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ULTRASTRUCTURAL STUDY OF UNENCAPSULATED VERTEBRATE MECHANORECEPTOR TERMINALS FACILITATED BY DOUBLE STAINING AND RESECTIONING OF THICK PLASTIC SECTIONS [1]

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A set of techniques for localization of unencapsulated sensory receptor terminals in plastic-embedded tissue is described. The tissue is pinned flat in fixative and flat-embedded in a small amount of medium-soft Epon. Pieces of the specimen are remounted so that tangential thick sections (2—3 μm) can be cut. These sections are stained with a mixture of 15 ml of 0.5% toluidine blue, 1% sodium borate; 10 ml of 2% *p*-phenylenediamine, and 5 ml of acetone. Stained sections are examined with a light microscope and brown-stained sensory receptor regions are located. Sections to be examined in the electron microscope are remounted by inverting a capsule of medium-hard Epon over them, and polymerized blocks are removed from the slide after heating it. Thin sections cut from remounted thick sections are stained with uranyl acetate and lead citrate. This procedure has been used successfully to locate aortic baroreceptors and very small apparent nerve endings in the atrium of the rat as well as stretch receptors in dog trachealis muscle. All of these receptor endings stain brown and are surrounded by light blue collagen and darker blue Schwann cells.

INTRODUCTION

The usual method of locating sensory terminals for study of their ultrastructure involves cutting thick (usually 0.5—1.0 μm) and thin sections of the same tissue block embedded in plastic. Tranum-Jensen (1975) used *p*-phenylenediamine (PPD) or toluidine blue (TB) to stain 2 μm sections of the mini-pig atrium in order to locate large mechanoreceptor end-organs before cutting thick and thin sections through them. This type of procedure can be quite tedious because one usually wishes to examine the thick sec-

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tions frequently for areas to thin-section. Other disadvantages of this procedure include loss of the thin sections for examination in the light microscope and the requirement for a tissue block small enough for thin-sectioning.

Recently, several methods for remounting thick sections so that they can be thin-sectioned have been developed (Campbell and Hermans, 1972; Bretschneider and Burns, 1979; Butcher et al., 1979). However, the problem of staining sensory terminals in thick plastic sections remains. Böck and Gorgas (1976) were able to stain thick sections (0.5—1.0 μm) of Araldite-embedded carotid baroreceptors with a combination of silver and blue stains. Araldite, however, is extremely viscous, has high electron density, and produces sections with poor contrast (Hayat, 1970).

Horseradish peroxidase (HRP) has now been used to locate sensory terminals (Mesulam and Brushart, 1979), but in this procedure it is necessary to inject HRP into the dorsal root ganglion and allow the animal to survive for 1—3 days before sacrifice. A long period of iontophoresis is usually necessary for cobalt staining of sensory nerve endings (El-Bermani and Chang, 1979). The HRP and cobalt methods are necessary for tracing of pathways, but for routine ultrastructural work they may be less desirable. A new method for fixation of methylene blue-stained neurons appears promising (Tsuji, 1979), but its usefulness for ultrastructural studies of mechanoreceptor terminals has not yet been demonstrated and several hours may be necessary to stain nerves with methylene blue before fixation.

During the course of our structural investigations of rat aortic baroreceptors, it became important to be able to locate the sensory terminals relatively rapidly. We have now developed a set of procedures for routine use in investigating the structure, at light and electron microscopic levels, of the terminals of complex unencapsulated mechanoreceptors and the connective tissue surrounding them. The methods can be used in the standard electron microscopy laboratory with a minimum of new supplies and no special equipment such as a high voltage electron microscope (Watari et al., 1977). TB and PPD (Pease, 1964) are used in combination to make the sensory terminals visible in thick Epon sections, from which thin sections are cut.

MATERIALS AND METHODS

The animals used for most experiments were male Wistar—Kyoto strain rats, of various ages from 7 to 48 weeks. After light ether anesthesia, sodium pentobarbital (Nembutal, 30—40 mg/kg) was administered intraperitoneally. The aortic arch and nerve were dissected as described by Brown et al. (1976) and were placed in a small plastic culture dish partially filled with Sylgard 182 encapsulating resin (Dow Corning, Midland, Mich.) and containing fixative consisting of 3% glutaraldehyde in 0.05 or 0.1 M piperazine-N,N'-bis(2-ethane sulfonic acid) (PIPES) buffer, pH 7.4 (Baur and Stacey, 1977). The aortic arch was dissected so that the region containing the aortic baroreceptors (Krauhs, 1979) remained and was pinned flat (without stretching) with

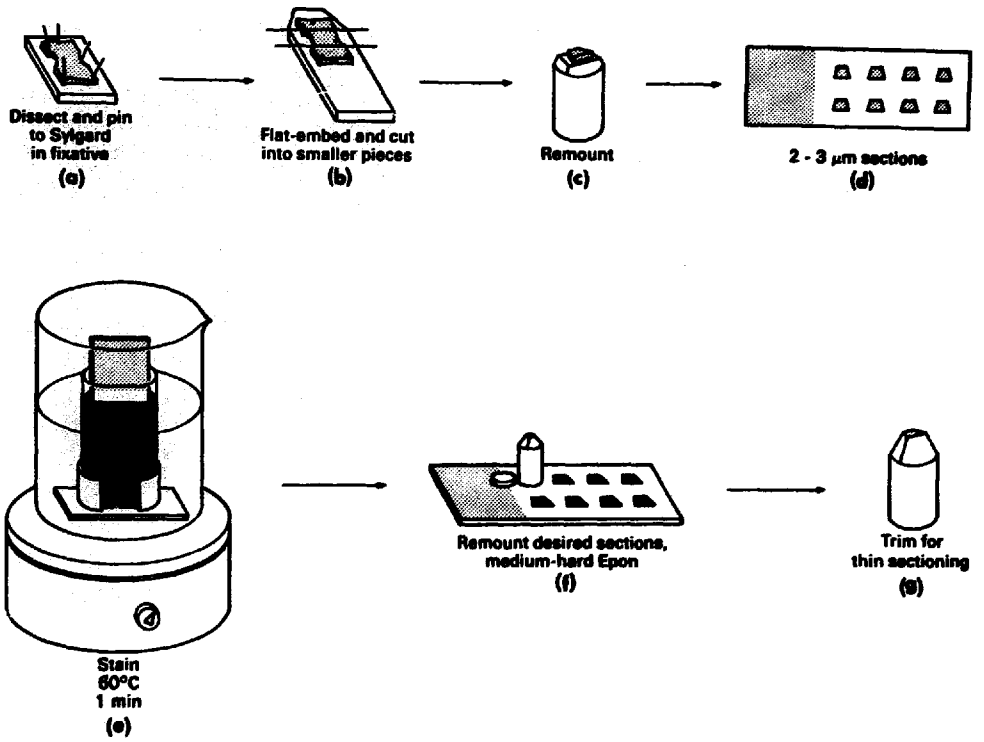

Fig. 1. Steps in the procedure for sectioning, staining and resectioning thick sections of flat-embedded tissue.

fine insect pins to a small flat piece of Sylgard (Fig. 1a). The orientation of the tissue could be marked by putting different numbers of pins on the dorsal and ventral sides. The piece of Sylgard to which the specimen was pinned was placed in a vial of fresh fixative and left overnight at 5°C.

Specimens were rinsed in buffer, postfixed in 1% OsO_4 in buffer, and dehydrated in ethanol series (pins were removed during the first 100% alcohol change). They were infiltrated with propylene oxide and a medium-soft Epon mixture containing 55.0 g of Epon 812, 50.0 g of dodecenyl succinic anhydride (DDSA), and 12.6 g of nadic methyl anhydride (NMA), plus 2 ml of 2,4,6-tri(dimethylaminomethyl)-phenol (DMP-30) (Luft, 1961 and B.N. Christensen, personal communication). Specimens were flat-embedded in a small amount of this resin, which was allowed to polymerize overnight at 60°C. Usually a razor blade was used to cut several pieces measuring about 1.5 × 2 mm from the original block (Fig. 1b) and each of these was remounted on a blank Epon stub (Fig. 1c) with Eastman 910 adhesive (Eastman Chemical Products, Kingsport, Tenn.) so that tangential sections could be cut. These blocks were sectioned (usually completely) at 2–3 μm with glass knives on a Sorvall MT-2B ultramicrotome and the sections were placed on drops of 15% ethanol in distilled water on glass slides ('subbed' if desired)

(Fig. 1d). Sections were dried thoroughly on a hot plate (low heat) before staining.

The staining solution consisted of 15 ml of 0.5% TB, 1% sodium borate; 10 ml of 2% PPD, and 5 ml of acetone. (If the PPD stock solution has not 'aged' for several weeks, results are better if the acetone is omitted.) Fresh stain should be made every day. After filtering, the stain was placed in a single slide stainer (Ted Pella, Tustin, Calif.) with a stirring bar, partially immersed in a beaker of water heated to 60°C on a hot plate/stirrer (Fig. 1e). Slides were immersed in the staining solution for 1 min, rinsed in distilled water and allowed to dry on a hot plate. The sections were examined with a light microscope to locate baroreceptor terminals. Drawings were made for subsequent location of areas to be thin-sectioned.

Sections selected for ultrastructural examination were remounted in the following manner. A medium-hard Epon mixture containing 26.7 g Epon 812, 7.0 g DDSA and 25.6 g NMA, plus 0.75 ml of DMP-30 (Luft, 1961) was prepared and used to fill BEEM (*not* gelatin) capsules (Better Equipment for Electron Microscopy, Bronx, N.Y.). The harder resin mixture was used to provide a more sturdy base for thin-sectioning, and capsule size 3 was most satisfactory for remounting. A filled capsule was inverted over each thick section to be resectioned (Fig. 1f) and the resin was allowed to polymerize overnight at 60°C. The new blocks were removed from the slides by placing each slide on a hot plate (65°C) for about 30 sec, then quickly sliding a razor blade under the inverted capsule and carefully prying it off. Each block was trimmed to include the area of interest (Fig. 1g); the stain allowed one to do this with the aid of a light pipe and dissecting microscope. Thin sections (60—70 nm) were cut with a diamond knife and collected on oval slotted (1 × 2 mm) copper grids coated with Formvar. They were stained with saturated aqueous uranyl acetate for 10 min and with Reynolds' lead citrate for 5 min. Sensory terminals were observed with Philips 201 and 300 transmission electron microscopes.

RESULTS

We can now use the light microscope to survey a relatively large area at low magnification, identify smaller regions in which sensory endings are likely to be found, and locate the terminals themselves with a 40X objective. Tissue components stain as follows with the double stain:

Sensory terminals: brown
Collagen: light blue in longitudinal sections, pale in cross-sections
Elastin: dark blue
Muscle: various shades of brown
Fibroblast-like perineurial cells: blue
Schwann cells: blue
Nuclei of Schwann cells and fibroblasts: bluish-brown, granular
Neuron somae: reddish violet

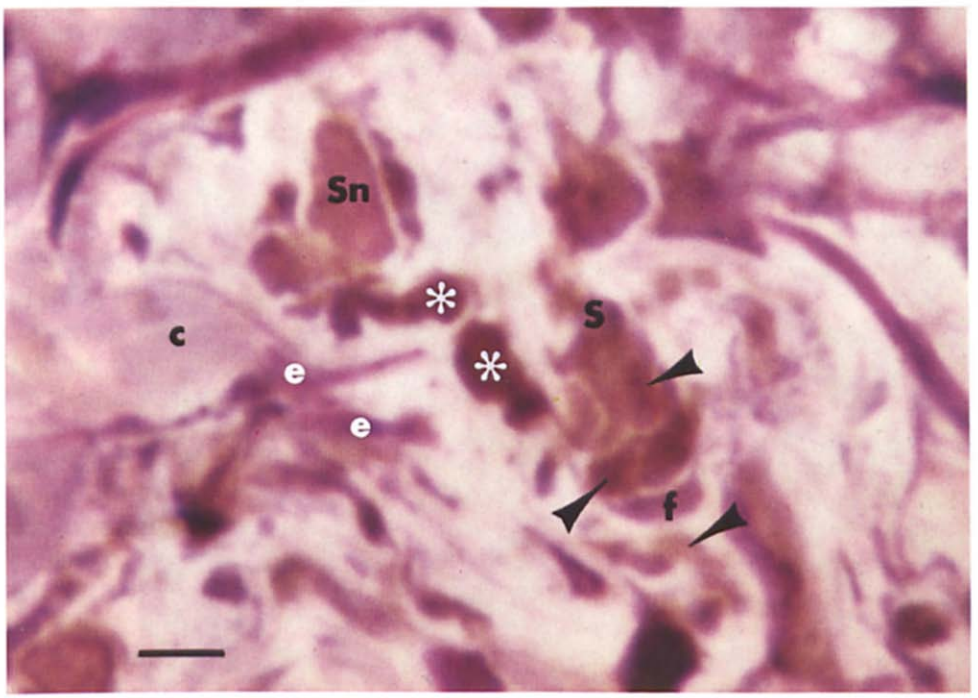

Fig. 2. Light micrograph of a 3 μm thick Epon section stained with toluidine blue and *p*-phenylenediamine, photographed using a Leitz light microscope with a Nikon AFM camera attachment. The violet color actually appears blue at lower magnification; mounting the section with water for oil immersion viewing appears to change its color. The structures indicated by asterisks are receptor terminals shown in Fig. 3. Arrowheads, other structures shown by electron microscopy to be receptor terminals; S, Schwann cell; Sn, Schwann cell nucleus; c, collagen; e, elastic lamina; f, fibroblast-like perineurial cell. The pale areas consist of extracellular matrix and collagen cut in cross-section. Bar indicates 3 μm.

Fat: golden brown
Red blood cells: dark brown.

The sensory terminals we have studied are surrounded by blue connective tissue; since the terminals stain brown, they contrast well with collagen, Schwann cells, and elastin (Fig. 2). The stain combination was found to be far superior to either stain used alone. Once the remounted section has been trimmed to form a very small block face, the sensory endings can be located easily in the electron microscope (Fig. 3). If desired, one can photograph each thick section to be thin-sectioned so that structures can be matched precisely (Figs. 2 and 3). The stain is stable in sections without mounting medium for at least 8 months.

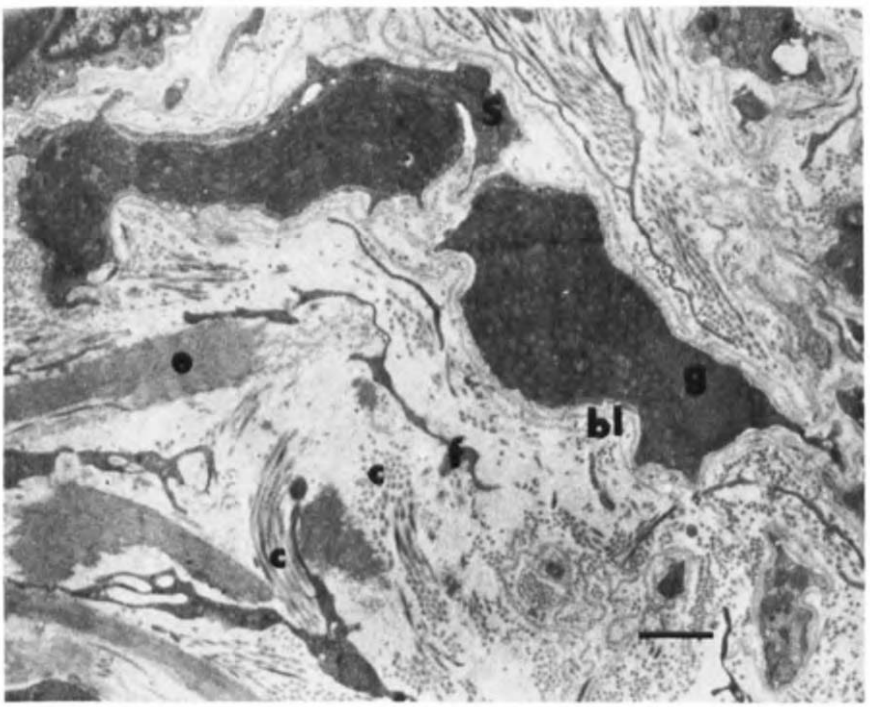

Fig. 3. Electron micrograph of a thin section from the thick section in Fig. 2. The thin section was further stained with uranyl acetate and lead citrate. The structures indicated by arrows in Fig. 2 are shown to be full of mitochondria and glycogen (g). This is a characteristic typical of mechanoreceptor terminal regions, including presumptive aortic baroreceptors in the rat (Krauhs, 1979). bl, basal lamina; c, collagen; e, elastic lamina; S, Schwann cell; f, fibroblast-like perineurial cell. Bar indicates 1 μm.

DISCUSSION

Since other components of the tissue may stain various shades of brown, a certain amount of exploratory investigation may have to be done with a new type of tissue until the sensory terminals can be recognized by their shape and relationship to other cells as well as by their color. Use of oil immersion allows more certain identification of sensory terminals in the light microscope but is not recommended for sections to be resectioned, although sections can be 'mounted' in water with a coverslip for viewing under oil. We have used the described methods to locate sensory terminals in dog trachealis muscle and very small apparent nerve endings in connective tissue in the rat atrium. (These structures will be described in future articles to be published elsewhere.)

A number of factors must be optimized for best results with these techniques. First, the procedures were designed for tissue which can be pinned out flat and is relatively thin. A flat orientation and tangential sectioning

greatly facilitate the following of nerve fibers through the tissue. The method has been used successfully (without pinning) with dog trachealis muscle (about 1.5 mm thick), and perfusion fixation can probably be used for thicker tissues.

Use of PIPES buffer instead of cacodylate (0.1 M, pH 7.4) appeared to give more satisfactory results; the brown color of cacodylate-fixed sensory terminals was more difficult to distinguish from that of erythrocytes and fat. The two concentrations of PIPES gave similar results.

Different section thicknesses may be appropriate for different tissues, although 2—3 μm has worked well for at least 3 preparations in our hands.

Stirring and use of a water bath appear to make staining more uniform. Staining the thick sections sometimes decreases contrast in the thin sections and it is necessary to stain with heavy metal stains longer than usual. It may be particularly difficult to produce good contrast in small unmyelinated fibers. Sometimes a fine precipitate can be seen in the thin sections, particularly on collagen; this appears to result from an interaction of lead citrate with one or both of the light microscopy stains in the tissue. This can be avoided by staining only half the thick sections with TB and PPD, but it is difficult to trim a block containing one large thick section unless it has been stained.

The chemical basis for the success of the combined stain is uncertain. At least some of the brown color of the terminals is probably due to osmium staining, because in sections stained with TB alone, the terminals appear somewhat brown. Use of PPD in addition to the TB makes these brown areas appear much darker. PPD is thought to combine with osmium that is bound to tissue but has not been oxidized (Hayat, 1975), and this might be the reason for the further darkening. Although we have used another double staining method involving basic fuchsin and TB (Bourne and St. John, 1978) with the aorta, use of PPD and TB appeared to provide better contrast between connective tissue and sensory terminals.

The resectioning method we use is very similar to that of Bretschneider and Burns (1979). We have found that if the proper capsule is used, it can be inverted over the section.

The procedures we have described should be useful for ultrastructural studies of a number of complex mechanoreceptors. Such structures have been neglected by neuromorphologists partly because their terminals are so difficult to locate by the usual procedures for electron microscopy.

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