

Immunocytochemistry with internally labeled monoclonal antibodies

(serotonin/neuropeptides/hybridomas)

A. CLAUDIO CUELLO*, JOHN V. PRIESTLEY*, AND CÉSAR MILSTEIN†

*Departments of Pharmacology and Human Anatomy, South Parks Road, Oxford OX1 3QT, England; and †MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England

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ABSTRACT One of the advantages of the production of monoclonal antibodies by tissue culture methods is that they can be internally labeled by using appropriate radioactive amino acid in the culture fluid. Thus, radioactive immunological probes of high specific activity can be prepared. Here we report applications of these internally labeled monoclonal antibodies for the direct localization of immunoreactive sites in the central nervous system of the rat at both light and electron microscopic levels (radioimmunocytochemistry). We explored the combined use of radioimmunocytochemistry with immunoenzymatic methods for the simultaneous detection of two antigenic sites: substance P and serotonin or substance P and enkephalin. In neurobiology this procedure could help to clarify certain aspects of transmitter-specific synaptic interactions and the coexistence of neuroactive substances in single neuronal cell bodies or nerve terminals. We also describe the application of radioimmunocytochemistry with internally labeled monoclonal antibodies to quantify the immunoreactions in discrete microscopic areas.

The importance of immunological methods in neurobiology is well illustrated by their contributions to the biochemical anatomy of the mammalian nervous system and by the introduction of new concepts, such as the coexistence of neurotransmitter substances in single neurones (for review see ref. 1). The power of immunological methods for the detection, characterization, and assay of substances in biological specimens has been enhanced greatly by the development of methods permitting the derivation of permanently established cell lines secreting monoclonal antibodies of predefined specificity (2). In recent years, we have developed and applied monoclonal antibodies against neurotransmitter substances for the study of some of the problems described above. More recently, we have started to explore applications of monoclonal antibodies internally labeled during biosynthesis *in vitro* with radioactive amino acid precursors; ³H-labeled anti-substance P designated ³H-NC1/34, and anti-serotonin, designated ³H-YC5/45, monoclonal antibodies have been used in immunocytochemical experiments (3, 4). Here we report further developments, which include the combination of radioautographic detection of the internally labeled antibodies with the peroxidase-antiperoxidase (PAP) technique (5), allowing the simultaneous dual localization of two antigenic sites both at the light and electron microscope level.

MATERIALS AND METHODS

Internally Labeled Anti-Substance P Monoclonal Antibody (³H-NC1/34). The hybrid myeloma clone NC1/34 HL (6) was used to produce internally labeled monoclonal antibody in the following manner. Approximately 2×10^6 cells from an exponentially growing culture were centrifuged, resuspended in Dulbecco's modified Eagle's medium supplemented with 5%

(vol/vol) fetal calf serum lacking the amino acid lysine, and pelleted by centrifugation. They were resuspended in the same medium, supplemented with 1 mCi (1 Ci = 3.7×10^{10} becquerels) of [³H]lysine per ml (specific activity 75–100 Ci/mmol; The Radiochemical Centre, Amersham, England), and incubated at 37°C in a water-saturated CO₂ incubator for 16–20 hr. The spent medium was dialyzed against large volumes of phosphate-buffered saline (pH 7.0), referred to hereafter as saline. The specific activity of the internally labeled monoclonal antibodies varied from batch to batch, but it was estimated to be in the range of 50–500 Ci/mmol of antibody.

Primary Antibodies. For the detection of serotonin immunoreactive sites, the monoclonal antibody YC5/45 HLK present in culture supernatants was used. This antibody is the product of a rat × rat hybrid myeloma prepared with spleen cell of a rat immunized against a serotonin-bovine serum albumin conjugate (7, 8).

For [Leu]enkephalin immunoreactive sites, a rabbit polyclonal antibody was used, generously provided by Richard Miller (University of Chicago). The characteristics of this antibody have been reported elsewhere (9).

Light Microscopic Radioimmunocytochemistry. Male adult Wistar rats weighing ≈250 g were anesthetized with equithesin and perfused through the heart or abdominal aorta with freshly made 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brains were removed, kept in a fixative for 2–4 hr, and then left in the same buffer containing 5% sucrose for periods ranging from 4 to 24 hr. Sections (10 μm) were cut on a cryostat, mounted on gelatin-coated slides, and then incubated with ³H-NC1/34 diluted from 1:1 up to 1:50 (vol/vol) in saline both with and without added Triton X-100 (0.2%). After incubation for 2 hr at room temperature or for 16 hr at 4°C, slides were briefly washed in saline and then air dried at room temperature. Slides were then dipped in Ilford L5 nuclear emulsion, diluted 1:1 in water (vol/vol), air dried, exposed for 4–10 days, and subsequently developed in Kodak D19. Alternatively some slides were dipped in Ilford L4 (1:4) and developed in Amidol.

Electron Microscopic Radioimmunocytochemistry. Rats were perfused as described above, except that the fixative also contained 0.05% glutaraldehyde. The brains were removed, kept in fixative for a further 2–4 hr, and then transferred to 0.1 M phosphate buffer containing 30% sucrose. After 8–24 hr, blocks of tissue were given a rapid freeze/thaw by immersing for a few minutes in liquid N₂ and then in phosphate buffer. Subsequently, 40-μm sections were cut on a Vibratome (Oxford Ltd.). Sections were then incubated in saline/0.2% Triton X-

Abbreviation: PAP, peroxidase-antiperoxidase.

100 (1 hr), in $^3\text{H-NCl}/34$ diluted from 1:1 up to 1:50 (vol/vol) (overnight at 4°C), and 1% glutaraldehyde (2 hr). Unless otherwise stated, incubations were carried out at room temperature. Between all incubations, sections were washed thoroughly with saline. Sections were then stained in 1% OsO_4 (1 hr), dehydrated, and embedded in Epon. For autoradiography sections were cut from suitable blocks, collected on celloidin-coated glass slides, stained with lead citrate, coated with carbon, and dipped in Ilford L4 1:5 (vol/vol) emulsion. Autoradiographs were developed with Kodak D19B after 4–8 weeks exposure.

Combined Substance P Radioimmunocytochemistry and Serotonin PAP Immunocytochemistry. Cryostat sections of the brain stem were prepared as for light microscopic radioimmunocytochemistry, except that the rats received $70\ \mu\text{g}$ of colchicine ($20\ \mu\text{l}$) injected into the brain ventricles 24 hours before perfusion. Antibody solutions (vol/vol) were made up in 0.2% Triton X-100/saline, except for the primary anti-serotonin antibody (YC5/45), which was made up in saline. The following incubations were carried out at room temperature with a wash in Triton X-100/saline between each incubation: 1:10 normal rabbit serum (30 min) to quench unspecific reactions, 1:300 YC5/45 (4°C , overnight), 1:10 rabbit anti-rat IgG (1 hr), 1:50 rat PAP (1.5 hr), 1:20 normal rat serum (1 hr), and 1:5 $^3\text{H-NCl}/34$ (1 hr). Peroxidase staining was then revealed by a 15-min incubation in 0.06% diaminobenzidine/0.01% H_2O_2 /0.05 M Tris-HCl, pH 7.6. Sections were then washed, postfixed in 1% glutaraldehyde (30 min), and processed for autoradiography as described for light microscopy.

Combined Substance P Radioimmunocytochemistry and Enkephalin PAP Immunocytochemistry. *Light microscopy.* Sections were processed as described in the previous section, except that the following antibody incubations were used: 1:10 normal goat serum (30 min), 1:200 anti-enkephalin plus 1:1 $^3\text{H-NCl}/34$ (1 hr), 1:10 goat anti-rabbit IgG (1 hr), and 1:50 rabbit PAP (1 hr).

Electron microscopy. Full details of variables involved in PAP ultrastructural immunocytochemistry are described elsewhere (10). Vibratome sections were prepared as before. Sections were then incubated at room temperature, unless otherwise stated, in the following solutions made up in saline: 1:10 normal goat serum (30 min), 1:200 anti-enkephalin (4°C overnight), 1:10 goat anti-rabbit IgG (1 hr), and 1:50 rabbit PAP plus 1:1 $^3\text{H-NCl}/34$ (overnight).

Sections were then incubated in diaminobenzidine plus H_2O_2 and postfixed in glutaraldehyde, as before. The sections were processed for electron microscopic autoradiography as described. Processed areas were those in which good PAP-enkephalin immunostaining was observed. Other details of experimental conditions employed in ultrastructural immunocytochemistry were as described (10).

Microscopy. For light microscopy, a Leitz Dialux 20 microscope was used equipped with NPL Fluotar objectives and set up for bright-field, dark-field, or phase-contrast illumination. Interference-contrast illumination (Leitz ICT) was also used and found to be the most suitable for examination of dually labeled sections.

For electron microscopy, a Phillips 201 electron microscope was used. When observing dually stained section, low-voltage (40 kV) and small-aperture (condenser lens, $100\ \mu\text{m}$; object lens, $20\ \mu\text{m}$) settings were used to enhance contrast of the diaminobenzidine reaction products.

Quantitation of immunocytochemical staining was carried out from the microscopic preparations by using the densitometer of a Quantimet 720 image analysis system. A $50\ \mu\text{m} \times 50\ \mu\text{m}$ area was set up on the variable frame and scale module, and a single-scan measurement was made of the integrated optical

density within this area. For each feature of interest a number of areas were scanned, and the mean and SEM of these values were computed.

RESULTS

Light microscopic radioautograms from sections incubated with $^3\text{H-NCl}/34$ revealed intense binding of the internally labeled monoclonal antibody. This binding correlated well with the distribution of substance P immunoreactive material as described with conventional immunohistochemical techniques (11, 12) or with the aid of antibodies of monoclonal origin (6). For example, intense binding was observed in the substantia nigra of the mesencephalon and particularly in the pars reticulata (Fig. 1a). Also intense radioimmunobinding was detected at the external borders of the nucleus interpeduncularis (Fig. 1a). Silver grains in the substantia nigra appear to be located in the neuropile of this nucleus. This cellular localization was corroborated when thin sections of the substantia nigra were prepared for high-resolution radioautography. These preparations resulted in good preservation of the ultrastructural details. As represented in Fig. 1b, clusters of silver grains were regularly noticed over nerve terminal profiles. The large majority of these nerve terminals displayed axodendritic synapses over characteristic large dendrites of the proximal region of the pars reticulata. Some pleomorphism of these nerve terminals was observed. In the raphe magnus of colchicine-pretreated animals, light micro-

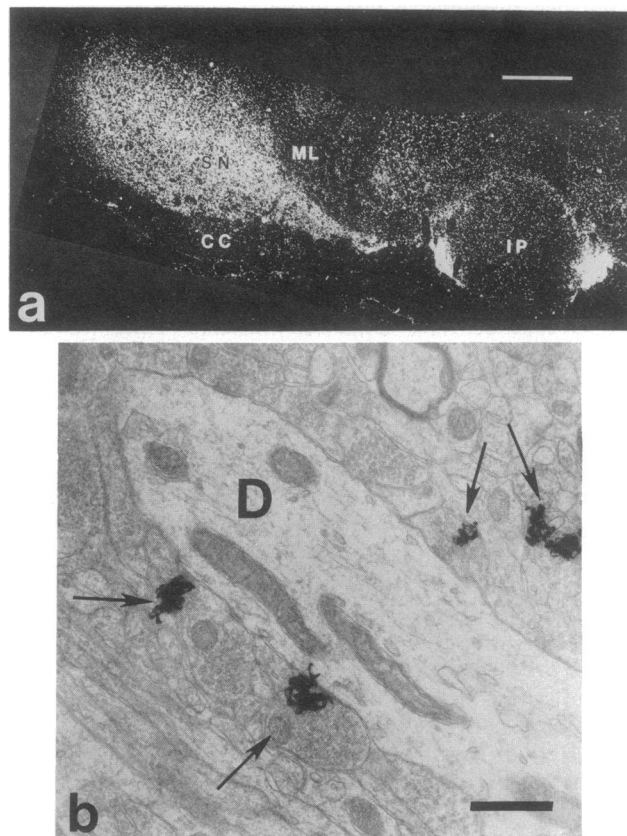


FIG. 1. (a) Light microscopic radioautograph (dark field) of rat brain stem, showing the binding of $^3\text{H-NCl}/34$ (radiolabeled anti-substance P antibody); SN, substantia nigra; IP, nucleus interpeduncularis; CC, crus cerebri; ML, medial lemniscus. (Scale bar = $100\ \mu\text{m}$.) (b) Radioimmunocytochemical preparation at the electron microscopic level of the rat substantia nigra (pars reticulata) with $^3\text{H-NCl}/34$. Clusters of silver grains are seen over nerve terminal profiles (arrows) in the neuropile. Three of these nerve terminals are establishing axodendritic synapses with a typical large dendrite (D) of this region of the brain. (Scale bar = $1\ \mu\text{m}$.)

scopic radioimmunocytochemistry with ^3H -NC1/34 revealed a number of substance *P* immunoreactive cell bodies.

In the lower medulla oblongata, the sites of greater concentration of ^3H -NC1/34 were the neuropile of the substantia gelatinosa of the spinal nucleus of the trigeminal nerve, of the nucleus of the tractus solitarius, of the lateral reticular formation, and to a lesser extent of the periventricular grey. The intense labeling of the substantia gelatinosa of the spinal nucleus of the trigeminal nerve can be seen in Fig. 2*a*. To explore the possible quantitation of the binding, we performed densitometric analysis of the radioautograms. The results in Fig. 2 show the differences in the binding of ^3H -NC1/34 between the tractus solitarius (incoming fibres), the substantia gelatinosa, and the nucleus of the trigeminal nerve. The differences among microscopically identifiable regions were more marked, applying higher concentrations of internally labeled antibodies.

These differences in the intensity of staining were much more marked than the difference obtained by PAP staining. Optical density measurements of NC1/34 binding by using the PAP technique (results not shown) revealed only 4-fold higher intensity in the substantia gelatinosa over the other areas, compared with 59- to 18-fold using radioimmunocytochemistry.

When radioimmunocytochemistry for substance *P* was combined with PAP immunocytochemistry for serotonin (YC5/45), it was seen that substance *P*-containing cells also contained serotonin. This is seen in Fig. 3, which shows cells with the characteristic diffuse brown staining due to PAP (serotonin) upon which was superimposed a high concentration of silver grains, indicating the presence of substance *P*.

At the light microscopy level, radioimmunocytochemistry with ^3H -NC1/34 for substance *P* and PAP for [Leu]enkephalin immunoreactive sites of the spinal nucleus of the trigeminal nerve revealed silver grains and peroxidase reaction products, the latter over a slightly wider area. At light microscopic level, it is not possible to resolve the cellular localization of these two

signals. Fig. 4*a* shows a case in which colchicine was administered, revealing [Leu]enkephalin immunoreactivity over local circuit neurones. Silver grains denoting substance *P* immunoreactivity are seen in the neuropile, but the synaptic interrelation cannot be established. It is possible that in spite of the species difference, the addition of both primary antibodies (anti-enkephalin and NC1/34) prior to the addition of anti-rabbit Ig and PAP conspires against resolution of the two signals. In an attempt to demonstrate these sites at the electron microscopic level, thin sections were prepared, combining the two procedures, but now the radioactive antibody was added at the end (Fig. 4*b*). Silver grains were restricted to neuropile. They were present in large nerve terminals (Fig. 4*b*) and also in smaller profiles. They were seldom closely related to enkephalin-immunoreaction terminals, and so far we have been unable to recognize axo-axonic synapses between these two transmitter-specific terminals.

DISCUSSION

This study demonstrates that the internally radiolabeled monoclonal antibody, prepared by culturing the hybrid myeloma NC1/34 in the presence of [^3H]lysine, is an excellent reagent for the direct detection of immunoreactive sites in tissue sections at both the light and electron microscopic levels. The distribution of the binding of ^3H -NC1/34 is similar to that described for substance *P* immunoreactivity detected by other methods. As expected, internal radiolabeling does not seem to affect the binding characteristics of the monoclonal antibody. The high specific activity obtained with the *in vitro* incorporation of radioactive precursors (up to 60 molecules of radiolabeled lysine can theoretically be incorporated in a single antibody molecule) results in an adequate signal of the immunoreaction. The advantages of internal labels over chemical labeling (e.g., iodination) have been discussed (3, 4). At the electron microscopic level, radioimmunocytochemistry has the

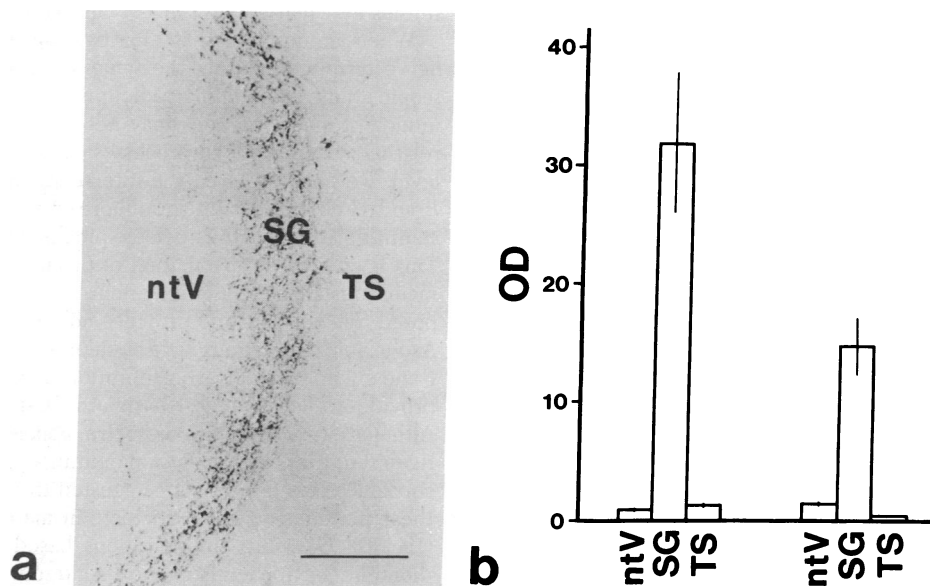


FIG. 2. (a) Light microscopic radioimmunocytochemistry with ^3H -NC1/34 of the lower medulla oblongata of the rat. Note intense labeling over the substantia gelatinosa (SG) of the spinal nucleus of the trigeminal nerve. Compare with radiolabeling in the spinal tract (TS) or superficial portions of the spinal nucleus (ntv). Interference contrast micrograph. (Scale bar = 100 μm .) (b) Integrated optical density to quantitate bound antibody in the microscopic areas described above. Note the relative differences in radiolabeling between the substantia gelatinosa (SG), the spinal tract (TS), and the spinal nucleus (ntv). Labeling in the substantia gelatinosa is from 18 to 59 times greater than labeling in the spinal tract or spinal nucleus, depending on the concentration of radiolabeled monoclonal antibody used: dilutions of cell culture medium supernatant containing radiolabeled monoclonal antibodies against substance *P* (^3H -NC1/34) were 1:20 (left columns; eight observations) and 1:50 (right columns; nine observations). OD, integrated optical density in arbitrary units. Bars indicate SEM.

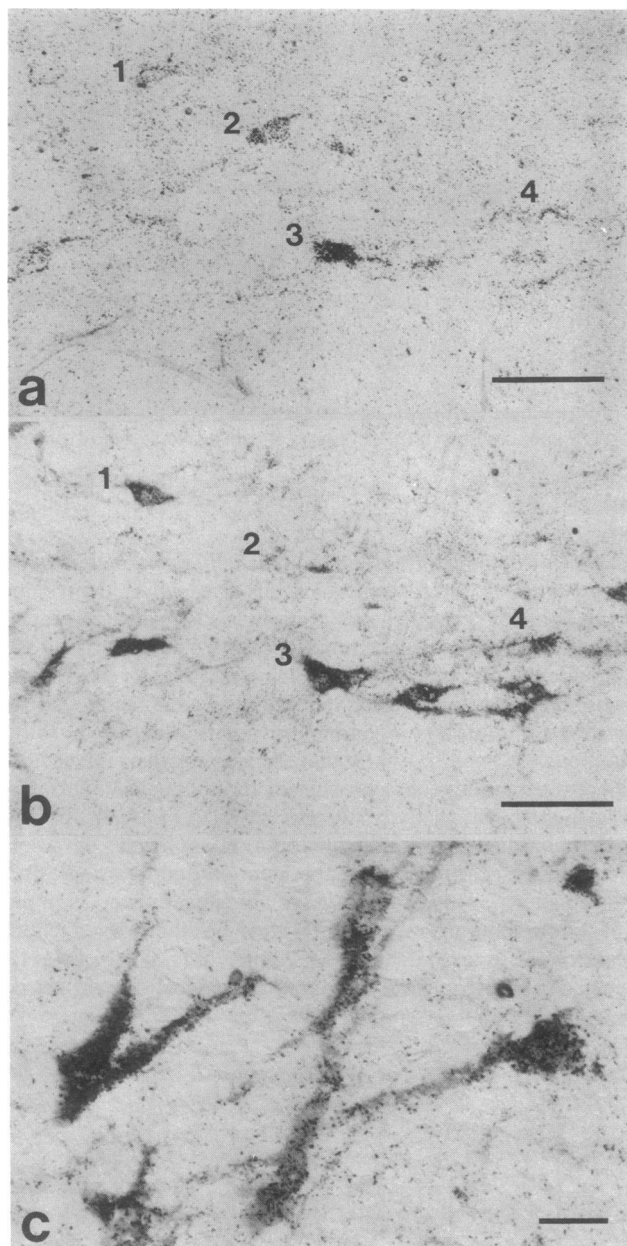


FIG. 3. Combined radioimmunocytochemistry and PAP immunocytochemistry; cryostat sections (10 μm thick) of the raphe magnus of the rat medulla oblongata. Antibodies were ^3H -NC1/34 (radiolabeled monoclonal anti-substance *P* antibody) and YC5/45 (anti-serotonin monoclonal antibody). *a* and *b* are consecutive serial sections. (*a*) Control. YC5/45 was adsorbed with excess serotonin. Note absence of immunoperoxidase reaction but intense radiolabeling over certain cell bodies (1–4), indicating the presence of substance *P* immunoreactivity. (Scale bar = 200 μm .) (*b*) Same preparation as *a* except that YC5/45 was not preadsorbed. Certain cells (1, 3, 4) observed in *a* appear in serial section and now show immunoperoxidase staining in addition to radiolabeling. This indicates that they contain serotonin as well as substance *P*. (Scale bar = 200 μm .) (*c*) High-magnification micrograph of a similar preparation to *b*, where silver grains (indicating substance *P* immunoreactivity) and homogeneous peroxidase reaction products (indicating serotonin immunoreactivity) appear over single neurons of the nucleus raphe magnus of the rat. (Scale bar = 20 μm .)

advantage that it involves minimum compromise between good ultrastructural preservation and antibody penetration because the internally radiolabeled monoclonal antibodies do not require large complexes of secondary antibodies or damaging enzymatic reactions.

The quantitative localization of immunoreactive sites by optical density measurements in local areas of autoradiograms could be of great assistance in measuring variations in the content of neuroactive substances, for instance in following lesions or drug treatment. The preliminary experiments reported here are most encouraging. The difference in the results obtained with the two antibody concentrations used in Fig. 2 suggests that the optical density in the areas of the spinal nucleus and spinal tract is mainly due to background and that the signal/noise ratio is significantly improved at higher antibody concentration. In this respect, both concentrations were much better than quantitative optical measurements using the PAP method. The high signal/noise ratio emphasizes the potential sensitivity of radioimmunocytochemistry. Further evidence of the sensitivity of the approach is provided by radioimmunocytochemical detection of serotonin sites with the monoclonal anti-serotonin antibody ^3H -YC5/45 in a population of amacrine cells in the frog retina (unpublished data), where small amounts of this indolamine can be found by high-performance liquid chromatography (13).

The simultaneous detection of two different antigenic sites in a single preparation poses special problems, due to the need for different markers of the immunoreaction and to possible crossreactions, particularly when using developing antibodies. These problems are particularly acute at the electron microscopic level, and as far as we know, there is no satisfactory protocol applicable to preembedding ultrastructural immunocytochemistry, the method most frequently used for the detection of transmitter markers in the central nervous system (10). We have shown that by combining radioimmunocytochemistry with immunoenzymatic methods, it is possible to overcome these difficulties. Certain technical details are essential, and in particular it is necessary to ensure saturation of uncombined sites of the link antibody in the PAP sequence. At the light microscopic level, this procedure confirmed the coexistence of substance *P* and serotonin immunoreactive sites in neurones of the nucleus raphe magnus of the rat, as first described in the elegant studies by Chan-Palay *et al.* (14) and Hökfelt *et al.* (15).

When immunoreactivity for two substances is present in nerve terminals sharing the same synaptic field, light microscopic studies combining radioimmunocytochemistry and immunoenzyme techniques may not resolve the intricate interrelation of these cellular processes. At the electron microscopic level, we have explored the interrelations between substance *P* and [Leu]enkephalin immunoreactive profiles in the substantia gelatinosa of the nucleus spinalis of the trigeminal nerve. This is an area of termination of primary sensory fibres, with many substance *P*-containing neurones, where it has been proposed that this peptide provides nociceptive information (16). Axo-axonic interactions have been proposed between substance *P* and enkephalin-releasing inhibitory axons (17), and this would provide a neurochemical basis of a "gating" mechanism (18). Although it is difficult to draw firm conclusions from single antigen stainings, ultrastructural immunocytochemistry for either substance *P* or enkephalin suggested that terminals containing these peptides do not participate in many axo-axonic contacts (19, 20). Our initial observations based on simultaneous radioimmunocytochemistry and PAP immunocytochemistry confirm this result and further suggest that substance *P* and enkephalin-containing terminals may sometimes synapse onto a common dendritic profile. The use of such combined methodology might contribute to solve a number of outstanding problems related to transmitter-specific synaptic interactions in many regions of the brain. The same approach is of general application in areas other than neurobiology.

Finally, it is possible that radioimmunocytochemistry will be

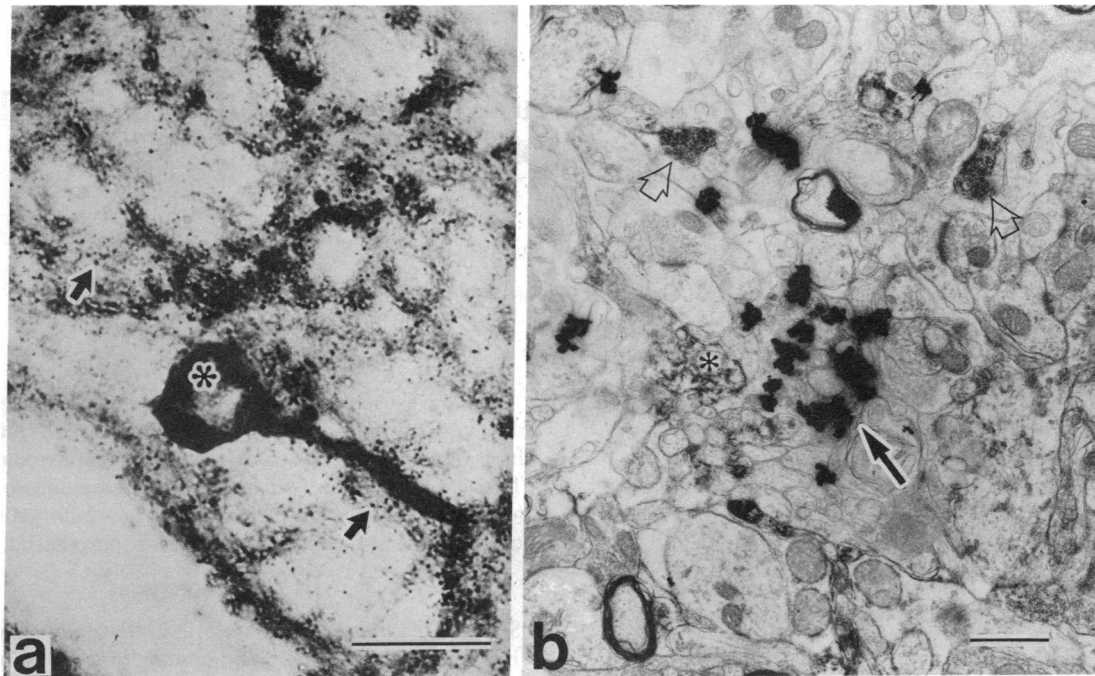


FIG. 4. (a) Combined radioimmunochemistry for substance *P* immunoreactivity and immunoperoxidase for [Leu]enkephalin immunoreactivity in the substantia gelatinosa of the spinal nucleus of the trigeminal nerve (rat lower medulla oblongata, colchicine-treated animal). Note presence of [Leu]enkephalin immunoperoxidase-stained neuron (asterisk) and silver grains (arrows) in neuropile, denoting binding of $^3\text{H}\text{-NC1/34}$. Bright-field micrograph. (Scale bar = 20 μm .) (b) Similar combined radioimmunochemical and immunoperoxidase preparation as in a but prepared from electron microscopy. Note clusters of silver grains over a nerve terminal in the centre of the field (solid arrow), indicating substance *P* immunoreactivity as revealed by $^3\text{H}\text{-NC1/34}$. Open arrows denote the presence of [Leu]enkephalin immunoreactive nerve terminals in the same field as revealed by immunoperoxidase. The asterisk indicates [Leu]enkephalin immunoreactivity in a neuronal process (presumptive dendrite). (Scale bar = 1 μm .)

useful to localize transmitters present in functionally identifiable neurones after intracellular injection of horseradish peroxidase (21, 22), where peroxidase cannot be further used in immunocytochemistry. As internally labeled antibodies are developed as radioautographs, it is possible that these two methods can be combined. This procedure, or related procedures, using internally labeled monoclonal antibodies would contribute to bridge the gap between biochemistry, form, and function in the nervous system.

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