Arnold, John M., William C. Summers, Daniel L. Gilbert, Richard S. Manalis, Nigel W. Daw, and Raymond J. Lasek (1974) *A Guide to Laboratory Use of the Squid Loligo Pealei.* Woods Hole, MA: Marine Biological Laboratory, 74pp.

VI. The preparation of the squid giant synapse for electrophysiological investigation

The anatomy of the giant synapse in the stellate ganglion of the squid *Loligo pealei* has been described by Young (1939 and 1973). The unique structure of this junction has permitted microelectrode techniques to be used in investigations concerned with presynaptic events in synaptic transmission (Kusano, Livengood, & Werman, 1967; Katz, 1969; Katz & Miledi, 1971; Llinás, Blinks, & Nicholson, 1972; Miledi, 1973). For discussions of the physiology of the squid giant synapse, the reader is referred to the writings of others (Eccles, 1967; Katz, 1969; Davson, 1970; Eccles, 1973); the review by Gerschenfeld (1973) is excellent.

The postsynaptic cell in the giant synapse is actually the giant axon which has been widely studied by neurophysiologists over the last several decades. A general description of the giant fiber system, of which the giant synapse and the giant axon are a part, is best given by Young (1938):

> A squid is capable of moving through the water either slowly by means of its fins or very fast by the expulsion of a jet of water through its funnel. It is this rapid movement which is produced by the activity of the giant nerve fibres. The muscles concerned in the movement are circular fibres of the mantle, serving to make its cavity smaller, and longitudinal fibres in the retractor muscles of the head and of the funnel, serving to pull these parts of the body back towards the mantle cavity. All of these groups of fibres can be activated from the single pair of giant nerve cells in the central nervous system which are termed the primary or *first order* giant cells . . . These do not innervate the muscles directly but branch and make synaptic contact with *second order* giant fibres, of which there are on each side five running to the retractor muscles of the head and funnel, and two to the stellate ganglion. In the latter ganglion the fibres branch and make synaptic contact with about ten *third order* giant fibres which pass to the muscles of the mantle. There is, therefore, one giant synapse on the path to the retractors but two on that leading to the muscles of the mantle.

The giant synapse is formed between the second-order giant axon that enters the stellate ganglion and the largest of the third-order giant axons — the giant axon which lies in the hindmost stellar nerve. Staub (1954) injected dyes intracellularly into the giant fiber system, and he was able to dramatically illustrate the anatomy of the giant synapse in which the second-order giant axon was stained red and the giant axon was stained blue. As described by Young (1939), the cell bodies of the third-order giant axons reside in the giant fiber lobe (Fig. 16) where axons from 30,000 cell bodies (Young, 1972) fuse to form one of the third-order giant axons. There are from nine to eleven stellar nerves radiating from each stellate ganglion; each stellar nerve contains, in addition to many small sensory and motor nerve fibers (Young, 1972), one third-order giant axon. These giant axons are motor fibers, and they innervate the mantle musculature (Young, 1938). Actually, the giant synapse which has been studied by most investigators is referred to as the distal synapse because there are additional synapses present which are more proximal to the cell

bodies of the third-order neurones. These junctions are referred to as the proximal or accessory synapses. Their presynaptic nerve is called the accessory giant axon, and there are no connections between it and the first-order neurone of the giant fiber system (Young, 1939).

Both proximal and distal synapses are excitatory (Young, 1938; Bryant, 1959), and they are similar to the frog neuromuscular junction (Katz, 1966) because, in the unfatigued preparation, one action potential arriving at a presynaptic nerve terminal causes enough transmitter to be released so that an action potential is then initiated in the postsynaptic cell. That is, no summation of postsynaptic potentials is necessary for synaptic transmission to occur.

Recently, Miledi (1972) has observed inhibitory as well as excitatory postsynaptic potentials while recording directly from cell bodies located in the stellate ganglion. These neurones are not part of the giant fiber system, but belong to the system of small nerve fibers which enter and leave the stellate ganglion along with the second- and third-order giant axons.

The dissection procedure has been described by Bullock (1948) and by Miledi (1967) in the method sections of their papers. Because this book is a laboratory guide, it is reasonable to present herein a somewhat more detailed description of this dissection, with emphasis placed on practical considerations, than would be appropriate for inclusion in the methods section of a scientific article. What follows is a description of the dissection which proceeds in two stages: 1) the gross dissection and 2) the fine dissection. In addition, an experimental chamber is described in which an axial wire, first used in this preparation by Hagiwara and Tasaki (1958), can be inserted into the giant axon and then placed into the postsynaptic region of the giant synapse.

This dissection procedure has allowed the author to visualize the giant synapse well enough to observe and penetrate the presynaptic nerve terminal with a microelectrode for either voltage recording or current injection, to have as many as three microelectrodes in the postsynaptic cell, and to use an axial wire for current injection during voltage clamp experiments of the postsynaptic membrane (Manalis, 1973).

The reader is encouraged to develop improvements over what will now be described because this is, of course, only one approach to working with this preparation.

The giant synapse fails easily when deprived of oxygen (Bryant, 1958). Therefore, the entire dissection should be performed in sea water which has been filtered, chilled to 5°C, and saturated with oxygen.

Darkfield Dissection Stand

In both the fine and gross dissections, it is very important to illuminate the preparation laterally while using a black background, i.e., to use darkfield illumination. Brightfield illumination or reflected light does not allow one to discriminate between structures nearly as well as does darkfield illumination. The top of the darkfield dissection stand consists of a piece of ¾″ plywood with a 5½″ hole cut in it and with the front edge cut so that one can rest his elbows while dissecting (Fig. 14). The wooden top is supported by four legs, each 4″ long. The dissection stand is made to be put onto a table or a laboratory bench.

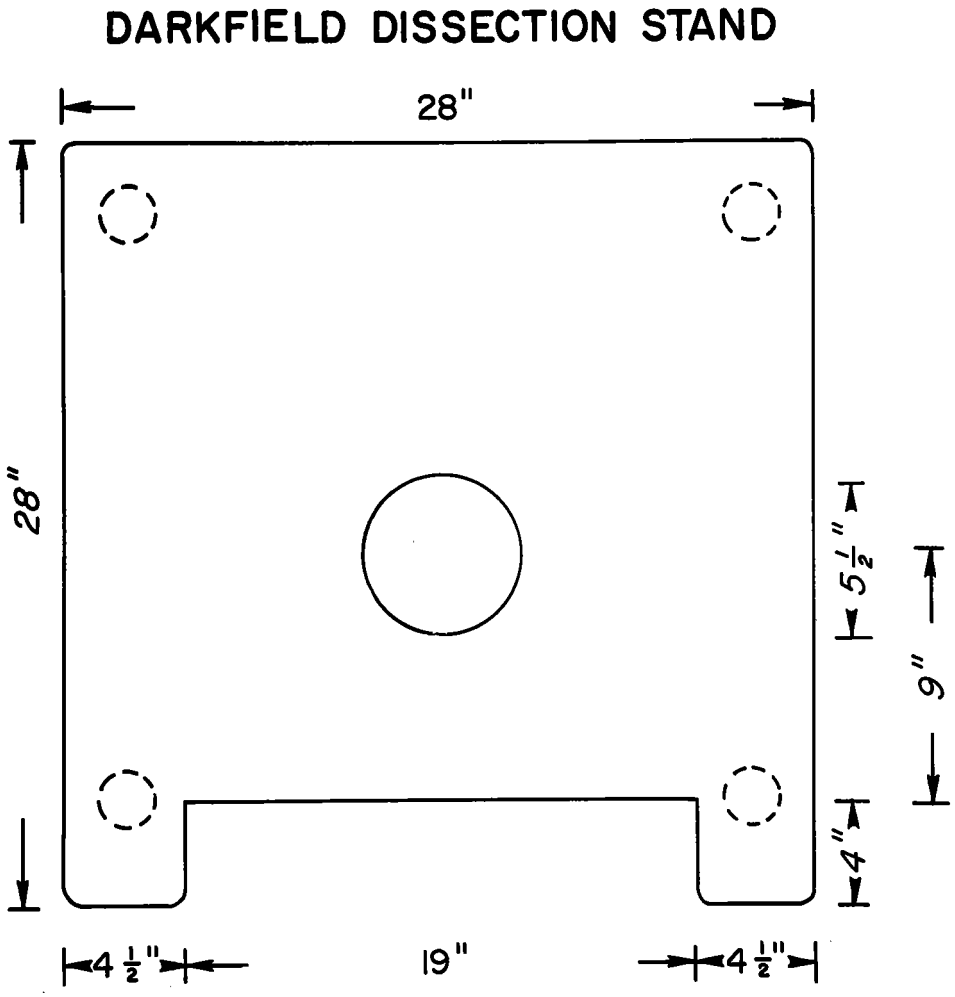

FIGURE 14

The top of the darkfield dissection stand is shown. The circles drawn with dashed lines represent four legs, each 4″ long. Centered beneath the 5½″ hole should be a circular fluorescent lamp fixture with an 8″ (O.D.) lamp attached. Both the fixture and stand are to lie on the same surface, a table top or a laboratory bench.

A circular fluorescent lamp (8″ O.D.) is attached to its appropriate metal fixture, which should have been painted with flat black enamel. The fixture is placed beneath the hole in the wood in order to provide for the darkfield illumination.

Size of Squid

Most success has been found when small to medium squid have been used (mantle length ranging from 10-15 cm). On occasion, preparations have been ob-

tained from very small squid in which the mantle length was 5 cm (see also Miledi, 1967). In these preparations, the anatomy of the stellate ganglion can be seen easily when viewed with a dissecting microscope with a magnification of 100x and when darkfield illumination is used. Studying the structure of ganglia from these very small squid would be a good way for one to become familiar with this preparation. The connective tissue enveloping the giant synapse is more dense in large squid, making it quite difficult to visualize the prenerve terminal, as can be done in preparations from small to medium squid. In addition, impalement with microelectrodes is more difficult when large amounts of connective tissue are present.

Gross Dissection

A Pyrex baking dish (13½" x 8¾" x 1½") is readily available and is quite suitable to serve as a dissecting dish. The bottom of the dish should contain a ¼" layer of Tackiwax (Cenco); a 5½" circle of wax should be removed to allow for illumination of the preparation when the dish is placed on the darkfield dissection stand. The remaining wax is used to attach the mantle during dissection and also to secure the free ends of ligatures at appropriate times during the dissection. The mantle is attached to the wax through the use of three sets of push pins and hooks made from insect pins. A ligature is tied to each push pin, and the other end of the ligature is fastened to the metal hook. The length of the ligature can be shortened by winding it around the push pin.

Obtain a squid from the storage tank using a soft, fine mesh net. Hold the squid and wait for its neck to extend from the mantle cavity. Have a pair of scissors in your other hand; and, with one cut, decapitate the squid as its neck extends. Put the squid into the dissecting dish, which is placed over the hole on the dissecting stand, and quickly fill the dish with oxygen-saturated sea water which has been previously filtered and cooled to 5°C. Open up the animal by making a midventral (the term *midventral* is used in its functional sense) incision, starting at its anterior end. The mantle can be held easily with toothed forceps. Next remove the skin from the mantle. Removal of the skin is necessary in order to clearly visualize the stellate ganglia and related structures.

Lay the external surface of the mantle downward with the tail nearest the rear of the dissecting stand. Anchor the mantle to the wax on the bottom of the dissecting dish with the three push pins and hooks; one hook is attached to the midline at the tail, and the remaining two hooks are each attached to the right and left edges of the mantle at its anterior border (Fig. 2).

Grasp the funnel with forceps and remove it and its adjoining two muscles (m. retractor infundibuli) using scissors. Cut the loose connective tissue that connects the gills to the mantle. Grasp the ink sac in a manner which minimizes the possibility of ink spilling into the dissection area, and proceed to remove the remainder of the viscera except the yellowish digestive gland (liver) which is located in the midline dorsal to the ink sac. Make a midline cut through the connective tissue sheath that covers the digestive gland, and, with a pair of toothed forceps, grasp the caudal end of the digestive gland and remove it from the nuchal cartilage (brain case or digestive gland capsule) in one cephalad motion. The presynaptic nerves (the pallial nerves), in which reside the second-order giant and the accessory giant axons, can easily be seen on either side of the midline lying in the cephalic region of the nuchal cartilage. Each presynaptic nerve proceeds caudal for 1-2 cm before it exits through a foramen in the nuchal cartilage to join its respective stel-

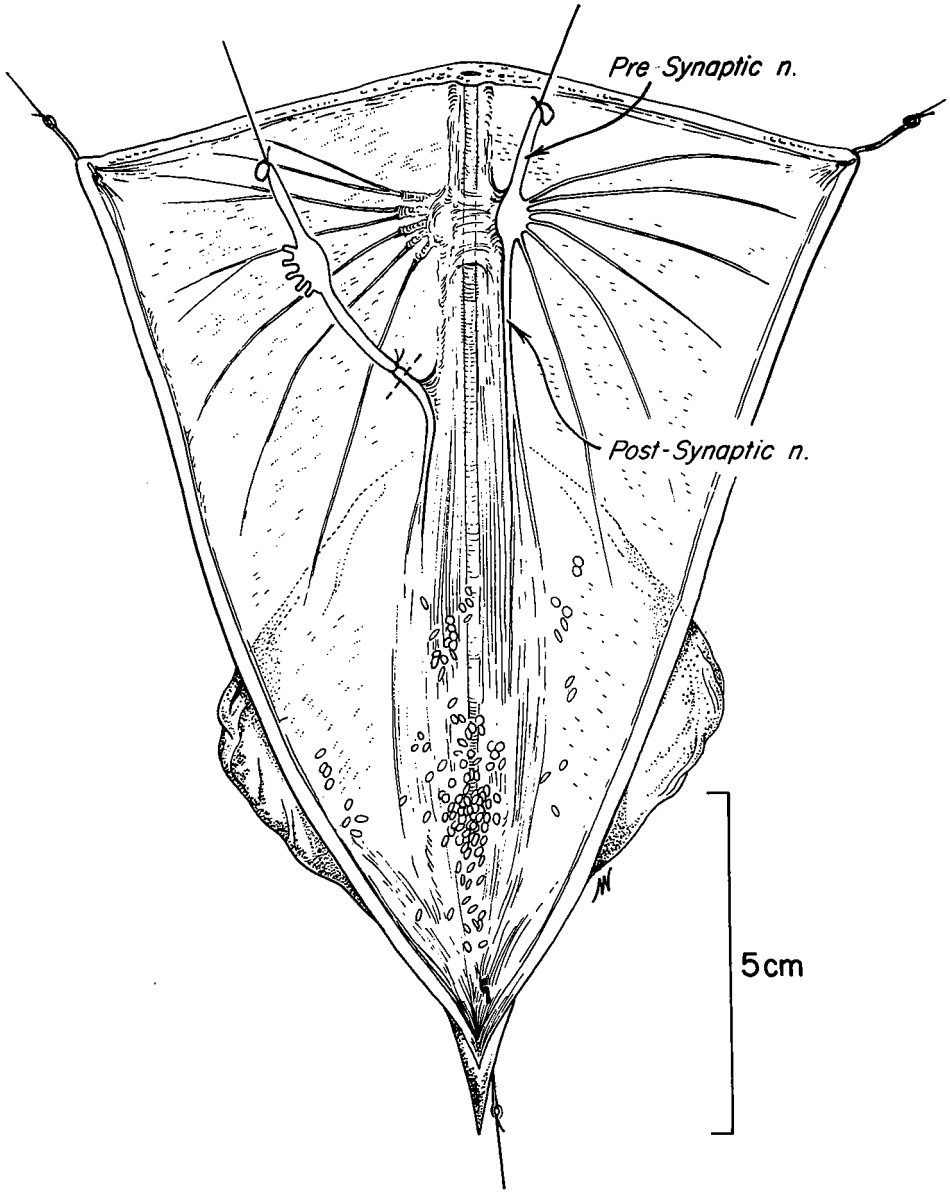

FIGURE 15

This is a diagram that was made from a photograph taken during the gross dissection. The nuchal cartilage, normally lying between the two stellate ganglia, has been removed. One stellate ganglion is shown *in situ* with its stellar nerves intact; all of the stellar nerves in the other ganglion have been transected except for the largest one (referred to as the post-synaptic nerve). About a 2 cm length of the postsynaptic nerve is dissected free. A ligature is tied, and the nerve is transected distal to the tie, thereby finishing the gross dissection for that ganglion. Actually, as described in the text, the gross dissection should be done with the tail pointing away from, not towards, the dissector.

late ganglion. With a small pair of scissors, carefully trim each wall of the nuchal cartilage to a point about 1-2mm ventral to the foramen through which the presynaptic nerve passes. Apply ligatures around the central end of each of the presynaptic nerves. Insert one ligature into the wax so as to remove its attached nerve from the dissection area while the other nerve is being dissected free up to its foramen. Grasp the wall of the nuchal cartilage with a small pair of toothed forceps just cephalad to the foramen in order to secure the preparation while a sharp #11 scalpel blade is gently pushed through the foramen, starting at the inside opening. Any injury is minimized by this procedure because the actual stretching of the tissue is only very slight when the #11 scalpel blade is used. Actually, in order to facilitate the opening of the foramen, a small amount of stretch should be applied to the presynaptic nerve before cutting with the scalpel blade; this can be done by inserting its ligature into the surrounding wax. Remove the piece of cartilage that formed part of the ventroanterior wall of the foramen. Now, the presynaptic nerve can be dissected free to where it joins the stellate ganglion by applying a small lateral stretch to the nerve while cutting through the connective tissue surrounding the ganglion close to its medial border. Cut also the nerve (interstellate commissure) that connects the two stellate ganglia. Free the other nerve in the same manner.

The remainder of the nuchal cartilage can be removed by grasping its cephalad end with forceps while cutting its connective tissue attachments to the mantle. Remove the clear and rigid pen which lies in the midline. Care should be taken not to cut or injure the two giant axons, one on each side of the midline, as they emanate from their respective stellate ganglia. Gently lift one stellate ganglion from the mantle surface with the ligature attached to its presynaptic nerve, and cut the stellar nerves, which radiate from the ganglion to innervate the mantle, except the largest one, which is the postsynaptic nerve (Fig. 15). Note that portions of the mantle should twitch following the transection of each of the stellar nerves. The fin nerve, which is to be removed during the fine dissection, runs along the medial side of the proximal region of the hindmost stellar nerve to which it is attached by a connective tissue sheath. The fin nerve is also attached to the medial borders of the stellate ganglion and the presynaptic nerve trunk. Dissect free about 2 cm to the giant axon plus accompanying small nerve fibers and fin nerve. This entire nerve bundle is referred to as the "postsynaptic nerve" in Fig. 15; it important to remember, however, that the giant axon itself is actually the postsynaptic cell that is to be studied. Tie a ligature around the most caudal portion of the postsynaptic nerve, and transect it distal to the ligature (see Fig. 15). Put this preparation in oxygen-saturated sea water, and store it at 5-10°C. Remove the other stellate ganglion and accompanying nerves in the same manner. This completes the gross dissection. The entire procedure should take around fifteen minutes.

Fine Dissection

The fine dissection is done in a Petri dish that is 10 cm in diameter and 2 cm deep. This dish should be modified in the following manner (Fig. 16): two glass posts — one at 12 o'clock and the other at 6 o'clock — can be securely anchored to the Petri dish by using silicon rubber cement (GE, RTV-108). Tackiwax should be placed near each glass post so that ligatures can be anchored into the wax during the dissection. The Petri dish should be immersed in a shallow ice water bath so that the fine dissection can be performed at 5-10°C. The glass dish used for the gross dissection can serve as a water bath which rests on the darkfield dissection stand. In order to prevent the Petri dish from sliding on the bottom of the water

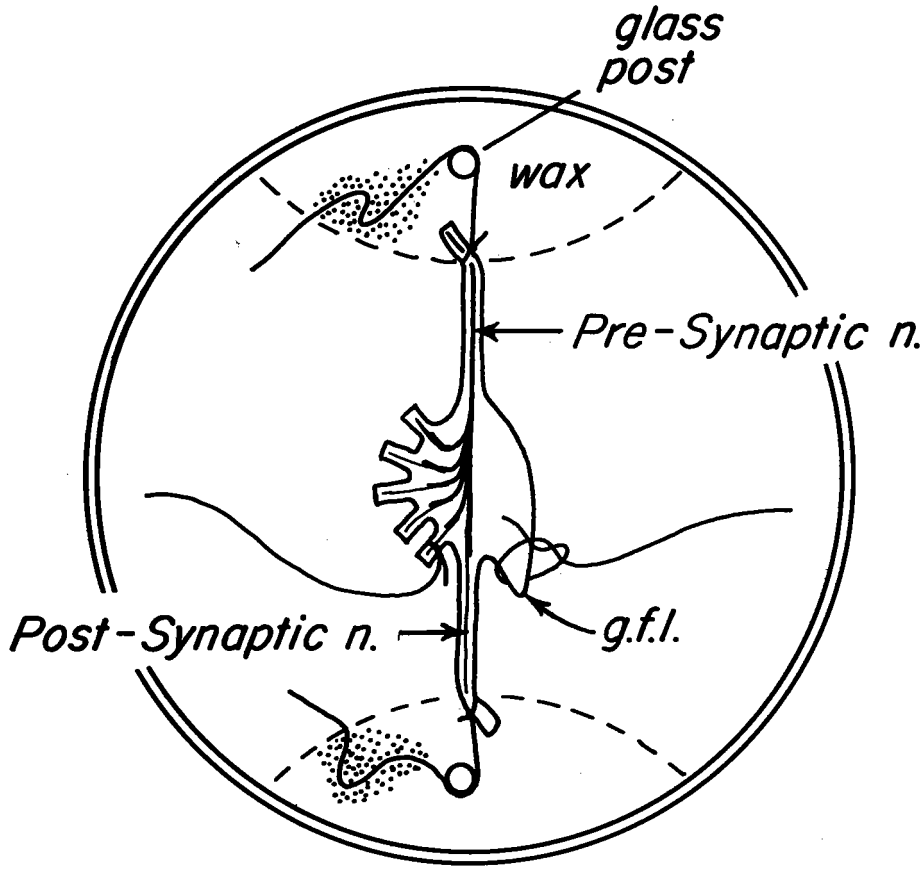

FIGURE 16

A top view is shown of the Petri dish that is used for the fine dissection; the ventral surface of the stellate ganglion is diagramatically illustrated. The preparation is anchored to the dish by wrapping the ligature attached to the presynaptic nerve one half turn around one of the glass posts and then inserting the ligature into the wax which surrounds the glass post; the remaining ligature from the postsynaptic nerve is similarity anchored to the opposite mound of wax. In this manner, a slight longitudinal pull on the ganglionic preparation results. As the fine dissection proceeds, the preparation apparently stretches somewhat. The resulting slack can be taken up by repositioning the ligatures in the wax. Of course, care should be taken so as not to damage the preparation by excessive stretching. Two additional ligatures are added during the fine dissection. One is tied to the tip of the giant fiber lobe (g.f.l.) and the other is tied to the proximal stump of the stellar nerve nearest the giant axon. These ligatures serve to mount the preparation in the experimental chamber (Fig. 17).

bath during the fine dissection, small amounts of silicon rubber cement, which serve as small rubber feet, should be put on the bottom surface of the dish.

The use of a dissecting microscope with a magnification of 20-30x is necessary during the fine dissection. Fill the Petri dish with oxygen-saturated sea water. Put one of the preparations in the dish. Wrap one ligature one half turn around a glass post and insert the ligature into the wax; secure the remaining ligature, arranging the preparation with the presynaptic nerve at 12 o'clock and the postsynaptic nerve at 6 o'clock (Fig. 16). First, remove the epithelial and loose connective tissue that surrounds the stellate ganglion and the remaining portions of the cut stellar nerves. This can best be accomplished through the use of two pairs of microdissecting tweezers (Dumont #5, stainless steel); only use microdissecting scissors when the tissue to be removed cannot gently be separated from the ganglion. Excessive cleaning of the ganglion is not recommended because the giant synapse is easily damaged by mechanical manipulation. Tie one ligature (6-0 silk) to the tip of the giant fiber lobe and a second one to the remaining portion of that stellar nerve which is closest to the giant axon. These two ligatures are to be used in mounting the preparation in the experimental chamber. Separate the fin nerve from the proximal region of the stellar nerve. Transect the fin nerve; grasp the central end, and dissect it free from the proximal part of the stellar nerve, the medial border of the stellate ganglion, and the presynaptic nerve.

If it is desired to insert an axial wire into the postsynaptic cell, the small nerve fibers which lie next to the giant axon in the stellar nerve should be removed at the site in which the wire is to enter the giant axon, which is usually about 1½ cm from the stellate ganglion. The fine dissection takes about thirty minutes.

Experimental Chamber

The finely dissected preparation is put into a small beaker filled with oxygen-saturated sea water, and the preparation is then transferred to the experimental chamber (Fig. 17). The preparation lies in the experimental compartment (bath) on a glass plate, the underneath surface of which is part of an internal compartment which is connected to a constant temperature circulator (c.t.c., see Fig 17). Experiments can be performed from 4-18°C. The preparation is perfused with oxygen-saturated sea water, which enters the experimental compartment by gravity and leaves by suction. A reference electrode, consisting of a coil of Ag-*AgCl* in 1% agar made with 3M KCl, is enclosed in a glass tube, one end of which is bent in order to make contact with the experimental compartment. The other end of the electrode is covered with silicon rubber cement in order to secure the Ag wire to the glass tube and to minimize dessication of the agar. The insert on the right in Fig. 17 shows how the preparation is mounted using the four ligatures each of which is inserted into a separate pool of wax (indicated by the stippling) with microdissection tweezers. The ventral surface of the stellate ganglion faces upward. The giant axon leaves the experimental compartment through a small slot in the right wall; the axon bends slightly towards the bottom of the chamber after passing through the slot. In this manner, an axial wire, which is attached to a micromanipulator and which is parallel to the longitudinal axis of the giant axon, can be inserted into the axon from the right after a small hole has been made in it at its bend with a microdissecting scissors.

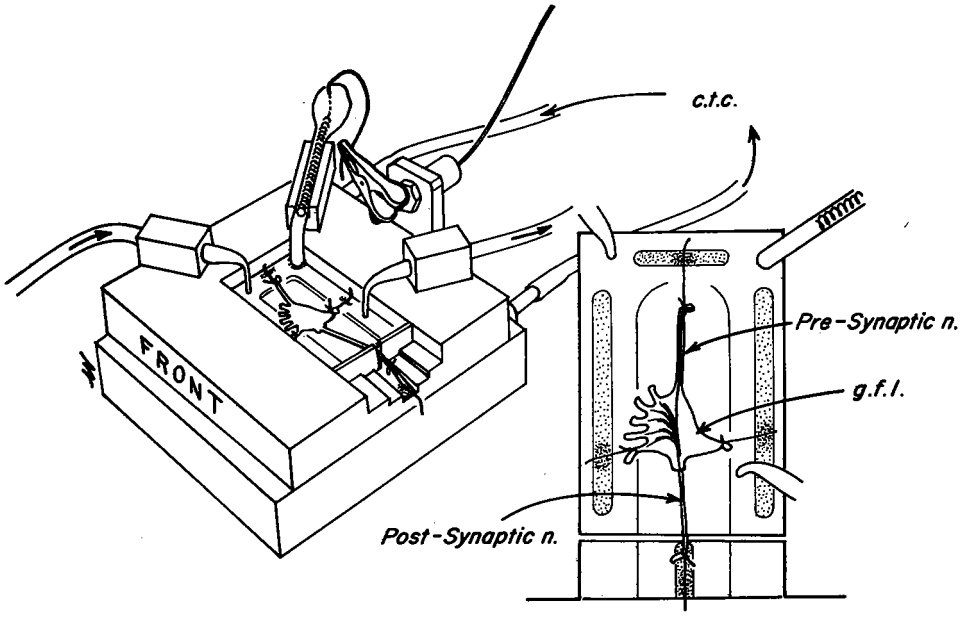

FIGURE 17

The essential features of the experimental chamber for use with the squid giant synapse are shown. The chamber was made from Lucite. See text for further explanation.

Extracellular Stimulation of the Presynaptic Nerve

The presynaptic nerve can be stimulated extracellularly through the use of a suction electrode (Florey & Kriebel, 1966) or through the use of a bipolar electrode made with enamel-coated platinum alloy wire (#851 Platinum, 33 micrometers O.D., Sigmund Cohn). This wire is so flexible that it should be mounted within glass sleeves (glass tubing, 1.0 mm O.D.), one for each wire. Each wire protrudes about 7 mm from the end of its glass sleeve in order to make contact with the presynaptic nerve, which lies in the experimental compartment (Fig. 17) and which is bathed in sea water. The two glass sleeves should be bent so that they can be attached with wax to the end of a rod the other end of which is attached to a micromanipulator. The thin enamel-coated wire can be attached to conventional hook-up wire through the use of miniature male and female contacts (Amphenol 220-PO2 and 220-S02). The only uninsulated portions of this stimulating electrode which are in contact with the experimental compartment are the two ends of the platinum alloy wires which are brought against select regions of the presynaptic nerve. The advantages of this bipolar electrode are as follows: 1) it is unnecessary to have the prenerve lie in a separate and moist compartment so that it can be stimulated; 2) the stimulus artifact is smaller than it would be if monopolar stimulation were used because the bipolar electrode can be made part of an isolated electrical circuit; 3) it is often possible to microposition this electrode so that either the second-order giant or the accessory giant axon can be selectively stimulated within the presynaptic nerve.

The preparation should be illuminated with a darkfield, or, if not, at least some kind of lateral illumination should be used in order to visualize the giant synapse adequately. It is very helpful to direct your light source onto an adjustable mirror; the light is then reflected into a darkfield condenser which is positioned as close as possible to the bottom of the experimental chamber. (A darkfield condenser can be made from a brightfield condenser by putting a circular piece of black tape in its center).

The author has also performed experiments on the giant synapse while the preparation plus its four attached ligatures (Fig. 16) were mounted onto a ¼" Lucite rod. It was not possible, however, to use an axial wire when the preparation is mounted in this manner. Experiments on the frog neuromusclar junction with the preparation being wrapped around a rod were first reported by del Castillo and Katz in 1957 (see also Stefani & Schmidt, 1972).

The location of the giant synapse varies among different preparations. The prenerve terminal can be dorsal, ventral, or to either side of the giant axon; sometimes the prenerve terminal appears to form a spiral around the giant axon. One can usually succeed in penetrating the nerve terminal only when it is close to the ventral surface of the stellate ganglion. When the nerve terminal is ventral to the giant axon but is some distance from the ventral surface of the ganglion, some investigators attempt to reveal the nerve terminal by microdissection; others start another preparation. Although this kind of microdissection has been successful, it is very easy to damage the giant synapse through this process.

Probably the most important fact to remember in performing experiments with the squid giant synapse is that it is very oxygen-dependent (Bryant, 1958); therefore, all solutions should be saturated with oxygen.

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