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A NOTE ON THE USE OF PICRIC ACID–PARAFORMALDEHYDE–GLUTARALDEHYDE FIXATIVE FOR CORRELATED LIGHT AND ELECTRON MICROSCOPIC IMMUNOCYTOCHEMISTRY

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Abstract—The buffered picric acid–paraformaldehyde fixative originally recommended for electron-microscopy[7,9] and which has since been used occasionally for light-microscopic immunocytochemistry, has been supplemented with glutaraldehyde and used as primary fixative for the perfusion of rat brains. In the basal ganglia and preoptic area, substance P, somatostatin and leu-enkephalin immunoreactive material was localized with the unlabelled antibody enzyme method in thick sections cut from freeze–thaw treated blocks. Good penetration of the antibodies without the use of detergents and the light background of the osmium-treated sections allowed the selection for electron-microscopy of immunoreactive structures as small as individual boutons that had been identified at the light-microscopic level.

It is suggested that the procedure may be useful for electron-microscopic sampling of immunoreactive structures occurring infrequently over a large area or for the electron-microscopic study of light-microscopically classified neurons.

Neurons in the central nervous system identified at the light microscopic level by selective staining, such as the Golgi method or intracellular injection of tracers, are increasingly used for electron-microscopic study of their fine structure and synaptic connections (see for example refs 1, 2, 6). The advantage of this method is that neurons can be classified and then identified and parts of the axonal and dendritic field can be sampled for examination of their synaptic connections in the electron-microscope. This approach has not been widely applied to neurons or their processes revealed selectively by immunocytochemical staining. The main reason for this is the poor penetration of antibodies in aldehyde-fixed material that is used most commonly for the unlabelled antibody–enzyme[8] electron-microscopic method.

In an effort to apply our previously described combined tracing methods at the synaptic level[5,6] to immunocytochemically stained neurons, we experimented with various fixatives. One of the fixatives reported here gave satisfactory depth of penetration of the antibodies, strong immunostaining and good preservation of fine structure.

EXPERIMENTAL PROCEDURES

Preparation of tissue sections

Fixation experiments were carried out using several species of mammal, and antisera to numerous neuropeptides were employed. We report here the final procedure adopted and illustrate it with the localisation of enkephalin, somatostatin and substance P immunoreactive material in the brain of two CFY strain male albino rats. One of the rats received an injection of 75 μg colchicine (BDH) in 20 μl sterile saline into the lateral ventricle 24 h before perfusion. Both animals were perfused through a needle in the left ventricle of the heart, under chloral hydrate anaesthesia (350 mg/kg i.p.), with Tyrode's solution (gassed with a mixture of 95% O_2, 5% CO_2) for 1 min, followed by approximately 200 ml fixative over 30 min at room temperature. One litre of fixative was made up by mixing in the following order; 500 ml 0.2 M sodium phosphate buffer (pH 7.4), 150 ml saturated picric acid in distilled water, 348 ml paraformaldehyde solution containing 40 g of depolymerized paraformaldehyde (TAAB) and 2 ml of 25% glutaraldehyde (TAAB). The final pH was between 7.2 and 7.4 and the final concentrations were paraformaldehyde (4.0%), glutaraldehyde (0.05%) picric acid (0.2%). After perfusion the brain was removed from the skull and fixed by immersion in the same fixative for 1–3 h at 4°C. Areas of interest were cut into blocks (approximately 2 × 4 × 5 mm) and washed in several changes of 0.1 M phosphate buffer pH 7.4, followed by 10% and 20% sucrose dissolved in the same buffer until the blocks sank. They were frozen in liquid N_2 and thawed in 0.1 M phosphate buffer (room temperature). Sections (70 μm) were cut on a Vibratome (Oxford Instruments) and washed overnight at 4°C in 0.1 M, pH 7.4 phosphate buffer.

Immunocytochemical procedure

Incubation of sections was carried out in the following order at room temperature unless otherwise stated: 1 h in 20% normal goat serum (Cappel); 1 h wash; overnight at 4°C in one of the following specific sera, rabbit anti-somatostatin (Otsuka Pharmaceutical Co. Ltd.) Lot No. IG-1

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diluted to 1:50, rabbit anti-substance P diluted to 1:1000, rabbit anti-leu-enkephalin diluted to 1:500; 3 × 40 min wash; 2 h in goat anti-rabbit IgG (Miles) diluted to 1:40; 3 × 40 min wash; 2 h in rabbit peroxidase–antiperoxidase complex (Cappel) diluted to 1:100; 3 × 40 min wash. Phosphate buffered saline was used for all the washes and antibody dilutions. One per cent normal goat serum was included in all specific sera.

For the localisation of peroxidase activity, sections were pre-incubated for 15 min in 0.05% 3,3'-diaminobenzidine tetra-HCl (Sigma) dissolved in 0.05 M Tris–HCl buffer, pH 7.4, followed by 6 min in the same solution containing 0.01% hydrogen peroxide. The sections were then washed in the Tris buffer for 5 min, then in 0.1 M phosphate buffer, pH 7.4 (2 × 15 min) and then treated with 1% or 2% OsO_4 in phosphate buffer for 1 h. This was followed by a wash in the phosphate buffer, dehydration in a series of alcohols and immersion in Durcupan (Fluka) resin for one day. To enhance contrast for electron-microscopy, 1% uranyl acetate was included in the 70% ethanol for 40 min. Lead staining of ultrathin sections was not used.

To test for specificity of the immunocytochemical reaction, each antiserum was adsorbed to an excess of the respective synthetic peptide overnight at 4°C. This treatment abolished all immunostaining. The immunohistochemical use of the antisera employed in this study has been reported elsewhere.[3,4]

Correlated light- and electron-microscopy of the same structure

The sections were placed on glass slides with a little Durcupan resin under a cover slip and cured for two days at 56°C. The resulting slides can be stored permanently. Selected immunoreactive neurons and fibres were drawn and high resolution photographs were taken of those parts of the neurons intended for electron-microscopy. In order to re-embed a selected area for electron-microscopy the coverslip was removed by pushing a razorblade between the thin layer of resin and the glass. A small piece of the section was cut out with scalpel while warming it gently on a hot plate, and lifted from the slide by inserting a razorblade under it. This piece of resin-embedded tissue was then placed in a flat bottomed polythene capsule (TAAB) reduced to 5 mm in height, which was then filled up with fresh resin. A coverslip was placed onto the open top of the capsule in order to obtain an optically smooth surface. After curing, the coverslip and the polythene was removed and the cylinder of resin could be placed on a slide with the end containing the specimen facing the objective lens of the light-microscope. During ultrathin sectioning the positions of stained neurons or processes in the block were checked directly under the light-microscope after each 40–60 section series. During electron-microscopy the high power light-micrographs helped in identifying stained structures.

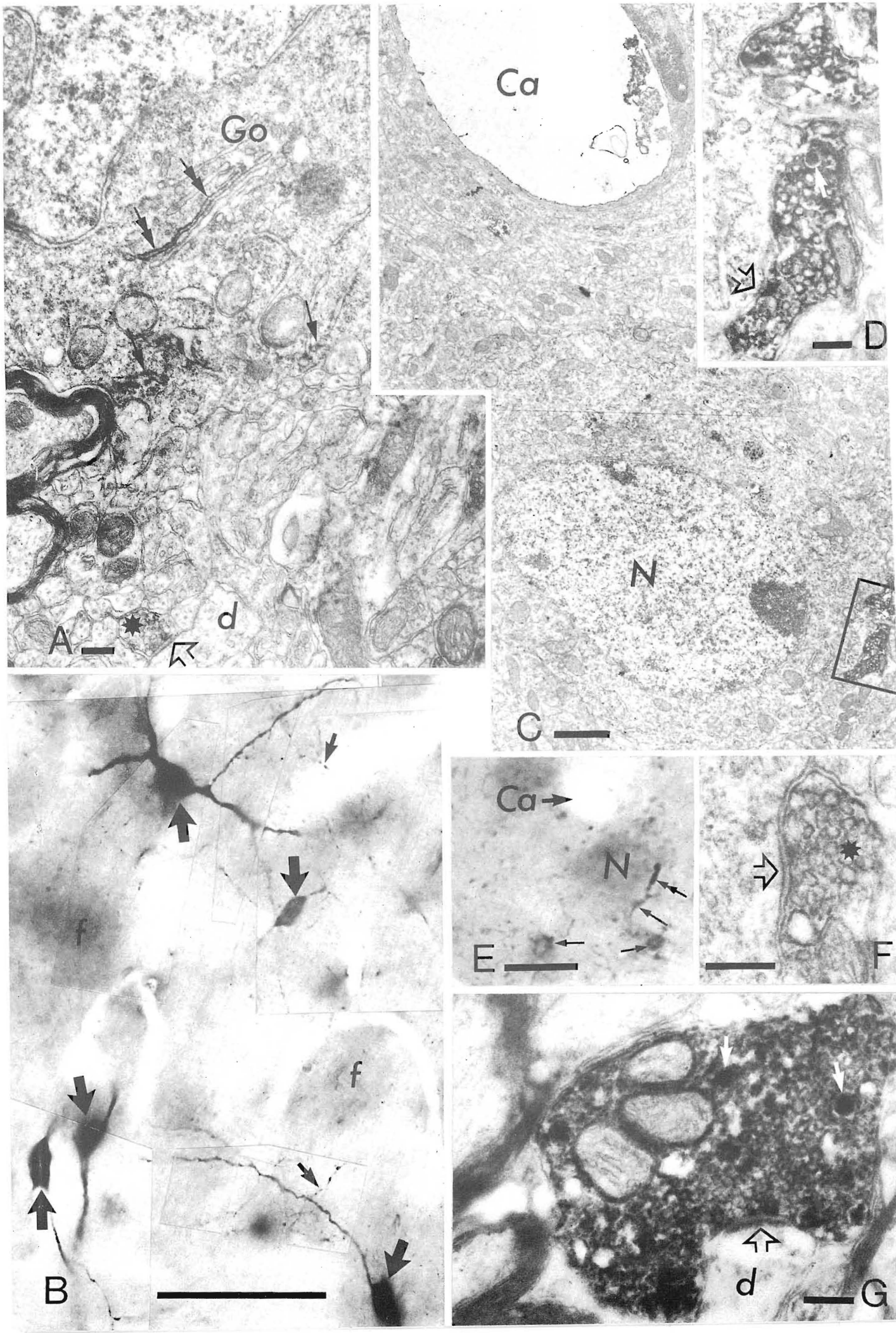
Serial ultrathin sections were collected on formvar coated single slot (2 × 1 mm) grids. Electron-micrographs were taken at 60 kV on Philips 201C and JEOL 100B electronmicroscopes, using 20 or 30 μm objective aperture.

RESULTS AND DISCUSSION

In the light-microscope, the 70 μm thick sections had a yellow to brown colour and immunoreactive structures were strongly contrasted by their darker brown or black colour. The sections were similar in many ways to those obtained with rapid Golgi methods; thus myelinated axons and fiber bundles were dark brown due to the treatment with osmium, but they were easily distinguishable from immunoreactive structures (Fig. 1B). Capillaries and unstained neurons could be identified (Figs 1B,E). The penetration of antibodies was somewhat variable from experiment to experiment but was usually between 10 and 40 μm. Thus in a 70 μm thick section complete staining could be obtained. The inclusion of picric acid in the aldehyde mixture greatly enhanced the immunostaining, while the use of freeze-thaw procedure increased the penetration. For these reasons neurons and their processes could be drawn and photographed in great detail.

Using the correlated light- and electron-microscope approach, any immunostained structure, even an individual bouton could be traced and found in the electron-microscope (Figs 1C–E). The addition of a low concentration of glutaraldehyde to Zamboni's original fixative[9] greatly improved the fine structural preservation. All cellular detail and organelles could be recognised (Figs 1A,C,D,F,G). Synaptic structures and

Fig. 1. Light (B, E) and electron micrographs (A, C, D, F, G) of immunoreactive structures in the rat brain using the picric acid–paraformaldehyde–glutaraldehyde fixative. A. Somatostatin immunoreactive perikaryon and a lightly immunoreactive bouton (star) in the neostriatum. The bouton makes a symmetrical synapse (open arrow) with a dendrite (d). In the perikaryon immunoreactivity is associated with a single saccule (double arrows) of the Golgi apparatus (Go) and with the rough endoplasmic reticulum (arrows). B. Osmium treated section suitable for electron-microscopy in the preoptic area showing somatostatin immunoreactive perikarya with dendrites (large arrows) and varicose fibres (small arrows). Myelinated fiber bundles (f) show up as dark patches. Colchicine-treated animal. C–F. Correlated light (E) and electron (C, D) micrographs of a small neuron (N) in the neostriatum. The neuron (N) and a capillary (Ca) are landmarks for correlation. Leu-enkephalin immunoreactive fibres and varicosities (arrows) are seen in E, one of them (double arrow) in close proximity to the neuron (N). The same bouton is shown in the framed area in C and at higher magnification in contact (open arrow) with the neuron in D. White arrow shows an immunoreactive large granulated vesicle. F. Another lightly immunoreactive bouton (star) forms symmetrical synapse (open arrow) with the same perikaryon at a different level. G. Substance P immunoreactive bouton making an asymmetrical synapse (open arrow) with a small dendrite (d) in the entopeduncular nucleus. White arrows indicate immunoreactive large granulated vesicles. Scales A, D, F, G, 0.2 μm; B, 50 μm; C, 1 μm; E, 10 μm.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the different types of synapse were similar to those obtained by standard electron-microscopic procedures using aldehydes and OsO_4 as fixatives. Both symmetrical or Gray type 2 (Figs 1A,D,F) and asymmetrical or Gray type 1 (Fig. 1G) synapses could be identified. In the immunoreactive boutons the peroxidase reaction end-product was present in large granulated vesicles (Figs 1D,G) as well as on the surface membrane of all intracellular structure and on the inner surface of the plasma membrane. It is significant that antigens could be localised within membrane-limited particles such as large granulated vesicles or Golgi saccules (Fig. 1A) without the use of membrane-active agents.

We have used this procedure to study the synaptic relationships of immunostained axons and cell types which occur infrequently in large nuclei like the neostriatum (unpublished). The selection of light-microscopically identified neurons for electron-microscopy greatly facilitated the study of these rare structures which in random ultrathin sections could not have been found in the electron-microscope.

This procedure was developed empirically, so it is not entirely clear which factors are responsible for the satisfactory penetration, fine structural preservation and retention of antigenicity. Nevertheless, it seems that the use of picric acid not only allows the use of low concentration glutaraldehyde but also improves fine structural detail and through the preservation of immunogenicity provides better immunostaining than aldehydes alone. Increasing the concentration of glutaraldehyde results in reduced immunostaining and less penetration, but glutaraldehyde is indispensable when the freeze–thaw procedure is employed. Penetration is greatly enhanced by the freeze–thaw treatment and although this adversely affects preservation, the fine structural detail is still fairly good. It is hoped that this method will be of value in the study of other parts of the central nervous system.

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