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Immunocytochemical demonstration of monoamine oxidase B in brain astrocytes and serotonergic neurons

(central nervous system/rat/monoamine)

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ABSTRACT **An antiserum to monoamine oxidase B (MAO-B) was used to define the distribution of this metabolic enzyme in the adult rat brain immunocytochemically. MAO-B is specifically located in two major central nervous system cell classes, astrocytes and serotonin-containing neurons. Double-immunofluorescence experiments using antisera to glial fibrillary acidic protein and MAO-B showed that both protoplasmic and fibrillary astrocytes throughout the brain contain MAO-B, whereas oligodendrocytes do not contain the enzyme. Areas lacking a blood–brain barrier, such as the specialized circumventricular organs, also contain MAO-B-positive cells. A double-immunofluorescence experiment using antisera to serotonin and MAO-B enabled the positive identification of neurons containing both molecules. The catecholamine-containing neurons of the brain did not contain detectable amounts of MAO-B. The specific distribution of MAO-B in the adult central nervous system indicates that the role of MAO-B in monoamine metabolism may be more specifically defined than previously believed.**

Monoamine oxidase [MAO; amine oxidase (flavin containing), amine:oxygen oxidoreductase (deaminating) (flavin-containing), EC 1.4.3.4] activity in the central nervous system (CNS) can regulate levels of monoamine neurotransmitters and other biogenic amines; and alterations in MAO activity have been implicated in some nervous disorders (1, 2). MAO activity has been subdivided on a pharmacological basis into two types: MAO-A has a higher affinity for the substrates norepinephrine and serotonin (5-HT) and the inhibitor clorgyline, and MAO-B has a higher affinity for the substrates phenylethylamine and benzylamine and the inhibitor deprenyl. Recent biochemical evidence indicates that these activities are mediated by two distinct mitochondrial proteins that have different molecular weights (3–5). Both types of MAO activity can be measured in all CNS regions, but it has been difficult to elucidate the cellular distribution of MAO with current pharmacological and biochemical methods because of the diversity of neuronal and nonneuronal cell types in the brain. By using an antiserum directed against MAO-B and immunocytochemical methods, we have localized MAO-B specifically to astrocytes and serotonergic neurons in the rat brain. The distribution of this enzyme suggests a role in regulating levels of specific neurotransmitters and their precursors as they enter the CNS or are released from synapses.

MATERIALS AND METHODS

Subjects. Eight adult female Sprague–Dawley albino rats (Charles River Breeding Laboratories) were prepared for immunocytochemical analysis by using the modified fixation protocol of Berod *et al.* (6) to enhance the preservation of the antigen. Deeply anesthetized animals were perfused transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 6.0) and then with 4% paraformaldehyde in 0.1 M sodium borate buffer (pH 11.0). The brains were immersed in 20% sucrose in 0.1 M sodium phosphate buffer (pH 7.2), rapidly frozen after at least 48 hr, and sectioned on a cryostat at −20°C.

Immunocytochemistry. Every third section, 10 μm thick, through the entire brain was collected on a gelatin-coated slide and immunostained by using rabbit preimmune serum or antiserum prepared against pure bovine MAO-B (ref. 7; unpublished data; MAO-B was obtained from James Salach and Walter Weyler). Sections on slides were incubated for 5 min with 1% sodium borohydride in 0.1 M sodium phosphate (pH 7.2) and then for 3 hr at 37°C with either MAO-B antiserum or preimmune serum diluted 1:100 in a staining buffer of 0.45 M NaCl/20 mM sodium phosphate, pH 7.4/0.3% Triton X-100/10% normal swine serum. The sections were washed for 30 min with the staining buffer and exposed to rhodamine-conjugated swine anti-rabbit IgG (Dako, Accurate Scientific) diluted 1:40 in the staining buffer. Sections were washed for 30 min and placed under a coverslip with a glycerol/0.1 M sodium phosphate, pH 7.2 (3:1), solution. The slides were examined on a Zeiss photomicroscope equipped with epifluorescence and photographed on Kodak Plus-X film using 1- to 2-min exposures. Some tissue sections were processed by using the peroxidase–antiperoxidase procedure (8, 9). The cell cultures (see below) were fixed with 4% paraformaldehyde in 0.1 M sodium borate buffer (pH 11.0) for 90 min at 4°C, rinsed extensively, and then stained as described above for the tissue sections. Double-labeling experiments were performed with a mouse antiserum prepared against pure bovine MAO-B (unpublished) and either rabbit antiserum specific for 5-HT (Immunonuclear, Stillwater, MN) or rabbit antiserum directed against glial fibrillary acid protein (GFA; provided by L. F. Eng, Veterans Administration Hospital, Palo Alto, CA). The GFA antiserum has been used previously in this laboratory to specifically localize astrocytes in tissue sections (8, 9). The specificity of these antisera to 5-HT and GFA antisera is well documented (10–12). For double staining, sections were incubated for 3 hr at 37°C with both mouse antiserum to MAO-B diluted 1:50 in staining buffer and rabbit antiserum to 5-HT diluted 1:100 or antiserum to GFA diluted to 1:200 in staining buffer. The MAO-B immunoreactivity was visualized with rhodamine-conjugated goat anti-mouse IgG diluted 1:40 in staining buffer. The 5-HT or GFA immunoreactivity was detected with fluorescein-conjugated goat anti-rabbit IgG diluted 1:40 in staining buffer. The slides were incubated

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Abbreviations: MAO, monoamine oxidase; CNS, central nervous system; 5-HT, serotonin; GFA, glial fibrillary acid protein.

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in both antisera for 1 hr at 37°C and then washed extensively prior to examination. No staining was visualized in control experiments in which the mouse antiserum was used in combination with the goat anti-rabbit IgG or the rabbit antiserum used with the goat anti-mouse IgG.

Astrocyte Cultures. Cell cultures were prepared from new born Sprague–Dawley rat hippocampus or cerebellum. Dissected tissue was finely minced in Ca^{2+}-, Mg^{2+}-free Earle's balanced salt solution containing DNase at 1 mg/ml (Sigma). The tissue was then incubated in balanced salt solution containing trypsin at 0.25 mg/ml (Flow Laboratories, McLean, VA) for 20 min at 37°C. The cells were dispersed by careful trituration and plated onto polylysine-coated glass coverslips in 30-mm dishes at an initial density of 10^6 cells per dish. The cells were maintained in complete Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, penicillin at 20 units/ml, and streptomycin at 20 mg/ml (GIBCO).

RESULTS

Nonneuronal Localization of MAO-B. Astrocytes stained positively for MAO-B in the adult rat CNS. No staining was present in any sections when preimmune serum was substituted for the MAO-B antiserum (Fig. 1*d*) or when the diluted antiserum was preabsorbed with 0.8 nM purified MAO-B. MAO-B immunoreactivity was localized within the cytoplasm and processes of both protoplasmic and fibrous astrocytes in all areas of the brain (Fig. 1 *a*, *c*, and *e*). The morphology of these cells conforms precisely to classical descriptions of CNS astrocytes (13, 14). Double staining with MAO-B antiserum and a known astrocytic cell marker, GFA (for review, see ref. 12), confirmed the astrocytic nature of the MAO-B-positive cells (Fig. 1 *a* and *b*). Oligodendrocytes were not MAO-B positive. The distribution and morphology of the immunoreactive glial cells in the rodent brain were also similar to those of cells stained with another astrocytic marker, S-100 protein (15). Glial end-feet situated in perivascular arrays or along the pial surface were prominent in the immunofluorescent material. In addition, there was extensive staining of fibrous astrocytes in fiber tracts including the corpus callosum, internal capsule, optic tract, olfactory tract, and pyramidal tract. Other nonneuronal MAO-B-positive cells were the Bergmann glia of the cerebellar cortex (Fig. 1*c*) and the tanycytes, remnants of embryonic radial glia, situated along the third and fourth ventricles. Ependymal cells lining all the ventricles were also immunoreactive (Fig. 1*e*), but the cells of the choroid plexus were MAO-B negative. Intense MAO-B immunoreactivity was present in cells of the circumventricular organs that lack a blood–brain barrier (16), including the organum vasculosum of the lamina terminalis, the subfornical organ, and the area postrema.

Neuronal Localization of MAO-B. Neurons in only two areas of the rat CNS showed MAO-B immunoreactivity. Cells located in the brainstem raphe and scattered cells in the hypothalamus were MAO-B positive and correspond to the previously described serotonergic cell groups of the rodent brain (17, 18). Double labeling of cells with antisera against 5-HT and MAO-B showed that almost all neurons that stained positively for 5-HT were also MAO-B positive (Fig. 2 *a* and *b*). A few neurons that were immunoreactive only to MAO-B were interspersed among the double-labeled cells. No other neurons in the rat CNS, including the catecholamine-containing cells of the substantia nigra and locus coeruleus (Fig. 1 *e* and *f*), were visualized in the MAO-B-stained sections. The MAO-B immunoreactivity in the 5-HT-containing neurons was distributed throughout the somata and dendritic processes. Unequivocal identification of MAO-B-stained axons was not possible at the light microscopic level. Thus, double-labeled axons were not evident, although dendrites were stained with both antisera (Fig. 2 *a* and *b*). In addition, no labeling of neuronal processes was detected in regions heavily innervated by 5-HT-containing neurons, such as the suprachiasmatic nucleus and the interpeduncular nucleus.

Cytoplasmic Distribution of MAO-B. Cell cultures of the neonatal rat hippocampus and cerebellum, grown for 10 days, were stained for MAO-B in an attempt to elucidate the intracellular distribution of the immunoreactivity. Cells that exhibited typical astrocytic morphology were also MAO-B positive (Fig. 2*c*). Double staining with GFA confirmed the astrocytic nature of the MAO-B-positive cells (Fig. 2 *c* and *d*). More than 99% of all processes in these cultures were GFA positive, a result identical to the recent characterization of postnatal cerebellar cell cultures (19). The reaction product in the MAO-B-positive cells appeared to be specifically localized to the mitochondria scattered throughout the astrocytic cell bodies and processes (Fig. 2 *c* and *e*). The distribution of MAO-B within the astrocyte cytoplasm was identical to the staining visualized recently by others for many cell types by using fluorescent dyes that have been shown to be specifically sequestered within mitochondria (20) or antibodies to a second well-characterized mitochondrial enzyme, cytochrome *c* oxidase (21).

DISCUSSION

Pharmacological analysis of MAO-A and MAO-B activities in the CNS has indicated a differential distribution of those activities in different regions of the brain (for review, see refs. 1 and 2). The use of this approach to elucidate the cellular distribution of the molecules mediating MAO-B activity has been difficult. The present results demonstrate the specific highly localized distribution of MAO-B immunoreactivity in two different cell classes in the CNS, astrocytes and serotonergic neurons. Since active MAO molecules are located in the outer mitochondrial membrane, our results demonstrating mitochondrial immunoreactivity are consistent with the conclusion that the antigen being recognized is MAO.

Specificity of MAO-B Antiserum. The antiserum used here appears to react selectively with rat MAO-B and not with rat MAO-A (unpublished data). As is the case for all immunocytochemical studies, we cannot determine the sensitivity limit for our MAO-B antiserum in tissues. Further, we cannot completely eliminate the possibility of some crossreactivity of the MAO-B antiserum with very high levels of MAO-A. However, the immunocytochemical specificity for MAO-B is strongly supported by our finding (unpublished) of an absence of staining in cell lines and rat tissues shown by ourselves and others to contain high MAO-A activity and little or no MAO-B activity, such as C6 glioma cells, spleen, and superior cervical ganglia (refs. 22 and 23; unpublished data). In addition, the dopaminergic neurons of the substantia nigra, believed to contain substantial levels of MAO-A activity (24, 25), are unstained in our material. The absence of MAO-B immunoreactivity in the central catecholamine neurons implies a negligible role for this enzyme in affecting transmitter levels in these neurons, particularly when compared with data demonstrating specific increases in norepinephrine, when MAO-A, and not MAO-B, is inhibited (26, 27). Currently, an antibody specific to MAO-A is not available to us; thus, the distribution of MAO-A in the CNS and the coexistence of MAO-A and MAO-B in the same cells cannot be determined at this time.

Functional Implication of MAO-B Localization. The presence of immunodetectable MAO-B in 5-HT-containing neurons would seem to indicate a possible role for this enzyme in controlling levels of this neurotransmitter and its precursor in these

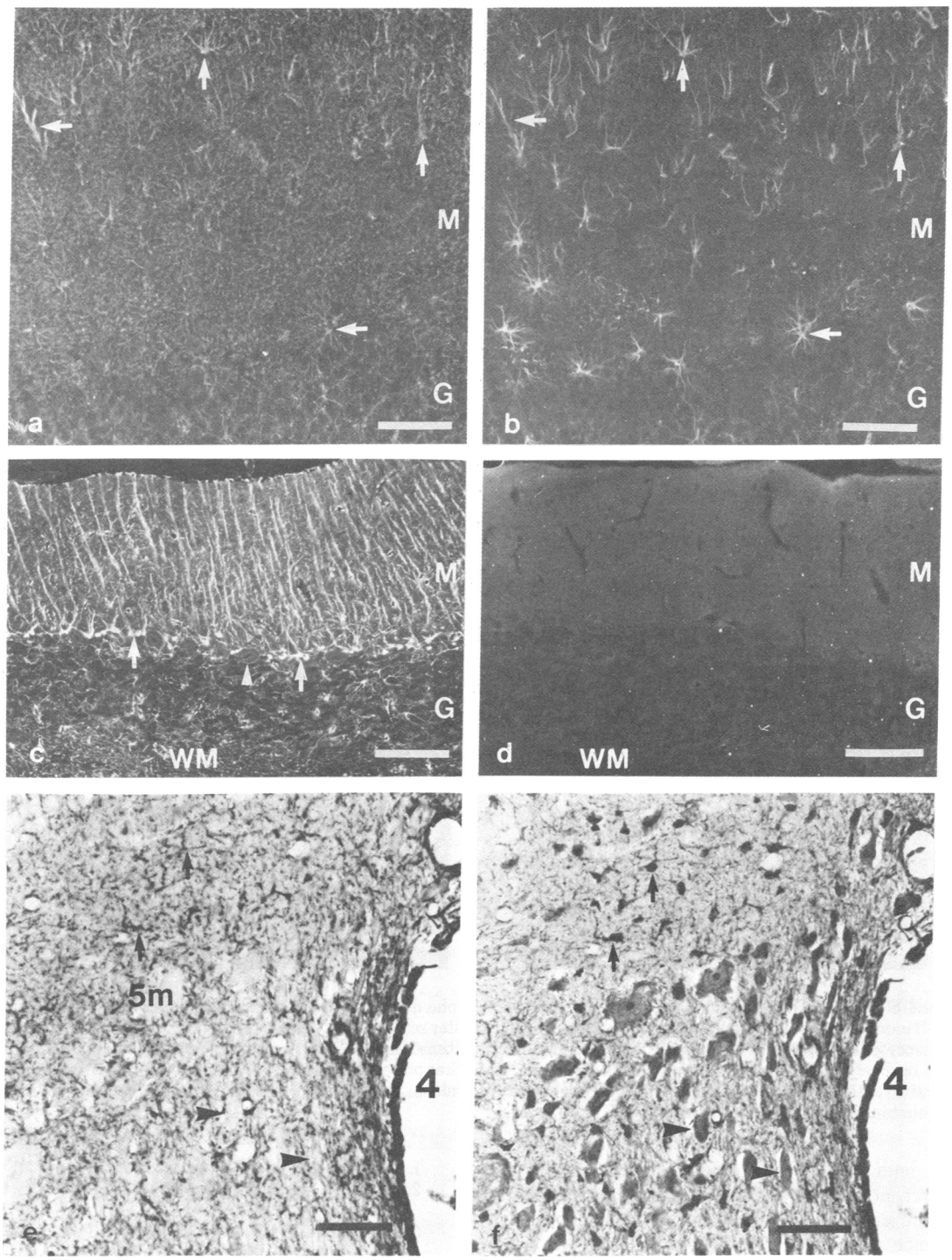

FIG. 1. Immunocytochemical localization of MAO-B-positive astrocytes. (*a*) Protoplasmic astrocytes (arrows) in the molecular layer (M) and granule cell layer (G) of the hippocampal dentate gyrus are MAO-B immunoreactive. (*b*) Treatment of the same section with GFA antiserum shows that identical cells are stained, thus demonstrating the astrocytic nature of the MAO-B-positive cells. (*c*) MAO-B-positive Bergmann glial cell bodies (arrows) are interspersed among negative-staining Purkinje cells (arrowhead). The Bergmann glial fibers extend through the entire molecular layer (M). G, granule cell layer; WM, white matter. (*d*) A control section incubated with preimmune serum shows no staining. (*e*) Astrocytes in the pons (arrows) and ependymal cells lining the fourth ventricle (4) show MAO-B immunoreactivity in the peroxidase–antiperoxidase-processed section. The large cells of the mesencephalic trigeminal complex and the norepinephrinergic locus coeruleus neurons (arrow heads) are completely devoid of staining. (*f*) An identical section was counterstained with cresyl violet to illustrate more clearly the location of the locus coerulus neurons (arrowheads). Note the appearance of glial cell bodies (arrows) whose processes are stained in Fig. 2*e*. Bars = 50 μm.

CNS cells. The localization of MAO-B to 5-HT-containing neurons was unexpected, particularly because of the somewhat lower affinity of 5-HT for MAO-B as compared with MAO-A. However, at the relatively high concentrations of 5-HT present in serotonergic neurons, these differences in substrate affinity may not be significant. It remains to be determined whether these neurons also contain MAO-A.

The presence of MAO-B in areas lacking a blood–brain barrier, within astrocytes and in tanycytes that have direct contact with the cerebrospinal fluid, has important functional impli-

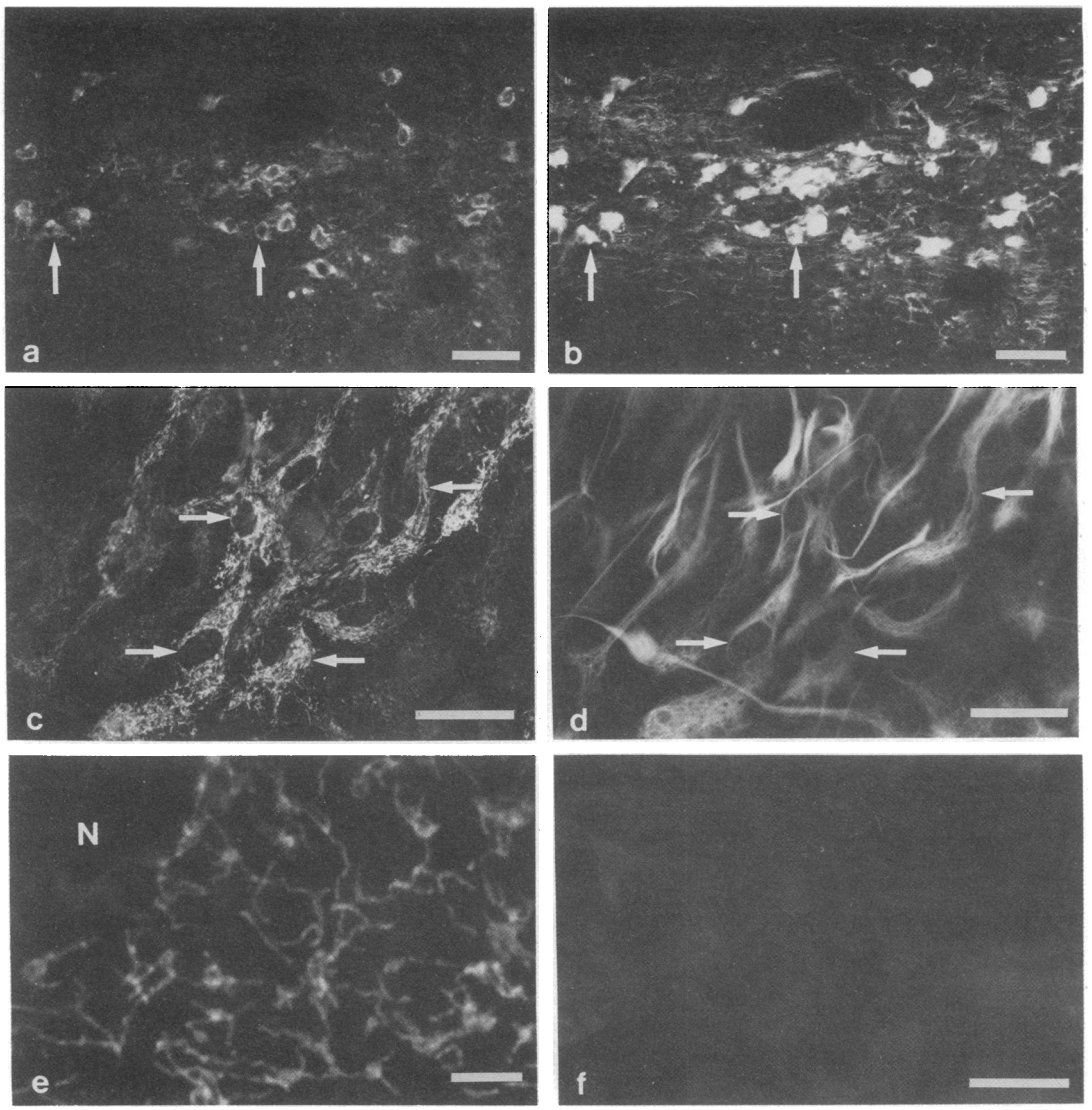

FIG. 2. (*a* and *b*) 5-HT/MAO-B double-labeled neurons in nucleus raphe dorsalis. Arrows indicate groups of cells that show obvious double staining. MAO-B immunoreactivity (*a*) was viewed by using a rhodamine filter set, and 5-HT immunoreactivity (*b*) was viewed by using a fluorescein filter set. (*c*) Astrocytes (arrows) from newborn rat hippocampus exhibit intense MAO-B immunoreactivity. (*d*) The same culture was stained with GFA antiserum to show that identical cells are also GFA positive. (*e*) Higher power examination of the intracellular staining shows that the distribution is identical to that of mitochondria scattered throughout the cytoplasm. N, nucleus. (*f*) No immunoreactivity is visible in a control culture stained with preimmune serum. Bars = 50 (*a–d* and *f*) or 10 (*e*) μm.

cations. Since both catechol-*O*-methyltransferase (28, 29) and MAO-B are present in glial cells that invest the brain capillaries and circumventricular organs, these enzymes may control in part the entrance of circulating biogenic amines into the brain. An additional role for MAO-B may be related to the intimate association between astrocytic processes and synaptic terminals. Thus, the presence of MAO-B may facilitate degradation of monoamine neurotransmitters released from terminals. Detailed morphological investigations of the specific distribution of MAO-B and MAO-A should further differentiate their roles in normal physiological functions and their possible participation in neuropsychiatric disorders.

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