Experimental Neuroanatomy

A Practical Approach

Edited by

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Combined approaches to experimental neuroanatomy: combined tracing and immunocytochemical techniques for the study of neuronal microcircuits

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1. Introduction

Most of the chapters in this volume have so far dealt with individual techniques in experimental neuroanatomy at the electron microscopic level. From these techniques we can identify projection neurones and examine the morphology of their afferent terminals. Moreover, the structural characteristics of the post-synaptic target of a set of anterogradely labelled terminals can be analysed from material containing immunostained structures, or intracellularly labelled neurones. These approaches in themselves produce valuable information concerning neuronal elements within the microcircuits and networks of the brain. However, the amount of data obtained using such approaches is limited in the sense that the identified structure is examined 'in isolation'. In order to establish the position of an identified neurone, or population of terminals within the neural network in relation to other neuronal elements, it is necessary to know the *nature* of the terminals afferent to a labelled neurone, or the *nature* of the post-synaptic targets of identified terminals. By this we mean that it is necessary to know the origin, neurochemical nature, and pattern of innervation of the synaptic terminals afferent to an identified neurone. Similarly, it is important to know the morphology, the chemical nature and the synaptic output of the neurones that are post-synaptic to a specific population of labelled terminals. Thus, to establish the microcircuitry or neuronal networks of a particular area of the brain it is necessary to *combine* the individual experimental approaches in a single experimental animal.

In several of the chapters, points at which individual experimental or histological techniques can be combined have already been described. The object of this chapter is to describe in detail some of the ways in which individual experimental approaches can be combined. The combinations of individual techniques will not be dealt with in an exhaustive manner since they are virtually limitless, often the only limitation being the ingenuity of the experimenter. The main emphasis will be on combinations of different tract-tracing techniques (anterograde and retrograde), and combinations of tract-tracing techniques with immunocytochemistry.

2. Combination of retrograde labelling with pre-embedding immunocytochemistry

The main objectives of this combination are two-fold.

(a) The identification of some chemical characteristic (usually relating to the nature of the transmitter) of a population of projection neurones.

(b) The identification of the transmitter characteristics of terminals *afferent* to a population of projection neurones.

The approach to this combination is first to administer the retrograde tracer to the animal and after the appropriate survival time the animal is perfuse–fixed. Sections containing the injection site and transport sites are cut and reacted to reveal the transported marker, and then subjected to immuno-cytochemistry (*Protocol 1*). Although various retrograde tracers (gold-labelled markers and cholera toxin conjugated to various markers) can readily be combined with pre-embedding immunocytochemistry (see Chapter 2), the most commonly used retrograde tracer and the easiest to combine with immunocytochemical techniques is horseradish peroxidase conjugated to wheatgerm agglutinin (WGA-HRP).

The choice of fixative for animals to be used for combined retrograde labelling and immunocytochemistry depends almost entirely on the sensitivity to fixation of the antigen that is to be localized by immunocytochemical means, since most retrograde markers are fairly robust and in particular, horseradish peroxidase activity is preserved in the tissue exposed to a wide variety of fixatives. The reader is therefore referred to Chapter 5 and should refer to both the manufacturers' recommendations and publications in which the same antiserum or antibody preparation has been used. In practise, it is generally the case that low concentrations of glutaraldehyde and relatively high concentrations of paraformaldehyde are required. The chromogen used in the immunoperoxidase reaction is usually diaminobenzidine (DAB) and the chromogen used to reveal the retrogradely-transported WGA-HRP is tetramethylbenzidine (TMB) (*Figure 1A*). The combined procedure may also be applied using DAB as the chromogen for both of the peroxidase reactions (*Figure 1B*).

Protocol 1. Combination of retrograde labelling with pre-embedding immunocytochemistry

1. Prepare and inject by pressure or iontophoresis WGA-HRP solution. Use a 0.5–7% solution in 0.9% NaCl solution (see Chapter 2).
2. Allow the animal to survive for 24–48 h.
3. Perfuse–fix the animal (see Chapter 1, *Protocol 3*) with a mixture of paraformaldehyde and glutaraldehyde (see Chapter 1, *Protocol 2*).
4. Perfuse the animal with cold phosphate buffer (0.1 M, pH 7.4), same volume as the fixative (see Chapter 1, *Protocol 2*).
5. Dissect the brain from the skull. Cut areas of interest, (i.e. injection sites and transport sites) into 5 mm-thick blocks and store in PBS at 4°C (see Chapter 1, *Protocol 1*) until sectioning.
6. Section areas containing the injection sites and the transport sites at 50–70 μm on a vibrating microtome (see Chapter 1, *Protocol 4*), and collect the sections in cold PBS.
7. *Optional*. Treat the sections for 15–20 min with 1% sodium borohydride (see Chapter 5, *Protocol 6*).
8. Wash several times in PBS, and then 0.1 M phosphate buffer at pH 6.2.
9. Incubate the sections to reveal the injected and transported WGA-HRP using TMB as the chromogen in the peroxidase reaction.[a] Prepare the TMB reaction mixture in the following manner:
   - dissolve 10 mg TMB in 5 ml of ethanol
   - dissolve 250 mg of ammonium molybdate (VI) tetrahydrate in 100 ml 0.1 M phosphate buffer pH 6.2
   - mix 2.5 ml of the TMB solution with 97.5 ml of the ammonium molybdate solution
10. Incubate the sections in this mixture for up to 40 min adding sufficient of 0.3% H_2O_2 every 5–10 min to give a final concentration of 0.003% (200 μl per 20 ml).
11. Wash sections 3 × 5 min in 0.1 M phosphate buffer at pH 6.2.
12. Stabilize the TMB reaction product by incubating for 5–8 min in an ice-cold solution consisting of:
    - 50 mg DAB[b] in 50 ml phosphate buffer (0.1 M, pH 7.4)
    - 2 ml of a 1% aqueous solution of cobalt chloride
    - 333 μl of a 0.3% solution of hydrogen peroxide per 10 ml of the mixture
13. Wash the sections for 3 × 5 min in PBS.
14. Separate the sections that will be prepared for light microscopy alone from those for electron microscopy.

Protocol 1. *Continued*

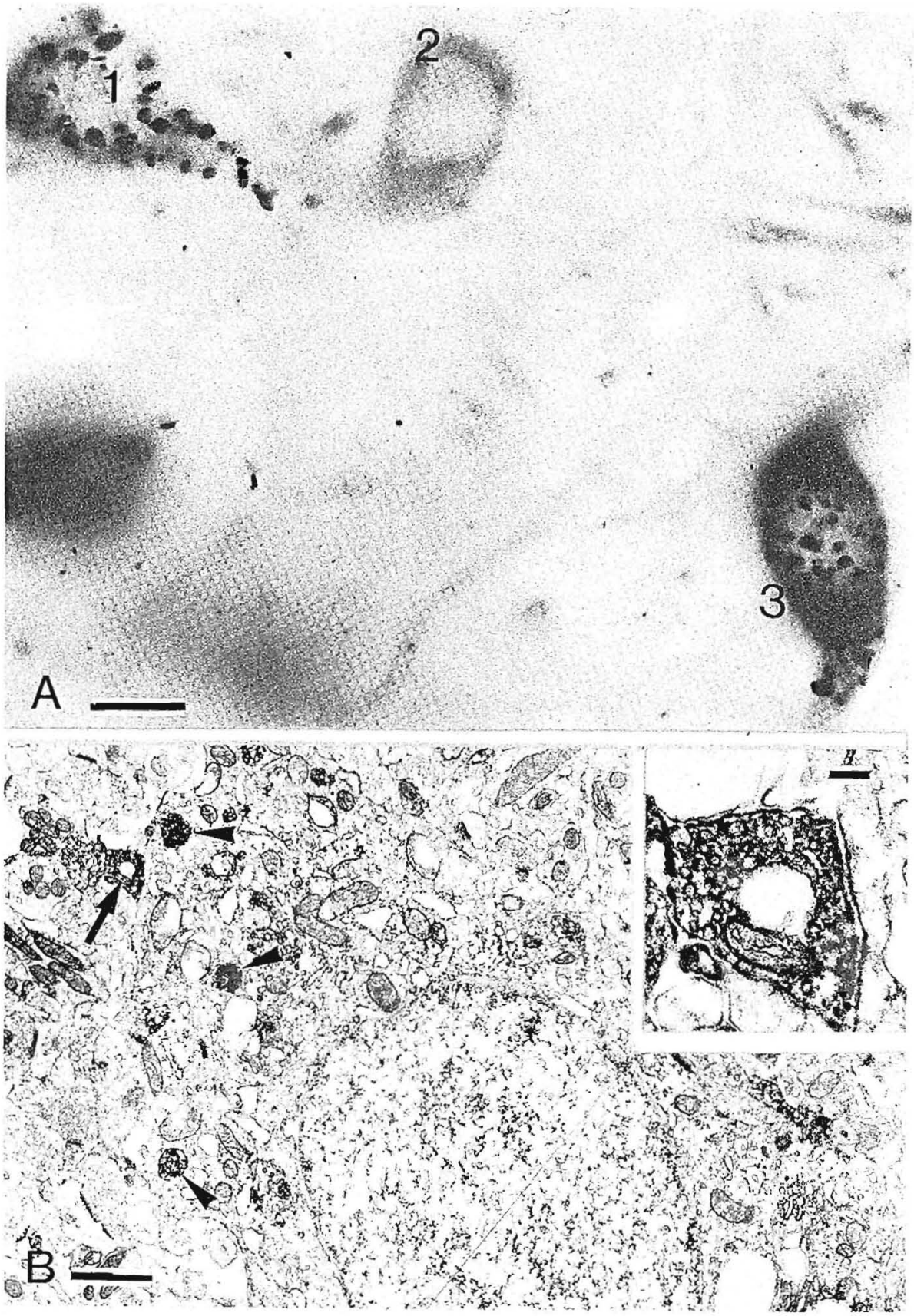
15. *Optional.* Freeze–thaw the sections that will be prepared for electron microscopy in liquid nitrogen (see Chapter 5, *Protocol 4*). Treat sections for light microscopy with Triton X-100, and include Triton in the primary antibody solution (Chapter 5, *Protocol 5*).
16. Stain the sections immunocytochemically to reveal the antigen of interest using the peroxidase anti-peroxidase method (see Chapter 5, *Protocol 8*), or the avidin–biotin–peroxidase method (see Chapter 5, *Protocol 9*).
17. Mount the sections prepared for light microscopy on gelatin-coated slides (see Chapter 1, *Protocol 5*), dehydrate, and apply coverslips (see Chapter 1, *Protocol 6*).
18. Post-fix the sections prepared for electron microscopy in osmium tetroxide (see Chapter 1, *Protocol 7*), dehydrate, and mount on microscope slides in an electron microscope resin (see Chapter 1, *Protocol 8*).

[a] See Chapter 2 for alternative protocols to reveal WGA-HRP.
[b] See *Safety note 3* in Chapter 5 (p. 119).

2.1 Appearance of the staining

The outcome of this combination are sections that contain both retrogradely labelled neurones and immunostained structures. At the light microscopic level, the retrogradely labelled neurones are recognized by the large dark blue granules of the TMB reaction product (*Figure 1A*). The immunostained structures will contain the typical DAB peroxidase reaction product, i.e. a brown, amorphous substance. After treatment with osmium tetroxide the characteristic location and the texture difference between the two reaction

Figure 1. Combination of retrograde labelling and pre-embedding immunocytochemistry (*Protocol 1*). (A) is a light micrograph of a section of the rat globus pallidus that contains a retrogradely labelled neurone (1), an immunostained neurone (2), and a neurone that is both retrogradely labelled and immunostained (3). The retrogradely labelled neurone contains the large granules of TMB reaction product and has a relatively clear cytoplasm, the immunostained neurone contains the amorphous DAB reaction product, and the double stained neurone has both the granules of TMB reaction product and the amorphous DAB reaction product. (Substantia nigra of rat injected with WGA-HRP; globus pallidus incubated to reveal transported HRP and then immunostained by the ABC method to reveal parvalbumin.) (B) is an electron micrograph of a neurone retrogradely labelled with WGA-HRP. It contains DAB reaction product that is present in multi-vesicular bodies (arrowheads). The neurone has a bouton apposed to it (arrow and inset) that is immunostained by the PAP method and revealed using DAB. It contains the typical amorphous, DAB immunoreaction product (see also Chapter 5). (Substantia nigra of rat injected with WGA-HRP, striatum incubated to reveal retrogradely-transported HRP using DAB as the chromogen, and then immunostained by the PAP method for substance P immunoreactivity, also using DAB as the chromogen.) Scale markers: (A) 10 μm, (B) 1 μm, inset, 0.1 μm.


1
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A
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products is maintained. Thus, it is possible to identify immunostained perikarya that contain the retrogradely-transported WGA-HRP on the basis of the colour and location of the reaction products; the perikaryon will have an overall brown appearance and contain the granules of the TMB reaction product (see *Figure 1*). Similarly it will be possible to identify immunostained terminals that are apposed to retrogradely labelled neurones. The sections may also contain, if the connection is reciprocal, terminals anterogradely labelled with the TMB reaction product.

At the electron microscopic level the two reaction products are distinguishable. The DAB reaction product is an amorphous floccular precipitate associated with the internal plasma membrane and external membrane of subcellular organelles, whereas the TMB reaction is crystalline, more electron dense, and in the form of clumps dispersed in the cytoplasm (see *Figure 2*). When DAB is used as the chromogen for *both* peroxidase reactions the two are distinguished on the basis of location (*Figure 1B*). Thus the DAB reaction product in retrogradely labelled structures is present in secondary lysosomes or multivesicular bodies and appear at low magnification as round granules (*Figure 1B*).

2.2 Analysis of the material

The examination of the afferent synaptic input to retrogradely labelled, immunostained neurones, or the examination of immunostained terminals that form synapses with retrogradely labelled neurones can be carried out in two ways. First, areas that are dense in labelled structures can be re-embedded (see Chapter 1, *Protocol 9*), and ultrathin sections scanned in the electron microscope to identify the labelled structures. The second approach is to examine individual retrogradely labelled neurones that are themselves immunostained and/or apposed by boutons that are immunostained, by correlated light and electron microscopy. Thus a neurone is identified, first at the light microscopic level, after it has been drawn and photographed the same neurone is then re-embedded and examined in the electron microscope (see Chapter 1). In this way, the problems of sampling of relatively rare events at the electron microscopic level are overcome.

2.3 Applications

This combination of procedures using this or similar methods has been used successfully in the analysis of microcircuits in many areas of the brain. For example, the afferent synaptic input has been characterized of neurones in the basal forebrain of the rat that were themselves characterized on the basis of immunoreactivity for choline acetyltransferase and their projection to the cortex (1). Similarly, by combining immunocytochemistry with the retrograde transport of WGA-HRP from the substantia nigra, it has been demonstrated that neurones in the striatum of the rat that project to the substantia nigra

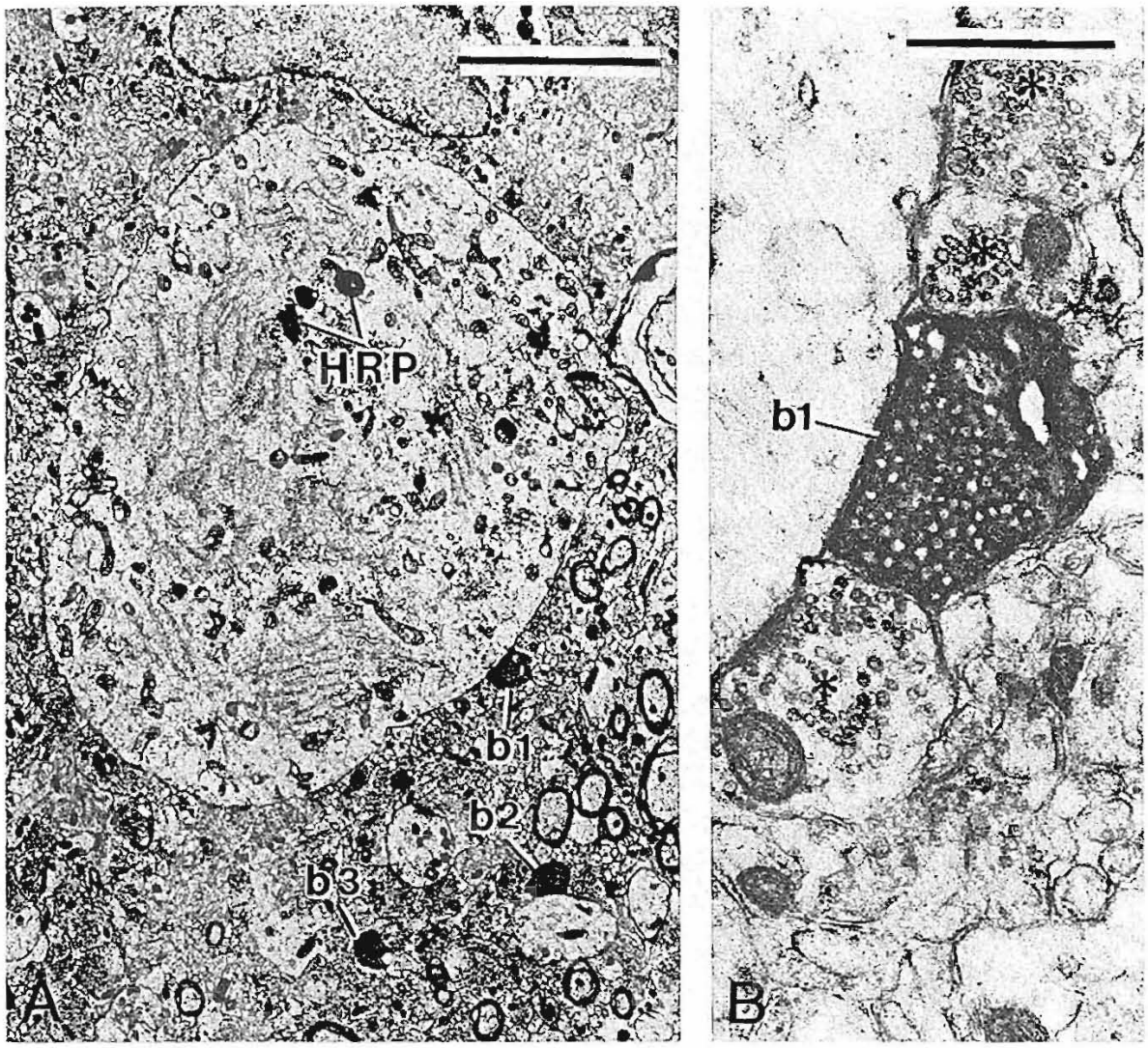


Figure 2. Anterograde transport of PHA-L combined with the retrograde transport of WGA-HRP (*Protocol 3*). (A) Electron micrograph showing a retrogradely labelled perikaryon in the rat substantia nigra after injection of WGA-HRP in the superior colliculus. The crystals of TMB reaction product are indicated by HRP. This perikaryon is contacted by a PHA-L immunoreactive bouton (b1) that has been anterogradely labelled after injection in the striatum. The association between the retrogradely labelled cell and the anterogradely labelled bouton is shown at higher magnification in (B). Note in (A) the presence of two other PHA-L positive terminals in the neuropil (b2 and b3). In (B) the asterisks indicate non-immunoreactive terminals that form contacts with the retrogradely labelled perikaryon. Scale markers: (A) 5.0 μm, (B) 1.0 μm.

receive synaptic input from terminals that are immunoreactive for choline acetyltransferase or for substance P (2, 3) (*Figure 1B*).

2.4 Limitations and controls

One limitation of this procedure is that the retrograde marker generally only labels the perikaryon and the most proximal part of the dendritic tree. Thus the synaptic input to the major part of a labelled neurone cannot be examined, and indeed it may be that it is in the distal dendritic tree that a particular class

of terminals make synaptic contact. One way to overcome this is by combining the procedure with Golgi-impregnation (see Chapter 1, *Protocol 12*). The combination of Golgi-impregnation with retrograde labelling and immunocytochemistry has been successfully applied to the analysis of the chemical nature of inputs to identified projection neurones in the basal ganglia (2–4). The outcome is that *all parts* of a retrogradely labelled neurone can be examined in the electron microscope. This combination however, suffers from the drawback that we have no control over which neurones will be impregnated by the Golgi procedure. A further limitation is the problem of false-negative immunostaining, since reagents to reveal retrogradely labelled neurones penetrate into the tissue more readily than immunoreagents.

The most important control experiment in combined procedures that involve more than one peroxidase reaction is to ensure that the chromogen of the second peroxidase reaction does not become deposited at the site of the first peroxidase reaction. Thus, the first peroxidase reaction must go to completion and the peroxidase molecules should not react with the second addition of hydrogen peroxide and the second chromogen. In practise this is a rare event, the peroxidase molecules are generally obscured by the first reaction product and are no longer available to react with the second application of substrate and chromogen. Nevertheless, controls should be performed to test for this and areas should be included that will contain neurones that are *only* retrogradely labelled and neurones that are *only* immunostained (see reference 1 and *Figure 1A*). The controls should also include sections that are incubated through the whole double procedure, but with omission of the primary antiserum or the whole double procedure, but first of all inhibiting the transported peroxidase (see Chapter 5).

3. Combination of retrograde labelling with post-embedding immunocytochemistry

The main objectives of the combination of retrograde labelling with post-embedding immunocytochemistry are the same as those for the combination of retrograde labelling with pre-embedding immunocytochemistry. The advantage of this combination (*Protocol 2*) over the previous combination, is that the problems of penetration of the immunoreagents (see Chapter 6) are overcome. An outcome of this is that in optimally fixed material the analysis of immunostained terminals in contact with a retrogradely labelled neurone can be carried out in a quantitative manner (5) since the likelihood of false negative immunostaining of terminals is minimal. To analyse the chemically characterized, (i.e. immunostained) synaptic input to a population of retrogradely labelled neurones the approach is to prepare ultrathin sections of a retrogradely labelled neurone, and then immunostain the ultrathin sections by the immunogold method (see Chapter 6, *Protocol 3*).

From a theoretical point of view, the combination may also be used to identify the chemical characteristics of a population of projection neurones, although this combination has so far not been applied. The approach is to cut semi-thin sections of resin-embedded retrogradely labelled neurones and immunostain them for the antigen in question (see Chapter 6, *Protocols 1* and *2*). The remainder of the neurone that is left in the resin block can then be sectioned for electron microscopy. A similar method to this has been used extensively to chemically characterize the post-synaptic targets of intracellularly filled neurones (6, 7).

Protocol 2. Combination of retrograde labelling with post-embedding immunocytochemistry

1. Prepare tissue with retrogradely labelled neurones under study according to steps **1–13** in *Protocol 1*. The preferred chromogen for the peroxidase reaction is TMB but others may also be used.
2. Post-fix the sections in osmium tetroxide (Chapter 1, *Protocol 7*), dehydrate, and embed them on microscope slides in resin (Chapter 1, *Protocol 8*).
3. Select retrogradely labelled neurones of interest on the basis of light microscopic analysis, and re-embed in blocks of resin for ultrathin sectioning (Chapter 1, *Protocol 9*).
4. Cut ultrathin sections and collect on coated, gold, or nickel single-slot grids (see Chapter 1).
5. Immunostain the ultrathin sections by the immunogold method for the antigen under study, according to *Protocol 3* in Chapter 6.
6. Examine in the electron microscope.

3.1 Appearance of the staining

The outcome of the combined procedure are sections that contain retrogradely labelled neurones identified by the presence of peroxidase reaction product (see *Figures 1* and *2A*; see also Chapter 2), and also structures that have a high density of immunogold particles associated with them, thus identifying them as immunopositive (see *Figures 3* and *5*; see also Chapter 6). The criteria for the characterization of a structure as immunopositive in the post-embedding immunogold method are described in Chapter 6.

3.2 Analysis of the material

The retrogradely labelled neurones identified by the presence of the TMB (or other chromogen) reaction product are examined in serial sections to determine whether they receive synaptic input from axonal terminals that are

immunostained. Because of the lack of problems of penetration of the immunoreagents, once it is established that the antigen is retained in the material, the immunostained input to retrogradely labelled neurones can be quantified (5). Thus the proportion of terminals in contact with a retrogradely labelled neurone that display a particular chemical characteristic can be determined. Similarly the proportion of neuronal membrane that is occupied by boutons of a particular chemical characteristic can be determined.

3.3 Applications

One example of the application of this procedure is the characterization and quantification of the afferent input to neurones in the medial globus pallidus of the rat that project to the cortex. These neurones were identified as part of the basal forebrain by virtue of their projection to the cortex, and were found to receive input from terminals that are immunoreactive for the inhibitory amino acid transmitter, GABA. This GABA-containing input was shown to account for greater than 70% of the afferent input to these cells (5).

3.4 Limitations and controls

The major limitation of this combination is that the antigen must survive the post-fixation with osmium and/or the dehydration and embedding in resin. The number of antigens to which the post-embedding procedure and hence this combined approach can be applied, is therefore limited (see Chapter 6). Embedding the tissue in resin *without* post-fixation with osmium tetroxide increases the number of antigens that can be studied in this way.

Since it is unlikely that the TMB reaction product within perikarya or dendrites will affect immunostaining in terminals that are afferent to the labelled structures, the controls that are required relate to the immunostaining procedure, and the reader is referred to Chapters 5 and 6.

4. Combination of anterograde labelling and retrograde labelling

This combination of procedures is used to determine the origin of the synaptic terminals in contact with neurones characterized on the basis of their projection site. Although various techniques have been developed over the last few years (8), the anterograde transport of PHA-L combined to the retrograde transport of WGA-HRP is the most sensitive approach to be used for such investigations (see *Protocol 3*).

Protocol 3. Anterograde transport of PHA-L[a] combined with the retrograde transport of WGA-HRP

1. Prepare and administer PHA-L (see Chapter 3, *Protocol 1*).
2. Allow the animal to survive for 7–10 days.

3. Prepare and inject WGA-HRP (see Chapter 2).
4. Allow the animal to survive for 36–48 h.
5. Perfuse–fix the animal (see Chapter 1, *Protocol 3*) with a mixture of paraformaldehyde (2–4%) and glutaraldehyde (0.1–2.5%) (see Chapter 1, *Protocol 2*).
6. Perfuse the animal with cold phosphate buffer (0.1 M, pH 7.4; same volume as the fixative) (see Chapter 1, *Protocol 1*).
7. Dissect the brain from the skull. Cut in 5 mm-thick blocks and store at 4°C in PBS (see Chapter 1, *Protocol 1*) until sectioning.
8. Cut the brain areas containing the injection sites and the transport sites at 50–70 μm on a vibrating microtome (see Chapter 1, *Protocol 4*).
9. Collect the sections in cold PBS.
10. Treat sections for 20 min with 1% sodium borohydride (see Chapter 5, *Protocol 6*).
11. Wash the sections several times in PBS.
12. Process the tissue by means of the tetramethylbenzidine method to reveal the injected and transported WGA-HRP (see *Protocol 1*, steps **8–11**).
13. Stabilize the TMB reaction product with diaminobenzidine (see *Protocol 1*, step **12**).
14. Wash the sections several times in PBS.
15. Separate the sections that are for electron microscopy from those that are for light microscopy alone.
16. Process the tissue for the immunohistochemical localization of PHA-L at the light and electron microscopic level (see Chapter 3, *Protocol 2*, steps **6–14**).
17. Post-fix sections for electron microscopy in osmium tetroxide, dehydrate, and mount on slides in resin (see Chapter 1, *Protocols 7* and *8*).
18. Mount sections prepared for light microscopy on gelatin-coated slides, dehydrate, and apply coverslips (see Chapter 1, *Protocols 5* and *6*).

[a] PHA-L can be replaced by biocytin in which case, biocytin is prepared, injected, and visualized according to *Protocols 3* and *4* in Chapter 3.

4.1 Appearance of the staining

The outcome of this method are sections containing retrogradely labelled perikarya characterized by the presence of large dark blue granules throughout the cytoplasm (see *Figure 1A*), and PHA-L immunoreactive axons and terminals that contain the brown diffuse DAB reaction product. In sections processed for electron microscopy, the colour of the DAB and the TMB

reaction products is the same, but they remain easy to differentiate by their location and texture. The DAB reaction product has an amorphous texture and is associated with axonal processes and terminals, whereas the TMB reaction product has a crystalline texture and is found in perikarya and dendritic shafts (*Figure 2*).

4.2 Analysis of the material

In order to examine in the electron microscope areas containing both sets of labelled elements, the sections are analysed carefully in the light microscope for the presence of retrogradely labelled neurones apposed by PHA-L immunoreactive terminals. Once they have been photographed and drawn, these neurones are re-embedded (see Chapter 1, *Protocol 9*) and re-sectioned on the ultramicrotome. They are then examined in serial sections in the electron microscope to identify synaptic contacts between PHA-L positive terminals and the retrogradely labelled cells (*Figure 2*). The appearance of the material subjected to this combined procedure is essentially the same as in the combination of retrograde labelling and pre-embedding immunocytochemistry (*Protocol 1*), as the retrograde marker and the PHA-L or endogenous antigen are localized in the same manner in both procedures.

4.3 Applications

This approach has recently been used to test the possibility that the projection neurones in the substantia nigra pars reticulata (SNr) receive direct synaptic inputs from the globus pallidus in the rat (9) (*Figure 2*). Iontophoretic injections of PHA-L into the globus pallidus led to the anterograde labelling of a rich plexus of varicose fibres in the substantia nigra. Electron microscopic analysis revealed that these varicosities are large terminals that contain many mitochondria and form symmetric synapses predominantly with perikarya and proximal dendrites of nigrofugal neurones of the SNr (9, 10).

4.4 Limitations and controls

Although this combination of techniques is powerful for analysing the microcircuitry of neuronal systems, it is important to be aware of the limitations and technical problems that may complicate the interpretation of the data that is obtained. One of the major limitations is the fact that the granules of TMB reaction product are found only in the proximal part of the retrogradely labelled cells. Therefore, it should be kept in mind that unlabelled small dendritic shafts contacted by PHA-L immunoreactive terminals may have a parent cell body that is retrogradely labelled. In order to circumvent this problem, the retrogradely labelled cells may be Golgi-impregnated (see Chapter 1, *Protocol 12*). Another problem that may complicate the interpretation of data is the presence of WGA-HRP labelled terminals in the area

of study. This will happen if the structure that has been injected with WGA-HRP is reciprocally connected with the area of study (for example the substantia nigra with the striatum), or if the retrogradely labelled cells in the area under investigation have recurrent axon collaterals. Thus, it is very important to be familiar with the texture of the TMB and the DAB reaction products in the electron microscope in order to be able to differentiate the WGA-HRP (TMB reaction product) from the PHA-L labelled (DAB reaction product) terminals. In general, the crystalline texture and the high electron density of the TMB-containing boutons make them relatively easy to differentiate from the amorphously stained DAB-containing terminals. A third limitation of this approach is the possibility that PHA-L is transported in the retrograde direction (see Chapter 3). This may complicate the interpretation of data if the brain area that received the PHA-L injection is reciprocally connected with the area under investigation and particularly, if the cells retrogradely labelled with PHA-L have recurrent axon collaterals. In such case it is not possible to determine the exact source of the PHA-L labelled terminals visualized in the area of study.

Control experiments to verify the specificity of the staining visualized with this protocol include:

- the localization of WGA-HRP alone
- the omission of the PHA-L antiserum from the incubation medium.

5. Combination of anterograde labelling and pre-embedding immunocytochemistry

The objectives of this combined approach are first, to identify the source of the terminals that form synapses on to neurones characterized by their chemical content and secondly, to study the synaptic relationships between a population of terminals characterized by its origin, and a population of terminals identified by its chemical nature on to single post-synaptic structures. The approach is to combine the anterograde transport of PHA-L or biocytin with the immunoperoxidase method. Both substances are localized by peroxidase reactions but using different chromogens (*Protocol 4*).

Protocol 4. Anterograde transport of PHA-L[a] combined with pre-embedding immunocytochemistry

1. Prepare and administer PHA-L (see Chapter 3, *Protocol 1*).
2. Allow the animal to survive for 7–10 days.
3. Perfuse–fix the animal (see Chapter 1, *Protocol 3*) with a mixture of paraformaldehyde and glutaraldehyde[b] (see Chapter 1, *Protocol 2*)

Protocol 4. *Continued*

4. Dissect the brain from the skull. Cut in 5 mm-thick blocks and store at 4°C in PBS (see Chapter 1, *Protocol 1*) until sectioning.
5. Cut the brain areas containing the injection sites and the transport sites at 50–70 μm on a vibrating microtome (see Chapter 1, *Protocol 4*).
6. Collect the sections in PBS.
7. Treat the sections for 20 min in 1% sodium borohydride (see Chapter 5, *Protocol 6*).
8. Wash the sections several times in PBS.
9. Separate the sections that are for electron microscopy from those that are for light microscopy.
10. Process the tissue for the immunohistochemical localization of PHA-L at light and electron microscopic levels (see Chapter 3, *Protocol 2*, steps **6–14**).
11. Mount the sections that include the PHA-L injection sites on gelatin-coated slides, dehydrate, and apply coverslips (see Chapter 1, *Protocols 5* and *6*).
12. Process the regions containing the PHA-L labelled terminals for the immunohistochemical localization of the antigen of interest, using the avidin–biotin–peroxidase (ABC) (see Chapter 5, *Protocol 9*), or the peroxidase anti-peroxidase (see Chapter 5, *Protocol 8*) methods.
13. In the sections processed for light microscopy, reveal the second antigen using the nickel-enhanced DAB method. Prepare the reaction mixture as follows:

    add 0.37 g of nickel ammonium sulphate ($NiSO_4$. $(NH_4)_2SO_4 \cdot 6H_2O$) to 100 ml of Tris-HCl buffer (0.05 M, pH 7.6)

    add 25 mg of DAB

    add 200 μl of hydrogen peroxide (0.3% stock solution) when the DAB is completely dissolved
14. Incubate the sections in this solution for 10–12 min and then wash several times in PBS.
15. For the sections processed for electron microscopy, reveal the second antigen using benzidine dihydrochloride (BDHC) as the chromogen for the peroxidase reaction (see Chapter 5, *Protocol 11*; see also *Safety note 4* in Chapter 5, p. 123).
16. Mount the sections prepared for light microscopy on to gelatin-coated slides, dehydrate, and apply a coverslip (see Chapter 1, *Protocols 5* and *6*).
17. Post-fix the sections prepared for electron microscopy in osmium tetroxide

(see Chapter 1, *Protocol 7*; but the osmium tetroxide must be diluted in PB 0.01 M, pH 6.8), dehydrate, and embed in an electron microscope resin on microscope slides (see Chapter 1, *Protocol 8*).

[a] PHA-L can be replaced by biocytin, in which case, biocytin is prepared, injected, and visualized according to *Protocols 3* and *4* in Chapter 3.

[b] The percentage of paraformaldehyde and glutaraldehyde in the fixative is adjusted to obtain optimal labelling for the second antigen since PHA-L can tolerate a wide range of fixatives.

5.1 Appearance of the staining

The outcome of this method are sections including PHA-L labelled terminals containing the brown amorphous DAB reaction product, and the BDHC-labelled structures which appear blue prior to osmium treatment and become blue/black or grey after osmium post-fixation (see *Figure 2A* in Chapter 5). In addition to the colour, a major difference between the DAB and the BDHC reaction products is the texture; the DAB deposit is amorphous whereas the BDHC reaction product is granular. In the electron microscope, the two reaction products also show marked differences. The DAB reaction product is amorphous and is associated with subcellular organelle membranes and the internal plasma membrane, whereas the BDHC reaction product is granular or crystalline and does not appear to have any particular association with the membranes of subcellulr organelles (see *Figures 3A*, and *5*, see also *Figure 2B* in Chapter 5). Moreover, the electron-density of the BDHC deposit is often much higher than that of the DAB deposit. These structural differences between the DAB and the BDHC reaction products at both light and electron microscopic level allow this approach to be used to investigate the synaptic relationships between a population of terminals characterized by their *origin*, and a population of neurones identified by their *chemical content* (*Figure 3A*). It also allows the analysis of two populations of axon terminals, one identified on the basis of origin and the other on the basis of chemical content.

5.2 Analysis of the material

In order to avoid scanning material in the electron microscope that does not contain labelled elements, it is recommended to analyse the material in the light microscope first. At the light microscopic level, it will be possible to identify BDHC-containing cells, (i.e. immunoreactive for the antigen of interest) that are good candidates for receiving synaptic inputs from DAB-containing, (i.e. PHA-L immunoreactive) anterogradely labelled terminals. After they have been drawn and photographed, these neurones are re-embedded and sectioned for electron microscopy. The material is then examined in the electron microscope to determine whether the anterogradely labelled terminals apposed to the immunoreactive cells that were visualized at the light microscopic level, form synaptic specializations.

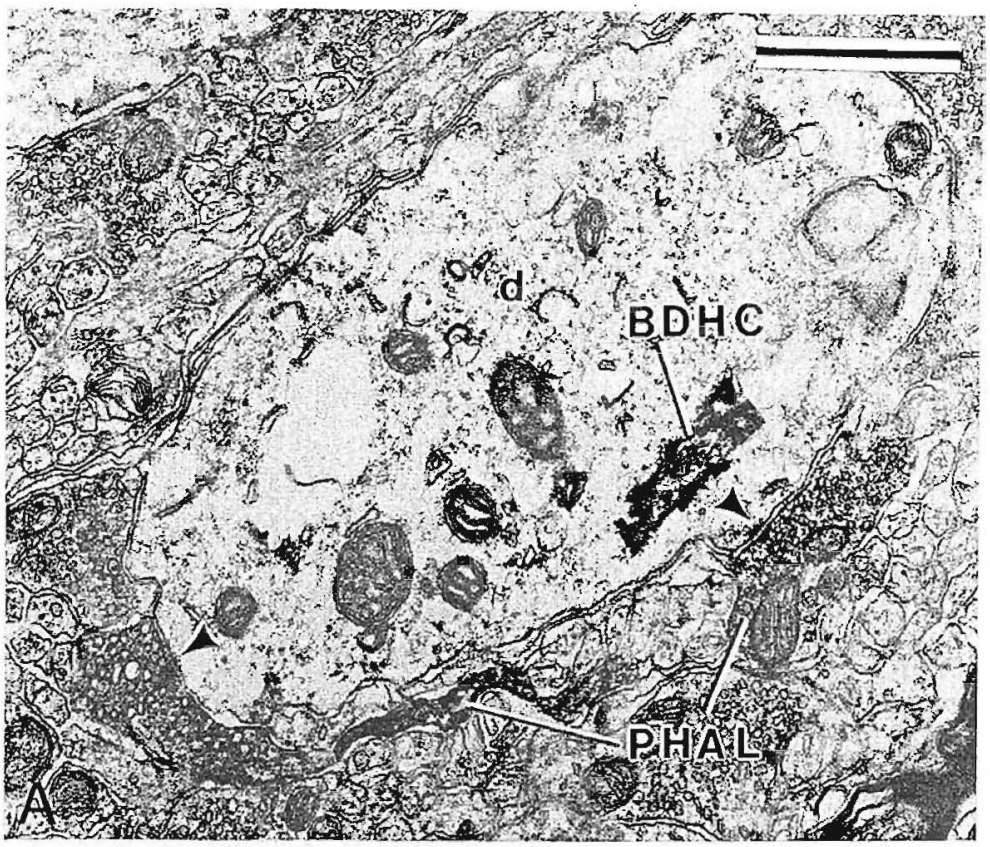
5.3 Applications

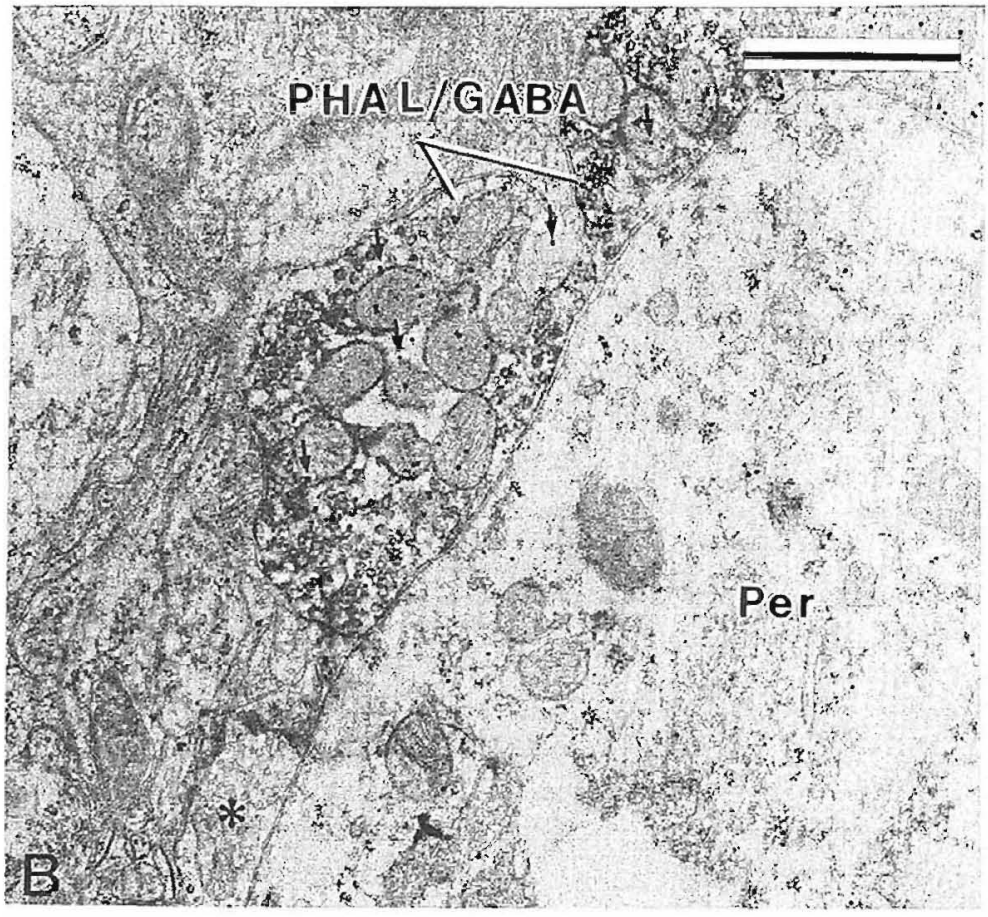
The application of this combination of procedures has demonstrated that tyrosine hydroxylase immunoreactive neurones, (i.e. dopaminergic neurones) in the substantia nigra of the rat receive synaptic input from terminals anterogradely labelled with PHA-L from the globus pallidus (9) (*Figure 3A*). More recently, this combination has been applied to the monkey striatum to study the organization of the synaptic afferents from the cerebral cortex on to parvalbumin-immunoreactive neurones (11). The results of this study revealed the existence of direct asymmetric synaptic contacts between the dendritic shafts of parvalbumin immunoreactive cells and the corticostriatal terminals.

5.4 Limitations and controls

Some technical problems may occur that complicate the analysis of the material obtained with this approach. One of the major problems is the poor penetration of the BDHC deposits into immunostained sections. It is important therefore, to collect the most superficial ultrathin sections of the block when cut on the ultramicrotome, since these are the sections that are most likely to contain *both* sets of labelled elements. Furthermore, the ultrastructural characteristics of the labelled elements in these superficial sections are often not as well preserved as in deeper sections. A second problem is the possibility that the BDHC has access to the peroxidase molecules associated with PHA-L, i.e. the possibility that the chromogen for the second peroxidase reaction had access to the peroxidase molecules of the first reaction. It is therefore important to carry out control experiments in which each primary antiserum is omitted in turn. When the antiserum that localizes the second antigen is omitted, the only labelling should be the PHA-L containing terminals with the DAB reaction product. When the PHA-L

Figure 3. (A) Anterograde transport of PHA-L combined with pre-embedding immunocytochemistry (*Protocol 4*). Electron micrograph of a TH-immunoreactive dendritic shaft (d) in the rat substantia nigra that is contacted (arrowheads) by two PHA-L immunoreactive terminals that have been anterogradely labelled after injection in the globus pallidus. In this experiment, the TH immunoreactivity was localized with BDHC whereas the PHA-L immunoreactivity was localized with DAB. Note that the BDHC and the DAB reaction products display different textures. (B) Anterograde transport of PHA-L combined with post-embedding immunocytochemistry (*Protocol 5*). Electron micrograph showing two PHA-L immunoreactive pallidal terminals that form synapses with a perikaryon (Per) in the rat subthalamic nucleus. The PHA-L immunoreactivity is indicated by the presence of the amorphous DAB reaction product in these boutons. This tissue has been processed by the post-embedding immunogold method to reveal GABA. The two PHA-L positive terminals are associated with a large number of gold particles (some are indicated by arrows) indicating that they display GABA immunoreactivity. Compare the density of gold particles over the PHA-L immunoreactive boutons with that over the GABA negative terminal indicated by an asterisk. Scale markers: 1.0 μm in (A) and (B).


d
BDHC
PHAL
A


PHAL/GABA
Per
B

antiserum is omitted, only the BDHC-labelled immunoreactive elements should be visualized. A further control experiment that should be carried out is to reverse the order in which the antigens are revealed, i.e. localize the endogenous substance first with DAB and the PHA-L positive terminals second with BDHC.

6. Combination of anterograde labelling and post-embedding immunocytochemistry

This combination of procedures is used for studying the chemical nature of a population of terminals characterized by its origin. The anterograde tracers that have been combined with post-embedding immunocytochemistry are WGA-HRP, PHA-L, or biocytin (8). Because of the advantages of PHA-L and biocytin for anterograde labelling of terminal boutons (see Chapter 3), *Protocol 5* describes the combination of post-embedding immunogold staining with the anterograde transport of these substances.

Protocol 5. Anterograde transport of PHA-L[a] combined with post-embedding immunocytochemistry

1. Carry out anterograde tracing with PHA-L or biocytin according to *Protocols 1–4* in Chapter 3.
2. Observe sections in the light microscope.
3. Draw, photograph, and re-embed areas of interest (see Chapter 1, *Protocol 9*).
4. Cut serial ultrathin sections on an ultramicrotome and mount them on coated (see Chapter 1, *Protocol 10*) single-slot nickel or gold grids.
5. Process a series of ultrathin sections for the post-embedding immunogold method to reveal the antigen of interest (see Chapter 6, *Protocol 3*).
6. Stain ultrathin sections with lead citrate (see Chapter 1, *Protocol 11*).
7. Observe in the electron microscope.

[a] PHA-L can be replaced by biocytin, in which case, biocytin is prepared, injected, and visualized according to *Protocols 3* and *4* in Chapter 3.

6.1 Appearance of the staining

The outcome of this procedure are ultrathin sections of anterogradely labelled axonal processes and terminals that are identified as PHA-L positive by the DAB immunoreaction product. The sections also contain structures, including axonal processes, neuronal perikarya, dendritic shafts, and glial cells that have gold particles overlying them. Furthermore, some DAB-

containing terminals, (i.e. PHA-L immunoreactive) may be associated with a large number of immunogold particles thus identifying as immunoreactive for the endogenous antigen (*Figure 3B*). The characterization of structures as immunopositive when using immunogold procedures has been discussed in detail in Chapter 6. In essence, a structure is considered immunopositive if the density of immunogold particles overlying it is at least five times greater than overlying the background, and that it remains constant in serial sections (*Figure 3B*).

6.2 Analysis of the material

As it has been suggested for the combined procedures described above, it is recommended to analyse the material at the light microscopic level first for the presence of anterogradely labelled terminals. Areas that are rich in PHA-L labelled structures are re-embedded and ultrathin sections cut. One grid out of two is stained with lead citrate and scanned in the electron microscope for the presence of DAB-containing (i.e. PHA-L positive) boutons. The grids adjacent to those that were found to contain PHA-L positive terminals are then processed for the immunohistochemical localization of the antigen of interest by the immunogold procedure (see Chapter 6, *Protocol 3*). The PHA-L positive boutons associated with a significant number of gold particles are photographed, and the density of gold particles is measured using a digitizing pad connected to a computer.

6.3 Applications

This approach has recently been used to demonstrate that a population of septo-hippocampal fibres, identified by the anterograde transport of PHA-L, display GABA immunoreactivity (12). It has also been used to show that terminals of neurones in the globus pallidus, anterogradely labelled with PHA-L that form symmetric synapses with neurones in the substantia nigra and the subthalamic nucleus, display GABA immunoreactivity (9, 13) (*Figure 3B*).

6.4 Limitations and controls

There are several limitations of this combined procedure.

(a) The limited number of antigens that can be localized by post-embedding procedures. Apart from small amino acids such as GABA and glutamate, the antigenicity of very few substances is preserved after osmium post-fixation, dehydration, and embedding in resin.

(b) The density of gold particles associated with anterogradely labelled terminals is often lower than that associated with non-labelled boutons. This is due to the fact that the peroxidase reaction product used to localize the first antigen (PHA-L) sometimes obscures the antigenic sites

of the endogenous substance (GABA or glutamate) to be localized. It is recommended to carry out post-embedding immunostaining on lightly stained anterogradely labelled terminals. It is often necessary to quantify the density of gold particles overlying the anterogradely labelled terminals to be sure that they display immunoreactivity. Nevertheless false-negative results will commonly occur.

(c) The electron density of the DAB reaction product in anterogradely labelled terminals is significantly reduced after post-embedding immunocytochemistry. For this reason it is sometimes difficult to ensure that the GABA-immunoreactive terminals do indeed contain the DAB reaction product. It is therefore recommended to collect a series of sections adjacent to those processed for the post-embedding immunostaining to verify whether the terminals that are associated with a large number of gold particles also contain the DAB reaction product.

Control experiments should be carried out to test the specificity of both immunoreactions. Thus, the primary antibody against PHA-L and the primary antibody against the endogenous antigen, (e.g. glutamate or GABA) should be omitted in turn from the immunohistochemical reactions (see Chapters 5 and 6).

7. Double anterograde labelling combined with retrograde labelling

The objective of this approach is to test the possibility that two sets of anterogradely labelled terminals arising from different sources form convergent synaptic contacts on to single post-synaptic neurones characterized by their projection site. Furthermore, this approach can be combined with the post-embedding immunogold method (see Chapter 6, *Protocol 3*) to determine some aspects of the chemical nature of the anterogradely labelled terminals (10). The approach is to combine the anterograde transport of both PHA-L and biocytin with the retrograde transport of WGA-HRP. Each of the markers is visualized using a peroxidase reaction, but the combined method takes advantage of the fact that different chromogens used for the peroxidase reactions have different colours, form, and location (*Protocol 6*). The same protocol shown in *Protocol 6*, with the omission of the WGA-HRP injection and processing, can be used to examine two sets of anterogradely labelled terminals without characterization of the post-synaptic neurone.

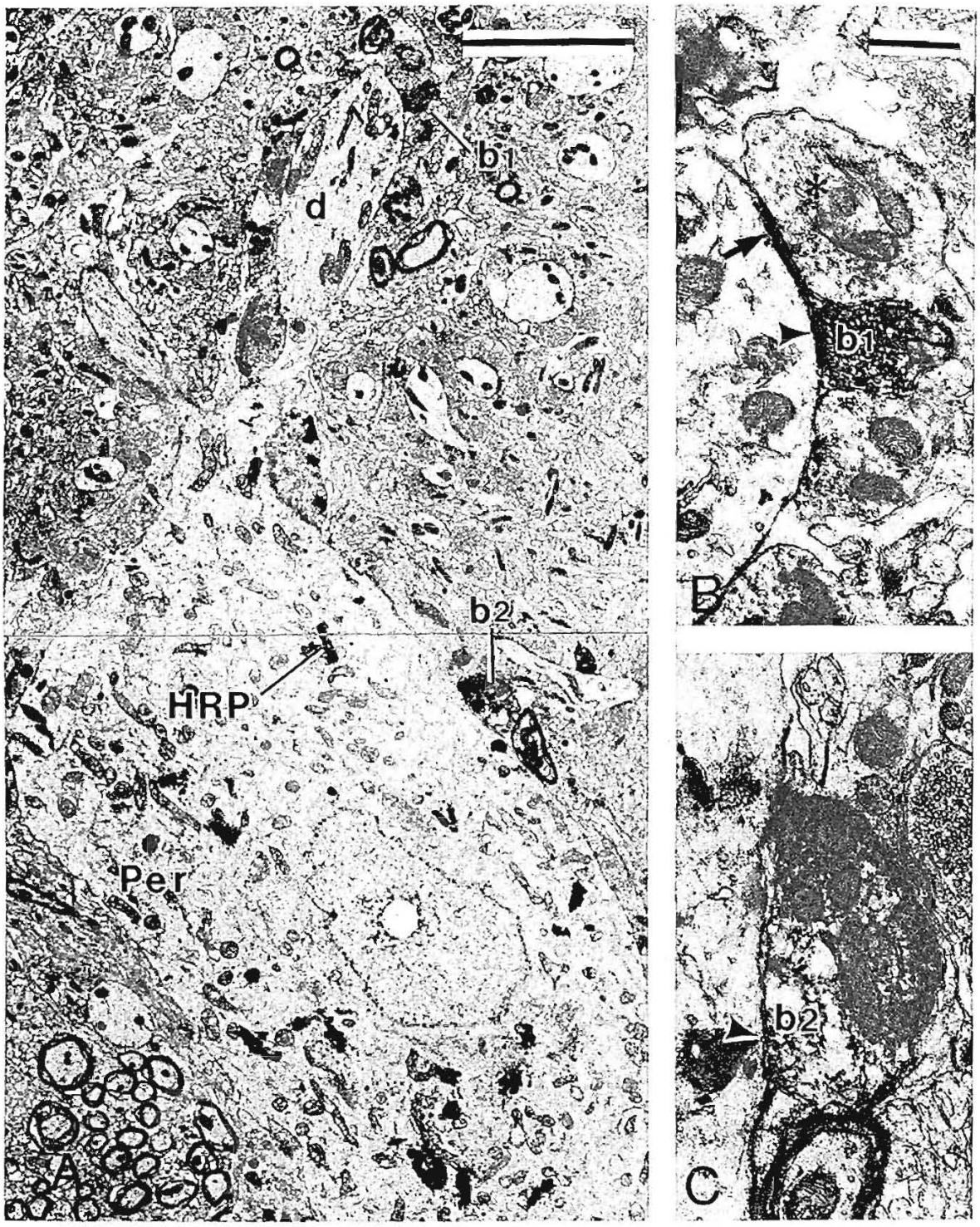
Protocol 6. Anterograde transport of PHA-L and biocytin combined with the retrograde transport of WGA-HRP

1. Prepare and administer PHA-L (see Chapter 3, *Protocol 1*).
2. Allow the animal to survive for 7–10 days.

3. Prepare and administer biocytin (see Chapter 3, *Protocol 3*) and WGA-HRP (see Chapter 2).
4. Allow the animal to survive for 24–48 h.
5. Perfuse–fix the animal (see Chapter 1, *Protocol 3*) with a mixture of paraformaldehyde (2–4%) and glutaraldehyde (0.1–2.5%) (see Chapter 1, *Protocol 2*), and then with cold phosphate buffer (0.1 M, pH 7.4) (see Chapter 1, *Protocol 1*).
6. Dissect the brain from the skull, cut in 5 mm-thick blocks and store at 4°C until sectioning.
7. Section the blocks at 50–70 μm on a vibrating microtome.
8. Treat the sections with 1% sodium borohydride for 20 min (Chapter 5, *Protocol 6*).
9. Wash the sections several times in PBS.
10. Process the tissue for the histochemical localization of WGA-HRP using TMB as chromogen for the peroxidase reaction (see *Protocol 1*, steps **8–12**).
11. Process the tissue for the histochemical localization of biocytin at light and electron microscopic level, using DAB as the chromogen for the peroxidase reaction (see Chapter 3, *Protocol 4*, steps **2–4**).
12. Process the tissue for the immunohistochemical localization of PHA-L (see Chapter 3, *Protocol 2*, steps **7–12**) using the nickel-enhanced DAB method (see *Protocol 4*, steps **13–14**) to localize the injection site, and for the sections containing transported PHA-L that will be analysed at the light microscopic level only. In sections prepared for electron microscopy, reveal the transported PHA-L using the BDHC method (see Chapter 5, *Protocol 11*).
13. Mount sections prepared for light microscopy on to gelatin-coated slides, dehydrate, and apply coverslips (see Chapter 1, *Protocols 5* and *6*).
14. Post-fix the sections processed for electron microscopy in osmium tetroxide (see Chapter 1, *Protocol 7*; but the osmium tetroxide must be diluted in PB 0.01 M, pH 6.8), dehydrate, and embed in resin on microscope slides (see Chapter 1, *Protocol 8*).
15. Examine the sections in the light microscope, draw and photograph regions of interest, re-embed, and cut ultrathin sections (see Chapter 1, *Protocol 9*).
16. Examine in the electron microscope.

7.1 Appearance of the staining

The outcome of this approach are two sets of anterogradely labelled terminals that can be differentiated from each other by the texture of the peroxidase reaction product associated with them (*Figure 4*). In the sections prepared for



light microscopy, the two sets of anterogradely labelled fibres are easily distinguishable since the brown DAB reaction product is distinct from the blue Ni-DAB reaction product. In the sections prepared for electron microscopy, the two reaction products are also distinguishable; the biocytin labelled terminals contain the amorphous DAB reaction product, whereas the PHA-L labelled terminals contain the crystalline BDHC reaction product (*Figure 4*). In some cases, because of the size of the labelled terminals, it is difficult to distinguish at the light microscopic level, the DAB from the BDHC labelled

Figure 4. Anterograde transport of PHA-L and biocytin combined with the retrograde transport of WGA-HRP (*Protocol 6*). Electron micrographs showing a retrogradely labelled nigrocollicular cell (indicated by HRP in (A)) that receives convergent synaptic inputs from the striatum (b1) and the globus pallidus (b2) in the rat substantia nigra. In this experiment, PHA-L was injected in the globus pallidus and biocytin was injected in the striatum. In the same animals, WGA-HRP was injected in the superior colliculus. The three markers were all localized by peroxidase reactions but using different chromogens. The biocytin was revealed using DAB as the chromogen, PHA-L was localized with BDHC as the chromogen, and the retrogradely-transported WGA-HRP with TMB as the chromogen. Note in (B) and (C) the difference between the DAB reaction product associated with b1 (B) and the BDHC reaction product associated with b2 (C). Both the striatal (DAB labelled, b1) and the pallidal (BDHC labelled, b2) terminals form symmetric synapses (indicated by arrowheads in (B) and (C)) with the retrogradely labelled cell. In (B), a non-labelled terminal (indicated by an asterisk) forms a symmetric synapse (arrow) with the nigrocollicular cell. Scale markers: (A) 5.0 μm, (B) and (C) 0.5 μm.

terminals. Nevertheless, in the electron microscope the two sets of terminals remain easy to distinguish (compare *Figure 4B* and *4C*). In addition to these two sets of anterogradely labelled terminals, the section will contain retrogradely labelled neurones characterized by the presence of large TMB crystals randomly distributed throughout the cytoplasm of the perikaryon and the dendritic shafts of labelled cells (*Figure 4A*; see also *Figure 2A* and Chapter 2 for TMB reaction product).

7.2 Analysis of the material

The best way to analyse this material is to carry out correlated light and electron microscopy (see Chapter 1). First, the area of study is scanned for the presence of retrogradely labelled cells that are apposed by biocytin and PHA-L labelled terminals. This cell is drawn and photographed before being re-embedded and cut in serial ultrathin sections. In the electron microscope, the retrogradely labelled cell is examined through its entire extent to determine whether the biocytin and PHA-L labelled terminals identified at the light microscopic level form synapses with this neurone. If necessary, ultrathin sections adjacent to those containing both sets of terminals and the retrogradely labelled cell can be processed for the post-embedding immunogold procedure to determine the chemical nature of the anterogradely labelled terminals (10).

7.3 Applications

This approach was developed to test the possibility that synaptic terminals from two different sources converge on to single post-synaptic targets characterized by their projection sites. We have recently shown that after injection of biocytin in the striatum and PHA-L in the globus pallidus, rich plexuses of both sets of anterogradely labelled terminals overlap in the ipsilateral

substantia nigra. In the electron microscope, it has been found that many projection neurones in the substantia nigra pars reticulata receive convergent synaptic inputs from the PHA-L labelled boutons arising from the globus pallidus and the biocytin positive boutons arising from the striatum (10, 14) (*Figure 4*). Moreover, we have also demonstrated, by using the post-embedding immunogold method for GABA, that both striatal and pallidal terminals, that converge on to single projection neurones in the substantia nigra, display GABA immunoreactivity (10).

7.4 Limitations and controls

The combination of three tract-tracing methods in one experiment inevitably has some technical limitations. First, the major limitation is the poor penetration of the BDHC reaction product into the tissue (see *Protocol 4*). As mentioned above, it is necessary to collect the first few micrometres of a re-embedded block to have all three sets of labelled elements on single ultrathin sections. Second, the ultrastructural features of the terminals localized with BDHC may be damaged (see *Figure 4C*). Third, it may be difficult to differentiate the BDHC reaction product from the TMB reaction product in the electron microscope. Fortunately, in many cases the location of the two deposits is largely different, i.e. the TMB deposit occurs in perikarya and proximal dendrites whereas the BDHC reaction product is found in anterogradely labelled terminals. However, the TMB reaction product may occur in terminals if the WGA-HRP is transported anterogradely to the area of study, or if the retrogradely labelled cells have recurrent axon collaterals (see *Protocol 3*). In such conditions it may be difficult to differentiate the PHA-L positive terminals (BDHC labelled) from the WGA-HRP containing terminals. It is therefore important to carry out control experiments in which the WGA-HRP only is revealed; this allows one to verify whether WGA-HRP labelled terminals occur in the material under investigation.

In addition to the localization of WGA-HRP alone, two other control experiments must be carried out to verify the specificity of the staining. First, reverse the order in which the anterograde tracers are revealed; i.e. localize PHA-L first with DAB, and biocytin second with BDHC. In such a case the PAP method must be used to localize the PHA-L immunoreactivity (see Chapter 5). Secondly, omit the ABC or the primary antibody against PHA-L in turn from the incubation medium. In the case where the ABC is omitted, localize the PHA-L immunoreactivity using the PAP method (see Chapter 5).

8. Double/triple immunocytochemistry at the electron microscopic level

The main objective of the double immunohistochemical procedure at the electron microscopic level is to determine the chemical nature of the synaptic

input to chemically characterized neurones. The same approach is used when the anterograde transport of PHA-L is combined with the pre-embedding immunocytochemistry for the transmitter in the post-synaptic target neurones (see *Protocol 4*). This method involves the use of DAB and BDHC as the chromogens for the peroxidase reactions to localize the two sets of immunoreactive structures (*Protocol 7*; see Chapter 5, *Protocol 11*). Furthermore, co-localization of two chemicals within single terminals can be carried out by combining this double immunohistochemical method with the post-embedding immunogold procedure in a manner similar to that described in *Protocol 5* (see Chapter 6, *Protocol 3*).

Protocol 7. Double/triple immunocytochemical labelling at the electron microscopic level

1. Perfuse–fix the animal (see Chapter 1, *Protocols 2* and *3*).
2. Cut the brain in 50–70 μm thick sections on a vibrating microtome (see Chapter 1, *Protocol 4*).
3. Collect the sections in PBS (see Chapter 1, *Protocol 1*).
4. Process the tissue for the localization of the first antigen using the ABC (see Chapter 5, *Protocol 9*), or the PAP (see Chapter 5, *Protocol 8*) method.
5. Reveal the antigen using DAB as the chromogen in the peroxidase reaction (see Chapter 3, *Protocol 2*, step **13**).
6. Wash the sections several times in PBS.
7. Process the tissue for the immunohistochemical localization of the second antigen using the ABC or the PAP method (see Chapter 5, *Protocols 8* and *9*).
8. Reveal the second antigen using BDHC as the chromogen in the peroxidase reaction (see Chapter 5, *Protocol 11*; see also *Safety note 4* in Chapter 5, p. 123).
9. Post-fix in osmium tetroxide (see Chapter 1, *Protocol 7*, but dilute the osmium tetroxide in PB 0.01 M, pH 6.8), dehydrate, and embed the sections in resin on microscope slides (see Chapter 1, *Protocol 8*).
10. Examine the sections in the light microscope.
11. Re-embed areas of interest (see Chapter 1, *Protocol 9*).
12. Cut ultrathin sections and mount them on coated (see Chapter 1, *Protocol 10*) single-slot nickel or gold grids.
13. Process the tissue for post-embedding immunocytochemistry (see Chapter 6, *Protocol 3*).
14. Examine the sections in the electron microscope.

8.1 Appearance of the staining

The outcomes of this approach are:

- neuronal elements labelled with the DAB reaction product resulting from the first immunocytochemical reaction
- neuronal structures containing the BDHC reaction product resulting from the second immunocytochemical reaction
- neuronal structures associated with immunogold particles
- neuronal elements containing either the DAB or the BDHC peroxidase reaction products and associated with immunogold particles

The two peroxidase reaction products are distinguishable as described above (see Section 5.1). In the electron microscope, double labelling of a single neuronal structure is indicated by the presence of either the DAB or the BDHC reaction product, together with a significant number of the immunogold particles (at least five times higher than the background) (*Figure 5*) (see *Protocol 5* and Chapter 6).

8.2 Analysis of the material

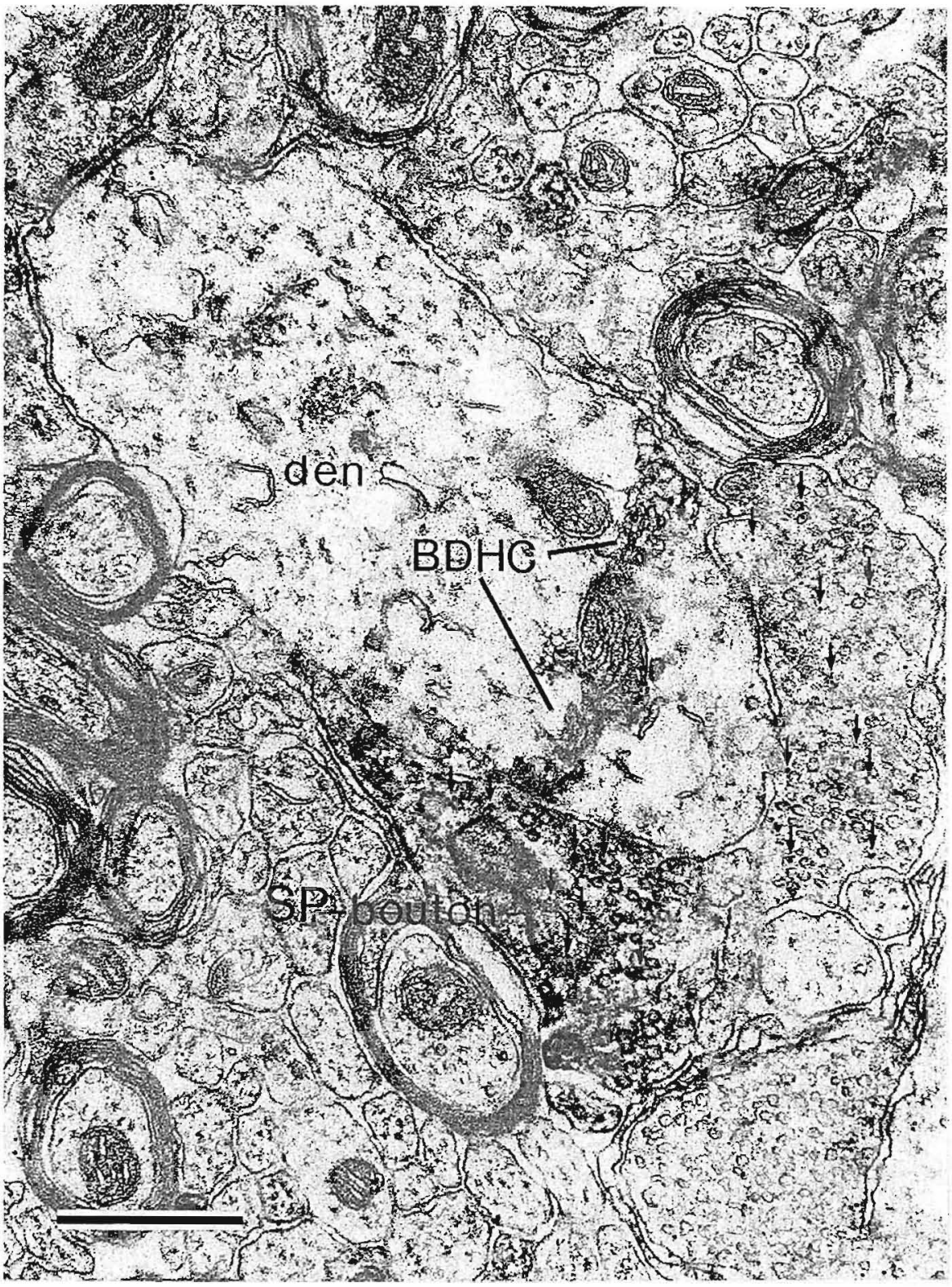
The approaches to analyse such material have been described in detail in Sections 5.2 and 6.2.

8.3 Applications

This approach has recently been used to demonstrate that substance P positive/GABA terminals form symmetric synapses with tyrosine hydroxylase immunoreactive dendrites in the rat substantia nigra (15) (*Figure 5*). In this experiment, sections of the rat substantia nigra were processed for the pre-embedding immunohistochemical localization of substance P and tyrosine hydroxylase (TH). The substance P immunoreactive structures were localized using DAB as the chromogen for the peroxidase reaction, whereas the TH positive elements were revealed using BDHC. Ultrathin sections containing both sets of labelled structures were then processed to reveal GABA by the immunogold method (see Chapter 6, *Protocol 3*).

The double peroxidase method, i.e. the use of both DAB and BDHC for chromogens in the peroxidase reactions, is used in several of the other

Figure 5. Double pre-embedding immunocytochemistry combined with post-embedding immunocytochemistry (*Protocol 7*). Electron micrograph of the substantia nigra of the rat reacted to reveal three neural antigens. It was reacted first to reveal substance P immunoreactivity using DAB as the chromogen for the peroxidase reaction, an axonal bouton (SP-bouton) contains the DAB reaction product. Secondly it was reacted to reveal tyrosine hydroxylase immunoreactivity using BDHC as the chromogen; the dendrite (den), con-



tains the reaction product (BDHC) which is partially bleached due to subsequent processing. Finally, the section was subjectd to the post-embedding immunogold method to reveal GABA; three boutons apposed to the dendrite, including the substance P immunoreactive one, contain relatively high concentrations of the immunogold particles (some of which are indicated by arrows) identifying them as GABA-immunoreactive. Scale marker: 0.5 μm.

combined procedures described in this chapter, see *Protocols 4* and *6* (see *Figures 3A* and *Figure 2B* in Chapter 5).

8.4 Limitations and controls

This combination of immunohistochemical techniques suffers from all the disadvantages mentioned above for the *Protocols 4–6*.

Control experiments including incubations in solutions from which the primary antisera to localize the first, the second, and the third antigens are omitted in turn must be carried out to ensure the specificity of the immunostaining (see Chapters 5 and 6).

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