A Combined Retrograde Transport and Immunocytochemical Staining Method for Demonstrating the Origins of Serotonergic Projections

ROBERT M. BOWKER, KARIN N. WESTLUND, MERRY C. SULLIVAN, and JOE D. COULTER

Marine Biomedical Institute and the Department of Psychiatry and Behavioral Science, and the Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550-2772

Received for publication March 16, 1982; accepted March 25, 1982 (OA 82-151SA)

Key Words: Horseradish peroxidase; Serotonin; Retrograde transport; Immunocytochemistry; Spinal cord.

Principle of Technique

In recent years considerable interest has been directed toward defining projections of neurons containing specific neurotransmitter substances. As a result, numerous techniques have been developed that use fluorescent retrograde cell markers (2,6,10,11,19) or horseradish peroxidase (HRP) (3,4,13,14,17) combined with the fluorescence histochemistry (2,17,19), immunohistochemistry (6,10), or the unlabeled antibody, peroxidase-antiperoxidase (PAP) method (4,5,14) to identify the projections of neurotransmitter-defined neurons. While these techniques have contributed to the advancement of our basic understanding of the anatomical organization of certain pathways, they are often laborious and inconvenient or the existence of the different cell markers is relatively short-lived. A technique (4) has been developed in our laboratory that combines the retrograde transport of horseradish peroxidase (HRP) or of wheat germ agglutinin conjugated to HRP (WGA-HRP) with the PAP method of Sternberger. This technique can be employed to determine the origin of neurotransmitter pathways as well as pathways containing other putative neurotransmitter/modulator substances. In the present article, these methods are applied to determining the origin of descending serotonergic pathways to the spinal cord (4,5), but the techniques can be readily adapted to studies of other systems (15,20).

Instruments

Equipment for HRP injections
- Stereotaxic apparatus; David Kopf; $1600
- Universal holder; $100
- Surgical instruments; $100-300
- Hamilton syringes—1 μl, 5 μl, or 10 μl; $18-37
- Freezing microtome (A060); Scientific Prod.; $3000
- Hollow polyethylene stoppers (17 × 11 mm, package of 24); Scientific Prod.; $3
- Disposable tissue culture plates (24-wells, 17.8 × 16 mm, 100 dishes per case); Costar; $83.

Chemicals

Perfusion
- Paraformaldehyde; Fisher Scientific; $14/500 g
- Sucrose; $2/5 lb.

Histochemicals
- Horseradish peroxidase (HRP); Miles; $120/100 mg
- Cobalt chloride (CoCl2); Sigma; $7/100 g
- 3,3-Diaminobenzidine-HCl; Fisher Scientific; $6/5 g
- 3-Amino-9-ethylcarbazole; Sigma; $6/50 g
- Dimethylformamide; Sigma; $8/liter.

Immunocytochemicals
- Anti-serotonin; Immuno Nuclear Corp.; $115/0.1 ml

1Supported by National Institutes of Health grants NS 12481 and NS 11255.
Goat anti-rabbit immunoglobulin G (IgG); Cappel Labs.; $12/2 ml
Rabbit peroxidase-antiperoxidase (PAP); Cappel Labs.; $59/1 ml
Normal goat serum; Grand Island Biol.; $7/100 ml
Triton X-100; Sigma; $4/100 ml.

Procedure

Injection of Animal

In anesthetized animals, HRP or wheat germ agglutinin conjugated to HRP (WGA-HRP) is injected via a glass micro-pipette (tip 50–100 μm) attached to a Hamilton syringe. The quantity of HRP injected will vary depending upon the system studied. For our purposes, small quantities (0.1–0.2 μl) of a 25–50% solution of HRP or 0.1% solution of WGA-HRP are deposited into the spinal cord at each penetration (a range of 6–15 penetrations may be made bilaterally over 2–3 spinal segments). Further detailed descriptions of different delivery systems, retrograde transport, and survival times can be obtained from other sources (12,16).

Perfusion

2. Solutions.
   a. Physiological saline (PS)
      - NaCl; 9 g; distilled water; 1000 ml
      - Note: 300–500 ml of PS is warmed to 35–38°C before perfusing the animal. In our laboratory the solution is warmed to 45–50°C so that when poured into the perfusion apparatus and then infused, the saline solution has cooled to a temperature ranging between 35–38°C for perfusion of the animal.
   b. Fixative
      - paraformaldehyde; 30–38.5 g; distilled H₂O; 1000 ml
      - 0.1 M phosphate buffer (see Appendix); 100–200 ml
      - Note: Cool solution to 4°C. The fixative solution can range between 3.0% and 3.8% paraformaldehyde with no apparent difference in degree of fixation or retrograde labeling with HRP or WGA-HRP.
   c. Sucrose solution
      - sucrose; 300 g; distilled water; 1000 ml
      - 0.1 M phosphate buffer; 100–200 ml.

Perfusion Procedure

The animals are deeply anesthetized with pentobarbital, and the limbs affixed to a wire grate with tape. After exposure of the heart, the right ventricle (or atrium) is cut open and the left ventricle is punctured with a blunt 14–18 gauge needle, which is connected to the perfusion apparatus containing the saline, and is held in the ascending aorta. For rats, 300–500 ml of warm physiological saline is infused until the effluent remains clear for 30–60 sec. This phase of the procedure usually takes 2½–5 min. For cats and monkeys, 800–1000 ml of warm saline is infused.

The perfusion solution is then changed from warm saline to the cold 3.0–3.8% paraformaldehyde solution. In our experience, the first movements of the animal produced by the infusion of paraformaldehyde fixative are a subtle withdrawal of the mandible, which serves as a fairly reliable indicator of a successful perfusion of the brain. The blunt 14–18 gauge needle is then clamped in place in the aorta with a large hemostat. The connective tubing is placed in an ice bath to maintain the temperature of the fixative between 5–12°C. The rate of perfusion by the fixative is adjusted so that the infusion of the 1100–1200 ml is completed within 40–60 min. We have arbitrarily set 60 min as the maximum length of time that the brain and spinal cord tissues are in contact with the paraformaldehyde solution. At this time, 1 liter of cold (5–12°C) 30% sucrose solution is infused to prepare the tissues for sectioning. Perfusion with the sucrose solution is usually completed within 60–90 min. The brain and spinal cord are removed immediately and placed in a small amount of the sucrose solution. Tissue sectioning (20–25 μm) on a freezing microtome can begin immediately or can be delayed until the next day. For larger animals, e.g., cats and monkeys, 2.5–3.0 liters of both fixative and sucrose solutions are employed.

HRP Histochemistry

Solutions

- a. 0.1 M phosphate buffer (pH 7.4) (see Appendix)
- b. Tris/HCl buffer (pH 7.2–7.4) (see Appendix)
- c. 0.5% Cobalt chloride; 1.5 g
      - Tris/HCl buffer (see Appendix); 300 ml
      - Freshly made solution is bright pink in color
- d. 0.020% 3,3′-diaminobenzidine hydrochloride (DAB); 50 mg
      - 0.1 M phosphate buffer; 250 ml
      - 30% hydrogen peroxide; 30–50 μl.

HRP Histochemistry Procedure

The sectioned tissues are collected in cold phosphate buffer and rinsed two to three times in Tris/HCl buffer prior to incubating them in 0.5% CoCl₂ for 5–10 min. The sections are then rinsed two to three times in Tris/HCl buffer and two to three times in phosphate buffer before reacting them for HRP histochemistry. At this phase of the procedure the sections have a very light gray or bluish cast to them. The sections are then incubated in 0.02% DAB for 10–15 min without H₂O₂ and then 15 min in 0.02% DAB with H₂O₂. To ensure complete exposure of tissues to the DAB solution, the tissue sections are agitated frequently during the reaction. The sections are rinsed in 0.1 M phosphate buffer two to three times at the end of the histochemical reaction. To abbreviate this procedure the sections can be incubated in DAB with H₂O₂.
for 15 min. At this time retrograde labeling can be evaluated by examining a few of the wet sections under the microscope (low magnification, bright-field optics). Usually in the wet tissue sections, the black HRP granules are visible only in the larger sized neurons. The tissue sections are next processed for immunocytochemistry.

**Immunocytochemistry**

**Solutions**

a. 1% normal goat serum
   0.1 M phosphate buffered saline (PBS); 250 ml
   Triton X-100; 0.050 ml
   normal goat serum; 2.5 ml

*Note:* This solution is used as diluent for antisera and for washes between steps in the immunocytochemical procedure.

b. 3% normal goat serum
   0.1 M phosphate buffered saline; 250 ml
   gelatin; 1.8 g
   Triton X-100; 0.050 ml
   normal goat serum; 8.0 ml

*Note:* Heat PBS solution to 50–60°C, dissolve gelatin, and then let cool and add normal goat serum.

c. Goat anti-rabbit IgG

*Note:* Dilute IgG 1:10 in 1% normal goat serum.

d. Rabbit peroxidase-antiperoxidase (PAP)

*Note:* Dilute rabbit PAP 1:80 in 1% normal goat serum.

**Immunocytochemistry Procedure**

a. Tissue sections are placed in hollow polyethylene stoppers and are incubated in an antiserum raised to serotonin for 24–48 hr at room temperature with continuous or frequent agitation. The dilutions of serotonin antiserum (ImmuNo Nuclear) used in our laboratory range from 1:2000 to 1:10,000. Incubation of the tissue sections in the hollow polyethylene stoppers serves two purposes: 1) the wells conserve the quantity of antiserum utilized during each experiment and 2) they are a convenient and efficient mechanism to process the tissue sections for immunocytochemical staining. Following incubation in the 5-hydroxytryptamine (5-HT) antiserum, the contents of the wells, after several rinses, are transferred to a small compartmentalized (12 x 12 x 12 mm) tray having a fine nylon mesh attached to its base. This tray with its fine mesh permits thorough washing of the tissue sections and then allows the incubation of the sections in the immunocytochemical reagents during the PAP staining procedure. In addition, by having the tray compartmentalized, tissue sections from one or more animals can be processed simultaneously.

b. Wash repetitively 6–10 times in 1% normal goat serum in 0.1 M PBS.

c. Incubate tissue sections in 3% normal goat serum, 0.75% gelatin in PBS for 30 min. This is to "block" nonspecific staining. (While this step may be omitted if nonspecific staining is not a problem, it also serves as an additional washing step.)

d. Incubate in goat anti-rabbit IgG in 1% normal goat serum in 0.1 M PBS for 30 min.

e. Wash 6–10 times in 1% normal goat serum in 0.1 M PBS.

f. Incubate in 3% normal goat serum, 0.75% gelatin in 0.1 M PBS for 30 min. See c above.

g. Incubate tissue sections in rabbit PAP diluted 1:80 in 1% normal goat serum in 0.1 M PBS for 30 min.

h. Wash 6–10 times in 0.1 M phosphate buffer.

i. Incubate sections in 0.02% DAB and 0.01% H₂O₂ in 0.1 M phosphate buffer for 6 min.

j. Wash well in 0.1 M phosphate buffer. *Note:* Instead of DAB, one can use 3-amino-9-ethylcarbazole as the chromagen. Dissolve 20 mg of 3-amino-9-ethylcarbazole in 5 ml of dimethylformamide and mix with 95 ml of 0.05 M acetate buffer (pH 5.0) containing 30–50 μl of 30% H₂O₂. React for 3 min and rinse in distilled water. Reaction product is a bright red instead of the brown stain seen with DAB (9).

k. Mount and cover slip in alcohols, xylene, and Permount. The red reaction product seen after the 3-amino-9-ethylcarbazole reaction must be mounted in glycerin and distilled water (4:1).

l. Control experiments—in control experiments the serotonin antiserum is preabsorbed with 5-hydroxytryptamine HCl (Sigma). An excess of 5-hydroxytryptamine HCl is incubated with 5-HT antiserum (dilution 1:2000) at 37°C for 1 hr and then for 18–24 hr at 4°C. After centrifugation (2000 × g for 15 min) to remove any precipitate, the preabsorbed antiserum is incubated with tissue sections at room temperature for 18–48 hr. No staining indicative of positive 5-HT immunoreactivity should be observed in any tissue sections using antiserum preabsorbed with 5-hydroxytryptamine concentrations greater than 6.5 μM (2.5 mg per ml of the diluted antiserum).

**Examination of Slides**

The tissue sections can be viewed under bright-field microscopy. Neurons retrogradely labeled with HRP or WGA-HRP from the spinal cord injection site contain black punctate granules distributed throughout the cell body and proximal dendrites, while the cytoplasm is clear or smoke tinted (Figure 1A). The amount of HRP or WGA-HRP reaction product varies, with some neurons having only a few HRP granules while others are completely filled with reaction product. Neurons heavily labeled with black reaction granules can obscure the presence of the immunocytochemical staining, while neurons very lightly labeled with HRP are difficult to detect under
Figure 1. Photomicrographs illustrating examples of HRP-labeled (A), serotonin-stained (B), and double-labeled (C) neurons. (A) Photomicrograph shows black punctate granules (triangles) with a relatively clear or unstained cytoplasm. Bar = 40 \mu m. (B) Photomicrograph of serotonin-stained neuron shows a relatively homogeneous staining to both cytoplasm and dendrites (open arrows). Bar = 25 \mu m. (C) A serotonin-stained neuron (open arrows) is shown to contain black HRP granules (triangles) within both cytoplasm and dendrites. Bar = 20 \mu m.

Advantages and Weaknesses

The major advantages of this technique are that both cell markers can be viewed simultaneously in the same tissue section and that this method is versatile in being applicable to other neurotransmitter systems. As a result, the relationships of the double-labeled cells to HRP-filled cells, to the nonprojecting immunoreactive neurons, and to cytoarchitectural borders of nuclei, in Nissl stained sections, can easily be seen under bright-field microscopy. In addition, the relationships of these identified cell populations to other immunoreactive neurons, following a second immunocytochemical processing with a different antiserum and chromagen can also be easily visualized. Both cell markers are relatively permanent with little fading, can be processed for electron microscopy, and are easily quantifiable in terms of relative numbers of neurons.

A number of variables become important, in the data quantification when using this double-labeling technique. The major variables include the amount of HRP or WGA-HRP injected, antiserum dilutions, and tissue section thickness. Insufficient penetration of the HRP histochemical solutions appears to be a minimal problem as tissue sections exceeding 100 \mu m in thickness can still be reacted successfully for HRP reaction product. On the other hand, penetration of the antiserum and other immunoglobulins into tissue sections is critical to the immunocytochemical staining (8). The results of the collected quantitative data will vary considerably depending upon the thickness of the tissue section. To illustrate this important variable, a rat was injected with a large quantity (4 \mu l of a 25–50% solution) of HRP over fifteen penetrations in the lumbosacral spinal cord and was perfused 2 days later. Sections of the brain stem were cut serially at 50, 40, 30, and 20 \mu m and then the sectioning cycle was repeated. The tissue sections were reacted for HRP reaction product, incubated in 5-HT antiserum for 24 hr at dilutions of 1:3000 and 1:6000
HRP RETROGRADE TRANSPORT AND 5-HT IMMUNOCYTOCHEMISTRY

(two dilutions for each thickness), and then processed for immunocytochemical staining. As shown in Figure 2, the percentage of raphe-spinal neurons that were stained for serotonin immunoreactivity decreases as the thickness of the tissue section increases. Furthermore, the intensity of the immunoreactive staining was extremely faint in many double-labeled cells (questionable in as many as one-third) seen in tissue sections cut at 30 and 40 μm. On the other hand, the majority of the double-labeled cells were clearly stained in 20 μm thick sections at these same 5-HT antiserum dilutions and incubation times. These findings indicate that markedly different results can be obtained depending upon the thickness of the tissue sections. Thus, in double-labeling experiments using the present technique tissue sections should be cut relatively thin (20–25 μm) in order to maximize the exposure of the retrogradely labeled neurons to the 5-HT antiserum and immunoglobulins. Other variables that probably enhance the penetration of the immunoglobulins are the greater concentrations of detergents and longer periods (48–72 hr) of antiserum incubations.

The relative sensitivity of the present method for the retrograde labeling of chemically identified neurons is further enhanced by employing lectins (WGA) conjugated to HRP. This increased sensitivity of the lectin conjugates appears to be due to differences in the uptake mechanisms in which the conjugates are internalized by specific binding to sugar moieties located on the plasma membrane, while free HRP appears to be endocytosed on the basis of the concentration gradient (7,8). The combination of the lectin conjugates with the immunocytochemical PAP method has particular usefulness in experiments requiring relatively small and restricted injection sites. By this means, greater numbers of retrogradely labeled cells are observed in comparison to those experiments utilizing free HRP (7).

The primary disadvantage of this technique is that the DAB used for the HRP reaction is a well-known carcinogen.

Appendix

Phosphate buffer: to make a 0.1 M solution of phosphate buffer at pH 7.4, use reagent grade mono- and dibasic sodium phosphate (Sigma).

- 2.4 g (monobasic)
- 11.4 g (dibasic)
- 1 liter distilled water

Phosphate buffered saline: add 9 g of NaCl to above solution.

Tris/HCl buffer: to make a 0.1 M solution of Tris/HCl buffer at pH 7.2–7.4, use reagent grade Trizma HCl and Trizma base (Sigma).

- 0.69 g Tris base
- 3.03 g Tris HCL
- 500 ml distilled water

Acknowledgments

The authors wish to thank Dr. H.W.M. Steinbusch for providing the serotonin antiserum used in the initial studies in their laboratory and Dr. W. Pagh for the gift of wheat germ agglutinin conjugated to horseradish peroxidase. They would like to express their appreciation of the excellent typing assistance of Phyllis Waldrop and Debbie Pavlu.

Literature Cited


Figure 2. Graph showing percentage of raphe-spinal neurons stained for serotonin as a variable of tissue thickness. Under identical incubating and tissue processing conditions the relative numbers of spinally projecting neurons stained for serotonin immunoreactivity increases as the thickness of the tissue section decreases.


17. Smolen AJ, Glazer EJ, Ross LL: Horseradish peroxidase histochemistry combined with glyoxylic acid induced fluorescence used to identify brainstem catecholaminergic neurons which project to the chick thoracic spinal cord. Brain Res 160:353, 1979

