ELECTRON MICROSCOPICAL IDENTIFICATION OF 3,3',5,5'-TETRAMETHYLBENZIDINE-REACTED HORSERADISH PEROXIDASE AFTER RETROGRADE AXOPLASMIC TRANSPORT

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Tetramethylbenzidine (TMB) histochemistry for retrogradely transported horseradish peroxidase (HRP) has been successfully applied in electron microscopical preparations in the guinea pig visual cortex. In ultrathin sections, the TMB-HRP reaction product appears as an accumulation of typical electron-dense crystals. These crystals were identified in the cytoplasm of labeled cells in the proximal portions of the dendrites, and in myelinated axons. It is demonstrated that the TMB-HRP method is well suitable to analyze the ultrastructure of labeled cells and their synaptology.

3,3',5,5'-Tetramethylbenzidine (TMB, Sigma) is the most sensitive chromogen in horseradish peroxidase (HRP) histochemistry, and visualizes an exceedingly higher number of neurons labeled after retrograde axonal transport than any other chromogen [6, 7]. Furthermore, it is less hazardous to the user than some of the other chromogens. Therefore, TMB is now widely used in light microscopical studies of neuronal connectivity. However, for electron microscopy, efforts failed to produce a stable, electron-dense TMB-HRP reaction product (ref. 1; personal communication by many colleagues). For that reason, the carcinogenic and less sensitive 3,3'-diaminobenzidine 4HCl (DAB) is still utilized [2, 4, 5], in spite of the well-known difficulties to identify unequivocally sparsely distributed particles of DAB-HRP reaction product on the ultrastructural level (Fig. 1). This study presents a modification of the TMB histochemical procedure which results in a stable and electron-dense TMB-HRP reaction product.

The present experiments were performed in the projection of layer V pyramidal cells of the primary visual cortex to the superior colliculus [3] in guinea pig. In 5 animals, unilateral stereotaxical injections of 0.1-0.4 μ l of a 30% aqueous solution of HRP (Serva) were made into the superior colliculus. Two days later, the animals were transcardially perfused with Ringer solution followed by a mixture of 1.25% glutaraldehyde/1% paraformaldehyde in phosphate buffer (pH 7.2, 0.1 M). The primary visual cortex was immediately removed and dissected in 100-200 μ m thick

Fig. 1. a: electron micrograph of a pyramidal cell in layer V treated with DAB after HRP injection into ipsilateral colliculus superior. This cell contains few dark granules which may represent DAB-HRP reaction product (stained with lead citrate). b: montage electron micrograph of a pyramidal cell in layer V of an animal which has not been injected with HRP nor treated with DAB. Dark granules are discernible similar to the granules in a (stained with uranyl acetate and lead citrate).

Fig. 2. Light micrograph of a flat embedded specimen (about 100 μm thick) of guinea pig primary visual cortex. Pyramidal cells in layer V labeled after HRP injection in colliculus superior, are discernible at

sagittal sections with a razor blade. After 3 washes of 30 sec each in phosphate buffer the thick sections were reacted with TMB solution according to Mesulam [6], i.e. a 20 min pre-reaction soak in TMB solution was followed by a 20 min incubation in the same TMB solution, with 0.03% H_2O_2 added. Histochemical procedures, carried out at room temperature or at 4°C , were without any noticeable difference in the appearance of the TMB–HRP reaction product. The sections were again rinsed in phosphate buffer and remained in the buffer during a short search for labeled cells. A great number of stained cells, including the proximal portions of their dendrites, were easily discernable under a magnification of 10 times (Fig. 2). Sections containing labeled cells were subdivided to blocs of 1 mm width. Following osmification in 1% OsO_4 in phosphate buffer for 30 min to 1 h these blocs were transferred immediately to 80% ethanol for 30 sec and further dehydrated in 90%, and 2 times in 100%, ethanol (each for 5 min), and in propylene oxide 2 times for 10 min each. Then the blocs remained for 12 h in a propylene oxide araldite mixture (1:1), in pure araldite for another 12 h and were embedded in fresh araldite. When the time of osmification was reduced to 30 min, labeled pyramidal cells could easily be recognized in flat embedded specimens under low magnification, and photographs could be taken (Fig. 2). It turned out to be important to use phosphate buffer instead of Tris buffer in the above solutions. This resulted in a black TMB–HRP reaction product at the light microscopical level, in contrast to the blue reaction product reported by Mesulam [6]. As low percentage alcohols were found to solubilize the TMB–HRP reaction product, these alcohols were strictly avoided, and the time in the higher alcohols was reduced to a minimum.

In semithin sections ($2\ \mu\text{m}$), HRP-labeled cells were identified by their content of brownish-yellowish TMB–HRP reaction product which was accumulated in the cytoplasm around the nucleus and in the proximal dendrites (Fig. 3). In some cases, the myelinated axon of an identified neuron also contained TMB–HRP reaction product (Figs. 3 and 4). In ultrathin sections the TMB–HRP reaction product appeared as an accumulation of electron-dense crystalline structures (Fig. 5a,b). Thus, labeled cells were immediately visible at low magnifications. The amount and size of these crystals is subject to variation. In general, the electron-dense reaction product seems to be joined closely to cell organelles, especially to fibrillar structures, to the rough and smooth endoplasmic reticulata, to the Golgi apparatus, and to the outer membranes of mitochondria (Fig. 5a,b). In heavily labeled cells,

first sight by their dense accumulation of TMB–HRP reaction product. Osmification was reduced to 30 min.

Fig. 3. Semithin section ($2\ \mu\text{m}$) through layer V. Four TMB–HRP labeled cells (1, 2, 3, 4) are discernible. Arrows point to the labeled axons of cells 1 and 4 (weak counterstaining with methylene blue).

Fig. 4. TMB–HRP labeled myelinated axon. Arrows point to the crystalline reaction product (lead citrate stain).

arrays of crystals arise which might extend beyond the cell membranes. Sometimes holes exist in the cytoplasm surrounding the crystals, probably produced by crystallization forces during the histochemical reaction (Fig. 5a). Occasionally,

Fig. 5. a: montage of electron micrographs through the soma and proximal dendrite of the labeled pyramidal cell 4 shown in Fig. 3. Note the accumulation of electron-dense crystalline TMB-HRP reaction product in the somatic and the dendritic cytoplasm. Arrowheads indicate artifactual holes probably caused by the TMB-HRP crystals (without staining). b: labeled (D) and unlabeled (d) apical dendrites originating from different pyramidal cells (stained with lead citrate).

TMB–HRP crystals were detected in the myelinated axons of the retrogradely labeled neurons (Fig. 4).

Conventional double staining of ultrathin sections with uranyl acetate and lead citrate was avoided. Weak labeling was overshadowed by uranyl acetate due to increased electron-density. Staining with lead citrate alone for 3 min [8], however, improved the visibility of even minute crystals, thus allowing the identification of weakly labeled cells and of isolated dendritic profiles.

In summary, we consider the TMB–HRP reaction product as a reliable tracer to identify retrogradely labeled neurons in the electron microscope. The tissue and the cells in general are well preserved, permitting the recognition of the fine structure of the labeled cell and its synaptology. Exceeding crystallization and occasional tissue damage might occur but can probably be avoided by further modification, i.e. changes in the pH of the buffer or reduction of H_2O_2 concentration in the incubation medium, or changes of the reaction time. Nevertheless, the TMB–HRP method is superior to the DAB–HRP method in identifying weakly labeled neurons since a single TMB–HRP crystal can be recognized as such, unequivocally, whereas normally occurring dark inclusions might be mistaken for DAB–HRP label, or vice versa (Fig. 1). This facilitates quantitative approaches as even lightly TMB–HRP labeled neurons can be readily acknowledged.

It will be shown by Schönlitzer and Holländer (in preparation) that the present method is equally well suited for the demonstration of anterogradely transported HRP in axons and their terminals.

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