Van Leeuwen, Buijs, Pool and Pach (eds.) Molecular Neuroanatomy

CHAPTER 21

Immunocytochemical demonstration of GABA in physiologically characterized, HRP-filled neurons and their postsynaptic targets

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1. Introduction

The combination of intracellular recording and marking of neurons with horseradish peroxidase (HRP) has greatly extended the understanding of neuronal circuits. This approach is suitable for the reconstruction of the axonal and dendritic arborizations of neurons over several millimeters, thus providing a more complete picture of individual cells than was possible with previous methods. In addition the direct correlation of electrophysiological and structural data becomes possible. In most cases the interpretation of the possible role of identified neurons would greatly benefit from a knowledge of their biochemical characteristics, especially their transmitters. The identification of the transmitters of the postsynaptic cells would also be useful because each neuron or afferent system is likely to contact several neurochemically, morphologically and physiologically distinct neuronal populations.

In the vertebrate nervous system, with few exceptions (Aghajanian and Vandermalen, 1982; Bornstein et al., 1986; Grace and Bunney, 1983; Reaves et al., 1983; Weiler and Ammermuller, 1986), information about the transmitters of physiologically characterized cells and their postsynaptic targets has largely been predicted from separate neurochemical, pharmacological or histochemical experiments. In order to provide direct information about the neurochemical characteristics of intracellularly recorded cells, we combined intracellular HRP filling with light and electron microscopic post-embedding immunocytochemistry. Following intracellular recording, the tissue containing the HRP-filled cells is processed for electron microscopic analysis. Thus the afferent and efferent synaptic connections of the physiologically characterized, HRP-filled neurons can be studied at the ultrastructural level. In addition, the connections of these same cells can also be

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established during the recording of the cell's electrical activity, by electrical stimulation of their input and output pathways.

The application of immunocytochemistry to cells that had been processed for conventional electron microscopy will be illustrated here with examples using an antiserum to gamma-aminobutyric acid (GABA). The development of antisera to amino acid neurotransmitters opened up new possibilities for their immunocytochemical localization in the central nervous system (Seguela et al., 1984; Somogyi et al., 1984; Storm-Mathisen et al., 1983). The antibodies are usually raised against amino acid-aldehyde-protein conjugates which resemble the form of the amino acid in aldehyde fixed tissue. This could explain why epitopes produced in the tissue during fixation of amino acids seem to be less sensitive to tissue processing conditions than those of most proteins and peptides. The particular property that has proved most advantageous is that fixed amino acids, and in particular GABA, are recognized by antibodies following routine osmium tetroxide treatment, dehydration and epoxy resin embedding for electron microscopy (Somogyi and Hodgson, 1985). The procedures however should not be restricted to the localization of amino acids, since there is an increasing number of other molecules that can be localized under similar conditions (Bendayan et al., 1986; Graber, 1985; Hearn et al., 1985; Theodosis et al., 1986; Van den Pol, 1985).

Two methods will be described:

(1) Post-embedding immunocytochemistry, using the unlabeled antibody enzyme method, is carried out on semithin (0.5–1 μm thick) sections which are cut from osmium treated 80 μm thick sections containing the physiologically characterized HRP filled neuron (Freund et al., 1985; Kisvarday et al., 1985). First the synaptic contact(s) is(are) identified between the physiologically characterized presynaptic cell and its postsynaptic target by correlated light and electron microscopy. Semithin sections are then cut from parts of the postsynaptic neurons still in the thick section, and the presence or absence of the epitopes is established in the postsynaptic cell. Several antisera can be tested on consecutive sections.

(2) Post-embedding immunocytochemistry, using immunogold methods, is carried out on ultrathin sections cut from the 80 μm thick osmium treated section containing the HRP-filled neuron (Somogyi and Soltesz, 1986). Alternate ribbons of sections are studied, either conventionally for the identification of synaptic connections, or after the immunogold reaction. Since it is necessary to remove the osmium from the sections prior to the immunoreaction, the HRP-filled processes are difficult to locate without studying the alternate non-immunoreacted grids. This method is suitable for demonstrating immunoreactivity both in the HRP-filled cells and in their postsynaptic targets. Alternate grids can be reacted for different epitopes or the same grid can be reacted for several epitopes using different sizes of colloidal gold.

2. *Methods*

A flow diagram of the combined procedures is provided in Scheme 1. The steps will be described below, together with technical comments on possible difficulties.

1. Intracellular recording and HRP-filling
   Electrophysiological characterization of connections
2. Perfusion fixation and removal of brain
3. Sectioning on Vibratome and washing of sections
4. HRP enzyme-histochemistry
5. Osmium treatment and flat-embedding in epoxy resin
6. Light microscopic analysis
   Photography, drawing and reconstruction of HRP-filled cells
7. Reembedding for electron microscopy
8. Electron microscopic analysis of synaptic connections

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Semithin sections
Immunoperoxidase method

9. EM identification of postsynaptic somata
10. Cut semithin sections of somata and mount on slides
11. Etching resin in ethanolic NaOH
12. Wash in ethanol and distilled water
13. Removal of osmium in sodium periodate
14. Wash in water and buffered saline
15. Blocking serum
16. Primary antiserum (to GABA) and wash
17. Bridge antibody followed by wash
18. PAP-complex followed by wash
19. Immunoperoxidase enzyme reaction
20. Wash, dehydration and mounting
21. Identification of immunopositive cells and correlation with electron micrographs

Ultrathin sections
Immunogold method

9. Cut serial EM sections onto Formvar coated gold grids
10. EM identification of postsynaptic element
11. Next grid containing the same element for immunoreaction
12. Periodic acid treatment and wash
13. Removal of osmium in sodium periodate
14. Wash in water and buffered saline
15. Blocking protein solution
16. Primary antiserum (to GABA) and wash
17. IgG or Protein A coated colloidal gold
18. Wash followed by uranyl acetate and lead citrate treatment for contrasting
19. Identification of immunopositive pre- and postsynaptic structures
20. Correlation with HRP filled profiles from non-reacted grids

Scheme 1. Flow diagram of combined procedures for immunocytochemical characterization of the afferent and efferent synaptic connections of intracellularly recorded neurons.

2.1. Preparation of animals, recording and intracellular HRP injection

Several reviews have described in detail the current techniques for intracellular recording and HRP filling (Brown and Fyffe, 1984; Kitai and Bishop, 1981). The

cells studied by us derive from the material of Martin and Whitteridge (1984), and the electrophysiological recording and HRP filling have been reported by them in detail. All the cells are from the visual cortex of the cat. Electrical stimulation of the cells through electrodes inserted along the visual pathway provided the basis for determining the mono- or polysynaptic nature of their thalamic input and the presence of a callosal input. Electrical stimulation was also used to determine the type of visual afferents (X or Y) providing their predominant thalamic input (Bullier and Henry, 1979; Martin and Whitteridge, 1984). For recording, glass electrodes filled with 4% solution of HRP (Boehringer, grade 1) dissolved in 0.2 M KCl and 0.05 M TRIS (pH 7.9) were used. The receptive field properties of the cells were recorded first extracellularly, using visual stimulation. The membrane of the cell was then penetrated, the receptive field was examined again, and the cell was filled with HRP using 2–4 nA positive current pulses, following the procedures of Friedlander et al. (1981).

2.2. Fixation and tissue processing

At the end of the recording experiment the animals were given a lethal dose of anesthetic, they were removed from the ventilation apparatus and were perfused through the heart first with saline, followed by fixative. The fixative consisted of 2.5% glutaraldehyde, 1% paraformaldehyde and 0.1 M sodium phosphate buffer (PB, pH 7.4). Several neurons were filled in each animal, thus 2–20 h elapsed between filling the cell and the fixation.

In general, a high concentration of glutaraldehyde in the fixative results in a good immunoreactivity for amino acids (Ottersen and Storm-Mathisen, 1984; Somogyi et al., 1985; Storm-Mathisen et al., 1983). However, even using 2.5% glutaraldehyde, as above, a considerable variation was produced in immunoreactivity and background staining from animal to animal. This indicates that for strong immunoreactivity the method of delivering the fixative to the cells is more important than the concentration of the crosslinking aldehyde. This is supported by our finding (Somogyi et al., 1985) that much lower glutaraldehyde concentrations, down to 0.05%, can produce excellent immunoreactivity. Using our antiserum to GABA we found no improvement at all by increasing the glutaraldehyde concentration to more than 2.5%, as recommended by others (Seguela et al., 1984; Ottersen and Storm-Mathisen, 1984).

Following the perfusion, 5–10 mm thick slices of the visual cortex were cut in the frontal plane and sectioned at 80 μm on a Vibratome (Oxford Instruments). The tissue was submerged in 0.1 M sodium phosphate buffer and the sections were collected in the same buffer, serially in trays. Thereafter the sections were washed free of fixative in three changes of buffer, 30 min each.

2.3. HRP enzyme-histochemistry

Most sections were processed according to the method of Hanker et al. (1977), supplemented with cobalt and nickel intensification (Adams, 1981) as described earlier (Martin and Whitteridge, 1984). The procedure is as follows:

(1) The sections are washed in sodium cacodylate buffer (0.1 M, pH 5.1) for 2×10 min. All subsequent steps are carried out at 4°C.

(2) They are preincubated for 15 min in a 5:1 mixture of intensifying solution (consisting of 0.4% $[NH_4]_2SO_4 \cdot NiSO_4 \cdot 6\ H_2O$ and 0.6% $CoCl_2$) and chromogen solution (consisting of 0.1% catechol and 0.05% *p*-phenylenediamine dihydrochloride dissolved in 0.1 M cacodylate buffer at pH 5.1).

(3) The sections are washed for 5 min in 0.1 M PB, at pH 7.4.

(4) The sections are incubated, with agitation, for 15 min in the chromogen solution in the presence of 0.01% H_2O_2.

(5) They are washed 2×10 min in 0.1 M PB.

Some sections were processed in 0.1 M PB, using 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) as the chromogen (Graham and Karnovsky, 1966) both with (Adams, 1977) and without cobalt intensification.

As far as the visualization of the intracellularly injected cells is concerned, the reaction of Hanker et al. (1977) is more sensitive than the DAB procedure. In the intracellularly injected cells GABA immunoreactivity remained equally detectable whether HRP was reacted with DAB or *p*-phenylenediamine/pyrocatechol. The use of heavy metal intensification does not affect the immunoreactivity.

2.4. Embedding and light microscopic analysis

Following the enzyme reaction the sections were washed extensively in 0.1 M PB and treated for 30–40 min with 1% OsO_4 dissolved in 0.1 M PB. They were washed again in PB and dehydrated in an ascending series of ethanol, followed by propylene oxide and embedding in Durcupan (Fluka) epoxy resin. We prefer to use this resin because of its hardness, but post-embedding immunostaining for GABA has been achieved using a variety of other resins, including Epon (Van den Pol, 1985), Spurr (Theodosis et al., 1986) and L.R. White (unpublished observation). Durcupan is made up by mixing with a spatula 10 g of component A (Epoxy resin), 10 g of component B (Hardener 964), 0.3 g of component C (Accelerator 964, phenol derivative, harmful) and 0.3 g of component D (Plasticizer). The sections are taken from propylene oxide, put into the resin contained in disposable aluminium foil wells and kept overnight at room temperature. The following day they are placed serially onto glass microscope slides, and covered with glass coverslips. Durcupan has high viscosity at room temperature and the osmium treated sections are very rigid and can easily break. Therefore the transfer of the sections is carried out on a warming table in order to increase the fluidity of the resin. Neither the slides nor the coverslips were specially cleaned, because during re-embedding for electron microscopy the cured resin has to be separated from the glass. This may be difficult when acid-cleaned glass is used. The use of siliconised slides and coverslips, or acetate foil instead of glass (Hollander, 1970; for review see Brown and Fyffe, 1984) has been recommended, but we have found the use of plain glass both convenient and satisfactory. The slides were then placed into an oven, and the resin cured at 56°C for two days. These specimens can be stored permanently.

We routinely enhance contrast en bloc during dehydration by including 1%

uranyl acetate in the 70% ethanol for 40 min. This does not interfere with immunocytochemistry for any of the antigens we have tested so far.

The HRP-filled cells were then drawn using a drawing tube, and in addition some of them were reconstructed in three dimensions using a computerized microscope system (Capowski and Rethelyi, 1982; Zsuppan, 1984). Complete reconstructions of the neurons can be done at this stage, and any feature of the cell can be recorded on light micrographs. Those parts of the dendritic and axonal fields that were subsequently resectioned for electron microscopy were photographed in small focal steps using 100 × oil immersion objective and these photographs were used to correlate the light and electron microscopic images of the same neuronal process.

2.5. Electron microscopic analysis of synaptic connections

Selected parts of the sections were re-embedded as described earlier (Somogyi et al., 1979). In order to transfer the resin embedded section from the slide to a resin block that can be fixed into the specimen arm of the ultramicrotome, first the cover slip is removed by inserting a razor-blade between the coverslip and the resin. Scoring the glass with a diamond pen once the blade is under the glass makes it possible to remove only part of the coverslip so the majority of the sections remain protected. We cut out only a small part (1–3 mm^2) of the section that contains the area of interest, using a scalpel and stereomicroscope. During this operation the slide is kept on a warming plate to make the resin softer. The small piece of section is then lifted from the slide with the tip of a scalpel or a pointed razor-blade. It is placed into a flat-bottom embedding capsule that is cut to about 5–8 mm height. The capsule is filled with resin and a coverslip is placed on top of the resin, parallel to the bottom in a way that ensures no air bubbles remain in the capsule. After the curing of the resin and removal of the coverslip the block is now in a cylinder that has an optically smooth, flat bottom. This cylinder can be placed on a microscope slide and examined in the light microscope with dry objectives any time during the subsequent trimming and sectioning.

2.6. Post-embedding immunoperoxidase techniques for semithin sections

When the axons of intracellularly HRP-injected neurons contact the cell bodies of other cells it is possible to localize the antigens in the somata of the postsynaptic neuron at the light microscopic level. First the presumed contact is located in the 80 μm thick section while it is still on the slide. Following re-embedding the synaptic contact is confirmed or rejected by electron microscopy. Alternatively axo-somatic contacts provided by the HRP-filled cell may be located during electron microscopy. In both cases immunocytochemistry is carried out on semithin sectons cut from the remaining part of the postsynaptic soma still in the block.

The steps of the immunocytochemical reaction are described below, expanded after Somogyi et al. (1984).

(1) The resin embedded, semithin sections (0.5–2 μm thick) are placed onto gelatin or egg albumen coated slides, flattened on a drop of warm water and gently

dried on a hotplate. Drying too quickly and high temperatures should be avoided, to prevent the sections sticking too tightly. The reaction is often uneven over the section, those parts that are not tightly stuck to the slide reacting much more strongly, presumably because the etching (see below) of the resin takes place from both sides. Uncoated slides can also be used, but sometimes the sections may float off. If the reaction is not carried out on the day of the sectioning the slides are kept in a 56°C oven overnight.

(2) Etching of the resin is carried out in ethanolic sodium hydroxide (Lane and Europa, 1965) in Coplin jars. The solution is made up at least a day before it is used by adding NaOH pellets to absolute ethanol. It is stirred several times in the first day and can be used for weeks. It turns brown but this is not counterindicative of its effectiveness. From here on, care should be taken to prevent the drying of the sections.

(3) Following etching the slides are washed in three changes of absolute ethanol and two changes of distilled water, 5 min each.

(4) Sodium periodate ($NaIO_4$, 1%, freshly made) is used to remove the osmium. Usually 7–10 min is sufficient, but if this treatment proves ineffective, the concentration of $NaIO_4$ can be increased or the duration of treatment extended. We have obtained good results using saturated $NaIO_4$ for up to one hour. This treatment is followed by two 5 min washes in distilled water.

(5) Two 10 min washes in Tris(10 mM)phosphate(10 mM)-buffered saline (TPBS) are applied. Other buffered salt solutions such as TBS, PBS can also be used, both here and in the subsequent steps.

(6) The slides are placed horizontally into a moist chamber, and all antibody solutions are applied to them in this chamber. We use a plastic box, with wet tissue in the bottom. Most of the buffer is drained from the slides. Only a thin layer of fluid should be left over the sections. Thereafter blocking protein solution is layered over them. Usually 10% normal serum of the species that produced the bridge antibody is used; in the case of GABA immunocytochemistry we use normal goat serum for 20 min, then the serum is drained.

(7) Antiserum to GABA, raised in rabbit, is overlaid for 1–2 h, diluted with 1% normal goat serum in TBPS, at dilutions from 1:1000 to 1:5000. The antisera we used have been raised to GABA conjugated to bovine serum albumen by glutaraldehyde, and they have been extensively characterized for crossreactivity with a large number of related molecules, both in a nitrocellulose test system and on sections of the brain (Hodgson et al., 1985; Somogyi et al., 1985). In this step, control sera can be used to replace the antiserum to GABA.

(8) Slides are rinsed with TPBS from a pressure bottle and washed in Coplin jars in 1% NGS for 2×10 min.

(9) Bridge antibodies (goat anti-rabbit IgG, Miles) diluted 1:30 with 1% NGS are applied for 40 min, followed by a rinse in TPBS and washes in 1% NGS for 2×10 min.

(10) Peroxidase-antiperoxidase complex (diluted 1:100) is applied for 1 hour, followed by two 15 min washes in TPBS and 10 min in 0.1 M PB. It should be noted that although we generally use the unlabeled antibody enzyme method (Sternberger

et al., 1970), other methods such as the use of peroxidase conjugated second antibody, avidin biotin methods or silver intensified gold methods produce equally good results on semithin sections.

(11) For the peroxidase enzyme method, the slides are preincubated in 0.05% DAB dissolved in 50 mM TRIS buffer (pH 7.0–7.4). 5 min later 1% H_2O_2 is added to a final concentration of 0.01% and the slides are incubated for a further 5–10 min. Thereafter they are washed in 0.1 M phosphate buffer for 3×5 min and the contrast is increased by treating them for 5 min in 0.01% osmium tetroxide in 0.1 M PB.

(12) The slides are washed 3×5 min in distilled water, dehydrated in ethanol, followed by xylene and covered with a synthetic mounting medium under coverslip.

The immunoreactive cells are dark brown and immunoreactive puncta corresponding to immunoreactive dendrites, axons and nerve terminals can clearly be recognized. Because the osmium is removed from the sections, the originally black or dark brown HRP-filled processes of the physiologically characterized cells become pale brown or ochre coloured, and they are more difficult to locate than before the immunoreaction.

2.7. Post-embedding immunogold technique for ultrathin sections; demonstration of GABA immunoreactivity

Serial sections are cut and picked up onto Formvar-coated, single slot, gold grids. Only 3–5 sections are placed on each grid, in order to have the same boutons represented on more than one grid. The immunocytochemical method follows procedure I of Somogyi and Hodgson (1985) with small modifications. Droplets of solutions for the immunocytochemical reaction are put on Parafilm in Petri dishes. The Parafilm is surrounded with wet paper tissue to avoid drying of the grids. Unless otherwise stated, the grids are floated on the droplets at room temperature, with the sections facing down. When transferred from one droplet to another the excess fluid is removed from the grids by filter paper, but the sections are never allowed to dry. All reagents and washing solutions are Millipore filtered (pore size 0.22 μm). The following steps are carried out:

(1) Pretreatment of the resin in 1% periodic acid (H_5IO_6, BDH Chemicals Ltd., freshly prepared) for 7–10 min.

(2) Three washes in double distilled water, by dipping the grids several times into vials, followed by 5 min. in double-distilled water.

(3) Removal of osmium in 1% sodium periodate ($NaIO_4$, BDH Chemicals Ltd., freshly prepared) for 7 min, as recommended by Bendayan and Zollinger (1983). For some specimens a longer time may be necessary; it is worth trying a series of different times when using unfamiliar material.

(4) Washing as in step 2.

(5) The grids are blotted with filter paper on the side opposite to the sections. From now on, unless otherwise stated, the grids are not dipped into any solution, but are just floated on the droplets. Occasionally, if a grid sinks in the droplet, it is put into the droplets in all subsequent steps with the sections facing upwards.

(6) Two 10-min periods in Tris (10 mM)-phosphate (10 mM) buffered isotonic saline (TPBS), pH 7.4.

(7) Blocking of non-specific reaction with either a solution of 5% normal goat serum for 20 min, if IgG-coated colloidal gold is to be the reagent later, or with a solution of 0.5% ovalbumin, if Protein-A coated colloidal gold is to be the reagent later.

(8) 1 to 2 min in TPBS.

(9) Antiserum to GABA for 1–2 h, produced in rabbit against a GABA-glutaraldehyde-bovine serum albumin conjugate (Hodgson et al., 1985). It is diluted with 1% normal goat serum at 1:1000 to 1:3000. For control studies some grids are reacted with the same antiserum diluted 1:1000 and preincubated for 4 h with GABA, or other amino acids attached to polyacrylamide beads with glutaraldehyde as described elsewhere (Hodgson et al., 1985).

(10) Washing in three changes of 1% goat serum diluted with TPBS, 15 min each.

(11) Three to 5 min in 0.05% solution of polyethylene glycol (mol wt 15000–20000, Sigma), dissolved in 50 mM Tris buffer, pH 7.0. This step is carried out to avoid the mixing of the TPBS with the protein-coated, colloidal gold in the next step.

(12) Goat anti-rabbit IgG coated colloidal gold (15 nm, Janssen Life Sci., Prod.), diluted usually to 1:10 to 1:40 depending on the batch, for 1–2 h. It is diluted with the same solution as used in step 11. In other experiments Protein-A coated, colloidal gold is used as reported earlier (Somogyi and Hodgson, 1985).

(13) Three dips in double distilled water, followed by 10 min in double distilled water.

(14) Staining with uranyl acetate and alkaline lead citrate to increase contrast for electron microscopy.

(15) Three dips in double distilled water and blotting with filter paper.

From each series only every second grid is incubated for immunocytochemistry. The incubation in sodium metaperiodate greatly reduces the electron density of the HRP reaction endproduct, probably by removing the osmium. This makes the localization of the HRP-filled processes and synaptic contacts difficult, therefore unreacted serial sections are indispensable for the efficient collection of a reasonable sample of HRP-filled profiles. The reduction of electron density was observed in both the 3,3′-diaminobenzidine and the *p*-phenylenediamine/pyrocatechol reacted HRP-filled processes. In lightly filled cells all traces of the HRP reaction disappeared and the processes could only be identified by their location. Therefore, the HRP-filled processes are first localized in sections on the non-incubated grids. Thereafter the same process is located in the consecutive sections which are reacted to reveal GABA, using capillaries, myelinated axons and other conspicuous structures as fiducial marks.

3. Application of the combined technique

The simultaneous demonstration of physiological, structural and biochemical properties in neuronal networks has several potential applications. The most basic

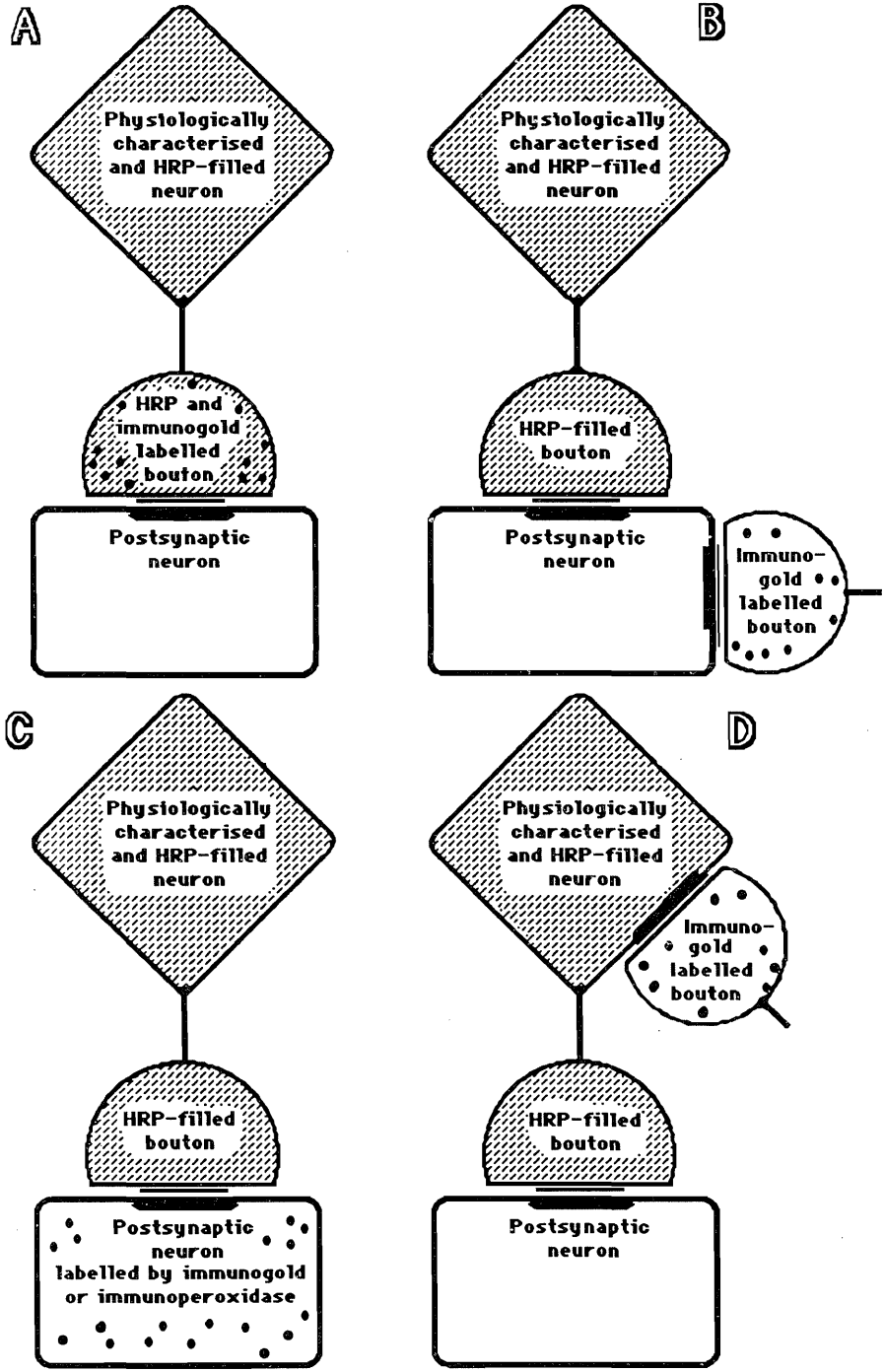

Fig. 1. Summary of some basic combinations of intracellular recording and marking studies with immunocytochemistry. In all cases the illustrated synaptic connections are established by electron microscopy. Additional, especially long range, connections can be studied using

situations are summarized in Fig. 1 but further combinations are clearly possible. Some of the circuits will be illustrated by examples from recent results obtained in the cortex using antisera to GABA. We have not yet completed a systematic study of the analysis of immunoreactive synaptic terminals on different parts of physiologically characterized cells (Fig. 1D), but this is now clearly possible. The limitations of the procedures and the potential for the use of other antisera will be discussed in section 4.

3.1. Demonstration of GABA immunoreactivity in intracellularly recorded neurons, cortical basket cells (see Fig. 1A for scheme)

In the cerebral cortex the majority of axo-somatic boutons are thought to originate from the so-called cortical basket cell (Jones, 1975; Marin-Padilla, 1969; Marin-Padilla and Stibitz, 1974; Ramon y Cajal, 1911; Szentagothai, 1973; 1983). Intracellular HRP filling studies combined with electron microscopy and the quantitative evaluation of their synaptic connections (Kisvarday et al., 1985; Martin et al., 1983; Somogyi et al., 1983) provided evidence for the existence of cells that establish up to 40% of their efferent synapses with the somata of other neurons. In the visual cortex of cat three types of basket cell have been revealed by intracellular HRP injection. The so-called "large basket cells" have axons running up to 800 μm from the soma tangentially in layers II–III, and a small radial projection to the infragranular layers (Martin et al., 1983; Somogyi et al., 1983). In contrast, the neurons called "clutch cells" terminate 100–300 μm from their somata and most of their terminals are in layer IV with a small projection to the infra- and supragranular layers (Kisvarday et al., 1985). Both types can be monosynaptically activated by thalamic afferents (Martin et al., 1983). A third type of neuron, the so-called "deep basket cell", was found recently to be situated in layers V–VI where it provides a long horizontal axon plexus, in addition to its radial ascending axonal tuft terminating in layer III (Kisvarday et al., 1987). All three cell types establish type II synaptic contacts and they make 20–30% of their synapses with the somata of other neurons (Kisvarday et al., 1985, 1987; Somogyi et al., 1983) the rest being with dendrites and dendritic spines.

On the basis of similarities between the identified basket cells and neurons immunoreactive for GABA (Somogyi and Hodgson, 1985; Somogyi et al., 1985) or the GABA-synthetic enzyme, glutamate decarboxylase (GAD) (Freund et al., 1983; Hendry et al., 1983; Ribak, 1978), it has been proposed that these neurons may use GABA as their transmitter (Kisvarday et al., 1985; Martin et al., 1983; Somogyi et al., 1983). This assumption, however, was based on indirect comparisons (see discussion Somogyi and Soltesz, 1986). In order to overcome the inherent uncertainties of the inferential evidence we tested directly for the presence of immunoreactive GABA in synaptic terminals of identified basket and clutch cells (Somogyi and Soltesz, 1986; Kisvarday et al., 1987), using the electron microscopic immunogold protocol described in section 2.

The selective distribution of colloidal gold indicated that some neuronal processes contained a high concentration of GABA. Immunoreactivity was found in terminals

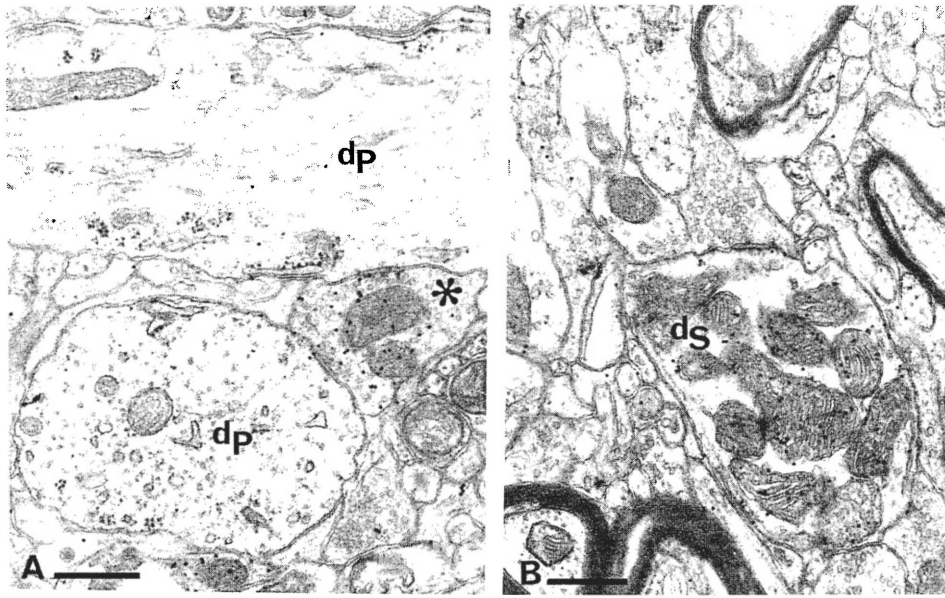

Fig. 2. Electron micrographs of GABA/immunogold-reacted sections from the striate cortex of cat. A. The selective accumulation of colloidal gold identifies a GABA-positive terminal (asterisk) establishing type II synaptic contacts with two dendrites (d_P) probably belonging to pyramidal cells. B. A large dendrite (d_S) probably originating from a smooth dendritic cell, is filled with GABA-positive mitochondria, as demonstrated by the selective accumulation of colloidal gold. Scale: 0.5 μm.

making type II or symmetrical synaptic contacts (Fig. 2). Some dendrites also displayed GABA immunoreactivity and in these cases the colloidal gold particles were mainly over mitochondria. Many GABA-positive dendrites were very rich in mitochondria (Fig. 2), and they probably belong to smooth dendritic cells. GABA immunoreactivity was also demonstrated in identified, HRP-filled boutons and in the dendrites of three clutch cells (Somogyi and Soltesz, 1986; Kisvarday et al., 1987). The GABA-immunolabeled basket cell terminals made synapses with somata, dendritic shafts and dendritic spines. Dendritic spines that received a synapse from a GABA-positive basket or clutch cell bouton also received a type I synaptic contact from a GABA-negative bouton (Fig. 3). This indicates that selective interaction can take place on single spines between a GABAergic basket cell terminal and another input. A few of the postsynaptic dendrites were also immunoreactive for GABA, indicating synaptic interactions between GABAergic basket and other GABA-containing cells. The fine structural characteristics of the majority of postsynaptic targets suggested that they were pyramidal and spiny stellate cells.

There was a great variation between the reactivity of the individual cells, just as there was variation in the general reactivity of the tissue from animal to animal. This is probably explained by differences in fixation, because in general, tissue showing poor ultrastructural preservation reacted poorly, and showed also the

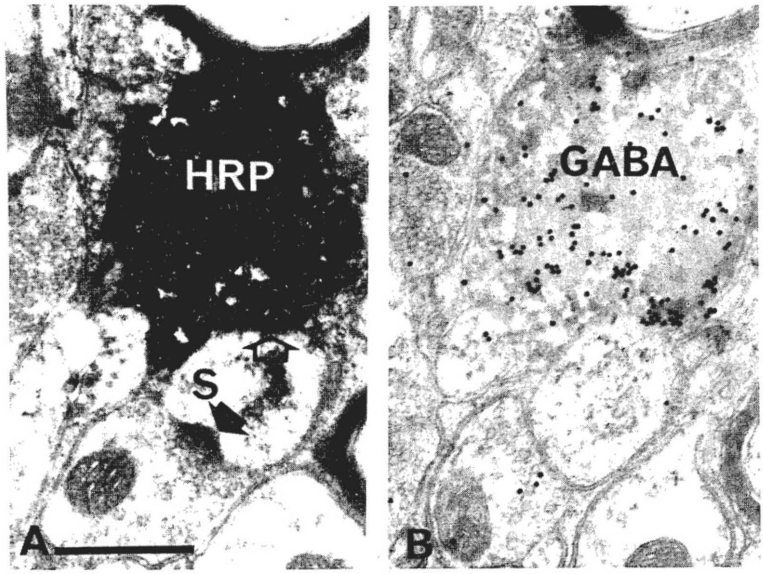

Fig. 3. Electron micrographs of serial sections from layer IV of the primary visual cortex of cat. An HRP-filled bouton originating from an intracellularly recorded clutch cell establishes a type II synaptic contact (open arrow) with a dendritic spine (s) which also receives a type I contact (filled arrow) from an unidentified terminal. The section in B was reacted to reveal GABA immunoreactivity; the selective distribution of gold particles over the clutch cell terminal demonstrates its high GABA content. Scale: 0.5 μm.

highest background deposition of gold. In the same reaction there was little variation between the boutons of an individual cell, but those sections of a bouton that contained many mitochondria usually exhibited higher gold density. Mitochondria usually show strong immunoreactivity for GABA, but only in those neuronal profiles whose other parts are also immunoreactive (Fig. 2). This immunoreactivity may be due to the endogenous GABA content of mitochondria, or could result from cytosolic GABA that was fixed to mitochondrial basic proteins. The dendrites of clutch cells were also immunoreactive for GABA (Fig. 4). Both proximal and distal dendrites were GABA-positive, most of the gold being deposited over mitochondria. Both clutch and basket cells have myelinated axons, and these also showed immunoreactivity for GABA (Somogyi and Soltesz, 1986).

These studies provided direct evidence for the presence of immunoreactive GABA in identified basket and clutch cells and the results strongly suggest that GABA is a neurotransmitter at their synapses. The orthodromic activation of basket cells by visual and electrical stimulation demonstrated that some of them receive mono-, others polysynaptic, thalamic input and they can be activated either by the Y or the X stream of thalamic afferents (Martin et al., 1983). One of the basket cells was also shown to receive callosal inout. The laminar distribution of the synaptic terminals of the different basket and clutch cells demonstrates that these GABAergic neurons, which have similar target specificity, segregate into different laminae, and

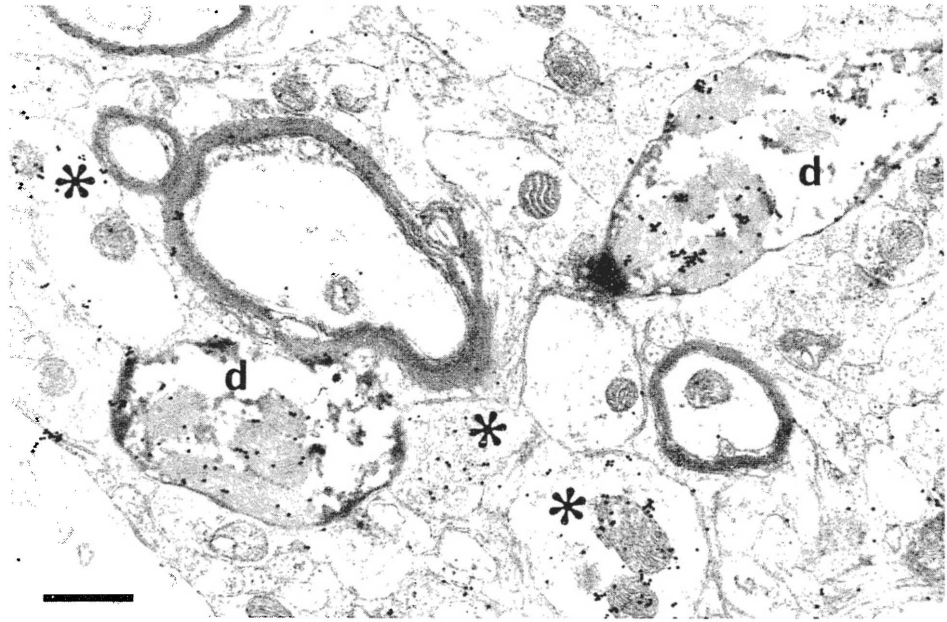

Fig. 4. Electron micrograph of a GABA/immunogold reacted section from the striate cortex of cat. The HRP-filled dendrite of an intracellularly recorded clutch cell is GABA-positive as are three vesicle containing boutons (asterisks). Scale: 0.5 μm.

that the same GABAergic cells can take part in both horizontal and radial interactions. Since the spatial distribution of their axonal arborization is largely complementary, our findings demonstrate that in the different cortical laminae different sets of basket cells use the same transmitter for similar operations.

3.2. Demonstration of GABA in postsynaptic targets of intra-cellularly recorded neurons (see Fig. 1C for scheme)

3.2.1. Immunogold method: targets of pyramidal cell axon collaterals

The identification of an antigen in the postsynaptic elements of intracellularly recorded cells may help to clarify hypotheses for their roles. For example there are numerous hypotheses for the role of the local axon collaterals of pyramidal cells. Most hypotheses predict that pyramidal cells activate specific classes of postsynaptic cells. According to one hypothesis the local axon collaterals may activate inhibitory interneurons thereby providing the structural basis of recurrent inhibition (Phillips, 1959; Stefanis and Jasper, 1964; Creutzfeldt et al., 1969). The major inhibitory transmitter in cortex is GABA. Thus we were able to test the above hypothesis by testing for the presence of GABA in structures postsynaptic to pyramidal cell axon collaterals (Kisvarday et al., 1986).

In the cat visual cortex two pyramidal cells in layer III were examined in the electron microscope. These cells were of special interest because of their clumped

axon arborization near, and also 0.4–1.0 mm from, the cell body, in register in both layers III and V. Of 191 synaptic contacts established by the axons, only one bouton contacted a cell body and that was immunoreactive for GABA. The major targets were dendritic spines (84 and 87%), which probably originated from other pyramidal cells, and the remainder were dendritic shafts. Only about a third of the postsynaptic dendrites tested showed immunoreactivity for GABA. The majority of the non-immunoreactive dendrites showed characteristics of those originating from pyramidal cells. The putative inhibitory cells therefore formed not more than 5% of the postsynaptic targets of these pyramidal cells. The results showed that the type of pyramidal cells with clumped axons studied by Kisvarday et al. (1986) make contacts predominantly with other pyramidal cells. Thus the primary role of both the intra- and intercolumnar collateral systems is the activation of other excitatory cells (Kisvarday et al., 1986). The presence of a small population of GABAergic cells amongst the targets, however, may be significant if they receive highly convergent input from a large population of pyramidal cells. Thus the possibility that a specialized GABAergic cell type responsible for recurrent inhibition exists is worth studying further.

In the previous section on basket cells we have already mentioned that a few of the dendrites postsynaptic to basket cells were also immunoreactive for GABA (Somogyi and Soltesz, 1986). These two examples illustrate that the immunogold-GABA method is well suited for determining the proportion of putative GABAergic targets postsynaptic to physiologically characterized axons.

3.2.2. Unlabeled antibody enzyme method: targets of thalamic X and Y axons in visual cortex

The specific visual afferents, arriving mainly from the lateral geniculate nucleus of the thalamus to the cortex, terminate principally in layer IV and to a lesser extent in layer VI (Rosenquist et al., 1974; LeVay and Gilbert, 1976). It has been known from degeneration studies that a small proportion of the terminals made synapses with the somata of non-pyramidal cells (Garey and Powell, 1971). This was confirmed in recent experiments (Freund et al., 1985) by examining the terminations of intracellularly recorded, HRP-filled and resin embedded thalamic axons. Furthermore, it was discovered that each thalamic axon made multiple contacts with the somata of about 7–10 neurons, which seemed similar in size and shape to a population of large GABA-positive cells demonstrated previously. Using electron microscopy of the HRP-filled axons, Freund et al. (1985) showed synaptic contacts between the thalamic terminals and the large somata. The cells postsynaptic to the intracellularly recorded axons were then tested for the presence of GABA. Semithin (0.5 μm) thick sections were cut from parts of the somata still in the block and reacted using the unlabeled antibody peroxidase technique described in section 2. Every cell that received somatic input from the axons was GABA-positive, and their fine structural characteristics agreed well with those of identified basket and clutch cells. Furthermore, it could be shown that X type afferents contacted GABA-positive somata that, as a population, were significantly smaller than the ones contacted by the Y axons.

This study demonstrated that GABAergic, presumably inhibitory, neurons are activated at the first stage of cortical processing, and that the cells are of a select population of GABA-containing neurons probably corresponding to basket and clutch cells. The size differences between the GABA-positive cells contacted by the two functionally distinct streams of visual afferent provide evidence for the segregation of parallel functional channels at the first step of processing in the primary visual cortex.

3.3. Convergence of HRP-filled and immunolabeled boutons onto the same postsynaptic cell (see Fig. 1B for scheme)

While analysing the postsynaptic elements of basket and pyramidal cells, we noted that GABA-immunoreactive terminals often contacted these dendrites and somata within the same section as that where the synapse from the intracellularly injected axon was located. In the case of the basket cells whose terminals were also immunopositive, this demonstrated the convergence of several GABAergic cells to the same neuron.

The terminals of pyramidal cells, however, were never immunoreactive for GABA, and all available evidence suggests that their direct effect on other cells is excitatory. Therefore the presence of GABA-containing and presumably inhibitory synapses near the synapses established by pyramidal cells suggests that their effect may be selectively reduced under certain circumstances. This would especially apply to the pyramidal terminals on dendritic spines, whose excitatory effect would be selectively reduced by appropriately timed input from the GABAergic bouton contacting the same spine. This arrangement could provide the basis for the "synaptic veto mechanism" (Koch and Poggio, 1983).

4. Limitations and future perspectives

The retrospective localization of GABA in electrophysiologically characterized, HRP-filled cells, which were embedded for electron microscopy, is possible because the epitope(s) remain recognizable by the antiserum following tissue processing. Other methods have also been used to localize the putative transmitter of physiologically characterized neurons in the central nervous system, and the transmitters of many identified neurons in invertebrates have been established. In the central nervous system of vertebrates dopamine has been localized in intracellularly recorded mesencephalic neurons by fluorescence microscopy, following the elevation of dopamine levels by intracellular injection of drugs (Grace and Bunney, 1983). Fluorescence microscopy and intracellularly injected fluorescent dyes were also used to determine the chemical nature of intracellularly recorded noradrenergic and serotoninergic neurons (Aghajanian and Vandermaelen, 1982; Weiler and Ammermuller, 1986). The neurons characterized in these procedures, however, could not be processed for electron microscopy.

Immunocytochemistry was employed to reveal neurophysin, enkephalin and arginine vasotocin immunoreactivity in magnocellular paraventricular neurons in

the hypothalamus (Reaves et al., 1983). In these studies, intracellularly injected Lucifer Yellow and Procion Yellow were used to locate the recorded cells. Immunofluorescence combined with intracellular recording and dye injection was also used in the peripheral nervous system to study the response properties of neurons containing different neuropeptides (Bornstein et al., 1986). Reaves et al. (1983) were also able to study the labeled cells in the electron microscope following deposition of DAB in the fluorescent dye-filled cells. HRP-filling has the advantage that it gives not only a very detailed image of the cell, but also the axonal arborizations can be followed over dozens of sections covering several millimetres in the brain. The specimens are also permanent and, using the post-embedding methods, several antigens can be localized in the same tissue.

It is not clear yet how generally applicable the demonstation of transmitter within the intracellularly recorded HRP-filled neurons will be. The impalement of the cell and the injection of HRP may cause damage that upsets the normal biochemical machinery for transmitter production. In the case of GABA its levels are known to increase post mortem in the brain (Balcom et al., 1975; Van der Heyden et al., 1978), thus any damage to the cell may even increase its GABA levels. Other transmitters however could decrease following HRP-filling, thus negative results concerning the intracellularly recorded cell, as generally in immunocytochemistry, should be treated with caution.

In order to achieve electron microscopic localization of synapses in most studies, the tissue will be osmium treated and embedded in resin. A wide range of antigens has been localized in nervous tissue following embedding into epoxy and other resins, but only few of these studies included osmium treatment. Of the neuroactive substances a few peptides such as LHRH (Van den Pol, 1985), oxytocin (Theodosis et al., 1986), neurophysin (Van den Pol, 1984, 1985) and the amino acids glutamate (Somogyi et al., 1986) and GABA (Somogyi and Hodgson, 1985; Van den Pol, 1985) have been localized in osmium treated tissue. Our experience is that many epitopes, especially on proteins, are sensitive to osmium treatment and embedding, and will be lost during processing of the tissue. Thus, the major limitation of the above procedures is that, at the moment, they can only be applied to relatively few antigens.

However, from new developments along several lines it can be predicted that the localization of more and more antigens will become possible under conditions that permit the analysis of synaptic connections.

Firstly, new resins are being introduced that preserve tissue antigenicity better. Both Lowicryl 4KM (Roth et al., 1981; Van den Pol, 1984) and LR White (e.g. Cohen, 1985) have great potential for immunocytochemical studies of the brain. Secondly, antibodies are developed to an increasing number of small neuroactive molecules that are resistant to tissue processing conditions following their fixation. Besides GABA, the localization of glycine (Van den Pol and Gores, 1986) and glutamate (Somogyi et al., 1986) has been achieved under post-embedding conditions in material similar to that used in the studies for GABA.

In this review, besides providing a detailed methodological description, I have also tried to illustrate the potential of the procedures. It should be clear that our

examples, related to one transmitter and only in one area of the brain, merely mark the beginning of future applications.

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