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NEUROTRANSMITTER-SPECIFIC PROJECTION NEURONS REVEALED BY COMBINING PAP IMMUNOHISTOCHEMISTRY WITH RETROGRADE TRANSPORT OF HRP

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SUMMARY

The use of PAP immunohistochemistry in combination with HRP retrograde transport is described, allowing the transmitter characterization of identified projection neurons. To assess the validity of this procedure the dorsal raphe nucleus has been studied. It has been possible in single sections to stain for 5-HT immunoreactivity cells which have been retrogradely labeled following injection of HRP into the striatum. The presence of such neurons and their distribution in the dorsal raphe demonstrated with this dual staining technique agrees very well with previous results obtained from separately performed retrograde labeling and histochemical or immunohistochemical staining. The procedure described has some advantages over other traditional and recently described methods and in addition should be applicable to electron microscopic studies.

INTRODUCTION

Since its introduction in 1972 by LaVail and LaVail[18], the retrograde transport of horseradish peroxidase (HRP) has been used extensively as a tool for studying neuronal connectivity[19]. However, this technique does not provide information on the neurotransmitter characteristics of the identified pathways. In an attempt to overcome this limitation various workers have combined retrograde transport of HRP with

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immunohistochemistry[20] or fluorescence histochemistry[2,4] in order to identify the transmitter used by a particular group of projection neurons. These techniques, however, are not ideal since they involve re-staining for HRP activity of the histochemically treated sections and the re-photographing of each section in order to identify any jointly HRP-labeled and histochemically stained cells. In addition, since the histochemical sequences ultimately depend on the localization of a fluorophore, these approaches are not applicable to electron microscopic studies. The recently introduced use of retrograde transport of fluorescent dyes[17] in combination with immunohistochemistry[5] or with fluorescence histochemistry[3] also suffers from some of these drawbacks. As a possible alternative approach we describe in this report the use of retrograde HRP transport in combination with the unlabeled antibody peroxidase–antiperoxidase (PAP) technique of Sternberger[30]. As a model system in which to explore this technique, the already well characterized serotonergic projection from the dorsal raphe to the striatum[12,22] was selected. For immunostaining we have used a newly developed monoclonal antibody which detects serotonin (5-HT) antigenic sites[6].

MATERIALS AND METHODS

Male Wistar rats (180–200 g) received 0.1 μl of a 20% HRP solution (Sigma, Grade VI) delivered over a period of 20 min using a stereotaxically positioned 1 μl syringe. A unilateral injection was made at coordinates A 6.7, L 3.0 and V 4.0 (from the pial surface) according to the atlas of König and Klippel[16]. After 24 h the rats were re-anesthetized and perfused through the ascending aorta with 15 ml of 0.1 M phosphate buffer, 5% sucrose, followed by 100 ml of 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer. The brains were removed and kept in the same fixative for a further 4 h and then transferred to the 0.1 M phosphate 5% sucrose for a minimum of 24 h. For subsequent staining 20 μm serial sections were cut on a Deites cryostat (Heidelberg, G.F.R.) and thaw mounted on gelatin-coated slides. Series of transverse sections were cut at approximately every 300 μm between levels corresponding to A 1.4 and P 2.0 of the atlas of Palkovits and Jacobowitz[25]. This covers the entire rostro-caudal extent of nucleus raphe dorsalis and includes the serotonergic cell groups B6 to B9 identified by Dahlström and Fuxe[10]. Sections were then stained for 30 min in 0.06% 3,3-diaminobenzidine, 0.01% hydrogen peroxide in 0.05 M Tris·HCl buffer (pH 7.6) to show up peroxidase activity. Following extensive washing in 0.1 M phosphate buffer, the immunohistochemical staining was then carried out. Unless otherwise stated, all antibody solutions were made up in phosphate buffered saline (PBS) and staining was carried out at room temperature. Sections were incubated in PBS containing 0.2% Triton X-100 for 15 min, followed by 30 min in 1/10 normal rabbit serum, 18 h at 4 °C in 1/300 monoclonal anti-5-HT[6], 1 h in 1/10 rabbit anti-rat IgG made up in PBS Triton, and finally 1.5 h in 1/50 rat PAP (Cappel Lab., U.S.A.). Between each incubation step the sections were washed in PBS for 30 min. Sections were then stained for a second time in the DAB/hydrogen peroxide solution (for 15 min), washed thoroughly, dehydrated and mounted under DEPEX. In each series of serial sections various controls were included. These consisted of: (a) sections

which were dehydrated and mounted immediately after the first staining for peroxidase; (b) sections incubated in monoclonal anti-substance P antibody[8], or in anti-5-HT antibody preabsorbed with 2 mg/ml of serotonin creatinine sulphate, instead of in anti-5-HT antibody; (c) sections which were incubated in the presence of a peroxidase inhibitor (1.5 h in 0.2% phenylhydrazine) instead of receiving the first staining for peroxidase.

In addition, the extent of HRP diffusion was checked by cutting sections at the level of the injection site and staining them for peroxidase activity. All sections were subsequently examined using both bright- and dark-field illumination, and also using interference optics (Leitz, Interference Contrast Device T).

RESULTS

Examination of the site of HRP injection confirmed that the centre of peroxidase activity was in the caudate putamen with diffuse staining also in the globus pallidus, capsula interna and in the basal parts of the surrounding neocortex. This is similar to that reported by other workers using relatively large injection volumes[12,20,21]. In sections stained only for HRP activity (control, Fig. 1a) retrogradely labeled neurons

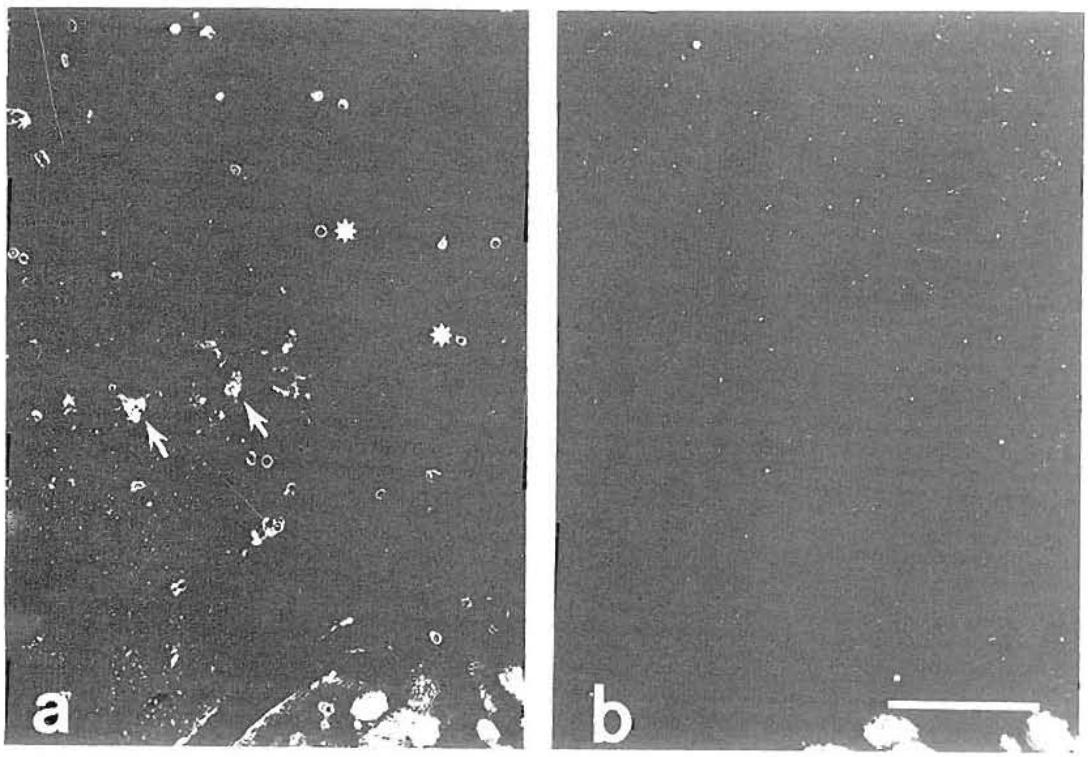

Fig. 1. Identification of HRP-filled, retrogradely labeled neurons using dark-field illumination. Arrows indicate labeled neurons, stars indicate non-specific peroxidase stained red blood cells. a: section processed for HRP activity without immunostaining (control a). b: section treated with a peroxidase inhibitor prior to successful immunostaining (control c). Peroxidase staining of retrogradely labeled cells and of red blood cells is abolished. Scale bar 100 μm.

were observed in nucleus raphe dorsalis and in the ipsilateral pars compacta of the substantia nigra. Such cells were localized using dark-field illumination (Fig. 1a). Those sections which had also been processed for 5-HT immunoreactivity had retrogradely labeled cells with a similar distribution, but in addition a large number of immunostained cells were seen. The two populations of cells were easily distinguishable since the reaction deposit following the immunoreaction has a diffuse homogeneous appearance, filling the cell body and proximal dendrites, whereas the reaction deposit formed by the HRP is of a distinct granular nature. One result of this difference in localization is that whereas the HRP labeled cells show up most easily using dark-field illumination, in contrast the 5-HT immunostained cells show up best using bright-field illumination (Fig. 2). Using interference optics at high magnification it is also possible to distinguish the two types of reaction deposit (Fig. 3). In this way, 3 types of cell were identified, namely those which stain only for HRP, those which stain only for 5-HT and those which stain for both HRP and 5-HT (Fig. 3).

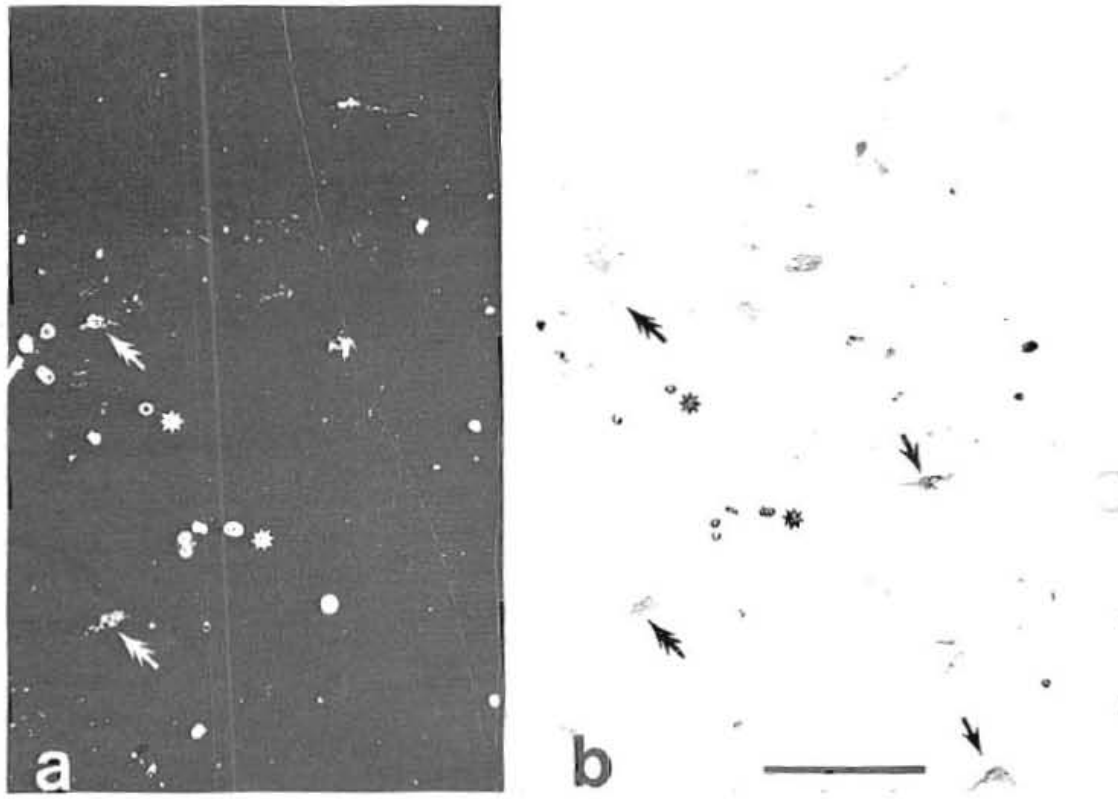

Fig. 2. Identification of HRP retrogradely labeled and 5-HT immunostained neurons in a single section using both dark-field (a) and bright-field (b) illumination. Double arrowhead: 5-HT immunostained cells with HRP labeling, visible using both dark-field and bright-field illumination. Single arrowhead: 5-HT immunostained cells without HRP labeling, visible only using bright-field illumination. Stars: non-specifically stained red blood cells, visible using both dark-field and bright-field illumination. Scale bar = 100 μm.

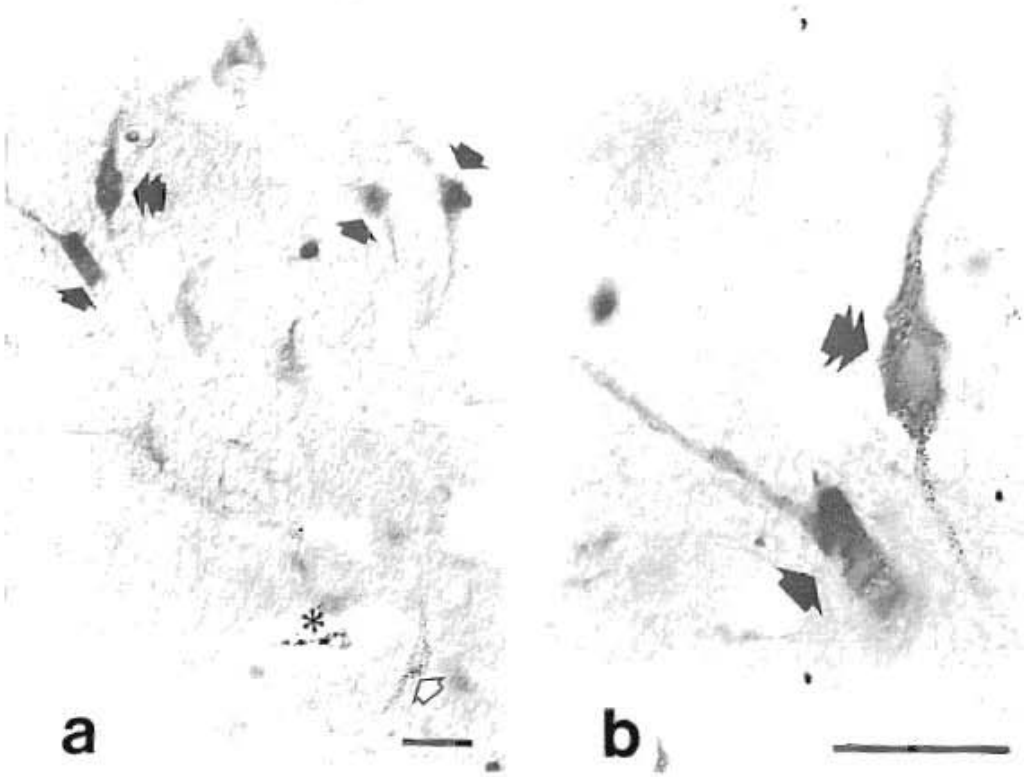

Fig. 3. Identification of HRP retrogradely labeled and 5-HT immunostained neurons in a single section using interference contrast illumination. a: filled single arrowhead: 5-HT immunostained cells without HRP labeling. Open single arrowhead: HRP retrogradely labeled cell without immunostaining. Filled double arrowhead: 5-HT immunostained cell with retrograde HRP labeling. Asterisk: HRP taken up non-specifically in an endothelial cell. b: high power of same. The jointly immunostained and retrogradely labeled cell is seen to have a large number of black dots on top of the diffuse immunostaining. These represent the HRP concentrated in secondary lysosomes. Scale bar = 25 μm.

The occasional labeled endothelial cell was also seen (Fig. 3a), but these were easily distinguished from retrogradely labeled neurons by the large and variable size of the peroxidase-containing granules in the former[24]. Preabsorption of the 5-HT antibody with serotonin abolished all immunostaining without affecting the HRP localization (Fig. 4). Similarly, the distribution of HRP-labelled cells was no different in those sections which had been immunostained for substance P (controls b). In these latter sections, substance P immunostaining was seen in pars reticulata of substantia nigra and in the ventrolateral borders of nucleus interpeduncularis. In control (c), in which sections were incubated in the presence of a peroxidase inhibitor prior to immunostaining, non-specific staining of red blood cells was abolished and no retrogradely transported HRP could be detected (Fig. 1b), although the 5-HT immunostaining was normal.

Within raphe dorsalis retrogradely labeled cells were mostly in the area just

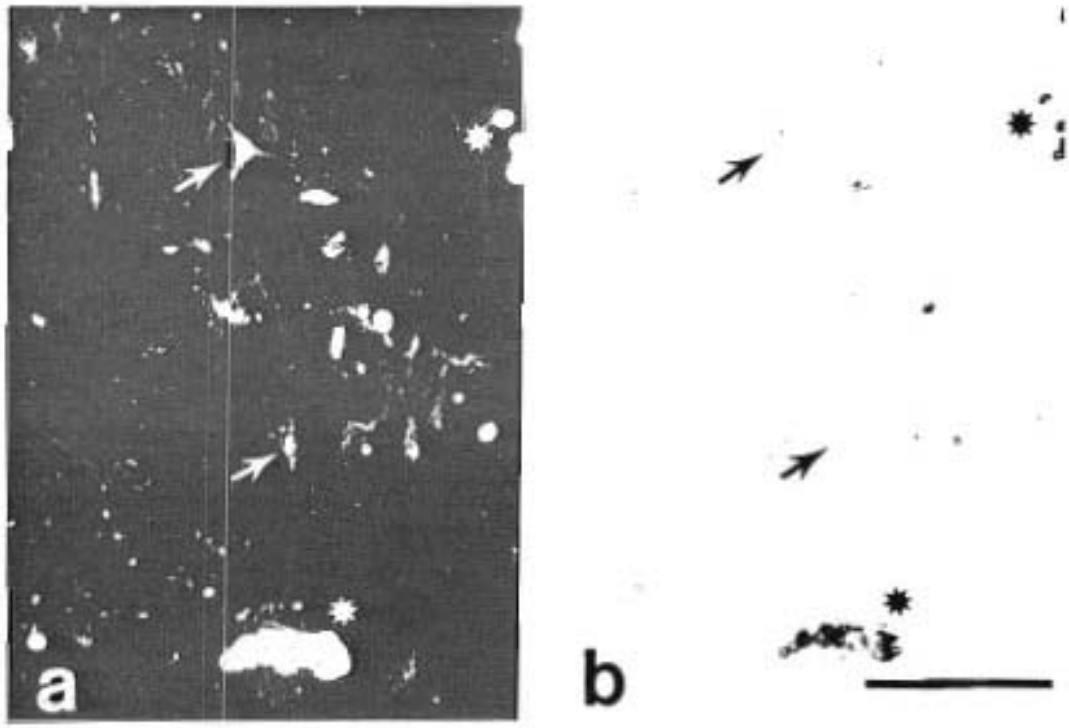

Fig. 4. Identification of HRP retrogradely labeled neurons in a control section incubated with 5-HT-absorbed monoclonal antibody (control b), using both dark-field (a) and bright-field (b) illumination. Arrows indicate HRP retrogradely labeled neurons, stars indicate non-specific peroxidase stained red blood cells. No immunostaining is visible under bright-field illumination because staining had been abolished by preabsorption of the antibody with serotonin. Scale bar — 100 μm.

dorsal to the nucleus originis nervi oculomotorii and at a level roughly corresponding to the most caudal portion of nucleus interpeduncularis, namely A 0.9–A 1.4 of the atlas of Palkovits and Jacobowitz[25]. Labeled cells were ipsilateral to the injection site, although occasional contralaterally labeled cells were observed. Immunostained cells were observed in all the cell groups previously characterized by Dahlström and Fuxe[10] and present in the areas here investigated, namely B6, B7, B8 and B9, but the jointly retrogradely labeled and immunostained cells were only seen in that area of the nucleus raphe dorsalis (B7) described above (Fig. 5). In this area and on the side ipsilateral to the injection, approximately 30% of the immunostained cells showed retrograde labeling. As indicated in Fig. 5, the dual labeled cells formed a distinct cluster within the nucleus with the prominent serotonergic group immediately ventral to the aqueduct not retrogradely labeled. Isolated retrogradely labeled cells which showed no immunostaining were observed (Fig. 3a), and the retrogradely labeled cells in pars compacta of the substantia nigra had no immunostaining.

DISCUSSION

Although retrograde transport of HRP has been combined with fluorescence

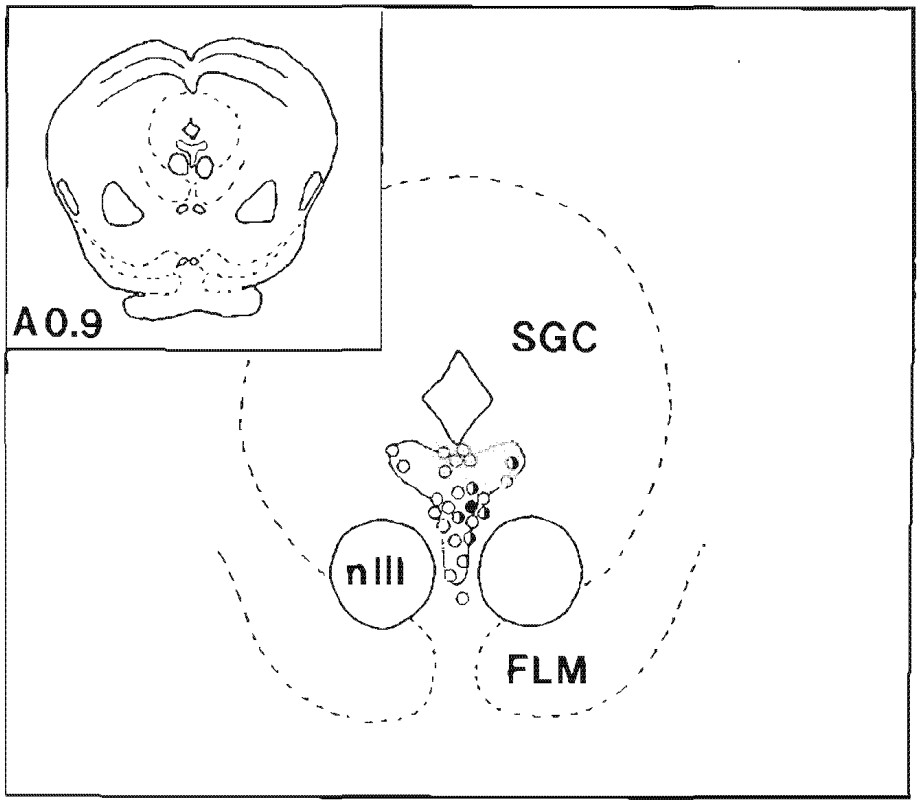

Fig. 5. Distribution of HRP retrogradely labeled and 5-HT immunostained neurons within nucleus raphe dorsalis. Open circles: 5-HT immunostained cells without retrograde HRP labeling. Filled circles: HRP retrogradely labeled cells without immunostaining. Half-filled circles: 5-HT immunostained cells with retrograde HRP labeling (dually labeled neurons). Inset indicates the level of raphe dorsalis at which dually (5-HT + HRP) stained cells were seen. SGC, substantia grisea centralis; FLM, fasciculus longitudinalis medialis; nIII, nucleus originis nervi oculomotorii.

histochemistry[2,4] and with fluorescence immunohistochemistry[20], it has not previously been used in combination with either labeled or unlabeled immunohistochemical procedures which have peroxidase as their final marker. In this paper we report such a use in combination with PAP immunohistochemistry in order to identify a population of 5-HT containing neurons in the dorsal raphe which project to the ipsilateral striatum. The retrogradely transported peroxidase and the immuno-associated peroxidase are revealed using the same staining procedure but distinguished on the basis of their microscopic appearance. In our preparations the reaction deposit due to the retrogradely transported HRP appears as distinct granules in the light microscope, probably reflecting its association with multivesicular and dense bodies[19]. This discrete localization is seen in lightly retrogradely labeled cells[28] and this light labeling is encouraged by the experimental conditions employed in this paper, namely long fixation in high paraformaldehyde[15,27] and use of relatively insensitive staining procedure employing DAB as electron donor[19,21]. In contrast the immunostaining has a diffuse appearance which possibly reflects the association of 5-HT with endoplasmic

reticulum and with synaptic vesicles[1,11]. Occasionally neurons may appear diffusely stained following HRP injection, especially if there is uptake by damaged axons of passage[13,14], and this might be confused with immunostaining. However, such cells would quickly be detected in control sections incubated with the absorbed 5-HT antibody, and when present, extra care should be exercised in interpretation. In this study the localization of retrogradely labeled immunostained cells described agrees very well with the results of separately performed retrograde labeling[12,22] and histochemical staining[10] studies in the dorsal raphe. The existence of some non-serotonergic raphe neurons projecting to the striatum, suggested by our identification of a few retrogradely labeled but not immunostained cells, is also in agreement with previous work[12]. In addition, Steinbusch et al.[29] has recently obtained very similar results to our own using the retrograde transport of fluorescent dyes. All this suggests that the dual labeling described here is a genuine representation of retrogradely transported HRP and serotonin immunostaining. This is further indicated by the presence in single sections of cells which contained either only 5-HT immunostaining or only HRP staining, and by the control sections in which it was possible to abolish the HRP activity and the immunoperoxidase activity independently of one another.

The fixation and incubation conditions employed here are suitable for immunohistochemical studies of most transmitters and related neuronal markers[7], so that the approach we have described should be useful generally for the transmitter characterization of retrogradely identified projection neurons. In addition, we are currently exploring the feasibility of employing this technique for those neuronal groups requiring cholchicine pretreatment in order to reveal neurotransmitter-related antigenic sites in the somata[5]. The procedure described here is relatively simple and has certain slight advantages over previously published methods in that it requires no re-staining and re-photography, and dually labeled cells can be quickly identified in single sections. Although two different electron donors could have been used to localize the two different peroxidase labels[23], this was not necessary and the use of DAB alone avoids the problems of solubility and of colour fade found with some other chromogens[17,21]. In addition, it should be possible to extend with only slight modification the present methodology to the ultrastructural level, since DAB produces an osmiophilic insoluble polymer which is suitable for use in electron microscopy. Following the light microscopic examination of plastic embedded material, sections could be re-embedded and processed for electron microscopy[23,26], thus allowing the ultrastructural identification of the dually labeled cells and in addition the transmitter characterization of nerve terminals which synapse on the soma and proximal dendrites of retrogradely labeled neurons. We have previously shown that the fixation and incubation conditions employed here are suitable for ultrastructural immunohistochemistry[9] and work is now under way to combine this with the retrograde HRP transport.

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