Combined Use of Silver Staining of the Retrograde Tracer WGAapoHRP-Au and Pre-embedding Immunocytochemistry for Electron Microscopy: Demonstration of Dopaminergic Terminals in Synaptic Contact with Striatal Neurons Projecting to the Substantia Nigra in the Rat

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Received for publication October 15, 1991 and in revised form January 23, 1992; accepted January 28, 1992 (IT2482).

We investigated the applicability of the pre-embedding immunoperoxidase technique to WGAapoHRP-Au retrograde tracing. After injection of the tracer into the substantia nigra of rat, the brain was fixed and cryostat sections were immunostained for dopamine. The sections were osmicated and silver-stained to amplify the colloidal gold particles. Products of both the immunoperoxidase staining and the silver staining could be detected and distinguished by electron microscopy at low magnification. The ultrastructure was so well preserved that synaptic characteristics could be investigated. Dopaminergic terminals were demonstrated to synapse with striatal neurons projecting to the substantia nigra. (J Histochem Cytochem 40:889-892, 1992)

KEY WORDS: Striatum; Dopaminergic afferent; Striatonigral neurons; WGAapoHRP-Au; Retrograde tracing; Silver staining; Pre-embedding immunocytochemistry; Electron microscopy; Rat.

Introduction

A conjugate of wheat germ agglutinin—enzymatically inactive horseradish peroxidase—colloidal gold (WGAapoHRP-Au) has been introduced by Basbaum and Menetrey (3) as a retrograde tracer. Advantages of this tracer are: (a) it can be detected with high sensitivity when visualized by silver staining of the gold particle in the tracer; (b) injection sites of the tracer can be made quite small; (c) the tracer persists in cells for long periods of time; and (d) retrograde tracing with this tracer can be combined with an immunoperoxidase technique, without the need to inhibit the enzymatic activity of the peroxidase in the tracer (1-3,7,8).

There are two problems with the use of this tracer in electron microscopic studies (2,3): (a) standard silver staining procedures are carried out at low pH, which is incompatible with good ultrastructural preservation, and (b) osmication of the tissue oxidizes the silver precipitate to silver salt, which is then lost in subsequent washing and dehydration steps. A new silver staining procedure, which is run at neutral pH and performed after osmication, has recently been introduced to make the tracer visible at the electron microscopic level (2). With this procedure ultrastructural preservation was good, and loss of the silver precipitate by osmication was eliminated (2).

To characterize the cytochemistry of the synaptic inputs to retrogradely labeled cells, it is necessary to combine immunocytochemistry with retrograde tracing at the electron microscopic level. For this purpose, WGAapoHRP-Au retrograde tracing with the new silver staining procedure has previously been combined with post-embedding immunogold staining (2). Unfortunately, high magnification is necessary to detect unintensified immunogold labels. Pre-embedding immunoperoxidase staining provides higher sensitivity than post-embedding immunogold staining and permits easier detection of signals by low-magnification electron microscopy (9). In the present study, we evaluated the applicability of the pre-embedding immunoperoxidase technique to WGAapoHRP-Au retrograde tracing with the new silver staining procedure.

Materials and Methods

Male Wistar rats (250–300 g) were used. Experiments were performed in
acCORDANCE WITH THE STANDARD FOR ANIMAL EXPERIMENTS AND ANIMAL CARE OF OUR INSTITUTION.

**Tracer Injection.** According to Basbaum and Menétry (3), WGAapoHRP-Au was made by coupling a conjugate of wheat germ agglutinin–enzymatically inactive horseradish peroxidase (Sigma, St Louis, MO) to colloidal gold. Under anesthesia with an intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight), a glass micropipette was stereotaxically inserted into the substantia nigra, and then 0.5 μl of WGAapoHRP-Au was injected by pressure.

**Tissue Preparation.** Two days after the tracer injection, the rats were anesthetized and perfused through the ascending aorta with 1% sodium bisulfite in 0.1 M cacodylate buffer (pH 7.4; 4°C). The striatum was dissected out, placed in the same fixative for 1 hr, and then immersed in 0.1 M phosphate buffer (pH 7.4; PB) containing 15% sucrose. Frontal sections of the striatum were cut on a cryostat at 50 μm and collected in 0.1 M PBS.

**Pre-embedding Immunocytochemistry of Dopamine.** Dopamine immunoreactivity was detected by use of the avidin–biotin–peroxidase method (6) with a mouse monoclonal antibody directed against conjugated dopamine (4). The specificity of this antibody has been previously described (4,3). Floating sections were incubated with: (a) monoclonal anti-conjugated dopamine antibody (diluted 1:20,000 in PBS) for 24 hr at 4°C; (b) biotinylated goat anti-mouse immunoglobulin G (Vector, Burlingame, CA; 1:1000 in PBS) for 1 hr at room temperature; and (c) avidin–biotin–peroxidase complex (Vector; 1:1000 in PBS) for 1 hr at room temperature. Activity of tissue-bound peroxidase was visualized by incubating the sections with 0.025% 3,3’-diaminobenzidine (Sigma), 0.6% nickel ammonium sul- fate (Nakarai, Kyoto, Japan), and 0.006% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.6). After each incubation step, the sections were rinsed in PBS for 30 min at room temperature.

**Osmication.** The stained sections were osmicated (1% osmium tetroxide in PB) for 1 hr at 4°C. The sections were then rinsed in PBS for 30 min at room temperature.

**Silver Staining of WGAapoHRP-Au.** According to Basbaum (2), the sections were silver-stained with a commercially available kit (Intense M; Arnersham, Poole, UK). Before and after this step, the sections were rinsed in PB.

**Electron Microscopy.** The sections were dehydrated in graded alcohols and flat-embedded in Epon. A small area containing the striatum was trimmed from the embedded section and sectioned on a Reichert ultramicrotome. Ultra-thin sections were collected on grids, stained with lead citrate and uranyl acetate, and examined under a Hitachi H5000 electron microscope.

**Results**

Figure 1 shows low-magnification electron micrographs of striatal neurons which were retrogradely labeled and were in contact with dopamine-immunoreactive axon terminals. The product of immunoperoxidase staining in labeled terminals was electron-dense and was distributed diffusely around synaptic vesicles and mitochondria (Figures 1A and 1B). Retrogradely transported WGAapoHRP-Au stained by silver was highly electron-dense, round in shape, and had a sharp outline (Figures 1A and 1B). Some retrograde labels were clustered over lysosomes (Figure 1A). It was easy to detect and distinguish the two kinds of labels at low magnification. The ultrastructure was well preserved, and this allows investigation of synaptic characteristics. Figure 1A shows symmetric synapses between the cell body of the retrogradely labeled neuron and two axon terminals, one of which is immunoreactive and the other non-immunoreactive.

Although we did not specifically make a quantitative study, it seems that almost all of the retrograde labels were localized in the cell bodies and proximal dendrites, but only a few were in the distal dendrites. Some dopamine-immunoreactive terminals were in contact with cell bodies in which retrogradely transported WGAapoHRP-Au was localized. Thus, dopaminergic terminals were demonstrated to synapse with striatal neurons projecting to the substantia nigra in the rat. However, no immunoreactive terminals were found to contact distal dendrites that possessed retrograde labels.

**Discussion**

We describe here a new method of ultrastructural double staining for both chemically identified afferent fibers and retrogradely labeled cells. This method is composed of two procedures: pre-embedding immunoperoxidase staining for dopamine (5) and WGAapoHRP-Au retrograde tracing with the silver staining procedure, which is run at neutral pH and performed after osmication (2). With our method it is easy to detect and distinguish labels of both immunocytochemistry and silver staining by low-magnification electron microscopy. Moreover, the ultrastructure was well preserved, and this allows investigation of synaptic characteristics. Our method therefore facilitates studies of the morphological relationship between retrogradely labeled neurons and chemically identified afferent fibers.

**Literature Cited**

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Figure 1. (A, B) Low-magnification electron micrographs of neurons in the striatum. Dopamine-immunoreactive axon terminals are indicated by arrows. Retrogradely transported WGAapoHRP–Au stained by silver is indicated by arrowheads and double arrowheads. Double arrowheads indicate retrograde labels which are clustered over lysosomes. Inset (a) shows a higher magnification of the boxed area in A. Note symmetric synapses (thick arrows) between the cell body of the retrogradely labeled neuron and two axon terminals, one of which is immunoreactive (thin arrow) and the other non-immunoreactive. N, nucleus of retrogradely labeled neurons. Original magnifications: A x 6000; a x 12,000; B x 7000. Bars = 1 μm.