Journal of Neuroscience Methods, 20 (1987) 271–281
Elsevier

NSM 00702

Research Papers

Coupled intracellular horseradish peroxidase–monoamine oxidase histochemistry: description of the technique and its application to the study of physiologically identified tuberomammillary neurons

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(Received 8 January 1987)
(Accepted 19 February 1987)

Key words: Monoamine oxidase; Intracellular horseradish peroxidase; Tuberomammillary nucleus; Histamine neuron; Intracellular staining technique

A technique is described which couples visualization of intracellular horseradish peroxidase (HRP) with histochemical localization of monoamine oxidase (MAO), permitting simultaneous study of histochemistry, morphology and physiology in a single neuron. Using this technique, individual neurons in the tuberomammillary nucleus of the hypothalamus were stained in a Golgi-like fashion, revealing their somatodendritic morphology in detail. The technique has general applications as a method for conferring cytochemical specificity to intracellular staining of other MAO positive neurons, to retrograde transport of HRP by MAO positive neurons, as well as for ultrastructural studies of positively stained neuronal elements.

Introduction

Intracellular staining techniques occupy a unique niche in neurobiology insofar as they permit simultaneous study of the physiological and morphological properties of neurons (Kater and Nicholson, 1973; Brown and Fyffe, 1984). Although early work using fluorescent dyes such as Procion yellow (Kravitz et al., 1968) yielded dramatic results, the technique was rarely applied to mammalian brain until the development by several laboratories of the intracellular horseradish peroxidase (HRP) technique (Cullheim and Kellerth, 1976; Jankowska et al., 1976; Kitai et al.,

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1976; Light and Durkovic, 1976; Snow et al., 1976). The advantages of the intracellular HRP technique, including extensive dendritic and axonal filling as well as its electron-dense reaction product, are dealt with elsewhere (Brown and Fyffe, 1984). One notable diadvantage has been the absence of methodology for combining intracellular HRP with other cytochemical stains. Double-labelling methods employing intracellular injection of fluorescent dyes followed by either immunohistochemistry (Smithson et al., 1984) or histofluorescence (Aghajanian and Vander-Maelen, 1982) have been successfully used to identify the transmitter status of physiologically characterized neurons. The present study represents, to our knowledge, the first attempt to add cytochemical specificity to the intracellular HRP technique.

Monoamine oxidase (MAO) is a mitochondrial enzyme which is localized to selected neurons and glia in the brain (Levitt et al., 1982; Kishimoto et al., 1983; Maeda et al., 1984; Westlund et al., 1985; Nakamura and Vincent, 1986; Arai et al., 1986). In particular, MAO has been histochemically localized to the somata of noradrenergic, serotonergic and histaminergic neurons (see Arai et al., 1986 for review). The existence of a coupled HRP procedure for MAO histochemistry (Graham and Karnovsky, 1965; Kishimoto et al., 1983), in which HRP is included in the reaction mixture, led us to predict that HRP, intracellularly injected into MAO-positive neurons, could be visualized by coupling its reaction to the presence of endogenous MAO.

The neurons of the tuberomammillary (TM) nuclei in the caudal hypothalamus were chosen for study because, while relatively little is known of their morphological and physiological properties, their cytochemistry is remarkable. The somata of these neurons stain positively for a plethora of neutrotransmitters and related enzymes, including histamine, histidine decarboxylase, glutamic acid decarboxylase, MAO, adenosine deaminase, Met-enkephalin heptapeptide, galanin, and substance P (for reviews, see Köhler et al., 1985; Senba et al., 1985; Staines et al., 1986). MAO has been repeatedly demonstrated in TM neurons both histochemically (Maeda et al., 1984; Tago et al., 1984; Nakamura and Vincent, 1986; Arai et al., 1986) and immunohistochemically (Westlund et al., 1985). Although MAO is found in only 60% of TM neurons (Staines et al., 1986), all MAO positive TM neurons appear to contain adenosine deaminase (Staines et al., 1986) and histidine decarboxylase (P.B. Reiner, unpublished observations). In the present study we present: (1) a new technique which couples histochemical specificity for MAO to the intracellular HRP technique; and (2) its application to the study of the morphology of physiologically identified TM neurons. Some of these data have been presented in preliminary form (Reiner et al., 1986).

Materials and Methods

The coupled intracellular HRP / MAO protocol

Male Wistar rats (250–300 g), anesthetized with urethane (1.5 g/kg, i.p., supplemented as needed), were used in this study. Cell bodies in the TM region were

identified by their extracellular physiological properties including antidromic activation from the cerebral cortex (Reiner and McGeer, 1987) using glass micropipettes filled with 5% HRP (Sigma, Type VI) in 0.05 M Tris buffer containing 0.25 M KCl. Following extracellular characterization, TM neurons were penetrated and HRP iontophoresed intracellularly using positive current pulses of 2–5 nA and 300 ms duration at 2 Hz. No more than one neuron was filled per side.

Following survival times of 1–5 h, rats were perfused transcardially with 0.01 M phosphate buffered saline (pH 7.4) followed by ice-cold fixative containing 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min. The brain was removed from the skull, blocked, and placed in cryoprotectant (30% sucrose in 0.1 M phosphate buffer) overnight at 4°C. Serial sections of 100 μm thickness were cut on a freezing microtome, collected in Tris buffer (0.05 M, pH 7.4) containing 15% sucrose and incubated according to the coupled intracellular HRP/MAO protocol, schematically shown in Fig. 1A and described in detail below.

Sections were preincubated for 10 min in a solution of 0.005% 3,3′-diaminobenzidine hydrochloride (DAB), 0.6% nickel ammonium sulfate, 0.065% sodium azide and 15% sucrose in 0.05 M Tris buffer; at the end of this preincubation period, 0.075% tyramine was added and the reaction permitted to proceed for 1 h at room temperature with constant agitation. This protocol is essentially identical with that of Maeda et al. (1984) with the omission of exogenous HRP.

Following termination of the reaction by washing in 0.05 M Tris buffer, sections were briefly scanned for the presence of HRP/MAO-positive neurons, and, following overnight washing, the section containing the cell body was subjected to a second MAO reaction according to the protocol of Kishimoto et al. (1983) (Fig. 1B). Thus, the incubation was carried out in a solution identical to that described above but also containing exogenous HRP (0.01%) in order to identify all MAO-positive neurons. Furthermore, nickel ammonium sulfate was omitted from the solution so that, in contrast to the blue-black reaction product of the coupled intracellular HRP/MAO reaction, the second reaction stained brown all remaining MAO-positive TM neurons.

Serial sections were mounted and dried on chrome-alum coated glass slides, and dehydrated according to a modification of the dimethyl sulfoxide (DMSO) protocol of Grace and Llinás (1985). Sections were washed briefly in water, then placed in 100% DMSO for 1 h, followed by 5 min each in 100% alcohol and xylene. Slides were coverslipped with permount, and examined under the light microscope. Camera lucida reconstructions were drawn from serial sections viewed on an Olympus BH-2 microscope equipped with a drawing tube using a 100 × oil immersion lens.

Controls

Controls were performed on sections of rat brain which had previously received large (0.1 μl) intratuberal injections of HRP. These were deemed more appropriate than sections containing intracellularly labelled neurons because of the possibility of false negatives using the intracellular HRP technique. The hypothalamus was sectioned at 30 μm and adjacent sections incubated in the following: (1) Standard HRP histochemistry (0.005% DAB, 0.65% sodium azide; 0.6% nickel ammonium

sulfate; 0.0075% H_2O_2). (2) The coupled intracellular HRP/MAO solution (i.e. without exogenous HRP). (3) The coupled intracellular HRP/MAO solution following a 15 min preincubation with 0.1 mM pargyline hydrochloride, a specific and irreversible inhibitor of MAO (Hellerman and Erwin, 1968). (4) The coupled intracellular HRP/MAO solution with the omission of the substrate tyramine.

Results and Discussion

The coupled intracellular HRP / MAO protocol

The rationale underlying the development of this technique is shown schematically in Fig. 1. The standard MAO reaction of Kishimoto et al. (1983) makes use of the production of H_2O_2 during oxidation of tyramine by MAO to oxidize DAB in the presence of exogenous HRP (Fig. 1B). The coupled intracellular HRP/MAO protocol capitalizes upon this same reaction, but with the omission of exogenous HRP (Fig. 1A). Rather, it makes use of HRP deposited intracellularly in vivo following physiological characterization; using the coupled intracellular HRP/MAO protocol, the reaction product is seen in tissue sections if and only if this neuron is MAO-positive. Addition of nickel ammonium sulfate results in production of a blue-black reaction product which provides superior detail to the standard brown DAB reaction product.

Eight neurons exhibiting the physiological characteristics of TM neurons (Reiner and McGeer, 1987) were wholly or partly recovered using the coupled intracellular

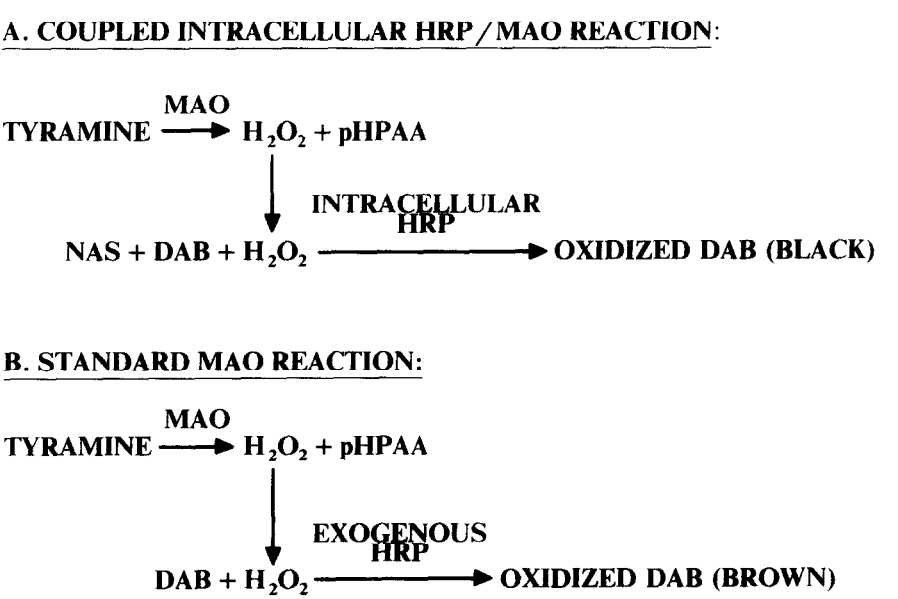

Fig. 1. Schematic representations. A: the coupled intracellular HRP/MAO reaction. B: standard MAO reaction. Abbreviations: MAO, monoamine oxidase; DAB, 3,3′-diaminobenzidine hydrochloride; HRP, horseradish peroxidase; NAS, nickel ammonium sulfate; pHPAA, *p*-hydroxyphenylacetylaldehyde.

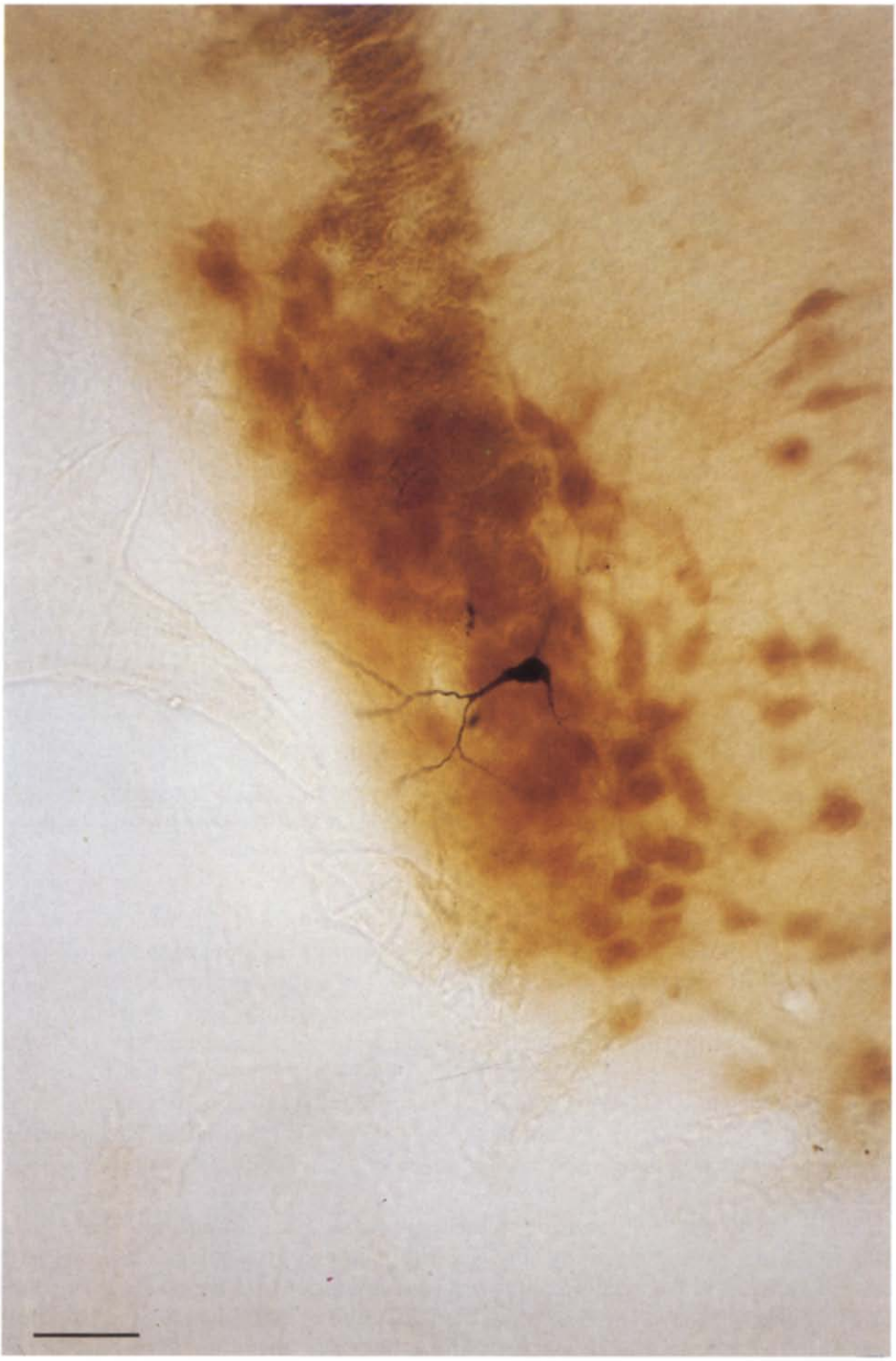

Fig. 2. A TM neuron stained by the coupled intracellular HRP/MAO protocol. The intracellularly stained neuron is black, while the surrounding MAO positive neurons of the TM nucleus are brown. Note that dendrites approach the pial surface. Calibration: 100 μm.

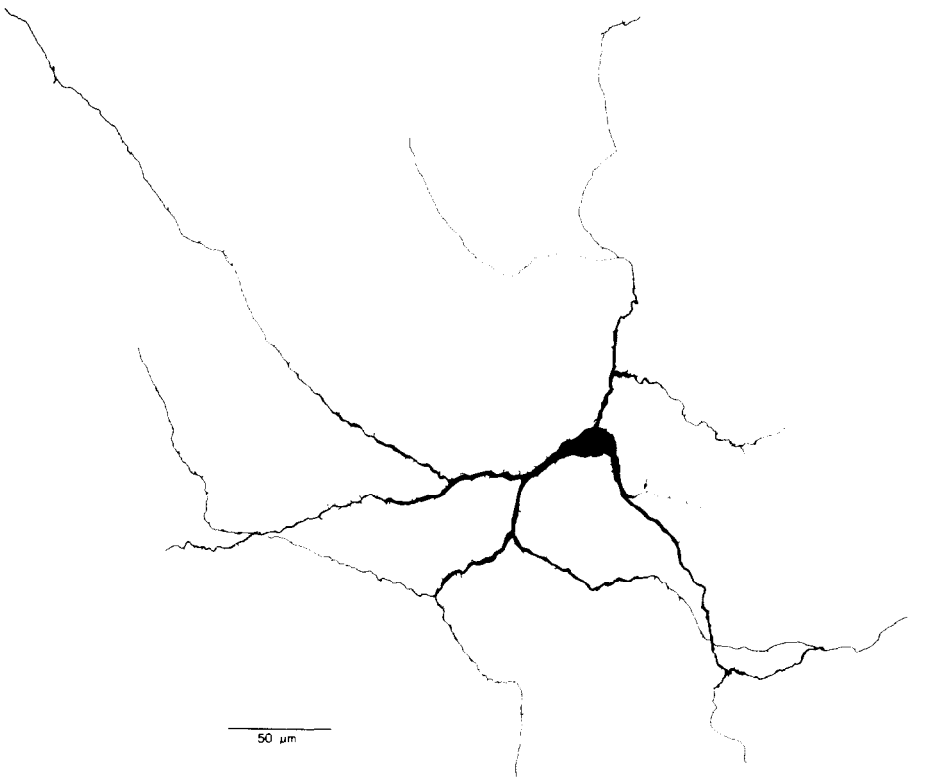

Fig. 3. Camera lucida reconstruction of a TM neuron stained by the coupled intracellular HRP/MAO protocol. This neuron was reconstructed from 4 adjacent 100 μm thick coronal sections. Arrow points to the presumptive axon.

HRP/MAO protocol. The soma and dendrites of positively stained neurons were dark blue-black (Fig. 2); occasionally, the presumptive axon could be identified. Camera lucida reconstructions (Fig. 3) revealed large, multipolar neurons with sparsely branching dendritic trees, a morphological pattern seen previously in TM neurons using either histidine decarboxylase immunohistochemistry (Hayashi et al., 1984) or Golgi staining (Wouterlood et al., 1986). Furthermore, as previously noted by others (Maeda et al., 1984; Köhler et al., 1985), data obtained using the coupled intracellular HRP/MAO technique clearly demonstrates that dendrites of TM neurons located well within the brain parenchyma extended to the ventral surface of the brain (Fig. 2).

The dendrites of TM neurons stained by the coupled intracellular HRP/MAO protocol were moderately spiny (Fig. 4). Although dendritic spines are not apparent on TM neurons in material prepared for conventional MAO histochemistry (Maeda et al., 1984; Arai et al., 1986), ultrastructural studies of TM neurons have clearly demonstrated the presence of dendritic spines on histidine decarboxylase immunoreactive elements (Hayashi et al., 1984; Wouterlood et al., 1986). These

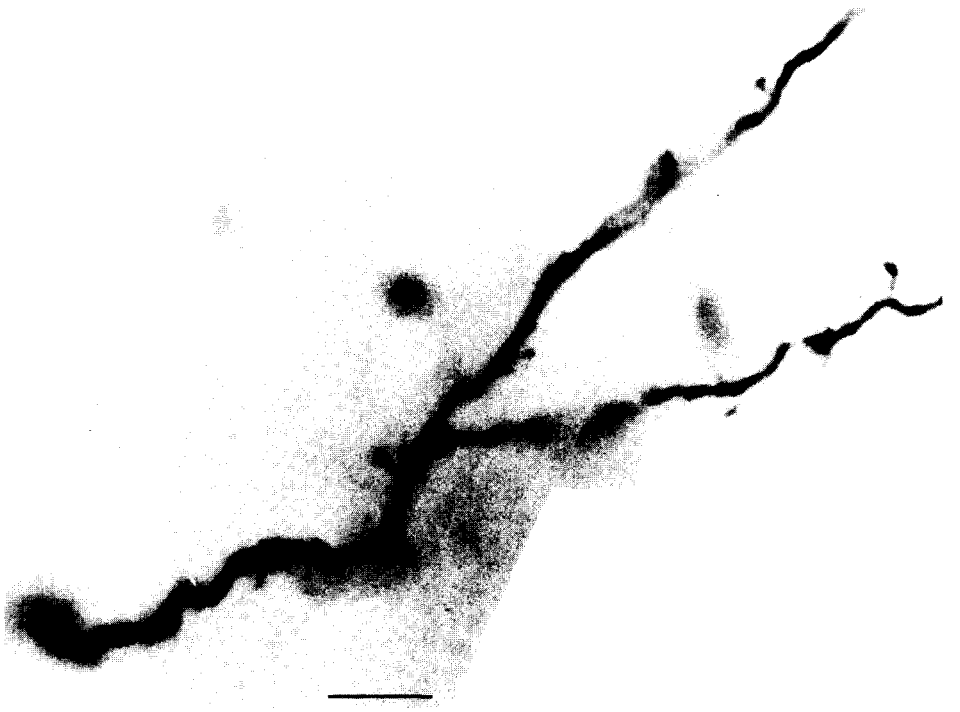

Fig. 4. Dendritic spines as revealed by the coupled intracellular HRP/MAO protocol. Calibration: 10 μm.

observations suggest that the coupled intracellular HRP/MAO protocol is either more sensitive than conventional MAO histochemistry or that the H_2O_2 produced by mitochondrial MAO within the soma and dendrites is able to react with HRP located in dendritic spines.

Controls

The major issue to be dealt with was the possibility that H_2O_2, produced by all MAO-positive neurons upon addition of the substrate tyramine, might diffuse through the tissue and react with intracellularly deposited HRP in non-MAO-positive neurons. The controls used for this study employed large intratuberal injections of HRP, followed by a variety of histochemical procedures to confirm the specificity of the protocol. The most important of these was incubation of the tissue according to the coupled intracellular HRP/MAO protocol. As can be seen in Fig. 5, although HRP is present throughout the tuberal region (Fig. 5A), only the magnocellular neurons previously shown to be MAO-positive (Maeda et al., 1984; Arai et al., 1986) are labelled when the coupled intracellular HRP/MAO protocol is used (Fig. 5B, C).

Additional controls were modelled after those described by Arai et al. (1986). No specific staining was seen after pretreatment with the irreversible monoamine

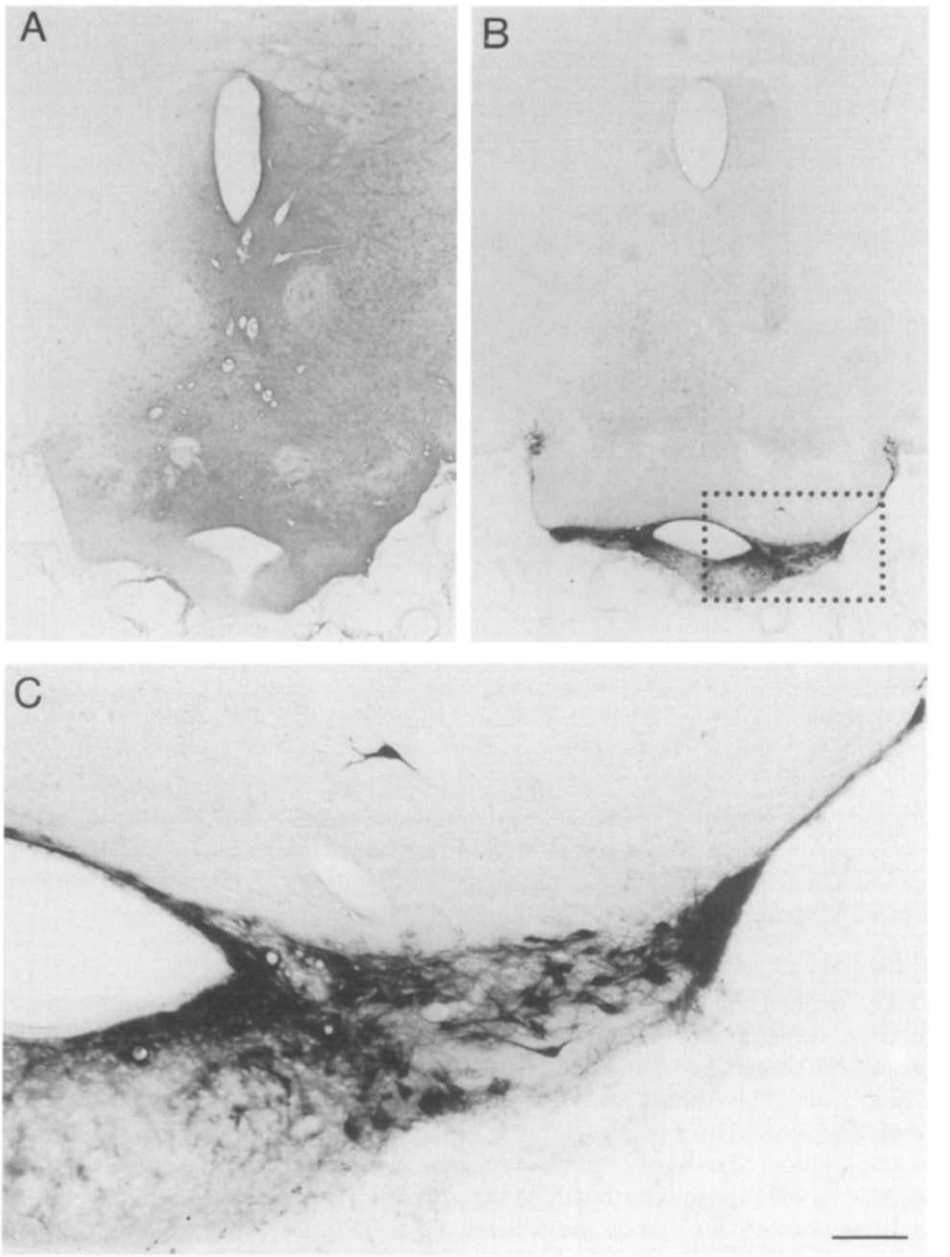

Fig. 5. Controls. A: a large hypothalamic injection of HRP can be seen to completely fill the tuberal region. B: adjacent section stained using the coupled intracellular HRP/MAO protocol. C: higher power view of area inset in B shows that only the magnocellular neurons of the TM nucleus and tanacytes are stained. Calibration: A and B, 600 μm; C, 100 μm.

oxidase inhibitor pargyline, suggesting that the reaction was indeed dependent upon the activity of MAO. Similarly, specific staining was absent following omission of the MAO substrate tyramine, ruling out non-specific oxidation.

General applications of the methodology

Glenner et al. (1957) first used histochemical techniques to localize MAO in tissues, employing direct reduction of tetrazolium salts by MAO. A major conceptual advance emerged from the studies of Graham and Karnovsky (1965), who developed the first 'coupled' peroxidatic reaction, using the production of H_2O_2 by MAO to oxidize semicarbazol dyes. Remarkably, it was not until recently that Kishimoto et al. (1982) modified this method by replacing the semicarbazol dye with DAB, thereby giving rise to an insoluble reaction product. The addition of nickel ammonium sulfate by Maeda et al. (1984) greatly improved the resolution of the technique, permitting detailed mapping of the distribution of MAO positive neurons in the brain (Arai et al., 1986). The development of the coupled intracellular HRP/MAO protocol thus represents a logical extension of MAO histochemical techniques applied to individual, physiologically identified neurons.

The coupled intracellular HRP/MAO technique applies a strategy in which substrate (i.e. HRP) for a particular histochemical reaction (i.e. MAO) is intracellularly deposited; its omission in the subsequent histochemical procedure specifically labels only neurons which contain intracellularly deposited substrate and the endogenous target of the histochemical reaction. A similar strategy was utilized by Grace and Bunney (1980) who intracellularly injected L-DOPA into dopamine neurons; the increased production of dopamine in those neurons was then detected by their relatively intense catecholamine histofluorescence. MAO-positive neurons are widely distributed in brain (Arai et al., 1986), and the protocol described in this paper is applicable to them all. In particular, it should prove useful for study of the structural and functional features of hypothalamic histaminergic neurons, as well as noradrenergic and serotonergic neurons of the hindbrain. The coupled intracellular HRP/MAO protocol should be applicable to MAO positive neurons in vitro, either in slice preparations or in culture. Furthermore, application of the same principles utilized in the present study should permit its use in combining retrograde HRP tracing with MAO histochemistry. Finally, it should be noted that the reaction product is electron dense, and that material prepared in this fashion can be taken to the ultrastructural level.

Acknowledgement

Supported by the Medical Research Council of Canada. P.B.R. is a Fellow of the MRC.

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