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Retrograde tracing of neural pathways with a protein-gold complex

I. Light microscopic detection after silver intensification

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Summary. In this study I have used a tracer complex made of wheat germ agglutinin horseradish peroxidase conjugate (WGA*HRP) coupled to colloidal gold for retrograde tracing of neuronal pathways at the light microscopic level. Visualization of the gold was achieved by silver precipitation (the gold silver intensification method) with gold particles acting as specific cores of nucleation. The presence of horseradish peroxidase in the protein conjugate allowed this method to be compared with classical histochemistry using tetramethylbenzidine as a chromogen. The gold silver intensification method proved to be reliable, specific and sensitive. It has been demonstrated to be useful with fixatives containing a high percentage of paraformaldehyde and compatible with histochemical procedures to show projections of transmitter specific pathways.

Introduction

In a previous report (Lee and Menétrey 1984), we described a new method which, through the coupling of gold particles to the wheat germ agglutinin-horseradish peroxidase conjugate (WGA*HRP), allows the demonstration of neural pathways at the electron microscopic (EM) level. In the present report, we take advantage of the gold silver intensification (GSI) methods of Roberts (1935) and Danscher (1981) to visualize gold particles in retrogradely labeled cells at the light microscopic (LM) level. This procedure provides a sensitive method for demonstrating retrogradely labeled cells at the LM level. It can be used in conjunction with immunohistochemical procedures to demonstrate projections of cytochemically characterized neurons.

Material and methods

Preparations of colloidal gold. Gold particles (6 to 12 nm) were prepared according to Muhlpfordi's procedure (1982). Briefly, 100 ml of hydrochloroauric acid solution (0.01% w/v in distilled water) was boiled under vigorous stirring. Reducing agents (2 ml of trisodium citrate and 100 µl of tannic acid, both 1% w/v in distilled water) were mixed in a separate beaker and rapidly added to the boiling solution. The reaction was complete (30 to 50 s) when the solution quickly turned dark-violet and then wine-red. Boiling and stirring were continued for five minutes after which the solutions were rapidly cooled under running water. Solutions can be kept at 4° C for several weeks if supplemented with 2% sodium azide (0.2% w/v).

Adsorption to protein. Horseradish peroxidase (HRP), wheat germ agglutinin (WGA) and wheat germ agglutinin-horseradish peroxidase conjugate (WGA*HRP), the three proteins most commonly used in tracing connections in the central nervous system (La Vail and La Vail 1972; Lechan et al. 1981; Gonatas et al. 1979) have all been tested for gold coupling but only WGA*HRP (Sigma) was satisfactorally conjugated. The pH range for gold-protein adsorption and the amount of protein needed to stabilize gold solutions against flocculations by salt were determined according to Geoghegan and Ackerman (1977) and Goodman et al. (1981). Good results were obtained for 60 µg of protein per milliliter of gold solution at pH 8.2.

The pH of the gold solution was raised to 8.2 by adding small quantities (app. 4 µl/ml solution) of potassium carbonate (0.2 *M*) and checked on one ml aliquot in the presence of 45 µl of 1% (w/v) polyethylene glycol (PEG, MW 20.000).

The gold solution was added to the diluted WGA*HRP (1 mg protein per ml of distilled water) and coupled under vigorous stirring in the proportion of 60 µg of protein per ml of gold solution. After 5 min filtered PEG was added in proportion of 1% (v/v) of the complete volume of the solution. This prevented aggregation of the complex.

The pellet was laid over a cushion of 8% sucrose in water and centrifuged for 120 mn at 18,000 rpm. The supernatant was aspirated and discarded and the soft pellet containing WGA*HRP-gold complex was gently resuspended in distilled water, to be later microcentrifuged. Consistent retrograde labeling was obtained when pellets had a final concentration of protein of at least 2.5%. This was expressed as the original weight of protein per the final volume obtained in the pellet. Such a pellet was used directly without further dilution. It can be kept at 4° C for several days with no detectable loss of sensitivity. Keeping it more than two or three weeks can lead to flocculation.

Animal preparation and tissue processing. Experiments were performed on 15 Sprague-Dawley rats deeply anesthetized with ketamine (75 mg/kg/IP supplemented with 35 mg/kg/IM as necessary). All tracer injections (0.2 to 1.0 µl) were done by pressure through micropipettes with tip diameter (15 to 30 µm). This reduced tissue damage during penetration. Injections made with iontophoresis were unsuccessful. Animals were perfused two days later with 200 ml of saline followed by 500 ml of fixatives. Fixatives consisted of 3% glutaraldehyde; 4% paraformaldehyde or 2.5 to 4% periodate-lysine-paraformaldehyde (MacLean and Nakane 1974), all in 0.1 *M* phosphate buffer (ph 7.4) at 4° C. Free floating (40 µm) frozen or vibratome sections were processed either for silver intensification or with tetramethylbenzidine (TMB) histochemistry (Mesulam 1982) for horseradish peroxidase (HRP). Silver intensification was performed in a dark box at room temperature (18°–22° C)

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as follows. After a brief rinse (5 mn) in sodium citrate buffer (0.1 *M*, pH: 3.8) sections were dipped in a physical developer for 60 min. Although sections can remain in the developer for a longer period of time, they should be removed when myelinated fibers begin to turn brown. Each 100 ml of physical developer consisted of: 60 ml gum arabic solution (50% w/v in distilled water) as a colloidal protecting agent, 10 ml citrate buffer (1 *M*, pH: 3.5), 15 ml hydroquinone (5.6% w/v in distilled water) as a reducing agent and 15 ml silver lactate (0.7% w/v in distilled water) to supply silver ions. Silver lactate solution was carefully protected against light and added just before the developer was used. After development, sections were washed once (5 mn) in phosphate buffer (pH: 7.4), put into sodium thiosulfate (2.5% w/v in phosphate buffer, pH 7.4) for 5 min and finally rinsed for 5 min in phosphate buffer pH 7.4. This silver intensification processing has been adapted from Danscher (1981) with buffer rinses replacing water rinses to improve tissue preservation.

Sections were either mounted, counterstained (neutral red), and coverslipped or processed for the immunohistochemistry with antisera to serotonin (5-HT) after pretreatment (30 mn) with 0.3% hydrogen peroxide to inactivate the HRP (Streefkerk 1972, Zehr 1978) in the WGA*HRP conjugate. This prevented enzymatic marking of retrogradely labeling of cells. Only immunoreactive cells showed the dark brown stain indicative of reaction with diaminobenzidine (DAB). Both the unlabeled antibody enzyme method (PAP) of Sternberger et al. (1970) and an avidin-biotin (Hsu et al. 1981) procedure were used.

Results

Injection sites

Injections (0.2 to 1.0 µl) were performed either in the lumbar spinal cord, the dorsal column nuclei or in the somatosensory cortex. Figure 1 shows such an injection (0.3 µl) in the somatosensory cortex. Typical injection sites consisted of both a dense black central core (visible without silver enhancement) (Fig. 1A) and a surrounding, lighter periphery, which appeared only with silver intensification (Fig. 1B). Neural labeled elements, could be easily recognized in this lighter periphery. The spread of the tracer complex was very limited. Injection volumes of up to 1 µl produced injection sites 1 mm or less in diameter. This limited spread of the complex is probably due to its size and/or the presence of heavy gold particles. The injection site spread revealed with TMB histochemistry is illustrated (Fig. 1C) for comparison. There is consistently a larger injection site with TMB histochemistry. HRP is thus localizable in the halo of the injection site that is apparently devoid of gold particles. This suggests either that there is a partial dissociation of the complex at the periphery of the injection site or that part of the protein was unlabeled and not completely eliminated during centrifugation.

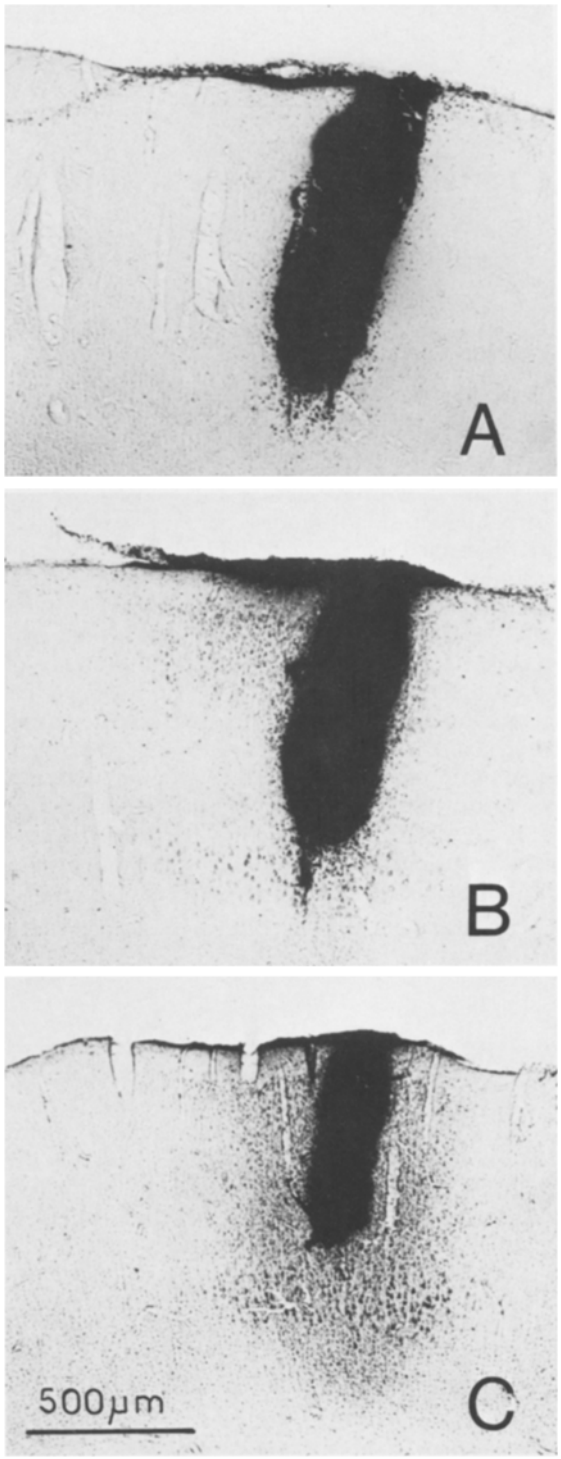

Fig. 1A–C. Example of an injection site of WGA/HRP-gold complex in the somatosensory cortex. **A** Injection as shown without histochemical processing for HRP or gold. **B** Injection after gold silver intensification. **C** Injection after TMB processing for HRP. Note that there was considerable shrinkage of the section after the TMB procedure

Retrograde transport

Examples of labeling. Figure 2 shows lamina VIII cells in the cervical enlargement after injections of protein-gold complex at lumbar levels and silver intensification. Labeled cells are easily recognized. The cell body and proximal dendrites contain round, black granules. Note that there is no background silver labeling.

Figure 3 shows labeled cells in the brainstem, mesencephalon and cortex after a bilateral injection of 1 µl of a 2.5% WGA*HRP-gold complex at the lumbar spinal level. Numerous labeled cell bodies and proximal dendrites, were easily detected in the raphé magnus and adjacent reticular formation (Fig. 2A), in the red nucleus (Fig. 2B) and in the pyramidal layers of the somatosensory cortex (Fig. 2C). Labelled cells could also be seen in the nucleus gigantocellularis, the dorsal column nuclei and the Edinger-Westphal nucleus. These cells correspond to those at the

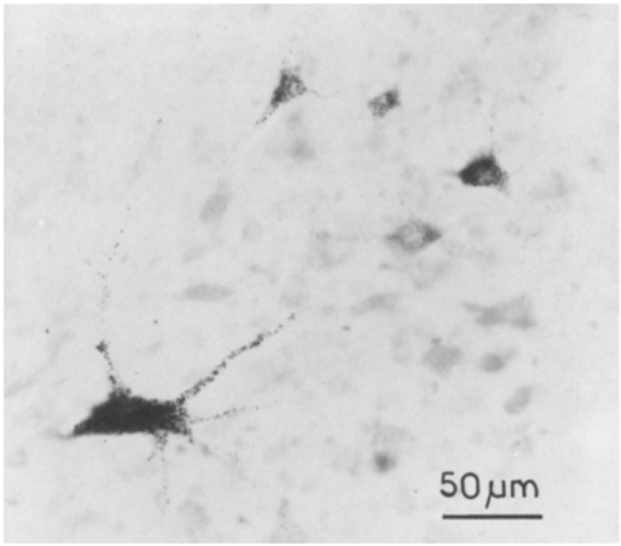

Fig. 2. Examples of lamina VIII cells in the cervical enlargement after lumbar injection of protein-gold complex. Bright field illumination. Counterstained with neutral red

origin of the brainstem and cortico-spinal projection systems. These results are particularly convincing since the injection was made at lumbar levels and was very restricted (total rostrocaudal extent of 1.5 mm).

Spinal motoneuronal retrograde labeling was also observed after tracer injection in either the gastrocnemius muscle or the sciatic nerve. In this latter case, however, the retrograde transport only resulted in few labeled motoneurones. The incorporation of the tracer after injections in bundles of fibers demonstrates that the protein-gold complex can be taken up by injured axons.

Sensitivity. To assess the sensitivity of the silver technique we counted the labeled cells in the red nucleus after tracer injections at lumbar levels. The numbers of labeled cells in adjacent sections were compared after silver intensification or TMB histochemistry in animals perfused with 3% glutaraldehyde, 4% paraformaldehyde or 4% periodate-lysine-paraformaldehyde. The number of retrogradely labeled cells was consistently higher (two to three folds) with the GSI method when the animal was perfused with fixative containing high paraformaldehyde. The fact that large number of retrogradely labeled neurons are found in paraformaldehyde perfused animals (even when the injection site was small) is of a particular value if this technique has to be used in conjunction with immunohistochemistry (see below).

Specificity. Of critical importance is the question of restriction of the marker to projection neurons. The labeling of Fig. 2 and 3 indicates that the marker did not leave the labeled cells since only structures known to project to the spinal cord were labeled. The location of this labeling precisely paralleled that observed in adjacent control sections reacted with TMB histochemistry. To ascertain that the silver reaction was related specifically to gold staining and not to degenerative processes which might lead to a perikaryal argyrophillic reaction (Gallyas 1982) we unilaterally injected the somatosensory cortex, made a lesion in the homologous part of the contralateral cortex and checked labeling in both ventrobasal complexes. Retrograde labeling was only observed on the injected side; thus the silver reaction specifically localizes gold particles.

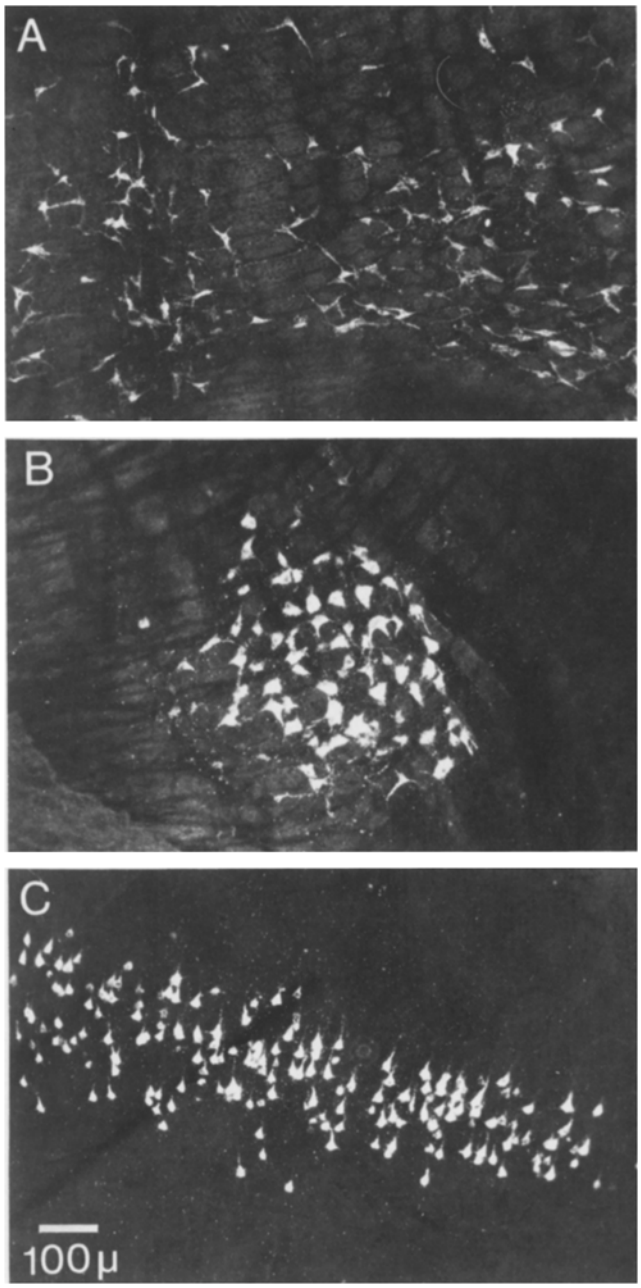

Fig. 3A–C. Retrograde cell labeling after a bilateral spinal injecton of WGA*HRP-gold complex at lumbar level. **A** Brain stem (nucleus raphé magnus and nucleus gigantocellularis) **B** Red nucleus **C** Pyramidal layer of the somatosensory cortex. Dark field illumination

Use in conjunction with immunohistochemistry. Figure 4 shows labeled cells as they appeared in the raphe magnus and adjacent structures following a bilateral spinal injection of the lumbar cord. The tissue was first processed with GSI and second for the immunohistochemistry. Two groups of cells were easily recognized in bright field illumination. Serotonin-immunoreactive cells that project to the spinal

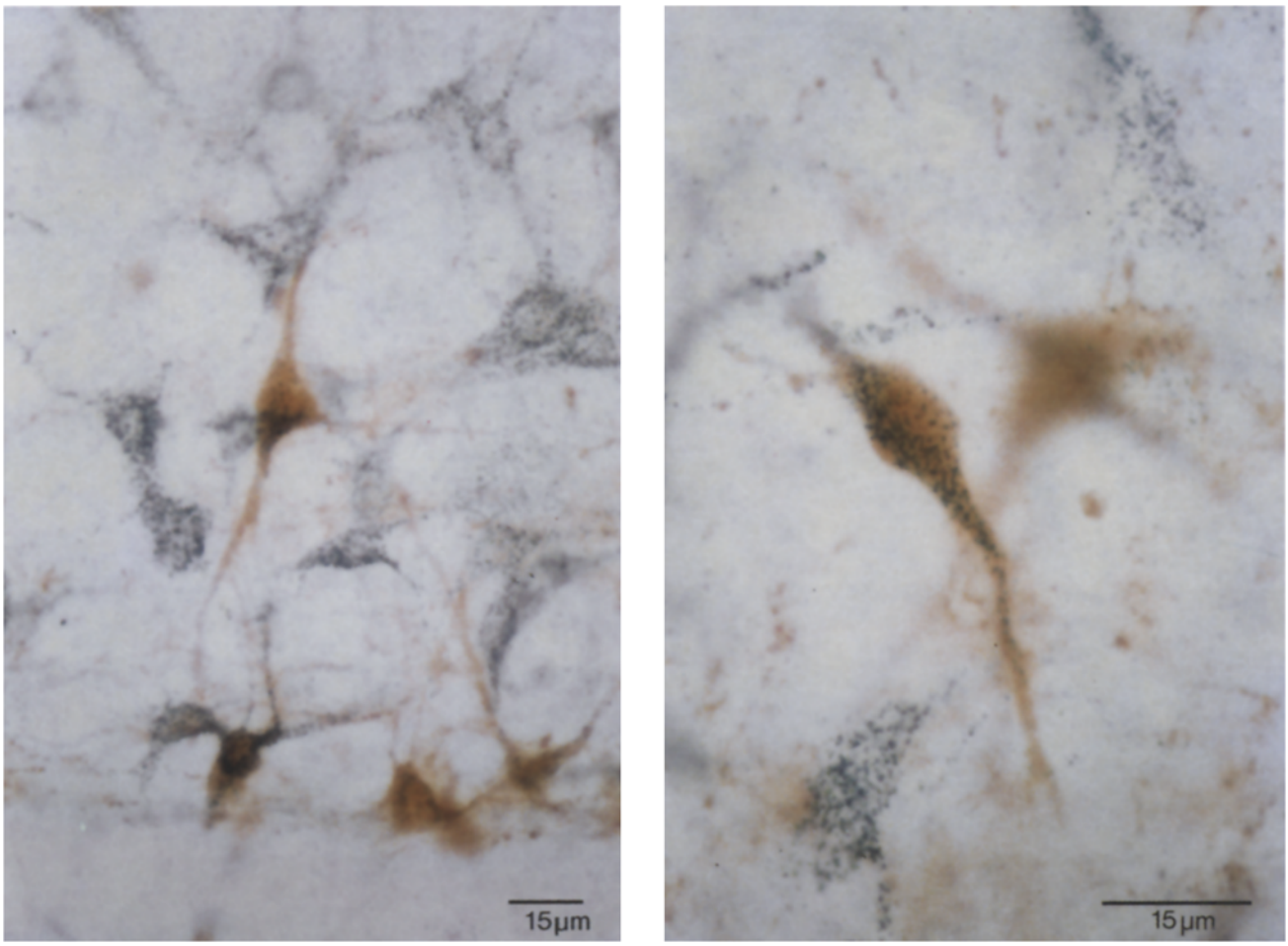

Fig. 4. Gold silver localization of retrogradely labeled cells after injection of protein-gold complex in the lumbar spinal cord combined with serotonin immunohistochemistry. Retrogradely labeled neurons contained only silver deposit (*grey dots*); double labeled cells contained grey dots on reddish brown background. Bright field illumination

cord exhibited reddish-brown stain as well as distinct round, black granules. These projecting cells constituted the large majority of the serotonergic cells. In fact, there were few nonprojecting 5HT-labeled cells. Other projecting cells contained only round, black granules that cover the entire cell bodies and proximal dendrites.

Anterograde transport

After tracer injections (0.3 to 1.0 µl) were administered in the somatosensory cortex or in the dorsal column nuclei anterograde transport of the protein-gold complex was never demonstrated in the pyramidal tract or the ventrobasal complex, respectively. Although TMB reaction product could be seen the silver labeling could not be detected. Similar observations were done in both the lateral geniculate body and the superior colliculus after tracer injection in the eye. Similarly the transganglionic transport of the protein-gold complex could not be demonstrated. Thus although the protein is transported in an anterograde fashion, the gold is apparently dissociated. Consequently, in the concentration range we used (2.5 to 5.0%), coupling gold to protein seems to produce a tracer that is specific for retrograde tracing.

Discussion

The present study demonstrates that gold particles coupled to protein can be very useful in retrogradely tracing neural connections at the light microscopic level when visualized by silver intensification (the gold silver intensification method or GSI method). The fact that gold coupling did not prevent retrograde transport of WGA*HRP is consistent with the observation of Schwab and Thoenen (1978) who described transport of gold coupled to tetanus toxin. The use of WGA*HRP as the protein probe however allowed us to compare the GSI method with the HRP histochemistry using tetramethylbenzidine as a chromogen (Mesulam 1982). We found the GSI method to be reliable, highly specific and sensitive, even after fixatives with a high percentage of paraformaldehyde. The sensitivity of this protein-gold procedure is related to several parameters including both the ability of and the specificity with which the terminals take up the protein-gold complex and transport it to the cell body. It is not known if coupling gold to protein substantially affects the incorporation of this latter by neural elements. The anterograde studies suggest that the gold is dissociated from the WGA*HRP after injection, perhaps through a degradative mechanism in the cell body.

This could result in trapped gold particles even though the WGA*HRP is still capable of being transported. Whether such a dissociation also occurs after retrograde transport is not known.

We believe that coupling gold to proteins offers a variety of possibilities in neuroanatomical retrograde studies. In addition to its use as a retrograde cell marker for electron microscopy (Lee and Menétrey 1984) it can be used for light microscopy after silver intensification. In this latter case, silver intensification can be done in conjunction with immunohistochemical procedures to demonstrate projections of cytochemically characterized pathways. Detection of double labeled cells is unequivocal. The procedure has several advantages over previously described methods (Bowker et al. 1983; Hockfelt et al. 1979; Rye et al. 1984). First, there is a high sensitivity in demonstrating retrograde labeling in tissue treated with fixatives that contain high percentages of parafomaldehyde. The latter usually reduces the sensitivity of retrograde labeling when HRP is detected by enzyme histochemistry (Malmgren and Olsson 1978) particularly if diaminobenzidine (Bowker et al. 1983) is used. In contrast paraformaldehyde is often essential for immunohistochemical studies (e.g. for peptides). Second, it is very simple to detect the silver granules on the reddish-brown background of immunoreactive cells with bright field illumination. Third, there is a complete absence of background silver labeling even after prolonged incubation. In contrast, in procedures based on differential horseradish peroxidase histochemistries (HRP as a retrograde cell marker and as a substrate for the unlabeled antibody peroxidase-antiperoxidase method) prolonged incubation in chromogens (either TMB or DAB) will produce background label indistinguishable from that in retrogradely labeled cells. Fourth, gold particles, whether enhanced by silver or not, are easily detected at the electron microscopic level since they are distinguishable from electron dense DAB reaction product. Thus EM colocalization of retrograde labeling and immunohistochemical product is possible. We are presently evaluating such possibilities.

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