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Postembedding Immunohistochemistry for Inhibitory Neurotransmitters in Conjunction with Neuroanatomical Tracers

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Abstract

This chapter is aimed at providing the reader with detailed instructions for combining postembedding immunohistochemistry for inhibitory transmitters with methods for characterizing neuronal connections at the ultrastructural level. Thus, it includes protocols for both retrograde and anterograde neuronal tracing that have been modified to facilitate electron microscopic examination of neuronal connectivity. In addition, it includes protocols for doing immunohistochemistry with antibodies to either glutaraldehyde-fixed gamma amino butyric acid or glycine on ultrathin sections. By employing these procedures together one can identify both the input/output characteristics of a neuronal structure and identify whether these synaptic connections utilize one of these inhibitory transmitters.

Keywords Ultrastructure, GABA, Glycine, Synapse, Terminal, Neuronal circuits

1 Introduction

It has long been clear that the nervous system employs different subsystems for activation of neurons and for inhibiting this activity. In almost every system, the realization that activity is critically shaped by inhibitory processes has motivated examination of the inhibitory circuits crucial to the functioning of the connections being examined. The role of inhibition has been further emphasized by the realization that activity in circuits can be modulated both by local circuit inhibitory neurons and long axon pathways that also employ inhibition. In this context, it is not surprising that from early on those studying the ultrastructure of the central nervous system wished to be able to differentiate excitatory from inhibitory synapses. It was for this purpose that Gray [1] divided synaptic terminals into Type 1 and Type 2 terminals. The former, which were presumed to be excitatory, contained spherical vesicles and made clearly asymmetric synaptic contacts with prominent postsynaptic densities. The latter, which were presumed to be inhibitory, contained flattened vesicles and made symmetric

synaptic contacts with modest postsynaptic densities. As useful as this classification scheme is, the fact remains that many terminals are not filled with patently flattened vesicles or homogeneously spherical vesicles. Instead, they contain variable numbers of pleomorphic vesicles making them difficult to peg as either inhibitory or excitatory. In addition, the degree of asymmetry in synaptic densities varies widely, and their classification is also affected by issues of plane of section [2, 3]. Thus, it has been very helpful that another approach to identifying inhibitory synapses, in the form of immunohistochemistry, has become available to resolve these issues at the ultrastructural level.

Immunohistochemical approaches originated from light microscopic studies that have used antibodies to elucidate the neurotransmitter specific architecture of the brain. Numerous investigators then took tissue in which the antibody was tagged with diaminobenzidine (DAB), and examined it at the electron microscopic level. If the goal is to simply identify the neurotransmitter in terminals in a sample, this approach is useful. However, most procedures for light microscopic immunohistochemistry utilize paraformaldehyde or formalin-fixed tissue. In addition, they employ detergents, usually 0.3 % Triton X-100, to partially solubilize cell membranes so that the large antibody complexes can gain access to the depths of the tissue. These two technical elements usually degrade the ultrastructure of the specimen, making it difficult to correlate the ultrastructure of labeled terminals and their postsynaptic targets with elements found in the normal ultrastructure of the region. The fixation problems can be ameliorated by including small amounts of glutaraldehyde in the perfusate (<0.2 %), or by postfixing in glutaraldehyde after the immunohistochemical steps. The detergent problem can be approached by using little or no Triton X-100, and then concentrating the ultrastructural examination on the outer few microns of the specimen.

A more direct approach to dealing with these shortcomings was taken by Somogyi and colleagues [4, 5]. They developed a postembedding immunohistochemical technique to provide transmitter identification with superior ultrastructural preservation. The first key element was to develop antibodies to antigens that have been fixed with glutaraldehyde. Thus, the tissue can be fixed to provide superior ultrastructural detail, but the antibody still recognizes the neurotransmitter even after its conformation has been changed by the fixation process. The second key element was to develop an approach that allows a small portion of the embedding resin to be stripped away from the surface of the ultrathin section using hydrogen peroxide, so that the antigen is made available for attachment by the antibody. Somogyi and colleagues used DAB as an electron-dense tag for the antibody. While easy to visualize, this approach is more technically challenging to employ and it somewhat obscures the ultrastructure. More recently, many labs have begun

to use a secondary antibody with a gold particle attached to it. This approach is somewhat simpler, and has the added advantage that it can be used in conjunction with neuroanatomical techniques that employ DAB as the electron-dense tag for neurons or terminals whose connectivity has been specified with tracers. Postembedding immunohistochemistry has been most widely used to identify inhibitory synapses and cells that contain either gamma amino butyric acid (GABA) or glycine as a transmitter. This is no doubt primarily due to the fact that antibodies to glutaraldehyde-fixed GABA and glycine are commercially available. However, it is also motivated by the chemical characteristics of these neurotransmitters. As small molecules, they are not well fixed by paraformaldehyde. Consequently, they tend to diffuse out of the tissue and thus present a difficult experimental target for conventional immuohistochemical techniques. Fixation with a perfusate containing between 1.5 and 2.0 % glutaraldehyde, in addition to 1.0 % paraformaldehyde, stabilizes these small molecules so that they can be found by the antibody. This approach has been used successfully in a wide variety of structures and systems [6–15].

Development of immunohistochemical techniques has proceeded in parallel with development of retrograde and anterograde neuronal tracer techniques. It has been particularly useful to combine these techniques in order to specify whether a particular cell population with a particular target utilizes a particular neurotransmitter [16–22]. Neuroanatomical tracers have been used at the electron microscopic level to allow the identification of both terminals via anterograde tracers and cells via retrograde tracers. More recently these techniques have been combined to specify monosynaptic input/output linkages between anterogradely identified terminals and retrogradely identified cell populations. Obviously it would be advantageous to further specify whether these linkages are inhibitory or excitatory. To do this, one can combine single or dual tracer neuroanatomical techniques and postembedding immunohistochemistry in order to allow terminals to be characterized at the electron microscopic level. We have had considerable success with this approach, and we offer here our procedures for its employment. Note, that this approach has also been used for glutamate. Since we have not used anti-glutamate antibodies, we will not discuss this further here, but the basic idea is the same.

Since individuals reading this chapter will come to it with different skill sets, we have organized the Materials and the Methods sections in a modular fashion, thus allowing the reader to pick and choose which elements they would like to attend to (*see* **Note 1**). An outline of these modules is provided here:

1. Perfusion and sectioning
2. Retrograde tracing
3. Anterograde tracing

4. Sampling, processing, and EM ultrathin cutting
5. Postembedding immunohistochemistry
6. Heavy metal staining
7. Analysis

2 Materials

2.1 Perfusion and Sectioning

1. Perfusion rinse: 0.1 M, pH 7.2 phosphate-buffered saline (PBS). Stock buffer concentrate, 0.4 M, pH 7.2 phosphate buffer (PB), contains 10.6 g of monobasic, monohydrate sodium phosphate [$NaH_2PO_4 \cdot H_2O$], and 56.0 g of dibasic potassium phosphate [K_2HPO_4] per 1.0 l of deionized water (dH_2O). Store refrigerated. This stock is diluted 1:3 with dH_2O to make 0.1 M working solution PB. PBS for the perfusion rinse is made by adding 0.85 g of NaCl per 100 ml of the working solution PB.
2. Mixed aldehyde fixative: 1.0 % paraformaldehyde and 1.5–2.0 % glutaraldehyde in 0.1 M, pH 7.2 PB (*see* **Note 2**). For each 1,000 ml of a 1.0 %/1.5 % mixture of fixative solution needed, place 10 g of paraformaldehyde [$(CH_2O)_n$] (Fisher T-353) into 100 ml of dH_2O in a flask. Then heat to 60 °C while stirring under a hood. Next, add 1.0 N NaOH, dropwise, until the solution clears (*see* **Note 3**). Cool below 30 °C and filter. For 1,000 ml of fixative, add 500 ml of dH_2O and 250 ml of 0.4 M, pH 7.2 PB to solution, and store at 4 °C. Before perfusion add 60 ml of 25 % glutaraldehyde [$OHC(CH_2)_3CHO$] (Fisher, 02957) and then 90 ml dH_2O measured in the same graduated cylinder for the 1.5 % glutaraldehyde fixative (*see* **Note 4**).

2.2 Retrograde Tracing

1. Retrograde tracers: 1.0–2.0 % Wheatgerm Agglutinin Conjugated to Horseradish Peroxidase (Sigma-Aldrich, L3892) (WGA-HRP) or 1.0–2.0 % Choleratoxin B-subunit conjugated to horseradish peroxidase (Sigma-Aldrich, C3741) (ChTB-HRP). Dissolved in dH_2O for both pressure and iontophoretic injections (*see* **Note 5**). Dissolve 0.005 g of tracer into 25 μl of dH_2O to make a 2.0 % solution.
2. Stock 0.4 M, pH 6.0 PB. This buffer contains 48.45 g of monobasic, monohydrate sodium phosphate [$NaH_2PO_4 \cdot H_2O$], and 8.56 g of dibasic potassium phosphate [K_2HPO_4] in 1.0 l of dH_2O. Store refrigerated. This is diluted 1:3 with dH_2O to make 0.1 M working solution (*see* **Note 6**).
3. Molybdate-TMB reaction solution A: This solution contains 1.95 g ammonium molybdate 4-hydrate, crystal [$NH_4Mo_7O_{24} \cdot 4H_2O$] in 780.0 ml of 0.1 M, pH 6.0 PB.

4. Molybdate-TMB reaction solution B: This solution contains 0.04 g of tetramethylbenzidine free base (TMB) $[C_6H_2(CH_3)_2 \cdot 4NH_2]_2$ (Sigma-Aldrich, T2885) in 20 ml of 100 % ethanol (*see* **Note 7**). Dissolve in covered beaker by stirring. Do NOT heat. This takes some time to go into solution. *N.B. TMB may be carcinogenic. All steps employing this chemical should be done using gloves and with the solutions sealed, for example, by placing plastic wrap over the reaction tray. Store used solution for proper disposal.*
5. 0.3 % Hydrogen peroxide: Dilute 0.3 ml of 30 % stock hydrogen peroxide $[H_2O_2]$ (Fisher, H325-100) in 45.5 ml of dH_2O. *Use gloves; do NOT mouth pipette.*
6. Stabilizer solution: 5.0 % Ammonium molybdate. Dissolve 30.0 g of ammonium molybdate 4 hydrate $[NH_4Mo_7O_{24} \cdot 4H_2O]$ into 600 ml of 0.1 M, pH 6.0 PB.
7. 0.1 M, pH 7.2 PB. See above.
8. Diaminobenzidine (DAB) protection solution: Dissolve 0.06 g of 3,3′-diaminobenzidine tetrahydrochloride $[C_{12}H_{14}N_4 \cdot 4HCl]$ (Sigma-Aldrich, D5637) (*see* **Note 8**) in 450 ml of dH_2O. Then mix with 150 ml of 0.1 M, pH 7.2 PB. *N.B. DAB is known to be carcinogenic. All steps employing this chemical should be done using gloves in a hood. Store used solution for proper disposal.*

2.3 Anterograde Tracing

1. Anterograde tracer: 10.0 % Biotinylated Dextran Amine, 10,000 MW (Invitrogen-Molecular Probes, D1956) (BDA). Dissolve BDA in dH_2O for both pressure and iontophoretic injections (*see* **Note 5**). Dissolve 0.005 g of tracer into 50 µl of dH_2O.
2. 0.1 M, pH 7.2 PB. See above.
3. Buffered 0.05 % Triton X-100: Stock solution of 0.3 % Triton X-100 is made by adding 0.3 ml of Triton X-100 [t-octylphenoxypolyethoxyethanol] (Sigma-Aldrich, A-100) into 99.7 ml of 0.1 M, pH 7.2 PB with a micropipette (*see* **Note 9**). The working concentration of 0.05 % is produced by diluting 15 ml of 0.3 % stock per 90 ml of 0.1 M, pH 7.2 PB.
4. 1:500 Avidin D-conjugated horseradish peroxidase solution (avidin-HRP): This is made by diluting 40 µl of avidin-HRP (Vector Labs, A2004) (*see* **Note 10**) into 20 ml of 0.05 % buffered Triton X-100.
5. Nickel cobalt diaminobenzidine solution: Dissolve 0.06 g of 3,3′-diaminobenzidine tetrahydrochloride $[C_{12}H_{14}N_4 \cdot 4HCl]$ (Sigma-Aldrich, D5637) (*see* **Note 8**) in 450 ml of dH_2O. Then, mix with 150 ml of 0.1 M, pH 7.2 PB. Immediately before using, stir in 6.0 ml of 1.0 % nickel ammonium sulfate

and 2.0–6.0 ml of 1.0 % cobalt chloride. These two 1.0 % stock solutions are made by dissolving 1 g of nickel ammonium sulfate [$NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$] into 100 ml of dH_2O, and 1 g of cobalt chloride [$CoCl_3$] into 100 ml of dH_2O. Both stock solutions are stored at 4 °C. *N.B. DAB is known to be carcinogenic. All steps employing this chemical should be done using gloves in a hood. Store used solution for proper disposal.*

2.4 Sampling, Processing, and EM Ultrathin Cutting

1. 0.2 M, pH 7.0 EM Phosphate buffer (EM PB): For 100 ml add 1.229 g of monobasic, dihydrate sodium phosphate [$NaH_2PO_4 \cdot 2H_2O$] (Fisher Scientific, S-381), and 1.739 g of dibasic sodium phosphate [Na_2HPO_4] (Fisher Scientific, S-374) to 97 ml of dH_2O.
2. 1.0 % Osmium tetroxide in 0.1, pH 7.0 M PB: Stock 2.0 % osmium tetroxide solution is made up by dissolving 1 g of osmium tetroxide [OsO_4] (EM Sciences, 19100) in 50 ml of dH_2O. The working solution is produced by mixing 1 part of the 2.0 % solution with 1 part 0.2 M, pH 7.0 EM phosphate buffer. (Filter before use.) *Osmium tetroxide is extremely reactive. It should only be used in the hood while wearing gloves and other protective gear. Store used solution and vials in a sealed glass container for proper disposal.*
3. Durcupan ACM Epoxy (EM Sciences, 14040)

2.5 Postembedding Immunohistochemistry

The TBS, TBS+BSA, and TBS+BSA+Tween 20 described in solutions 2–4 below should be made up fresh every time tissue is processed for postembedding. The staining process takes 2 days. Keep solutions refrigerated between days 1 and 2. The glutaraldehyde solution described in **step 7** can be made up in bulk and stored (*see* **Note 14**).

1. 0.1 M, pH 7.0 EM Phosphate buffer: Dilute 0.2 M EMPB (see above) 1:1 with dH_2O.
2. 0.1 M, pH 7.4 Tris-buffered saline (TBS). Add 0.76 g of Trizma (Sigma, T7693) and 0.85 g of NaCl to 100 ml of dH_2O in order to produce 100 ml of TBS.
3. TBS+bovine serum albumin (BSA): Take 50 ml of freshly made TBS and add 0.5 g of BSA (Sigma, A7906). Do not shake, but sonicate to put into solution.
4. TBS+BSA+Tween 20 (polyoxyethylenesorbitanmonolaurate): Take 40 ml of freshly made TBS+BSA and add 0.2 μl of Tween 20 (Sigma, P-137). Do not shake. Instead, sonicate to put into solution.
5. Primary antibody. For anti-GABA, use antibody to glutaraldehyde-fixed GABA raised in rabbits (Sigma, A2052). Dilute antibody 1: 250 in TBS+BSA+Tween 20 by adding 10 μl of antibody to 2.5 ml of TBS+BSA+Tween 20 (*see* **Note 11**).

For anti-glycine, use antibody to glutaraldehyde-fixed glycine raised in rabbits (Millipore, AB5020). Dilute antibody, 1:10, in TBS+BSA+Tween 20 by adding 100 μl of antibody to 1.0 ml of TBS+BSA+Tween 20 (*see* **Note 12**).

6. Secondary antibody: Goat anti-rabbit IgG conjugated to 15 nm gold particles (EM Sciences, 25113 (Aurion immunogold reagents)) in TBS+BSA+Tween 20. Dilute antibody 1:75 by placing 20 μl of it into 1.5 ml of TBS+BSA+Tween 20 (*see* **Note 13**).
7. 2.0 % Glutaraldehyde in 0.1 M, pH 7.0 EM PB: For 25.0 ml, use 12.5 ml of previously made PB. Add 10.5 ml of dH_2O and 2.0 ml of 25 % glutaraldehyde (Fisher Scientific, 02957-1). We use conventional glutaraldehyde instead of glutaraldehyde exclusively produced for EM, as we could not detect a significant difference in ultrastructural preservation in the past.

2.6 Heavy Metal Staining

1. Uranyl acetate solution: Make a 2.0 % solution by placing 0.2 g of uranyl acetate [$(UO_2(Ch_3COO)_2)$] (Ted Pella, 19481) in 10 ml of distilled (dH_2O) water. Filter before use.
2. Lead citrate solution: Add 0.40 g of lead citrate 3-hydrate [$Pb_3(C_6H_5O_7)_2 \cdot 3H_2O$] (Electron Microscopic Sciences 512-26-5), 0.30 g of lead nitrate [$Pb(NO_3)_2$] (Mallinckrodt, 5744), 0.30 g of lead acetate [$Pb(C_2H_3O_2)_2 \cdot 3H_2O$] (Mallinckrodt, 5688), and 2.00 g of sodium citrate [$Na_3C_6H_5O_7 \cdot 2H_2O$] (Mallinckrodt, 0754) to 82 ml of boiled, cooled dH_2O. Reagents are sonicated to place them into solution. The solution will remain cloudy until the sodium citrate is added. Add 18 ml of 1 N sodium hydroxide [NaOH]. Filter before use.

3 Methods

3.1 Perfusion and Sectioning

1. Animals are deeply anesthetized with sodium pentobarbital (50 mg/kg) only after the perfusion setup is entirely in place with all solutions loaded, and the surgeon has on protective eyewear, clothing (we prefer a plastic apron), mask, and gloves.
2. The perfusion rinse, 0.1 M, pH 7.2 PBS, is pumped through the vasculature by use of a peristaltic pump (Master Flex LS, Cole Palmer). To accomplish this, a blunt 13 G, 2¾ inch, stainless steel LNR needle (BD) attached to Tygon tubing with a Luer-Lock hub is advanced through the wall of the left ventricle, and threaded through the aortic valve until it lies in the base of the aorta. The needle is stabilized by clamping the ventricle around it with an Allis hemostatic forceps. The right atrium is opened with a fine scissors. The descending aorta is

clamped with a hemostat at the level just above the diaphragm, if one is studying the brain and not spinal cord.

3. Once the fluid leaving the atrium has cleared of blood, the fixative, 1.0 % paraformaldehyde/1.5–2.0 % glutaraldehyde in 0.1 M, pH 7.2 PB, is then pumped through the vasculature using the same apparatus (*see* **Notes 15** and **16**). The rinse and the first half of the fixative are run through the animal at a brisk pace to ensure that all the red blood cells are cleared. The remaining fix is then pumped through very slowly to maximize diffusion time and ensure complete tissue fixation. *N.B. Fixatives are toxic substances by definition. The perfusion should take place in a hood or on a down-draft table, and the fumes vented to the outdoors. Proper gloving, eye protection, and protective attire are a necessity.*
4. The head is removed from the body and placed in a stereotaxic head holder (Kopf). The dorsal skull is removed and the brain is blocked in the frontal plane using a micromanipulator armed with a # 22 blade attached to a pole on the micromanipulator arm. For best results when sectioning on a Vibratome, blocks should be kept at or below 1 cm in height.
5. A fiducial mark is placed in each block by driving a syringe needle through an unimportant area and leaving it there during the postfixation step [6].
6. The blocks are postfixed in the same fixative for 1–2 h at 4 °C and then stored overnight at 4 °C in 0.1 M, pH 7.2 PB.
7. Blocks are superglued to a glass plate attached to the Vibratome chuck. Then, sections are cut at 100 μm on a Vibratome (Leica VT 100S). They are collected serially and stored at 4 °C in 0.1 M, pH 7.2 PB.

3.2 Retrograde Tracing [23–26]

1. Retrograde tracer (1.0–2.0 % WGA-HRP or 1.0–2.0 % ChTB-HRP) is most conveniently injected by use of a 1.0 μl Hamilton syringe (*see* **Note 17**). For small injections, a glass micropipette can be attached to the syringe needle. Alternatively, a glass micropipette can be used to iontophoretically apply the tracer. The amount injected and the number of injections are determined by the size and shape of the target. Note that in dual tracer experiments this injection is actually made second, after the anterograde tracer injection, in a separate surgery.
2. Survival for transport of these tracers is usually 1 or 2 days, as WGA-HRP and ChTB-HRP travel by fast axoplasmic transport. The perfusion and cutting steps are described above.
3. Place sections in 0.1 M, pH 6.0 PB in a reaction tray in serial order (*see* **Note 18**). Rinse one time in 0.1 M, pH 6.0 PB is 10 min.

4. Combine molybdate-TMB reaction solutions A and B together in the dish and preincubate sections in this solution for 20 min at room temperature (~22 °C) with gentle agitation on a rotating table. Cover the reaction dish with plastic wrap to keep the alcohol from evaporating during the reaction steps.
5. Add 10 ml of 0.3 % H_2O_2 to the solution in the dish, and react for 20 min at ~22 °C with gentle agitation.
6. Repeat **step 5** two times.
7. Continue reaction overnight with gentle agitation at 4 °C in a sealed reaction dish (*see* **Note 19**).
8. Place sections in stabilizer solution, 5 % ammonium molybdate in 0.1 M, pH 6.0 PB, for 15 min with gentle agitation at ~22 °C.
9. Rinse twice in 0.1 M, pH 7.2 PB with gentle agitation at ~22 °C for 5 min (*see* **Note 20**).
10. Place in DAB protection solution for preincubation with gentle agitation at ~22 °C for 10 min. [*DAB is a carcinogen. **Steps 10–12** should be carried out in a hood.*]
11. React tissue by adding 6 ml of 0.3 % H_2O_2 to the DAB protection solution. Reaction is continued for 10–20 min with gentle agitation at ~22 °C (*see* **Note 21**). DAB *protection solution is carcinogenic and it should be properly disposed of* (*see* **Note 22**).
12. Rinse in 0.1 M, pH 7.2 PB, four times for 5 min each, with gentle agitation at ~22 °C. EM samples can be taken at this point, or the tissue can then be reacted to visualize biotinylated tracers (see below). Once EM samples have been taken, the tissue is mounted, counterstained, dehydrated, cleared, and coverslipped as a record of the areas sampled.

3.3 Anterograde Tracing [27–29]

1. Anterograde tracer (10.0 % BDA) is most conveniently injected by use of a 1.0 µl Hamilton syringe (*see* **Note 17**). For smaller injections, a glass micropipette can be attached to the syringe needle. Alternatively, a glass micropipette may be used to iontophoretically apply the tracer. The amount injected and the number of injections are determined by the size and shape of the target. Note that in dual tracer experiments this injection is made first, and the retrograde tracer injection is made in a separate surgery 1–2 days before sacrifice.
2. Survival for transport of BDA requires 7–21 days, depending on the length of the pathway being examined, as this tracer uses slow axoplasmic transport. The perfusion and cutting steps are described above.
3. Fill 12-well depression plates with 0.05 % Triton X-100 solution (500 µl per well) (*see* **Note 23**). For dual tracer studies,

transfer sections from the reaction tray into the wells. Gently agitate for 10 min at ~22 °C. Repeat two more times for a total of 30-min incubation.

4. Replace well solution with avidin-HRP solution. Gently agitate for up to 1 h at ~22 °C.
5. Continue gently agitation at 4 °C overnight in a sealed enclosure to avoid evaporation (*see* **Note 24**).
6. Transfer sections back into reaction tray and rinse in 0.1 M, pH 7.2 PB, three times for 10 min each, with gentle agitation at ~22 °C.
7. Preincubate in nickel-cobalt DAB solution for 10 min with gentle agitation at ~22 °C. *DAB is a carcinogen. **Steps** 7–9 should be carried out in a hood with gloves on. This solution is carcinogenic and it should be properly disposed of* (*see* **Note 22**).
8. React sections by adding 10 ml of 0.3 % H_2O_2. Reaction proceeds at ~22 °C with gentle agitation for at least 10 min, and possibly up to 30 min. The presence of cobalt chloride can produce strong background labeling (*see* **Note 25**). Some background staining is to be expected, but if the sections turn more than a light blue-gray tone, stop the reaction by proceeding to the rinse steps.
9. Rinse three times in 0.1 M, pH 7.2 PB. EM samples can be taken at this point. Once EM samples have been taken, the tissue is mounted, counterstained, dehydrated, cleared, and coverslipped as a record of the areas sampled.

3.4 Sampling, Processing, and EM Ultrathin Cutting

Tissue used for electron microscopy needs to be cut into rather small pieces to allow osmium to penetrate it. For example, we normally cut a macaque ciliary ganglion into 2–3 pieces. Cutting the brain into 100 μm sections accomplishes the same purpose. In addition, only a small sample can be cut with a diamond knife, so the area of interest is cut out with a razor blade or # 11 scalpel blade. The size of the sample is approximately 1–2 mm^2. *Remember to only work with osmium tetroxide vials in a hood. Use proper eye and hand protection. Store any solution containing any osmium tetroxide in a sealed glass container for proper disposal.*

1. Samples are collected into small glass vials filled with 0.1 M PB (pH 7.0). The following steps [2–8] include slow agitation on a rotator (EM Sciences, 65100-10).
2. Treat for 2 h with 1.0 % osmium tetroxide (OsO_4) in 0.1 M PB (pH 7.0) at ~22 °C.
3. Wash three times in dH_2O at ~22 °C.
4. Dehydrate with increasing concentrations of acetone for 10 min each (70, 90, 95 %) at ~22 °C.

5. Complete dehydration by rinsing three times in 100 % acetone at ~22 °C for 15 min each.

   In the next steps the samples are embedded in epoxy resin (*see* **Note 26**).

6. Place tissue in a 1:3 mixture of Durcupan and acetone overnight at ~22 °C.
7. Place in 3:1 mixture of Durcupan and acetone for 3 h at ~22 °C.
8. Place in 100 % Durcupan for 1 h at ~22 °C.
9. Embed in fresh Durcupan (100 %) and polymerize at 60 °C overnight.

   For the actual embedding, we use either (a) conventional plastic flat-embedding molds (EM Sciences) for small pieces of tissue like ganglia or (b) plugs of resin polymerized in Beem capsules for samples taken from 100 μm thick sections. *Any leftover resin should be polymerized before disposal.*

   (a) Fill molds with fresh Durcupan until they are about half full. Place samples at the front edge of the mold and complete filling the molds with Durcupan. Try to avoid or remove bubbles. Polymerize at 60 °C overnight.

   (b) Cut lids and bottom off the Beem capsules (retain lid). Place the remaining plastic tube without bottom onto the lid and fill with 100 % Durcupan. Polymerize at 60 °C overnight. Remove and retain lid. Remove the plug from the capsule. Make sure to create your plug at least one day before embedding.

   Place a small drop of fresh, fluid 100 % Durcupan onto the cutoff lid of a Beem capsule. Position sample in that drop. Press plug into the lid, sandwiching the sample between the lid and plug. Do not squeeze the sample, as this may cause mechanical damage to the tissue. Polymerize at 60 °C. Remove and discard lid.

10. Trim blocks using a single-edged razor blade.
11. For orientation purposes, take 1.0 μm thick semithin sections of already trimmed blocks on a microtome with a glass knife. Mount them on glass slides, dry on a heating plate, and stain them for 20 s with toluidine blue. Examine sections under a light microscope to determine the area of interest. Retrim blocks to include only the area of interest.
12. Cut ultrathin silver/gold sections (~90 nm thick) with a diamond knife on an ultramicrotome (Reichert Ultracut E) and mount them onto Formvar-coated nickel slot grids (EM Sciences, G2010-Ni).

3.5 Postembedding Immunohistochemistry

All immunohistochemical procedures are performed on Parafilm to create a clean bench space. Attach a piece of film onto the lab bench by running the dull end of a forceps over the edges of the Parafilm. Drops (~40–50 μl) of the respective staining or washing solution for the steps described below are placed onto the Parafilm with a Pasteur pipette (*see* **Note 27**). Float the grids on the drops with the sample side down, contacting the fluid. We recommend that one not try to process more than four grids at the same time. All steps are done at room temperature (~22 °C).

1. Rinse grids briefly with dH_2O.
2. Etch with 3.0 % H_2O_2 in dH_2O for 1 min.
3. Wash grids with dH_2O three times for 3 min each.
4. Treat the sections with 0.1 M, pH 7.4 TBS two times for 5 and 10 min each.
5. Pretreat in TBS+1.0 % BSA for 30 min.
6. Incubate in a solution of the primary antibody (e.g. rabbit anti-GABA IgG) in TBS+BSA+0.05 % Tween 20 overnight. Put a glass cuvette over your floating sections and seal with Parafilm, so that the drops do not evaporate overnight.
7. Rinse sections the next day with TBS+BSA+Tween 20 three times at 10 min each.
8. Incubate in secondary antibody (goat-anti-rabbit IgG conjugated to gold particles) in TBS+BSA+Tween 20 for 2 h (*see* **Note 28**).
9. Rinse grids again with TBS+BSA+Tween 20 three times for 10 min each.
10. Rinse with dH_2O three times for 3 min each.
11. Fix using a solution of 2.0 % glutaraldehyde in 0.1 M, pH 7.0 EM PB for 3 min.
12. Rinse tissue with dH_2O three times for 3 min each.

3.6 Heavy Metal Staining [30]

Prepare small, clean glass vials filled with ultraclean dH_2O before starting with these steps. For four grids, you will need six vials for each washing step between and after staining with uranyl acetate and lead citrate solutions. Wash by using a forceps to move each grid gently up and down within the solution contained in a vial at least ten times per step, before advancing the grid to the next vial (*see* **Note 29**). The two staining steps are again done on drops of the respective solution placed on Parafilm. All steps are done at room temperature (~22 °C).

1. Stain by floating grids, section down, on a drop of 2.0 % uranyl acetate for 3 min.
2. Wash in three vials and dry as described above.

3. Stain with calcinated lead citrate solution for 30 s. Put drops of the solution next to some sodium hydroxide pellets on the Parafilm to create a dry environment. Float grids on the solution and cover with a Petri dish. Do not breathe on the sections during the staining process. The exhaled carbon dioxide will result in lead precipitation.
4. Wash in vials and dry as described above.
5. Store in grid boxes.

3.7 Analysis

The gold particles attached to the secondary antibody appear as tiny, spherical dots or freckles over the labeled elements (Fig. 1, small arrows). The uniform size and appearance of the gold particles allow them to be distinguished from ribosomes and glycogen granules. Of course, not all GABA and glycine present in cells are necessarily involved in inhibitory synaptic transmission, as these molecules have other biochemical uses. In addition, as with all immunohistochemical processes, the antibodies can attach nonspecifically. Thus, it is necessary to determine which labeling actually corresponds to inhibitory elements. One particularly effective way of confirming that a profile is positively labeled is to find the same

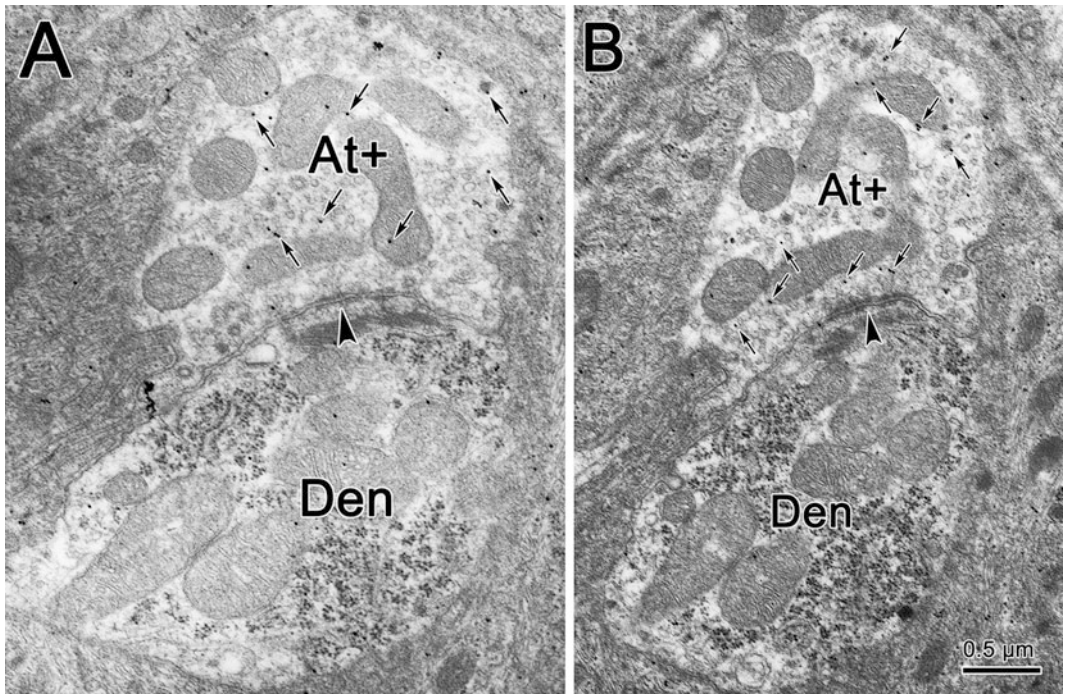

Fig. 1 Semiserial sections through a synaptic terminal in a macaque ciliary ganglion that have been labeled using postembedding immunohistochemistry for GABA. In both plates (**a**) and (**b**), numerous gold particles (*arrows*) are apparent over the terminal, indicating that it is GABA positive (At+). Synaptic density indicated by *arrowhead*. Den = dendrite. Scale in (**b**) = (**a**)

profile in semiserial sections. Background labeling should be fairly random, but if there are numerous gold particles lying over the profile in both sections, there is a high likelihood that it is labeled. Figure 1 shows such a pair, with numerous gold particles present over a GABA-positive terminal (At+) in the ciliary ganglion, but very few over other elements, such as the dendrite. Alternatively, one can quantitatively determine the background level of labeling and then establish a threshold for immunopositivity. For example, in recent studies [19, 21], we defined terminals exhibiting labeling at or below background as GABA negative and those with three times this threshold as GABA positive. Terminals with particle numbers lying between these values were placed in a non-characterized category. In this same tissue, we found that labeling in somata and dendrites was distinctly less prominent, and so we set the threshold at two times background. There are of course highly sophisticated image analysis systems for measuring particle density. However, we have found that a manual method is quite easy to use. We cut a square out of a piece of cardboard that is about the size of a typical axon terminal in our area of interest. We then slide it over the image making counts over areas that are clearly not heavily labeled. Counts from ten such areas are then averaged to define background level. Background is independently determined in this way for all the images from a single grid (*see* **Note 30**). This same square can then be placed over terminals, dendrites, or somata to allow them to be classified as positive or negative relative to this background level.

It is often useful to employ the postembedding immunohistochemical method to analyze the terminal populations contacting an identified output population. An example of this is shown in Fig. 2. The retrogradely transported tracer appears as electron dense, flocculent, crystals within the cytoplasm of the labeled cells, and dendrites. In some cases, it will nearly fill the cytoplasm of the labeled cell, as demonstrated in the labeled dendrite (Den*) in Fig. 2a and the somata (Soma*) in Fig. 2d. In other cases, there are just small patches of electron-dense material, as shown in the somata of Fig. 2b and the dendrite of Fig. 2e. The latter is preferable when identifying synaptic contacts (arrowhead) (*see* **Note 31**). In Fig. 2 the GABA-positive (At+) and GABA-negative (At) terminals are easily recognized along the membranes of somatic (Fig. 2b, d) and dendritic (Fig. 2a, e) profiles. Note the association of the gold particles with mitochondria. This association is often found in GABA-stained material. Even over non-labeled profiles, gold particles are generally more common over mitochondria.

Postembedding immunohistochemistry is also useful for identifying whether terminals from a defined source are inhibitory. Figure 3 shows examples of terminals labeled with biotinylated dextran amine (BDA). The BDA-labeled terminals (At*) contain granular electron-dense reaction product and are easily

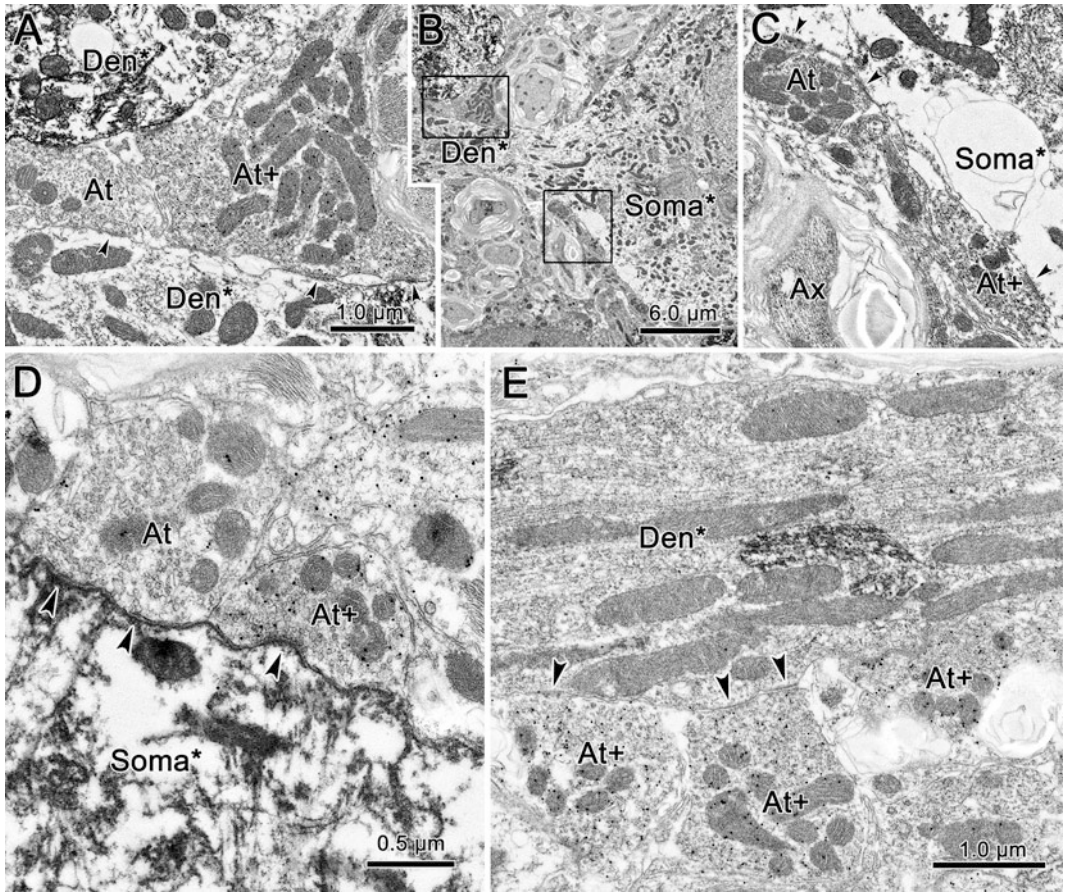

Fig. 2 Examples of combining retrograde labeling with postembedding immunohistochemistry. GABA-positive synaptic profiles (At+) are seen contacting (*arrowhead*) retrogradely labeled (ChTB-HRP) somata (Soma*) (**c**, **d**) and dendrites (Den*) (**a**, **e**). Low-magnification view in (**b**) shows sample areas for (**a**) and (**c**). Note electron-dense reaction crystals in postsynaptic elements and gold particles lying over GABA-positive terminals. At = unlabeled axon terminal. Ax = axon. Scale in (**a**) = (**c**)

discriminated from unlabeled terminals (At). In densely labeled examples (Fig. 3a, bottom and c) this product can fill the cytoplasm between the vesicles. In more lightly labeled examples (Fig. 3a, top and b) it surrounds the vesicles with an electron-dense fuzz. Those BDA-labeled terminals that are GABA positive have numerous gold particles over them (At*+) while those that are GABA negative (At*) do not (*see* **Note 32**).

Combining the anterograde and retrograde tracer techniques allow the investigator to make very forceful arguments about the input/output relationships within a nucleus. As shown in the lower magnification (a) and higher magnification (b) views in Fig. 4, both types of reaction product are present. Flocculent electron-dense crystals (arrows) are observed within this soma (Soma*) (Fig. 4a). The BDA-labeled terminal shown (At*) is completely filled with

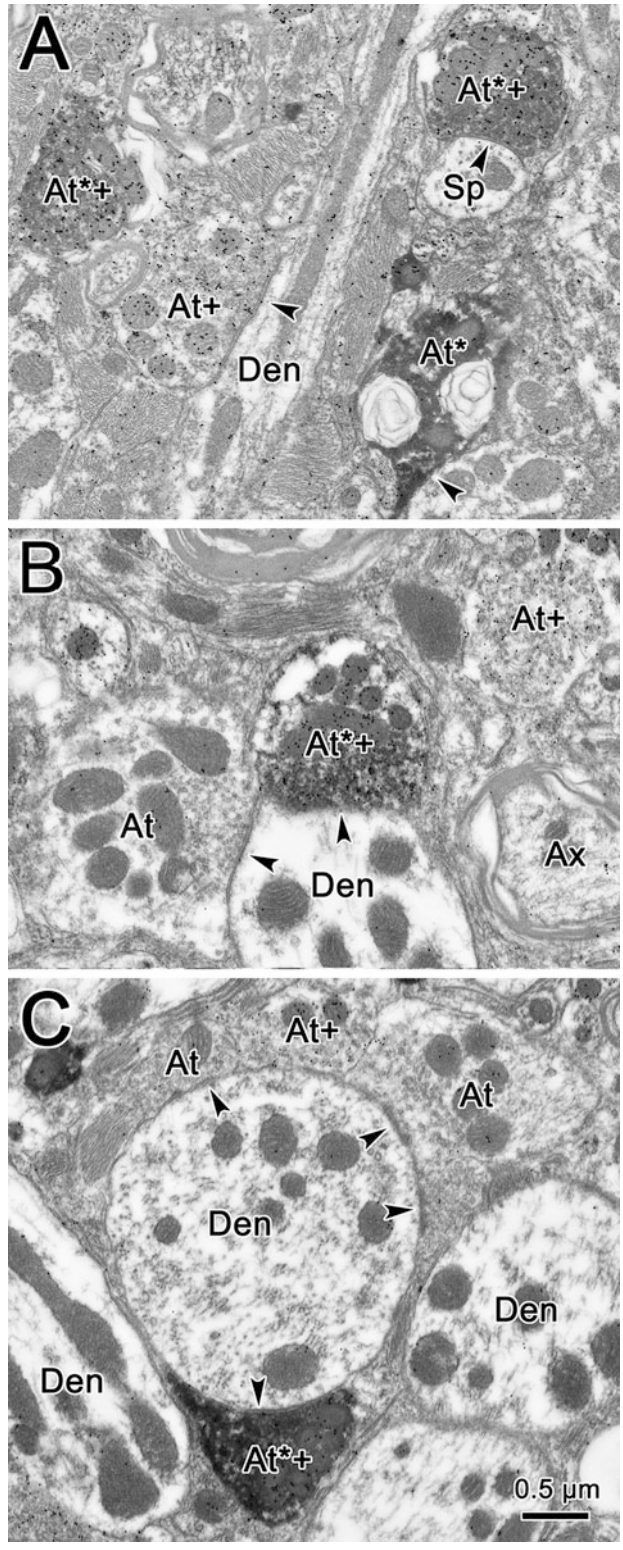

Fig. 3 Examples (**a**–**c**) of combining anterograde labeling with postembedding immunohistochemistry. Some GABA-positive profiles also are labeled with the anterograde tracer BDA (At*+), while others are not (At+). In addition, some BDA-labeled profiles are not GABA positive (At*). In each case, these terminals synapse (*arrowhead*) on unlabeled dendrites (Den). At = unlabeled axon terminal. Ax = axon. Scale in (**c**) = (**a**) and (**b**)

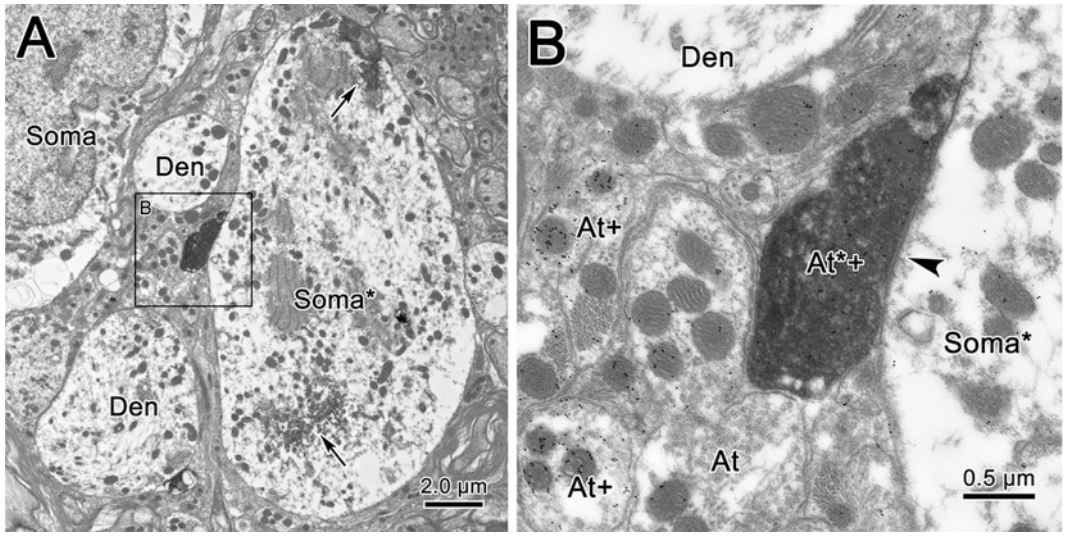

Fig. 4 Dual-tracer labeling in conjunction with postembedding immunohistochemistry. Low (**a**) and high (**b**) magnification views of a labeled synaptic contact (Box in **a**=**b**). A BDA-labeled synaptic terminal is seen contacting (*arrowhead*) a retrogradely labeled (ChTB-HRP) soma (Soma*). The densely labeled terminal also has gold particles over it, indicating that it is also GABA positive (At*+). At=unlabeled axon terminal, At+=GABA-positive axon terminal, Den=dendrite

reaction product. Nevertheless, it is still possible to visualize the gold particles indicating that this is a GABA-positive terminal (At*+), although identifying GABA-positive terminals that are not BDA labeled (At+) is considerably easier.

4 Notes

1. The solution amounts described below are for techniques developed for large feline and monkey brains. They can often be adapted for rodent work by moving the decimal points one place to the left for all of the volumes and weights.
2. Higher levels of glutaraldehyde will facilitate capture of the GABA or glycine molecules by the fix, but decrease the activity of horseradish peroxidase (HRP). Optimum fixation for HRP is with 1.25 % glutaraldehyde. So, lower levels, in the 1.5–2.0 % range, are used when doing retrograde tracing in conjunction with the postembedding immunohistochemistry. We have commonly used 1.5 % glutaraldehyde for anti-GABA experiments that are combined with retrograde tracers that employ horseradish peroxidase (HRP). We use 3.0 l of fixative for a macaque monkey perfusion and 2.0 l for a cat perfusion.
3. Do not allow the paraformaldehyde solution to heat above 60 °C. To do this, it is best to turn off the heater element

when the temperature reaches the vicinity of 50 °C, as the solution will continue absorbing heat from the hot plate. We use an Urbanti spiral ribbed funnel (Bell Art, F14648-000) and coarse #4 filter paper to speed the filtration process.

4. Since the glutaraldehyde is viscous, it tends to stick to the cylinder. This approach washes it into the fix. It is best to order the granular form of paraformaldehyde as the powder tends to become airborne and is breathed in.
5. We aliquot tracers into 0.5 ml microcentrifuge tubes (Fisher, 02-681-333) when we receive them and store them at 0 °C to maximize storage life. Once in solution, they are still fairly stable for up to a month, if refrigerated. We have described here the tracers we use most commonly, WGA-HRP, ChTB-HRP, and BDA. However, others also can be employed. Phaseolus vulgaris leucoagglutinin has also been successfully used as an anterograde tracer [21]. For short pathways, biocytin can be used instead of BDA [28, 29]. However, it should only be used with 24-h survival periods and should be injected in the same surgery as the retrograde tracer as it is quickly metabolized.
6. Of the various TMB protocols we reviewed when developing the approach, this one uses a pH closest to neutral, and it consequently is less damaging to the ultrastructure of the tissue. It also produces very little background staining and stunning signal.
7. Store TMB powder at 0 °C. Do not store solution B for more than overnight.
8. Store DAB powder at 0 °C. DAB dissolves much easier in water, than buffer, so the DAB solution is not buffered until after the DAB dissolves.
9. Triton X-100 takes a while to mix into solution even with rapid stirring. It can then be stored at 4 °C for long periods of time.
10. When received, avidin D-HRP should be stored at 4 °C.
11. The effective concentrations vary by lot. 1:250 is a good starting place, but it is likely that you will need to play with the concentration to maximize the signal-to-noise ratio for any individual antibody lot and for your tissue.
12. We have only limited experience with anti-glycine. This antibody provided us with usable data, but only at high concentrations. In the future, we plan to search for new sources and experiment with using higher glutaraldehyde concentrations in the fix.
13. Again, this is a suggested starting point for dilution. The investigator may need to play with this concentration to maximize the signal-to-noise ratio. We have also tried using 20 nm

gold particles. This makes finding the labeled terminals easier, but seems to lessen the number of gold particles present.

14. Make sure to use Millipore-filtered dH_2O for all solutions used for electron microscopy.
15. The tubing running through the pump is connected to two separate reservoirs (one for rinse and one for fix) through a Y-coupling. The tubes leading from the Y to the two reservoirs are each fitted with a quick-release hose clamp. This allows quick changeover from the rinse to the fix. The longer hose descending from the Y-connector is fitted with a stainless steel Luer-Lock hub at the open end. This is held in place with a screw-type hose clamp available from a local hardware store. One should carefully remove bubbles from the line before starting the perfusion. *Murphy's First Law of Perfusions* states: "Bubbles will inevitably lodge in the vessels leading to the area of interest, denying it fixative." The fixative is caught in a plexiglass tray. The animal lies on a perforated plexiglass platform that fits into this tray.
16. Different labs use different fixative temperatures. We are not convinced that this is a critical variable. We use room temperature wash followed by a cool fix, but this is due to the fact that the fix will cross-react if stored at room temperature. Similarly, there are many variations on the approach to the perfusion. We have come to believe that perfusions, because they have variable outcomes, engender superstitious behavior. The important variables are the animal, the skill of the experimenter, and the time before the fix hits the tissue. The latter two are obviously linked. Thus, we have directed our approach to simplifying the procedure, eliminating injections of cardiovascular agents, etc., and developing a clocklike precision to the process. We set out the instruments in order of use so that they are easy to pick up. The surgical procedure proceeds as follows:
    (a) The abdomen is opened with a scalpel.
    (b) The skin over the sternum is incised to meet the first incision.
    (c) The sternum is split using heavy scissors.
    (d) Then the diaphragm is cut with these heavy scissors.
    (e) Rib spreaders are used to stabilize the ribcage.
    (f) Delicate scissors are used to open the pericardium so that the heart can be slipped out.
    (g) The same scissors are used to stab a small hole in the wall of the left ventricle near the apex through which the needle of the perfusion tube is then inserted.

Even with the best technique, *Murphy's Second Law of Perfusions* will come into play. It states: "The quality of the perfusion is inversely proportional to the accuracy of the tracer injection."

17. We use a blunt-tipped Hamilton syringe and sharpen the needle tip with a sharpening stone, just enough that it can penetrate the brain, but with the ejection hole near the needle tip to maintain stereotaxic accuracy. For central injections of WGA-HRP or ChTB-HRP, we use volumes on the order of 0.01–0.03 μl for central injections. Ten times this amount (0.1–0.3 μl) is used for BDA injections. ChTB-HRP is reputed to give better dendritic filling, but we do not always see this.
18. We use a plastic tackle box with a hole drilled in the base of each section and nylon mesh screening (Small Parts, CMN-2000) adhered to the bottom with fiber glass resin (Bondo) for the reaction tray. The solutions are placed in a Pyrex reaction dish that holds the tackle box. Between steps, the tackle box is blotted on squares of bench coat paper. However, for smaller sections many individuals use a piece of plexiglass that has wells drilled in it as a reaction tray and place the solutions into a staining dish.
19. Sections will be yellow at the end of the reaction step, but this does not indicate a background problem.
20. For light microscopy, the sections can be mounted out of this buffer and counterstained with cresyl violet. The reaction product is a bright blue color that can be further enhanced by using crossed polarizers.
21. Reaction product can be seen to turn from blue to brown during this period. Once this transformation has occurred the sections can be moved into the rinse.
22. Soak reaction tray and glassware in 70 % ethanol with a little Chlorox to clean reaction product from them. Rinse thoroughly with tap water followed by dH_2O.
23. Probably most labs will use plastic multiwell culture plates for this step.
24. Depression plates are placed on a wet piece of bench coat in a closed plastic box to limit evaporation. If using multiwell culture plates, employ the cover.
25. The background staining is correlated with fixation quality: the poorer the fix, the worse the background. We divide sections into three series, so if the first series stains too darkly and the background is too high, we can lower the cobalt chloride levels in a subsequent series. We usually divide sections into three series, and reserve one series for light microscopic analysis.

26. For postembedding immunohistochemical staining, the tissue needs to be embedded in a resin that allows the antibody to penetrate the tissue in order to bind to the antigen one wants to detect, but at the same time results in good ultrastructural preservation.
27. Pick up and move the grids with a nonmagnetic Dumont forceps by grabbing them along the edge. It should be noted that other labs have successfully omitted some of the steps described below. Some labs omit the etching step when they can process the tissue immediately after sectioning. The glutaraldehyde fixation step seems to help increase signal in our hands, but other labs successfully omit this step.
28. If the grid sinks into the drop, it is no longer useable since immunogold has a high affinity for the Formvar film and so will produce a high level of background staining on the back of the film, obscuring positive label on the section.
29. To make sure that the last washing step of a grid is always performed in clean, not contaminated water, start with the first grid in vial 1, proceed to vial 2, and then vial 3. Dry grid and take off the remaining fluid by touching the edge of a piece of filter paper to it. Do not touch Formvar and sections. Next rinse the second grid as previously done with grid 1, but start in vial 2, and proceed to vial 4. The third grid will begin in vial 3 and proceed to vial 5, etc. Make sure that the grid remains vertical while moving through the rinse, so that the Formvar film is not ruptured. Do not break the surface of the water except to enter and exit the vial; the surface tension of the water adds stress to the rinse steps and can rupture the Formvar film.
30. We do not make background counts over the Formvar outside the specimen, because its attractiveness to the antibody and secondary is not the same as the tissue.
31. Of course, the extent of dendritic labeling will vary with the degree of retrograde labeling overall. However, it is our impression that one can often find patches of label in small, presumably distal, dendrites at the EM level, even when these distal dendrites do not appear to be labeled at the light microscopic level. We believe that small, lightly labeled dendrites do not have enough optical density to be visualized, but this is not the case at the EM level.
32. While the gold particles are relatively easy to see when working at the EM or on a monitor, they can be difficult to demonstrate in poster or publication pictures, due to the electron-dense background of the BDA. We find that using Photoshop to lighten the image and slightly decrease the contrast can improve their visibility.

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