Rapid Communication

Localization of Immunoreactive Tyrosine Hydroxylase in the Goldfish Retina with Pre-embedding Immunolabeling with One-nanometer Colloidal Gold Particles and Gold Toning

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The goal of this study was to develop an alternative to silver intensification for visualizing small colloidal gold particles by light and electron microscopy. The isolated goldfish retina was labeled with rabbit antiserum to tyrosine hydroxylase and 1-nm colloidal gold-conjugated goat anti-rabbit IgG. The gold particles were enlarged by toning with gold chloride, followed by reduction in oxalic acid. Dopaminergic interplexiform cells were clearly visible by light microscopy and, in lightly-fixed material treated with detergent, they were labeled in their entirety. Labeling was qualitatively similar, although less extensive, in material fixed and processed for electron microscopy. The labeled processes were apparent in ultra-thin sections viewed at low magnification, but the gold-toned particles were not so large that they obscured subcellular structures. The procedure apparently had no deleterious effects on the tissue, since the ultrastructural preservation was comparable to that seen with other pre-embedding immunolabeling methods. The technique was simple, reliable and, since the gold solutions were so dilute, relatively inexpensive. (J Histochem Cytochem 40:1465-1470, 1992)

KEY WORDS: Dopamine; Retina; Synapses; Goldfish; Colloidal gold; Intensification; Immunolabeling; Electron microscopy.

Introduction

Dopamine is a neurotransmitter of local circuit neurons in the vertebrate retina and is known to have a modulatory action, influencing visual adaptation and modifying the receptive field properties of retinal neurons (1). The dopaminergic neurons of teleost fish retinas have been particularly well characterized (14), and have proven to be useful models for dopaminergic neurons, both in other retinas and elsewhere in the central nervous system. The first step in a study to describe the circuits that regulate the activity of dopaminergic neurons was to develop an immunohistochemical double labeling technique for electron microscopy. It was preferable to label the retina before embedding, for reasons reviewed recently (4,10). First, since the dopaminergic neurons comprise only a small percentage of the retinal neurons and some of the neurons they contact also occur infrequently, it would be essential to select labeled regions by light microscopy before further analysis by electron microscopy (5,14). Second, although immunoreactive tyrosine hydroxylase has been localized previously with post-embedding immunolabeling at the light microscopic level (23), this technique has not been widely used for ultrastructural localization of this antigen. Finally, many of the antigens in neurons that contact the dopaminergic neurons are also easily extracted or denatured by the embedding process.

Colloidal gold is a logical choice as one of the labels, since the discrete gold particles are easily distinguished from the reaction product of horseradish peroxidase, the tracer most commonly used in electron microscopic immunocytochemical studies of the central nervous system. In various regions of the central nervous system, tyrosine hydroxylase (15), another neurotransmitter-synthesizing enzyme (19), and a neuropeptide (12) have been localized with relatively large colloidal gold particles. These particles do not diffuse well through thick sections of tissue fixed for electron microscopic immunohistochemistry and, more recently, colloidal gold particles of 1-5-nm diameter have been used instead (4,11,12; reviewed in 20). These particles are too small to be detected by light microscopy, and the smallest ones cannot be detected even by transmission electron microscopy, however, and it is necessary to enlarge these particles with silver. A number of problems have been reported with these methods, the major one being that some investigators find that the silver dissolves during osmium tetroxide post-fixation (1; and unpublished observations). This study was undertaken to determine whether gold particles could also be enlarged by toning with gold chloride and reduction with oxalic acid, a classic histochemical technique (16).

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Figure 1. Light micrographs at four levels of focus through a whole mount that was labeled with anti-tyrosine hydroxylase and 1-nm colloidal gold-conjugated second antibody and then treated with two cycles of gold chloride toning and oxalic acid reduction. (a) The boundary between outer plexiform and inner nuclear layers. (b) The middle of the inner nuclear layer. (c) The boundary between the inner nuclear and inner plexiform layers. (d) The proximal stratum of the inner plexiform layer. Original magnification x 300. Bar = 20 μm.

Materials and Methods

Common goldfish (Carassius auratus) approximately 10 cm long were obtained from a local supplier (Lilypons Water Gardens; Brookshire, TX) and maintained in aerated tanks. After dark adaptation overnight, they were sacrificed by decapitation, pithed, and enucleated according to a protocol approved by the University of Texas Medical School’s Animal Welfare Committee. The anterior half of the eye was removed and the retina was gently isolated from the eyecup and placed on a nitrocellulose filter. The retina was immersed for 2 hr at 20°C in Fixative 1, consisting of 4% paraformaldehyde and 3% sucrose in 0.06 M sodium phosphate buffer, pH 7.4 (PB). The retinas were removed from the filters and the vitreous humor was dissected away. The retinas were rinsed in PBS three times for at least 1 hr and incubated in rabbit antiserum to tyrosine hydroxylase (1012; Eugene Tech International, Allendale, NJ) diluted 1:1000 in PBS with 0.3% sodium azide and 0.3% Triton X-100 (Sigma; St Louis, MO) for at least 10 days at 4°C. After rinsing in PBS, the retinas were rinsed for 10 min in PBS with 0.1% bovine serum albumin (Sigma), 0.3% gelatin (Amersham; Arlington Heights, IL), 0.05% Triton X-100, and 0.3% sodium azide. They were incubated for 48 hr at 4°C with 1:30 goat anti-rabbit conjugated to 1-nm colloidal gold (Amersham) in the same buffer. After another rinse in this buffer, the retinas were rinsed in PBS and post-fixed for 60 min at 20°C in 2% glutaraldehyde (EM grade; Polysciences, Warrington, PA) in PB. The retinas were rinsed three times for at least 10 min each in HPLC-grade distilled water (Fisher Scientific; Fair Lawn, NJ), which was used in all the remaining steps, and were transferred to clean glass vials. In subsequent steps, all solution changes were made under a sodium safelight, and the toning and reduction were carried out in darkness. The retinas were toned with 0.05% gold chloride (Sigma) in water for 5 min, rinsed three times in water for 1 min, reduced in 0.05% oxalic acid (Sigma) in water for 5 min, and rinsed again in water. The last four steps were repeated for most retinas. In ordinary room light, the retinas were dehydrated and embedded in epon (Ted Pella; Tustin, CA) with vitreal side up on glass slides. Labeled neurons were photographed with a x40 objective in a Zeiss Axioptot.

The procedure for electron microscopic immunolabeling was similar in most respects, but light-adapted, 5-cm goldfish of the Black Moor variety were used. The retinas were fixed for 60 min in Fixative 1 with 0.05%
Figure 2. Electron micrographs of the outer plexiform layer that was labeled with anti-tyrosine hydroxylase and 1-nm colloidal gold-conjugated second antibody and then treated with one cycle of gold chloride toning and oxalic acid reduction. (a) Several labeled axons (arrowheads) are closely apposed to the distal side of an H1 cone horizontal cell. (b) A contact between a labeled axon and an H1 horizontal cell perikaryon. (c) A symmetrical synapse from a labeled axon onto a bipolar cell dendrite; arrowheads indicate the edges of synaptic membrane specializations. Original magnifications: a × 10,000; b,c × 20,000. Bars: a = 1 μm; b,c = 0.5 μm.

Glutaraldehyde and then post-fixed overnight in Fixative 2, consisting of 4% paraformaldehyde, 3% sucrose, 0.06 M PB, pH 10. The retinas were cut into 5-mm squares, treated for 60 min with 1% sodium borohydride in PBS, and rinsed three times for at least 10 min each in PBS. Triton X-100 was omitted, and the tissue was treated for 10 min each in a series of graded ethanol solutions in PBS (10%, 25%, and 40%) and rehydrated by applying the same solutions in reverse order. The tissue was post-fixed in 1% osmium tetroxide in PB for 60 min before rinsing in distilled water, and only one cycle of gold toning and reduction was done. The retinas were sectioned in the vertical plane and photographed in a JEOL 100 CX electron microscope.

Results
In retinas that had been lightly fixed and treated with detergent, dopaminergic interplexiform cells (DA-IPCs) appeared to be com-
Figure 3. Electron micrographs of the inner plexiform layer of the goldfish retina labeled as in Figure 2. Arrowheads denote synaptic specializations. (a) A synapse from an amacrine cell process onto a labeled dendrite. (b) A synapse from a labeled dendrite onto an amacrine cell process. (c) A synapse from a labeled dendrite onto a retinal ganglion cell dendrite. Original magnification × 20,000. Bar = 0.5 μm.
GOLD TONING OF 1-NM COLLOIDAL GOLD

were seen in the goldfish retina described previously (6,9,14,18,22-24), with two exceptions. First, the distinction between the typical symmetric synapses onto bipolar cell dendrites and less specialized contacts onto cone horizontal cells had not been described. Since osmium tetroxide was used as the primary fixative in one of the earlier studies (6), the postsynaptic densities may not have been as conspicuous. Another possibility is that the labeled processes were easier to analyze after gold toning, since they were not filled with peroxidase reaction product or damaged by toxic amines as in the earlier studies.

It was not surprising that dopaminergic axons made more than one type of contact in the outer plexiform layer, since this has been described previously in the inner plexiform layer of the goldfish (24) and in many other regions of the central nervous system (15). Second, the synapses onto retinal ganglion cell dendrites had not been described previously but, since the postsynaptic cells were not reconstructed from serial sections, their identification should only be regarded as tentative.

The primary goal of this study, however, was to develop a method for visualizing small colloidal gold particles before embedding. The use of gold chloride and oxalic acid for this purpose was first described by Feigen and Naoumenko (8), as part of a series of experiments on the mechanism of gold toning of silver in histochemistry. Metallic silver was replaced by minute “specks” of metallic gold (16), which were then toned with non-metallic gold and reduced with oxalic acid (8). They concluded, “apparently, the gold specks selectively catalyze the adsorption or the oxalic acid reduction of gold salts or complex.” Although the gold chloride-oxalic acid procedure has been used to reduce the electron density of Golgi-stained material (7) and to stabilize silver labeling (1,3), it has not been used to enlarge gold particles. More recently, a procedure was reported in which gold chloride was used to enlarge gold particles by approximately 50% in ultra-thin sections of plastic-embedded material (13). This technique has not been used before embedding, however, and it is uncertain whether the gold particles could be enlarged sufficiently to make them visible by light microscopy, as they were when the gold chloride was followed by oxalic acid.

Previously, thick sections of brain have been labeled before embedding with small particles of colloidal gold, which were then intensified by various silver techniques (11,17). Although this procedure has been used successfully in some studies (4,20,21), it has not been widely adopted. One of the most serious problems that has arisen is that the intensification is often done before the osmium tetroxide treatment, but the silver dissolves during that step in some instances (1; and unpublished observations). One author reports that silver intensification can be done after osmication, however (2). Another potential problem was that the tissue might be damaged by silver intensification in solutions that were not isotonic or at neutral pH (11). The gold toning in this procedure was done after 1 hr in 1% osmium tetroxide, when the tissue was no longer osmotically active and was protected by the extensive cross-linking. The osmium treatment had no apparent effect on the sensitivity or selectivity of the gold toning and oxalate reduction. This method provides an alternative to silver intensification for visualizing small gold particles before embedding.

Discussion

The tyrosine hydroxylase-immunoreactive neurons seen with this technique resembled dopaminergic interplexiform cells in goldfish retina described previously (6,9,14,18,22-24), with two exceptions. First, the distinction between the typical symmetric synapses onto bipolar cell dendrites and less specialized contacts onto cone horizontal cells had not been described. Since osmium tetroxide was used as the primary fixative in one of the earlier studies (6), the postsynaptic densities may not have been as conspicuous. Another possibility is that the labeled processes were easier to analyze after gold toning, since they were not filled with peroxidase reaction product or damaged by toxic amines as in the earlier studies.

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