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J Histochem Cytochem 2011 59: 258 originally published online 12 January 2011
DOI: 10.1369/0022155410397998

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HCS
Official Journal of The Histochemical Society

Journal of Histochemistry & Cytochemistry 59(3) 258–269

DOI: 10.1369/0022155410397998
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New Silver-Gold Intensification Method of Diaminobenzidine for Double-Labeling Immunoelectron Microscopy

Endre Dobó, Virág T. Takács, Attila I. Gulyás, Gábor Nyiri, András Mihály, and Tamás F. Freund

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Summary

The available methods for double-labeling preembedding immunoelectron microscopy are highly limited because not only should the ultrastructure be preserved, but also the different antigens should be visualized by reaction end products that can be clearly distinguished in gray-scale images. In these procedures, one antigen is detected with 3,3′-diaminobenzidine (DAB) chromogen, resulting in a homogeneous deposit, whereas the other is labeled with either a gold-tagged immunoreagent, or DAB polymer, on the surface of which metallic silver is precipitated. The detection of the second antigen is usually impeded by the first, leading to false-negative results. The authors aimed to diminish this hindrance by a new silver intensification technique of DAB polymer, which converts the deposit from amorphous to granular. The method includes three major postdevelopmental steps: (1) treatment of nickel-enhanced DAB with sulfide, (2) silver deposition in the presence of hydroquinone under acidic conditions, and (3) precious metal replacement with gold thiocyanate. This new sulfide-silver-gold intensification of DAB (SSGI) allows a subsequent detection of other antigens using DAB. In conclusion, the new technique loads fine gold particles onto the DAB deposit at a very low background level, thereby allowing a reliable discernment between the elements stained for the two antigens at the ultrastructural level. (J Histochem Cytochem 59:258–269, 2011)

Keywords

immunohistochemistry, double-labeling, silver intensification, gold toning, electron microscopy

To date, 3,3′-diaminobenzidine (DAB) (Graham and Karnovsky 1966) is by far the most frequently used chromogen for preembedding horseradish peroxidase–based immunohistochemistry, the use of which results in deposition of a polymer (DABp). Moreover, the reddish brown color of the DABp can be converted to darker hues of blue by addition of nickel or cobalt (Adams 1981; Hsu and Soban 1982), permitting double immunolabeling at the light microscopic level (Wouterlood et al. 1987).

Currently available double-labeling immunoperoxidase methods at the electron microscopic level feature an unequivocal distinction of the two separate antigen-containing sites by a post-immunohistochemical loading of the DABp with metallic silver for one antigen, which is followed by the detection of the other antigen with DABp alone. By this sequential staining, the first antigen-containing sites show heterogeneous granular deposits, whereas the elements labeled for the second antigen display homogeneous insoluble substances, which can be distinguished in the monochromatic electron micrographs. This changing of the appearance of the DABp can be achieved by means of many techniques, collectively called silver intensification. These techniques employ the catalytic property of DABp, which

Received for publication Jun 15, 2010; accepted for publication Oct 26, 2010

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leads to the reduction of silver ions to metallic silver (argyrophilia) by formaldehyde (Gallyas et al. 1982) or ascorbic acid (Merchenthaler et al. 1989) under alkaline or acidic conditions, respectively. The former was successfully applied not only for light but also for electron microscopic immunohistochemistry.

Yet, in our hands, use of variations of these techniques failed to provide satisfactory results in numerous experiments demanding double-labeling ultrastructural studies of brain samples. Although the first antigen was always detected as expected, the second antigen could often be visualized only at much lower sensitivity levels judged on the basis of the single-labeling experiments of the same area with the same antibody. The excessive number of false-negative structures could be due to (1) the endogenous silver-binding capacity of the tissues (Gallyas 2008), which may partially mask the second antigen and hinder spatial access of the immunoreagents to the epitopes, or (2) the side effects of pretreatments, which eliminate the nonspecific silver binding of the tissue (Gallyas et al. 1982).

Failure of the adequate detection of a second antigen, using the conventional silver intensification techniques, prompted us to seek another way of conversion of the homogeneous reaction end product to a granular one for the purpose of double-labeling immunoelectron microscopy, which would not rest on the argyrophilia of DABp. Instead, we conceived an expedient for using the argyrophilic property of the metal sulfides (Danscher and Zimmer 1978; Timm 1958), the endogenous form of which is absent from the brain. This approach seemed to be beneficial because special oxidative substances such as copper-catalyzed hydrogen peroxide (Gallyas and Merchenthaler 1988; Gallyas and Stankovics 1987) or thio-blocking pretreatments such as thioglycolic acid (Gallyas et al. 1982) or cysteine (Smiley and Goldman-Rakic 1993) could be avoided.

Our new technique incorporates three principal innovations. First, a powerful argyrophilic catalyst was formed from nickel ions chelated within DABp by neutralized sulfide treatment. Second, a modified gum arabic physical developer of Danscher (1981) was applied subsequently. Third, metallic silver was quantitatively replaced by gold(I) thiocyanate in a ratio presumed to be 1-to-1, unlike in the ratio of 1-to-3 when gold toning is carried out by gold(III) chloride (e.g., chloroauric acid).

This new sulfide-silver-gold intensification (SSGI) technique may provide significant advantages when compared with the other techniques. (1) It is the fastest silver intensification protocol available for DAB. (2) It does not require any pretreatment to suppress the endogenous argentaffin or argyrophilic properties of the brain samples. (3) It causes lower background than the corresponding other silver intensification methods, whereas the modification of the appearance of DAB is apparently as effective as in those. (4) It may provide better ultrastructure than other silver intensification techniques because it does not include solutions, which would impair tissue quality. Consequently, this may be the method of choice for silver intensification techniques in double-labeling immunohistochemical investigations when the two target proteins are known to be present in distinct cellular elements.

Here, we provide a detailed description of the new procedure and demonstrate its power by single- and double-labeling using antibodies against two neuronal (parvalbumin [PV] and cannabinoid receptor type 1 [CB_1]) and a glial (glial fibrillary acidic protein [GFAP]) marker on brain samples at both light and electron microscopic levels.

Materials and Methods

Animals

All experimental procedures were conducted according to the directive of the European Council (86/609 EEC) and to the Hungarian Animal Act. Specific approval of care and use of animals was obtained in advance from the Faculty Ethical Committee on Animal Experiments (University of Szeged).

Tissue Preparation

Male mice (C57Bl/6J, weighing 14–23 g, 30–52 days old) were deeply anaesthetized with an intraperitoneal injection of an anesthetic mixture, which was composed of 0.83% ketamine, 0.17% xylazine-hydrochloride, 0.083% promethazinium-chloride, 0.00083% benzethonium-chloride, and 0.00067% hydrochinonum, at a dose of 0.06 ml/10 g body weight. The animals were transcardially perfused with saline for 1 min, followed by approximately 200 ml fixative containing 4% formaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 30 min. Unless otherwise indicated, all reagents and materials used in this work were obtained from Sigma-Aldrich (St. Louis, MO).

The brains were removed from the skull and postfixed for 1 hr at room temperature. The samples were cut on a vibratome at 60 µm. The sections were rinsed in PB, then cryoprotected in buffered sucrose (10% for 30 min; 30% for 1–2 days) and stored at –70C until further processing.

Pretreatment of Sections

The sections were frozen by liquid nitrogen and thawed in 30% sucrose three times (Somogyi and Takagi 1982) to facilitate the penetration of immunoreagents. Sucrose was removed with six rinses of PB, 10 min each. Endogenous peroxidase-like activity of occasionally trapped red blood cells was blocked by 1% hydrogen peroxide in 0.05 M Tris-buffered saline (TBS; pH 7.6) for 15 min. After several rinses in TBS, the samples were pretreated with 10% normal goat or 10% horse serum in TBS for 1 hr. When a

Table 1. Combinations and Concentrations of Antibodies and Reagents Used in Experiments Shown in Figures 1 to 4

Labeled Antigens (First-Second)	First Primary Antibody	First Secondary Antibody	First Tertiary Reagent	First Labeling	Second Primary Antibody	Second Secondary Antibody	Second Tertiary Reagent	Second Labeling	Light Microscopic Figures	Electron Microscopic Figures
0-0 (control)		Biotinylated goat anti-rabbit IgG[a] (1/600)	Elite ABC[a] (1/500)	AuDABp		anti-Mouse ImmPRESS[a] (1/4)		NiDABp	2D	2D inset
PV-0	Rabbit anti-PV[b] (1/5000)	Biotinylated goat anti-rabbit IgG[a] (1/600)	Elite ABC[a] (1/500)	NiDABp					1A	
				AuDABp					1B	
GFAP-0	Mouse anti-GFAP[c] (1/2000)	anti-Mouse ImmPRESS[a] (1/4)		NiDABp					1C, 2A1	
				AuDABp					1D, 2A2	
GFAP-PV	Mouse anti-GFAP[c] (1/2000)	anti-Mouse ImmPRESS[a] (1/4)		AuDABp	Rabbit anti-PV[b] (1/5000)	Biotinylated goat anti-rabbit IgG[a] (1/600)	Elite ABC[a] (1/500)	NiDABp	2A3, 2B	3A
PV-GFAP	Rabbit anti-PV[b] (1/5000)	Biotinylated goat anti-rabbit IgG[a] (1/600)	Elite ABC[a] (1/500)	AuDABp	Mouse anti-GFAP[c] (1/2000)	anti-Mouse ImmPRESS[a] (1/4)		NiDABp	2C	3B, 3C
CB_1-PV	Rabbit anti-CB_1[d] (1/5000)	Biotinylated goat anti-rabbit IgG[a] (1/600)	Elite ABC[a] (1/500)	AuDABp	Mouse anti-PV[e] (1/8000)	anti-Mouse ImmPRESS[a] (1/4)		DABp		4

PV, parvalbumin; CB_1, cannabinoid receptor type 1; GFAP, glial fibrillary acidic protein.
[a]Vector Laboratories (Burlingame, CA).
[b]Baimbridge and Miller (1982).
[c]Chemicon (Temecula, CA).
[d]Bodor et al. (2005).
[e]Swant(P.O.Box 2660. CH-6501 Bellinzona, Switzerland).

concentration is described as a percentage, we imply that the solution was made using the weight-in-volume method in all cases (w/v %).

Single-Labeling Immunohistochemistry

The sections were incubated in primary antibody solution (Table 1; rabbit anti-PV, kindly donated by Dr. Kenneth G. Baimbridge [Baimbridge and Miller 1982], at a dilution of 1/5000; mouse anti-GFAP [Chemicon, Temecula, CA] at a dilution of 1/2000; or rabbit anti-CB_1, kindly donated by Dr. Ken Mackie [Bodor et al. 2005] at a dilution of 1/5000) at 4C for 2 days. The primary antibody solution was supplemented with 0.05% sodium azide. The unreacted antibodies were removed with six changes of TBS for 1 hr. Then, the sections were incubated in biotinylated goat anti-rabbit IgG (diluted at 1/600; Vector Laboratories, Burlingame, CA) for 4 hr at room temperature or anti-mouse ImmPRESS (diluted at 1/4; Vector Laboratories) overnight at 4C. After six changes of TBS for 1 hr, the samples that were reacted with the biotinylated antibody were incubated in Elite ABC reagent (1/500; Vector Laboratories) overnight at 4C.

After the horseradish peroxidase–conjugated reagents, the sections were washed first with TBS four times for 45 min, then with PB three times for 15 min, which was followed by preincubation in a solution containing 16 ml PB + 1 ml 2.5 mg/ml DAB + 6.66 mg ammonium chloride + 0.8 ml 0.05 M nickel ammonium sulfate hexahydrate for 20 min. The solution has to be filtered (e.g., on Reanal MN751 filter paper) before use. Then, 7.5 µl 0.03% hydrogen peroxide was added to 1 ml preincubation solution containing the sections, and the enzyme reaction was run for 30 to 60 min, stopped with PB, and then washed three times for 20 min. Those sections that were to be processed for double-labeling immunohistochemistry were posttreated with 1% hydrogen peroxide in PB for 10 min and then washed in PB (three times 10 min) and TBS (three times 10 min).

Sulfide-Silver-Gold Intensification (SSGI)

The sections were treated as follows:

1. Sulfide treatment for 5 min. The sodium sulfide solution was composed of 25 ml 0.2 M phosphate buffer (pH 7.4) and 0.2 ml stock solution of sulfide, the latter of which was prepared by dissolving 13.9 g sodium sulfide nonahydrate ($Na_2S{\cdot}9H_2O$, Mw: 240) in 20 ml water. (This sulfide solution, if stored tightly closed in the refrigerator, can be kept for some weeks without appreciable change.)
2. Rinse in 2% sodium acetate hydrate (SA) for four times 1 min. The sections were transferred into other vials at the last change of SA.
3. "Silver pretreatment" for 2 min. The last SA change was replaced by a solution, which was composed of one part 0.2% silver acetate ($AgC_2H_3O_2$; Fluka, St Louis, MO) and one part 2 M citrate buffer (CB; pH: 3.5–4), which was prepared by dissolving 23.5 g trisodium citrate dehydrate and 25.5 g citric acid monohydrate in 100 ml water (Danscher and Moller-Madsen 1985). This buffer can be kept at room temperature for about 2 weeks. The silver pretreatment is optional.
4. "Silver treatment" for 10 min. After either the second or the third step of the SSGI procedure, the sections were treated with an operating silver-containing solution, usually termed physical developer, which was always prepared right before use from three stock solutions: two parts gum arabic (GA; 50 g GA was dissolved in 100 ml water on a stirring hot plate, aliquoted, and stored in a deep freezer, where the solution can be kept indefinitely), one part freshly prepared 0.5% hydroquinone in CB, and one part 0.2% silver acetate. The silver treatment was carried out at room temperature or by using ice-cold solutions, under mild agitation at dimmed light.
5. Rinse in SA twice briefly.
6. Gold toning for 10 min. The gold-containing solution was freshly prepared by slow addition (drop by drop) of 0.2 ml 2% chloroauric acid ($HAuCl_4$) to 8 ml 1.5% potassium thiocyanate (KSCN) under vigorous vortex mixing at room temperature. At each drop, the solution turns red for seconds, then it returns translucent, and then it is ready to use.
7. Rinse in SA once briefly.
8. Thiosulfate fixation for 5 min. For removal of the ionic gold from the tissue, the sections were treated with 3% sodium thiosulfate pentahydrate.
9. Rinse in SA three times briefly.

Note for the procedure: From the last rinse in SA until the very end of the intensification procedure, all the glassware in contact with the sections has to be precleaned with a solution, usually referred to as Farmer's solution, which consists of nine parts 10% sodium thiosulfate pentahydrate ($Na_2S_2O_3{\cdot}5H_2O$) and one part 10% potassium ferricyanide ($K_3[Fe(CN)_6]$) for 30 min (Danscher and Moller-Madsen 1985). The Farmer's solution lasts some hours at room temperature. The glassware is washed with deionized water thoroughly.

Double-Labeling Immunohistochemistry

Although our new SSGI method has some advantages in comparison to previous single-labeling methods, it proves its greatest advantage when used in double-labeling experiments, before a second round of immunolabeling. The experiments were carried out according to the description in Table 1. Each step was performed similar to those in single-labeling experiments. In the CB_1-PV double-labeling experiment, the second immunperoxidase reaction was developed using DAB as a

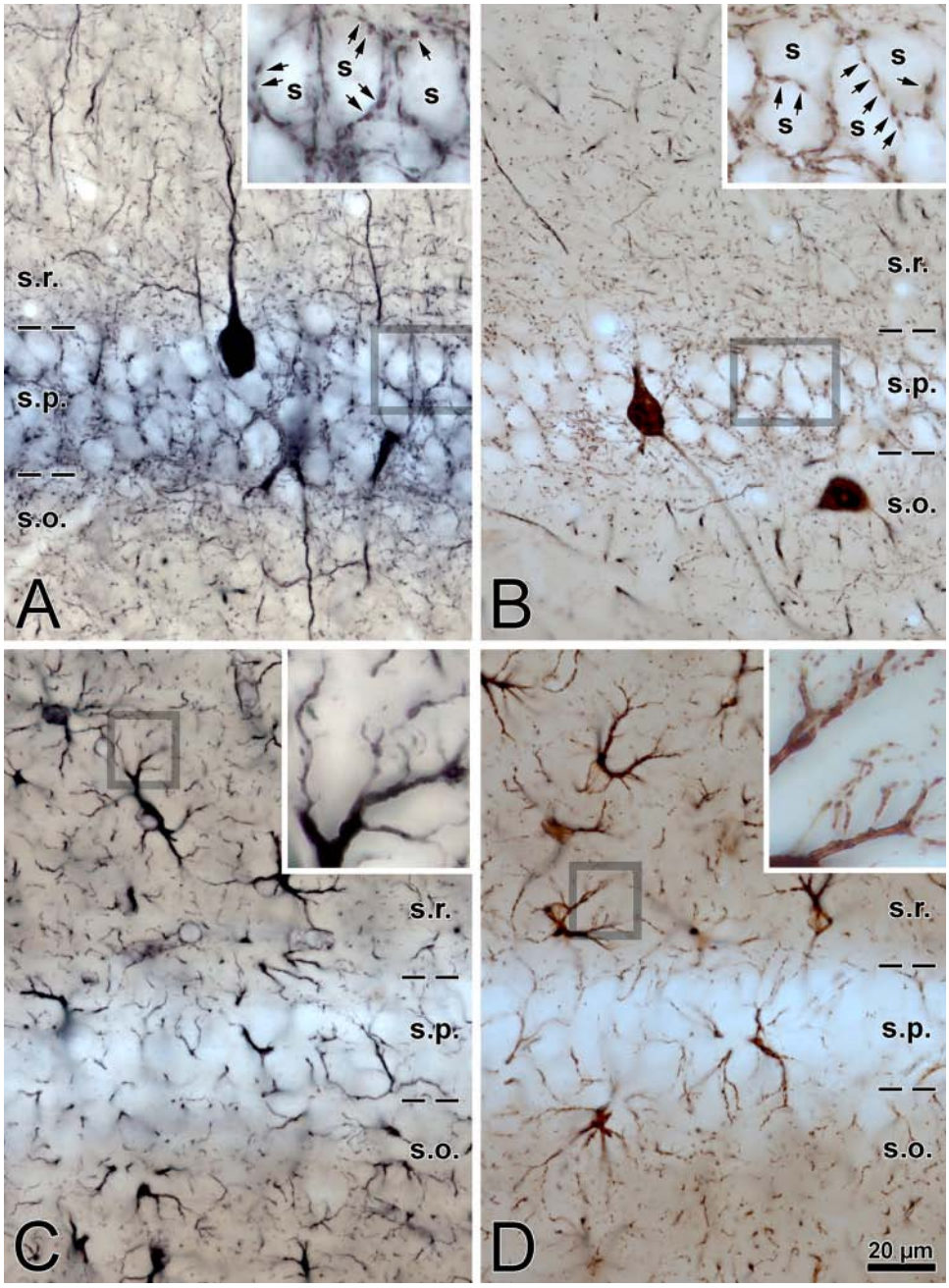

Figure 1. Color conversion of the primary reaction end product by the sulfide-silver-gold intensification (SSGI) technique. Light micrographs of the mouse hippocampus immunostained for parvalbumin (PV) (A, B) or glial fibrillary acidic protein (GFAP) (C, D). The antigen-containing sites were developed with 3,3′-diaminobenzidine (DAB) in the presence of nickel ions (A–D). Thereafter, the sections were processed according to the SSGI protocol (B, D). Numerous PV-immunoreactive processes were found throughout the hippocampus, many of which established basket-like arrays of boutons around the somata (s) of immunonegative pyramidal neurons. At higher magnification (insets, framed areas enlarged), the axon terminal (arrows) are better seen to be apposed to the perikarya. Application of the SSGI technique changed the color of the DABp significantly (B), but the overall staining intensity remained largely unaffected. Abundant astrocytic processes were revealed with NiDABp (C), the color of which was altered by the SSGI technique (D). s, soma; s.o., stratum oriens; s.r., stratum radiatum; s.p., stratum pyramidale.

chromogen. These sections were placed in a preincubation solution containing 1 ml 2.5 mg/ml DAB + 9 ml Tris buffer (pH 7.6) for 20 min, and then 5 µl 1% hydrogen peroxide was added to 1 ml preincubation solution containing the sections. We suggest that the first and the second immunoreactions are carried out consecutively, with the second primary antibody

applied only after finishing the SSGI development of the first one, to avoid the intensification having any negative effect on the second primary antibodies.

Light and Electron Microscopy

Sections were washed in PB and treated with 1% OsO_4 dissolved in PB for 1 min and then in 0.5% OsO_4 for 15 min at 4C. Samples were dehydrated in an ascending series of ethanol and finally cleared in acetonitrile, then flat-embedded in Durcupan (ACM; Fluka). During dehydration, the sections were stained with 1% uranyl acetate in 70% ethanol for 20 min.

Selected areas were reembedded and resectioned using an ultramicrotome (Leica, Wetzlar, Germany). Series of ultrathin sections (60 nm thick) were collected on Formvar-coated single-slot grids and counterstained with lead citrate (Ultrostain; Leica). Photos were taken with a Zeiss Axioplan2 light microscope (Carl Zeiss, Stuttgart, Germany) equipped with a DP70 Olympus camera or with a Hitachi 7100 electron microscope equipped with a Veleta CCD camera (Olympus Soft Imaging Solutions, Germany).

For light microscopic images (Fig. 1A–C and Fig. 2B–D), Image-Pro Express 6.0 software (Media Cybernetics, Silver Spring, MD, USA) was used to overlay and merge photos taken at consecutive focal planes of the same field of view. Figures were also edited using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA) to adjust brightness and contrast of the whole image.

Results

Light Microscopy

Single-labeling immunohistochemistry for PV and GFAP resulted in immunoreactive (IR) neurons and glial cells, as described by others (PV-IR neurons [Katsumaru et al. 1988], GFAP-IR astrocytes [Kosaka and Hama 1986]). In both cases, application of nickel in the developing solution rendered DABp bluish-black (NiDABp), which visualized not only the cell bodies but also the thin processes of both cell types at a very low background (Fig. 1A,C and Fig. 2A).

The sulfide treatment, the first major step of our silver intensification protocol, changed the color of the NiDABp to pale brown, fading the staining intensity greatly (not shown), even below the level obtained without addition of nickel to the simple DAB-containing developer without SSGI.

Immediately before the silver treatment, the sections were shortly soaked in a citrate-buffered silver ion-containing solution to facilitate the availability of free silver ions in the deeper parts of the free-floating specimen. In this solution, the sections darkened slightly. However, good results were also obtained when the silver pretreatment was omitted from the procedure, especially at the light microscopic level. All the images presented in this report, however, were taken of preparations that were "silver pretreated."

Application of silver ions in the post-DAB step—called "silver treatment" in Materials and Methods—retrieved the visibility of the cells nearly to the level of the NiDABp, depending on the duration (5–15 min) and, to a lesser extent, on the temperature (ice-cold to ambient) of the development. The ratio of the components was adjusted to allow a 10-min period of the treatment for the vast majority of the cases, which allowed good reproducibility and suitable color conversion. Some of the silver solutions tested (with higher concentrations and/or altered ratios of chemicals) permitted much shorter developmental times, but those tended to produce less uniform color conversion; heterogeneous staining quality, especially toward the middle portion of the sections; and concealment of fine neuronal and glial processes, close to the surface of the tissue specimens. The recommended developer solution, detailed in Materials and Methods, remains clear at room temperature on a generally illuminated bench for at least 20 min.

The third principle step ("gold toning"), which involves the use of freshly prepared gold solution that likely contains gold(I) ions reduced from gold(III), slightly increased the staining intensity. In addition, it also deepened the hue of the immunolabeled profiles by replacement of atomic silver deposits within the DABp with the other precious metal (gold-substituted, silver-intensified DABp: AuDABp).

The "thiosulfate fixation" step did not result in any noticeable change in the staining. Yet, application of thiosulfate is considered a necessary step to remove the nonspecific tissue-bound ionic forms of either silver or gold from the tissue samples, which later could be also reduced to metallic forms; thereby, the signal-to-noise ratio would fall dramatically, or even the staining would be spoiled.

In this methodological study, the staining intensity was not a major concern; rather, the distinct change of the electron microscopic appearance of the primary immunohistochemical reaction end product allowed the use of the same DAB chromogen for the second marker. Here, the experimental parameters of the recommended SSGI technique were set to convert the color of DABp without a net increase in staining intensity (Fig. 1B,D). Nevertheless, the SSGI produced a better visual contrast of the immunolabeling because the intensity of the dark cloud-like background decreased noticeably (Fig. 2A1,A2). In the brown immunopositive elements, occasional black granular deposits appeared. The final light-microscopic visibility of the immunopositive cells, before and after the SSGI technique, was similar, whereas the colors of the reaction end products were distinct (compare Fig. 1A,B and Fig. 1C,D).

In two sets of control staining experiments, the SSGI method was used on sections either without prior immunohistochemistry or only without the primary antibodies. In both of these negative control experiments, the tissue samples remained unstained. In addition, the tissue remained

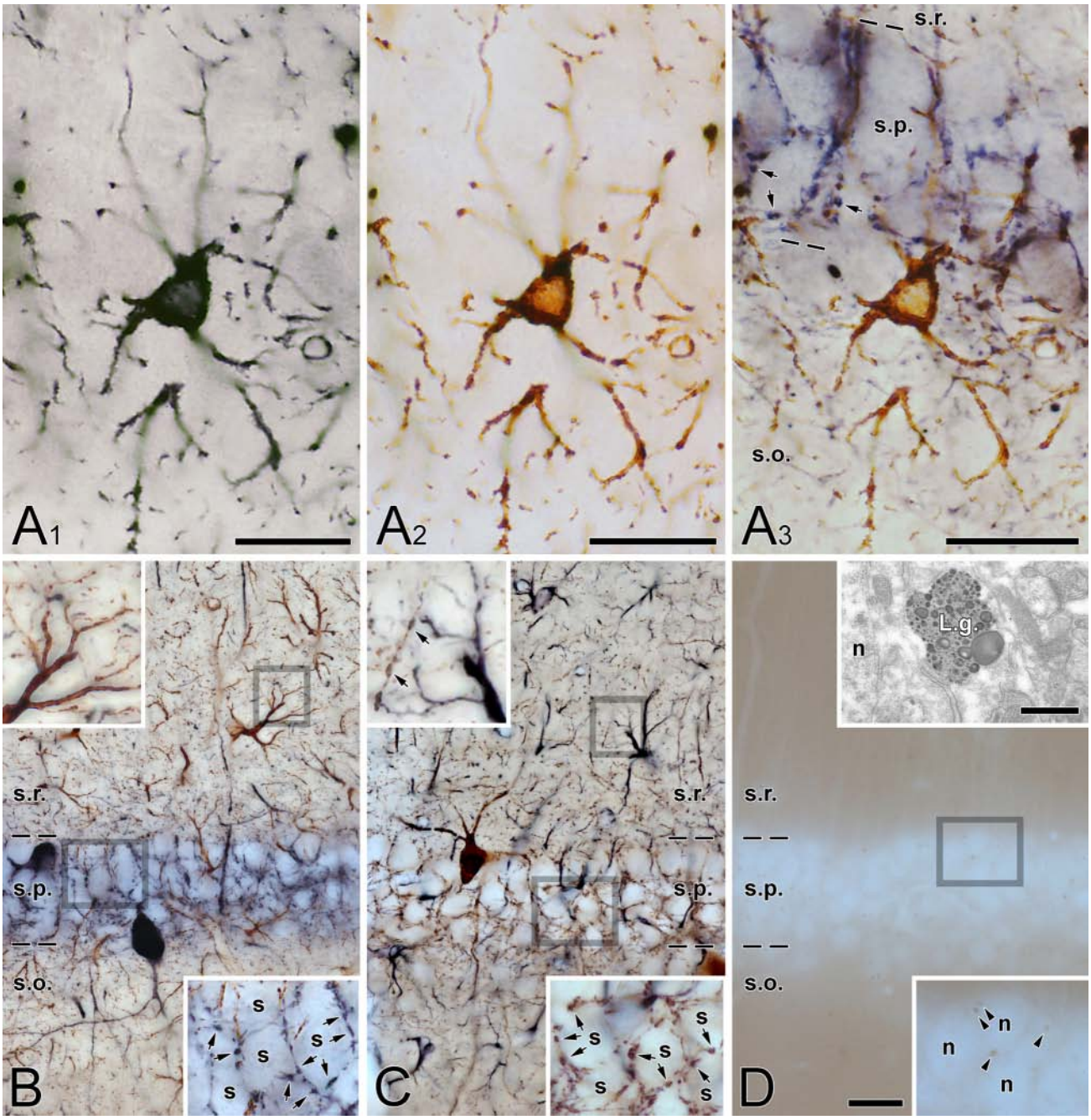

Figure 2. Application of the sulfide-silver-gold intensification (SSGI) technique in immunocytochemical double-labeling in vibratome sections from the mouse hippocampus. Double-labeling for glial fibrillary acidic protein (GFAP) and parvalbumin (PV) in A–C; control staining (without primary antibodies) in D. The first antigen was revealed with NiDABp, and then the sections were treated with the SSGI technique, resulting in dark reddish brown AuDABp. Afterwards, the second antigen was visualized with dark blue NiDABp. SSGI is demonstrated in the series A. The same section is immunostained first for GFAP (A1), then treated with the SSGI technique (A2), and thereafter immunostained for PV (A3). Arrows in A3 show PV-positive perisomatic boutons. The A1 and A2 micrographs were taken from the wet section mounted and temporarily coverslipped without dehydration; therefore, the focal planes could not be identical. The order of immunostainings for the antigens was indifferent to the quality of the stainings (B, C). The first antigens were GFAP in B and PV in C. Both neural tissue markers are clearly visible and distinguishable in both versions of double-labeling. The colors are best distinguished at higher magnification (see insets magnified from enframed areas in B and C). Arrows indicate labeled axonal varicosities. In the control section (D), diffuse staining and occasional pale puncta were found (lower right inset, slightly magnified from the enframed area). These puncta turned out to be lipofuscin granules in the electron microscope (upper right inset). l.g., lipofuscin granules; n., nucleus; s, soma; s.o., stratum oriens; s.r., stratum radiatum; s.p., stratum pyramidale. Bars in A-D = 20 μm, bar in the upper right inset of D = 0.5 μm.

translucent and retained its original size and shape during the entire procedure, unlike in the case of other silver intensification techniques of DAB (see Discussion for references). This indicated no more additional visible denaturing of the proteins than brought about by the primary aldehyde-based perfusion fixation (Fig. 2D shows the control without primary antibody; the other control was identical).

When the SSGI was applied in combination with immunohistochemistry, only a very slight, negligible background was found in every case.

Double-labeling immunohistochemistry was performed in a consecutive way, in which the detection of the first antigen was developed with Ni-enhanced DAB (Fig. 2A1); then the color was converted with the SSGI technique, resulting in AuDABp (Fig. 2A2); and finally the sites of the second antigen were revealed with Ni-enhanced DAB again (Fig. 2A3). In this way, the first antigen appeared in hues of brown, whereas the other antigen was stained in bluish-black, which could be distinguished from the previous color by the experienced eye. The color differences allowed distinction between the morphological elements with the separate neuronal and glial markers (Fig. 2B,C), even in the thin-caliber processes in most of the cases (insets in Fig. 2B,C).

Apparently, the order of immunostainings for the antigens (PV-GFAP vs GFAP-PV), used in this study to test the capacity of the SSGI method, was indifferent to the results (i.e., the labeling of the first antigen did not reduce the detectability of the second antigen).

No interference between the immunohistochemical treatments of the first and second detections was noticed. (1) The background of the double-immunolabeling was found not to exceed the virtual addition of background of the single-labeling experiments. This was also confirmed by means of omission of either one or both primary antibodies from the procedure for some sections, which were treated otherwise in the same way as those incubated in the presence of the primary antibodies (Fig. 2D). (2) The color of the first reaction end product was not modified (Figs. 2A3–C).

Electron Microscopy

Single-labeling immunohistochemistry for either PV or GFAP without subsequent silver intensification produced NiDABp, which appeared as homogeneous deposits in the ultrathin sections. Application of the SSGI method loaded variable-sized grains over the immunopositive elements. In all of the profiles, including those with the smallest noticeable amount of NiDABp, gold granules were also present. The penetration of the conversion was complete (i.e., gold granules were present even in ultrathin sections cut from the middle of the 60-µm-thick blocks). Careful ultrastructural investigation showed that unlabeled profiles did not contain precious metal. This was confirmed by negative experiments in which omission of the primary immunoreagents abolished the deposition of metallic gold particles (Fig. 2D).

In the ultrathin sections from the double-labeling free-floating brain samples, two types of end products were seen, which could be clearly distinguished (Figs. 3 and 4). One precipitation looked like DABp studded with roundish grains, and the other was the DABp alone. The order of detection of the neuronal and glial antigens did not affect the discernment between the markers.

As to the precipitation accuracy of the granules, that seemed to correspond to the localization accuracy of the original polymer. The final gold particles were usually confined to the cytoplasm limited by the protoplasmic and subcellular membranes. However, occasionally, some grains grew beyond the membrane (Fig. 3A and Fig. 4B), which, nevertheless, did not obscure the synaptic membrane.

Discussion

It is well known that the primary reaction end product of horseradish peroxidase, DABp, exhibits argyrophilia—that is, the polymer catalyzes the reduction of dissolved silver ions to submicroscopic metallic silver grains (also termed *nanocrystals*) (Danscher and Stoltenberg 2006) by dissolved organic molecules during an initial induction period. The grains possess autocatalytic capacity, by which the nanocrystals grow to a microscopic level (Gallyas 2008). The techniques based on this argyrophilia are referred to as silver intensification because metallic silver deposited on the DABp increases the visibility of the reaction end product.

Unfortunately, some tissue components may have at least as strong argyrophilia as DABp. Efforts have been made to enhance the argyrophilia of DABp (for references, see Newman and Jasani 1998) and to abolish that of the tissue proteins (for references, see Gallyas 2008). The former was usually achieved by development of tissue-bound peroxidase with DAB in the presence of nickel ions, which increases the power of the intensification procedures using formaldehyde at pH 10.5 (Gallyas and Merchenthaler 1988) or using ascorbic acid at pH 5.5 (Merchenthaler et al. 1989). The high pH, however, also enhanced nonspecific silver staining, which was abolished by means of rough oxidative pretreatment with copper sulfate and hydrogen peroxide (Gallyas and Stankovics 1987). This pretreatment, however, seriously damages the electron microscopic structure. Increasing the concentration of the protective colloid, tungstosilicic acid, sevenfold in the developer minimized the background (Przepiorka and Myerson 1986), and thus pretreatment was not needed at the expense of reduction in signal strength (unpublished observation, E.D.). The low pH condition supports the endogenous nonspecific silver staining to a much smaller extent than the high pH values; therefore, harsh pretreatments were not necessary because low pH causes less nonspecific sliver staining than high pH. Mullink et al. (1992) proved the ascorbic acid technique (Merchenthaler et al. 1989) to be satisfactory for light microscopic in situ hybridization. Interestingly, in the literature, we have not yet found

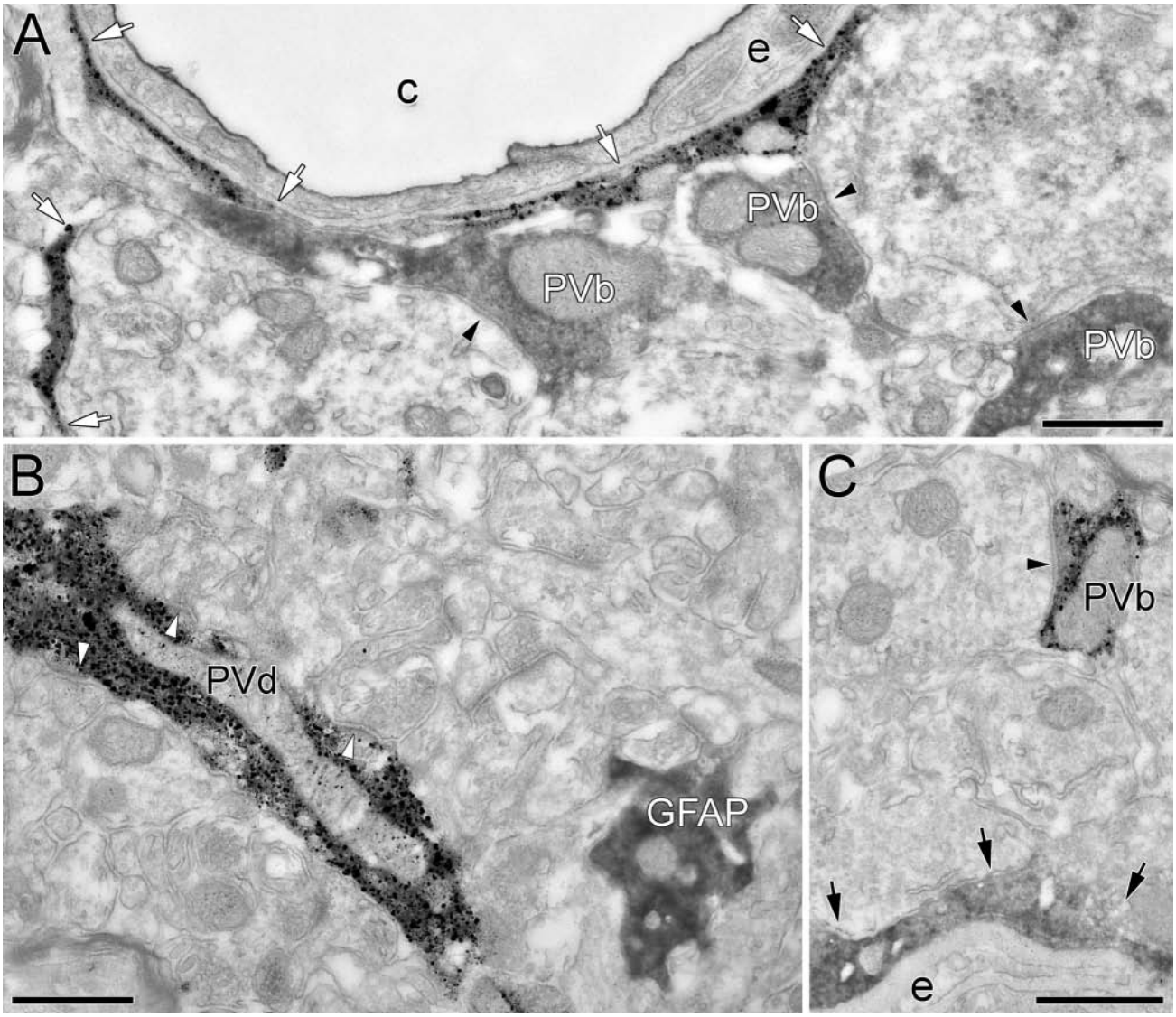

Figure 3. Clear distinction between the electron-dense NiDABp and AuDABp markers at the electron microscopic level in preembedding double-labeling immunohistochemistry. Electron micrographs were taken from ultrathin sections of mouse hippocampus. (A) Vibratome sections were stained first for glial fibrillary acidic protein (GFAP) by means of an immunoperoxidase method; treated with the sulfide-silver-gold intensification (SSGI) technique, resulting in granular precipitate; and then subsequently stained for parvalbumin (PV), developed with Ni-enhanced 3,3′-diaminobenzidine (DAB). White arrows demarcate fine GFAP–immunoreactive (IR) astrocytic processes, studded with gold particles. Well-preserved membrane (black arrowheads) of putative symmetrical synaptic active zones of PV-IR boutons (PVb) labeled by NiDABp. (B, C) Electron micrographs demonstrate the reverse order of double-labeling. First, PV-IR profiles are marked with punctate AuDABp, and then GFAP-IR structures are revealed with amorphous NiDABp. White arrowheads in B point to synaptic inputs onto PV-IR dendrites (PVd). Black arrows in C delineate GFAP-IR processes. c, capillary; e, endothelial cell. Bar = 500 nm.

references to this technique used in an immunoelectron microscopic study. The lack of this application may be tentatively explained by our observation that below pH 7, much less nickel ions are bound to DABp than at higher pH values, indicating that nickel ions chelated at higher pH would become mobile, and the freed nickel ions featuring variable oxidation states would cause foggy deposition of metallic silver.

Elimination of the background silver staining was accomplished most effectively by pretreatment with concentrated thyoglicolic acid (Gallyas et al. 1982). However, this substance at the recommended concentration heavily shrinks and distorts the sections, which may be reversible, but the impairment of the ultrastructure is permanent. This drawback was tried to be compensated by strong fixation (Liposits et al. 1982) and replacement of metallic silver with gold (Liposits

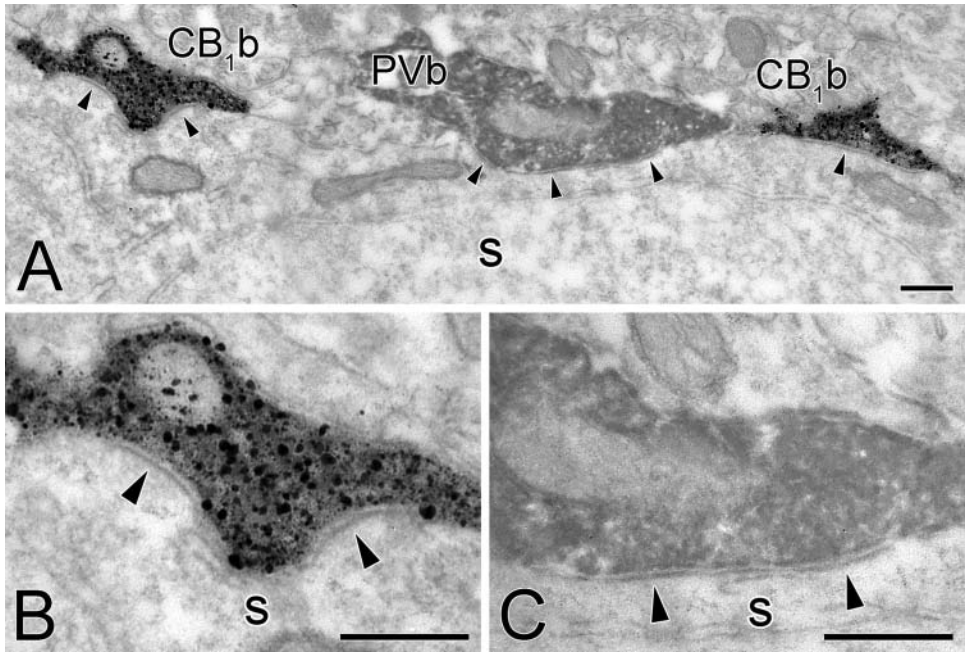

Figure 4. Application of the sulfide-silver-gold intensification (SSGI) technique for double-labeling immunoelectron microscopy. Cannabinoid receptor type 1 (CB_1) and parvalbumin (PV) were detected sequentially in the pyramidal layer of the mouse hippocampus. The staining for CB_1 was completed with the SSGI, resulting in many metal particles of various sizes (A, B), and then the same section was immunostained for PV, resulting in homogeneous NiDABp deposits. The perisomatic boutons (CB_1b and PVb, respectively) of the two different neuronal systems, which form symmetric synapses, can be clearly distinguished. The granules are confined to the CB_1-immunoreactive membrane-bound boutons. Few granules were precipitated in the mitochondria of immunopositive terminals but not in the immunonegative profiles. The arrowheads point at the postsynaptic membranes. s, soma of a neuron. Bars = 200 nm.

et al. 1984). This modification, termed *gold-substituted silver intensification*, resulted in a better contrast between the immunopositive and negative elements (Gorcs et al. 1986). In addition to immunohistochemistry, the thioglycolic acid technique (Gallyas et al. 1982) was also successfully applied for in situ hybridization (Liposits et al. 1991). It should be noted that the thioglycolic acid pretreatment is incompatible with NiDABp because chelated nickel ions are lost under highly acidic conditions.

A new method that increases the argyrophilia of DABp while decreasing that of the tissue was elaborated by Smiley and Goldman-Rakic (1993). The authors diminished the endogenous silver-reducing capacity by means of an intermediate step of 5% cysteine (pH 2.75) for 5 hr, interposed between the secondary and tertiary immunoreagents, the sites of which were developed with DAB as a chromogen. Thereafter, polymerized DAB was treated with sulfide solution, which enhanced the sensitivity of the silver developer for DAB. The recommended procedure ended with the replacement of silver in gold chloride solution. This silver-enhanced diaminobenzidine-sulfide (SEDS) technique resulted in easily visualized punctate metal deposits while maintaining a well-preserved ultrastructure. The small number of deposits was heterogeneous in size, shape, and distribution within the immunopositive profiles.

Each of the several widely different procedures of the silver intensification of DABp shows some distinct benefits such as enhancement of the staining intensity and/or color conversion, which are accompanied by modification of the ultrastructural appearance of DABp. According to our observations, the more powerful the silver intensification is, the harsher the required conditions are for quenching the tissue argyrophilia, and the poorer the ultrastructural preservation is. On the other hand, the less powerful the silver intensification is, the more equivocal color and ultrastructural modifications occur. Unfortunately, reliable color conversion and reproducible graining of the DABp along with good ultrastructural preservation are not provided by any of the aforementioned techniques.

Because the silver intensification of DABp, performed under mildly acidic conditions (pH 5.5), requires no pretreatments to quench endogenous tissue argyrophilia, we concentrated on this condition. We tested dampened variations of gum arabic solution of Danscher (1981), the original version of which was devoted to the detection of sulfide-precipitated metals. In our pilot experiments, DABp failed to possess catalytic activity for silver reduction. However, after treatment with neutralized sulfide, DABp exhibited weak to moderate silver reduction capacity, depending on the ratio and concentrations of the components in the Danscher's developer, whereas the

tissue remained unreactive. However, when the first immunoreaction was developed with DAB in the presence of nickel ions, sulfidized NiDABp dramatically increased the amount of silver on the immunopositive tissue sites. These observations strongly indicate that the catalyst is not DABp alone in this recommended procedure, but rather the exogenous metal sulfide plays the pivotal role in the silver development.

Thus, our SSGI technique may have limitations in experiments (e.g., Martinez-Galan et al. 2003) that combine, in the same tissue, immunohistochemistry and detection of metallic trace elements, including zinc, an important endogenous neuromodulator in glutamatergic neurotransmission, because the latter technique demands the use of sulfide-containing primary fixative. In a set of pilot experiments, we noticed that sulfide added to the primary fixative resulted in a weak staining of zinc in some limbic regions, such as the dentate gyrus. It should be noted that postfixation sulfide treatment does not interfere with the technique, as the control stainings proved.

Our primary purpose was the conversion of the homogeneous DABp to a punctate staining to be used in double-labeling immunoelectron microscopy, rather than a simple intensification, which would easily obscure the fine structural features. Therefore, in our recommended autometallographic silver solution, we dampened the power of the Danscher's developer by changing the concentrations: silver, from 0.085% to 0.05%; hydroquinone (reducing agent), from 0.567% to 0.125%; and CB (anionic protective chelator), from 10% to 25%.

Gold toning by gold chloride treatment is a frequently used posttreatment of silver enhancement techniques to prevent occasional loss of the signal (Basbaum and Menetrey 1987; Pohl and Stierhof 1998; Smiley and Goldman-Rakic 1993) due to oxidative osmium treatment. During the toning process, metallic silver is substituted with the chemically more inert gold, which is supposed to resist osmic acid. However, we experienced that gold chloride treatment itself also diminished visible amounts of the label, although the contrast between the immunopositive elements and the background was increased. This is in line with the observations of other authors that gold toning reduces the size of the silver-enhanced gold particles (Pohl and Stierhof 1998).

Compensation of the expected loss of signal is possible by slight "overshooting" in the silver staining. To avoid this inappropriate approach, we introduced a novel gold toning technique, which employs gold(I) ions instead of gold(III) ions. During stabilization of the signal, three silver atoms are replaced with not only one but rather three gold atoms, which results in a comparable opacity of the signal before and after gold toning. Furthermore, our gold thiocyanate solution is much more easily prepared than those described in the literature (Frederick et al. 1984; Peters 1955).

Our SSGI technique inherited some of the limiting features of similar methods. The question of colocalization cannot be investigated either at the light or electron microscopic level when the two target molecules are present in the same domain of the same cell. Yet, when the antigens are located in different cells, our technique offers advantages over the alternative methods, which can be used for preembedding double-labeling immunoelectron microscopy. These advantages include the highly reproducible modification of appearance of DABp with easy detectability and without compromise to good ultrastructural preservation. Moreover, the SSGI technique using the original Danscher's developer (Danscher 1981) may be a method of choice for other types of studies applying horseradish peroxidase as a detector molecule, including in situ hybridization and protein blotting as well.

Acknowledgment

The technical assistance of Mónika Kara, Andrea Kobolák, Katalin Lengyel, Katalin Iványi, and Győző Goda is greatly acknowledged.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

Disclosures

The author(s) disclosed receipt of the following financial support for the research and/or authorship of this article: This work was supported by the HHMI International Program, EU (LSHM-CT-2004-005166, LSHM-CT-2006-037315), NKTH-OTKA (CNK77793), and NIH (MH54671, NS030549). G.N. was supported by a János Bolyai Research Fellowship.

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