common inhibitory motoneurons in the metathoracic ganglion of the cockroach. *Can. J. Zool.* **51**:859–866.
12. PERACCHIA, C., and B. S. MITTLER. 1972. Fixation by means of glutaraldehyde-hydrogen peroxide reaction products. *J. Cell Biol.* **53**:234–238.
13. PITMAN, R. M., C. D. TWEEDLE, and M. J. COHEN. 1972. Branching of central neurons: intracellular cobalt injection for light and electron microscopy. *Science* (*Wash. D.C.*). **176**:412–414.
14. PURVES, D., and U. J. MCMAHAN. 1972. The distribution of synapses on a physiologically identified motor neuron in the central nervous system of the leech. An electron microscope study after the injection of the fluorescent dye Procion yellow. *J. Cell Biol.* **55**:205–220.
15. SELVERSTON, A. I., and M. P. REMLER. 1972. Neural geometry and activation of crayfish fast flexor motoneurons. *J. Neurophysiol.* **35**:797–814.
16. STIRLING, C. A. 1972. The ultrastructure of giant fiber and serial synapses in crayfish. *Z. Zellforsch. Mikrosk. Anat.* **131**:31–45.
17. STRETTON, A. O. W., and E. A. KRAVITZ. 1968. Neuronal geometry: determination with a technique of intracellular dye injection. *Science* (*Wash. D.C.*). **162**:132–135.
18. TAKEDA, K., and D. KENNEDY. 1964. Soma potentials and modes of activation of crayfish motoneurons. *J. Cell Comp. Physiol.* **64**:165–182.
19. ZUCKER, R. S. 1972. Crayfish escape behaviour and central synapses. III. Electrical junctions and dendrite spikes in fast flexor motoneurons. *J. Neurophysiol.* **35**:638–651.
20. ZUCKER, R. S., D. KENNEDY, and A. I. SELVERSTON. 1971. Neuronal circuit mediating escape responses in crayfish. *Science* (*Wash. D.C.*). **173**:645–650.