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Ultrastructural Localization of Tyrosine Hydroxylase in Noradrenergic Neurons of Brain

(immunohistochemistry/peroxidase–antiperoxidase/catecholamine)

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ABSTRACT **Tyrosine hydroxylase (EC 1.14.16.2), the enzyme catalyzing the rate-limiting step in catecholamine biosynthesis, was localized by electron microscopy within noradrenergic neurons of the nucleus locus coeruleus of the rat brain with a specific antibody to tyrosine hydroxylase labeled by the peroxidase–antiperoxidase immunohistochemical method. Labeled cell bodies and their processes were easily distinguished from unstained neuronal elements in the neuropil. The hydroxylase was only seen in the neuronal cytoplasm. Its distribution in processes was different from that in cell soma. In longitudinal sections of axons and dendrites, the peroxidase reaction product appeared as fiber-like strands aligned parallel with the plasma membrane. In cross section, the labeled structures had a diameter of 220 Å and exhibited an orderly distribution within the processes. The size and distribution of the peroxidase-stained structures suggest that they are neurotubules. In the perikarya, the cytoplasm was diffusely stained; the reaction was most intense on membranes of endoplasmic reticulum and Golgi apparatus, whereas lysosomes and mitochondria did not stain. The ultrastructural localization of tyrosine hydroxylase is consistent with biochemical data suggesting that the enzyme exists in different states in cell body and processes. The ultrastructural identification of enzymes subserving synthesis of neurotransmitters in central neurons and their processes may provide a useful tool in mapping the distribution of chemically specific synapses on identifiable neurons in brain.**

The enzyme tyrosine hydroxylase [EC 1.14.16.2; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase(3-hydroxylating)] catalyzes the rate-limiting step in the biosynthesis of catecholamines in specific neurons of the central and peripheral nervous systems and in the chromaffin cells of the adrenal medulla (1). In brain, the enzyme has been presumed to be contained within neurons that synthesize, store, and release the neurotransmitters dopamine, norepinephrine, and possibly epinephrine (1, 2). Prior evidence for the neuronal localization of tyrosine hydroxylase was indirect and based upon the detection of the enzyme by biochemical methods within those brain regions in which catecholamine-containing neurons had been demonstrated by specific histofluorescence (3, 4). Recently, the development in our laboratory of a specific antibody to tyrosine hydroxylase (5) has permitted us to localize the enzyme immunohistochemically (6) by the use of the peroxidase–antiperoxidase method (7). In this manner, we have directly demonstrated by light microscopy that the distribution of tyrosine hydroxylase is restricted: in the brain to cell bodies and processes of dopaminergic and noradrenergic neurons, in the peripheral nervous system to sympathetic ganglion cells, and in the adrenal medulla to chromaffin cells (6).

While the cellular distribution of tyrosine hydroxylase in brain has thus been established, the subcellular localization of the enzyme is unknown. Work by others in which the distribution of the enzyme in subcellular fractions of tissue homogenates has been analyzed biochemically has suggested that, in brain, the enzyme is present in both soluble and insoluble forms (8). It has been proposed (8) that in the cell body, the enzyme is primarily soluble, while in axon terminals an insoluble form predominates. However, even the best microdissection methods cannot sufficiently separate different portions of a neuron; and homogenates of areas of brainstem in which cell bodies of catecholamine neurons are concentrated not only contain the neuronal perikarya of these cells, but also their dendrites and emerging axons. Indeed, the subcellular localization of tyrosine hydroxylase within different portions of catecholamine neurons can only be determined by the application of histological methods that will both maintain the neuronal morphology and provide a specific label for detection of the enzyme by electron microscopy.

In the present study, we have therefore attempted to localize tyrosine hydroxylase ultrastructurally within noradrenergic neurons of the nucleus locus coeruleus of the rat brain by visualization of the electron-dense reaction product of antibody labeled with peroxidase–antiperoxidase (7). We report that with this method, tyrosine hydroxylase can be identified in these neurons by electron microscopy, and that the enzyme has a different subcellular distribution in perikarya and neuronal processes.

MATERIALS AND METHODS

Preparation of Antisera. Tyrosine hydroxylase was partially purified from the bovine adrenal medulla by a combination of precipitation with ammonium sulfate and column chromatography, as described (5). The antibody for tyrosine hydroxylase, which was produced in rabbits, was adjudged to be specific for the enzyme by the following criteria (5): (*i*) Immunoelectrophoresis of antibody run against either partially purified tyrosine hydroxylase from bovine or rat adrenal medulla yielded a single precipitin arc. No precipitin arcs formed when the antibody was run against other catecholamine-synthesizing enzymes purified from bovine adrenal medulla, including dopa decarboxylase (EC 4.1.1.28), dopamine-β-hydroxylase (EC 1.14.17.1), or phenylethanolamine *N*-methyltransferase

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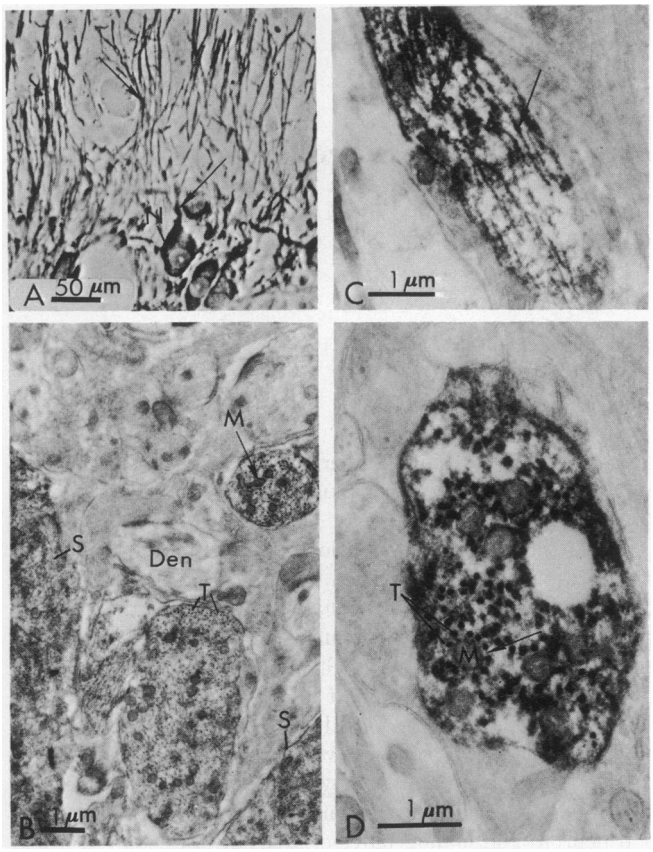

FIG. 1. Immunohistochemical localization of tyrosine hydroxylase in neurons in the nucleus locus coeruleus of rat brain stained by the peroxidase–antiperoxidase method. (A) Phase contrast photomicrograph of neurons (N) with darkly stained reaction product in the cytoplasm and processes (*arrows*). (B) Low magnification electron micrograph of peroxidase staining in neuronal soma (S) and processes. Note peroxidase-stained tubules (T) along the outer edge of a dendrite. The tubules form an ordered arrangement around mitochondria (M). Unstained dendrite (Den) is present in the neuropil. (C) Higher magnification electron micrograph of a longitudinal section through a neuronal process showing specific peroxidase staining in fiber-like structures lying parallel to the plasma membrane. (D) Cross section of a neuronal process showing selective peroxidase reaction product in circular dark structures (T) which are occasionally joined by cross-bridging (*arrow*) as they form a ring around a mitochondrion (M).

(EC 2.1.1.x). (*ii*) The antibody inhibited the activity of tyrosine hydroxylase in either purified or crude homogenates of bovine adrenal medulla and in homogenates of rat adrenals, superior cervical ganglia, or specific brain regions. (*iii*) The antibody did not inhibit the activities of dopamine-β-hydroxylase, dopa decarboxylase, or phenylethanolamine *N*-methyltransferase from bovine or rat adrenal gland.

Before incubation of the sections of tissue, the antiserum was diluted 1:20 with 0.5 M Tris-saline (pH 7.6) and adsorbed onto rat liver acetone powder (Sigma Chemical Co.). One milliliter of the diluted antiserum was incubated with 20 mg of rat liver powder for 2 hr at 37° and 8 hr at 4° followed by filtration through 0.2 μm Millipore filter.

Preparation of Tissue Sections. Male Sprague–Dawley rats weighing 150–180 g were anesthetized with pentobarbital (50 mg/kg) and perfused through the ascending aorta for 15 min with 1% (v/v) glutaraldehyde and 1% (v/v) paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.15 (9, 10). The fixative was warmed to 30–40° before perfusion. The heads were removed and placed in 2% glutaraldehyde–paraformaldehyde in cacodylate buffer for 2 hr before the brains were removed. Brains were sectioned on a vibrating microtome [Vibratome (11)] while immersed in 0.1 M sodium cacodylate buffer. Sagittal sections, 20-μM thick were taken about 1.5-mm lateral to the midline through the area containing the nucleus locus coeruleus, and were washed overnight in fresh buffer at 4° before immunohistochemical staining.

Peroxidase–Antiperoxidase Staining Procedure and Electron Microscopic Preparation. Sections were stained by a modification of the peroxidase–antiperoxidase method described by Sternberger for the staining of plastic embedded tissues (7). Sections were incubated sequentially in 30-min steps with antiserum to tyrosine hydroxylase, antibody against rabbit immunoglobulin G prepared in goat (Miles Lab) diluted 1:30 with Tris-saline at pH 7.6, and rabbit antiperoxidase bound to horseradish peroxidase, i.e., the peroxidase–antiperoxidase complex (generously supplied by Dr. L. A. Sternberger, Edgewood Arsenal, Md.). Each incubation with serum was followed by two 8-min washes with Tris-saline (pH 7.6) containing 1% normal goat serum (7).

The bound peroxidase was oxidized by reaction for 10 min with 0.5% 3,3′-diaminobenzidine (Sigma Chemical Co.) and 0.01% hydrogen peroxide in 0.1 M Tris buffer (pH 7.6) to form the brown reaction product (7). The area of the locus coeruleus, which was easily seen by light microscopy in each Vibratome section by its brown color, was dissected out of the section, using a fine blade, postfixed for 30 min in 2% osmium tetroxide in sodium cacodylate buffer (9), dehydrated, and embedded in Epon 812. Ultrathin sections were taken from the outer 1- to 2-μm surface of each 20-μm section for subsequent examination with a Phillips 200 electron microscope.

Controls. As controls for nonspecific staining, adjacent tissue sections were stained by the procedures described except for the substitution of antibody-free serum for the tyrosine hydroxylase specific antiserum, as described (6). The antibody-free serum was either: (*i*) pre-immune serum, i.e., serum collected from the rabbits before immunization with tyrosine hydroxylase, or (*ii*) blocked antisera, i.e., antisera in which the specific antibodies to tyrosine hydroxylase were removed by precipitation with amounts of purified adrenal tyrosine hydroxylase sufficient to remove reactivity by immunoelectrophoresis or enzyme inhibition. In the light microscope, tissues stained with control serum demonstrated a faint brown staining of all tissue elements. However, electron microscopy of the area of the nucleus locus coeruleus reacted with control serum failed to demonstrate neurons selectively marked by peroxidase reaction product.

RESULTS

In the light microscope, neurons in the nucleus locus coeruleus were clearly identified by the peroxidase reaction product in sections incubated with antiserum to tyrosine hydroxylase. The specific brown stain was restricted to the cytoplasm of the neuronal perikarya and to proximal parts of axons and dendrites (Fig. 1A).

Electron micrographs of comparable sections of the locus coeruleus incubated with antibody to tyrosine hydroxylase

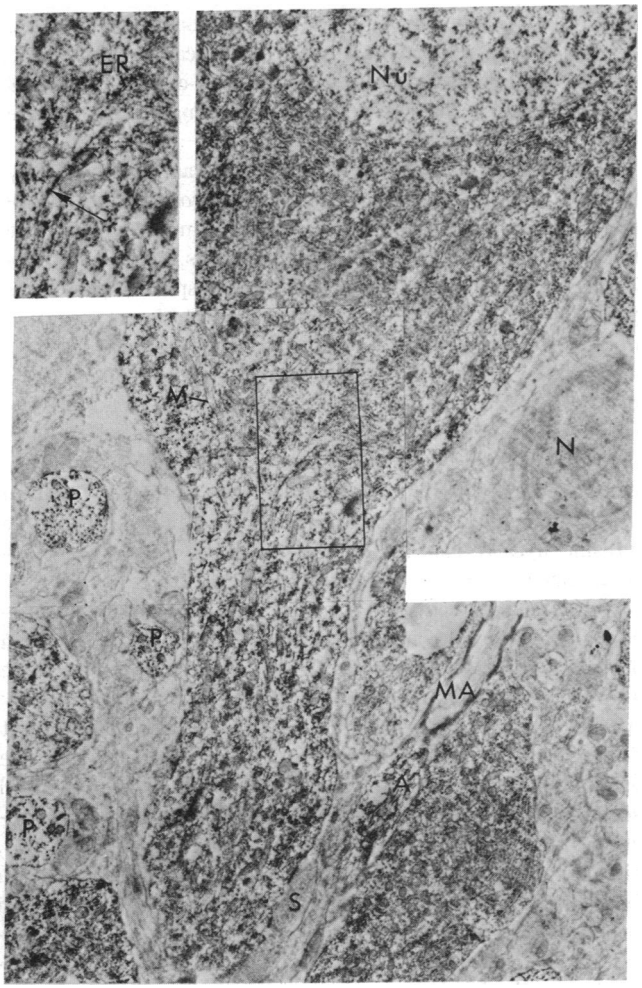

FIG. 2. Montage of a neuron in the nucleus locus coeruleus showing the electron microscopic localization of tyrosine hydroxylase by the peroxidase staining. The dense granular cytoplasmic staining can be traced from the lighter nucleus (Nu) into the proximal part of a neuronal process which, as indicated by the synapse (S), is a dendrite. The granular peroxidase stain is more intense on the outer surface of the endoplasmic reticulum (ER) and often forms a single strand (*arrow*) at the junction between the perikaryon and process, as shown in the insert in the upper left-hand corner of the figure. Numerous other processes (P) can be distinguished in the surrounding neuropil. Labeled axons (A) are unmyelinated, as compared to unstained myelinated axons (MA). Glia cytoplasm and nucleus (N) were unstained. ×6,000. Insert, ×7,200.

showed the processes and perikarya of neurons stained with electron-dense material (Figs. 1B, 2, and 3A). Among neuronal processes, only unmyelinated axons and dendrites (Figs. 1B–D and 2) were stained. In longitudinal sections through such processes (Fig. 1C), the dense reaction product was aligned in linear aggregates forming fiber-like structures usually arranged in parallel with the plasma membrane. The electron-dense material in each bundle consisted of a series of rounded globular subunits, which gave a beaded appearance to the strands. In cross section (Fig. 1B and D), the reaction product appeared in the form of darkly staining round structures with a mean diameter of approximately 220 Å. These structures often assumed an orderly arrangement within the processes and sometimes appeared on the electronmicrograph

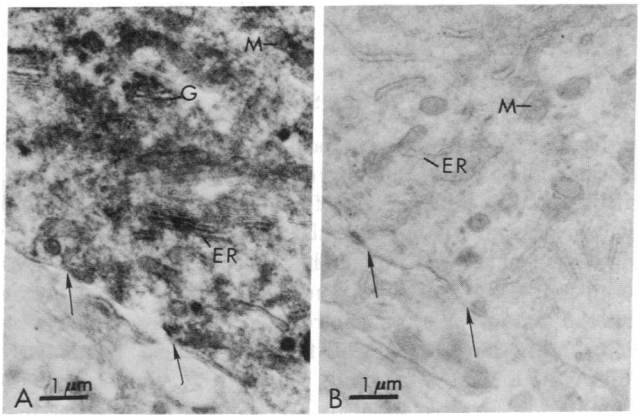

FIG. 3. Ultrastructural distribution of tyrosine hydroxylase in soma of noradrenergic neurons in locus coeruleus of rat brain. (A) Section incubated with specific tyrosine hydroxylase antisera labeled with peroxidase–antiperoxidase. Note dark peroxidase staining in the cytoplasm which can be clearly differentiated from the surrounding neuropil at the plasma membrane (*arrows*). The Golgi apparatus (G) and endoplasmic reticulum (ER) show some specific staining. However, a dark granular staining is present throughout the cytoplasm. (B) Section incubated with control serum labeled with peroxidase–antiperoxidase and processed in parallel with section seen in panel A. The soma, which is bounded by the plasma membrane (*arrows*), shows no enhanced staining over the surrounding neuropil. The organelles, such as the endoplasmic reticulum (ER) and mitochondria (M), are equally unstained.

in a circular arrangement around mitochondria (Fig. 1D). Occasionally they were joined to each other by cross-bridging. The structures that show the intense peroxidase staining in both longitudinal and cross sections through neuronal processes resemble neurotubules by virtue of their arrangement, size, and distribution (12). While occasionally the center of the structure was more lightly stained than the rim, in most instances the central lightly stained core that characterizes neurotubules in routinely stained electron micrographs (12) was not observed, possibly because of the density of the reaction product. The dense staining also made it impossible to determine if the antigen was localized on the surface or within the matrix of the tubules. Near the cell soma there was some labeling of the membranes of endoplasmic reticulum which extended into the neuronal processes (Fig. 2).

The distribution of tyrosine hydroxylase within the perikarya of locus coeruleus neurons differed in part from its localization in processes. In the perikarya the electron-dense particles of the peroxidase label was concentrated in the cytoplasm and lightly distributed in the nucleus of neurons (Fig. 2). While the generalized staining of the neuronal cytoplasm made it difficult to determine if the enzyme was localized in specific organelles, membranes of the Golgi apparatus, endoplasmic reticulum, and neurotubules appeared to stain, while those of mitochondria and lysosomes did not (Figs. 2 and 3A). A fine granular staining of neuronal but not glial nuclei was often seen. It was not restricted to catecholamine neurons, however, and also appeared in neurons that did not exhibit cytoplasmic staining by peroxidase. The nonspecific darkening of neuronal nuclei was retained when the rabbit antiserum was omitted from the staining procedure and thus may reflect a nonspecific staining by the peroxidase complex or simply a darkening of nuclear components by osmium.

In control sections (Fig. 3B), no cytoplasmic staining was observed.

DISCUSSION

In the present study we have localized by electron microscopy the catecholamine-synthesizing enzyme, tyrosine hydroxylase, within noradrenergic neurons of rat brain. Our ultrastructural identification of this intracellular antigen was facilitated by adaptation of several immunohistochemical techniques that permitted both the preservation of the neuron's fine structure and the deposition of an electron-dense marker of high specificity (13). Three technical features seemed particularly important. First, by sectioning tissue with a vibrating microtome, it was possible to cut relatively thin (20 μm) sections of unfrozen brain and, thus, to circumvent the cell damage caused by freeze-thawing when tissues are sectioned for immunohistochemistry on a freezing microtome (11). Second, the development by Sternberger (7) of the peroxidase–antiperoxidase method for labeling antibodies to specific antigens offered a cell marker of high specificity and resolution that was suitable for both light and electron microscopy. Finally, by staining sections with peroxidase–antiperoxidase-labeled antibody immediately after sectioning, it was possible to identify and dissect out the brain area to be sampled before embedding for electron microscopy. The latter procedure offers an enormous advantage in electron microscopy of brain, since the regional localization of the catecholaminergic neurons would be difficult to determine in unstained sections.

The present study has established that tyrosine hydroxylase is localized, as is norepinephrine (3), within the cytoplasm of catecholamine-containing neurons of the nucleus locus coeruleus. However, in accord with the biochemical evidence (8), the ultrastructural distribution of the enzyme is not uniform throughout the cell. Within axons and dendrites, tyrosine hydroxylase appeared to be selectively associated with neurotubules. That tyrosine hydroxylase is associated with a specific organelle in neuronal processes and is not free in the cytoplasm may explain the observation that most of the enzyme in subcellular fractions of neuronal terminals is particulate (8, 14). The association of tyrosine hydroxylase with neurotubules in axons may also correlate with the fact that in axons of peripheral sympathetic neurons, tyrosine hydroxylase is transported distally at relatively rapid rates of flow (15), since neurotubules have been primarily implicated in the fast axoplasmic transport of neuronal materials (12).

The association of tyrosine hydroxylase with neurotubules in axons is easily reconciled with the accepted hypothesis that the biosynthesis of the neurotransmitter norepinephrine takes place primarily within axon terminals in catecholaminergic neurons (16). The association of tyrosine hydroxylase with neurotubules in dendrites, however, is somewhat surprising. The finding may indicate that tyrosine hydroxylase, like nerve growth factor (17), may have a high affinity for neurotubular protein. Thus tyrosine hydroxylase may, in regions rich in neurotubules (i.e., processes), tend to associate with this particular organelle. Alternatively, it is conceivable that tyrosine hydroxylase specifically associates with neurotubules in the cell body and is then transported with them into both axons and dendrites. The neurotubule transport of materials in dendrites has been shown for certain amino acids (18). In dendrites, tyrosine hydroxylase is most likely catalytically active since catecholamines have been identified by histofluorescence in proximal dendrites of catecholaminergic neurons (3). The functional role of dendritic catecholamines is unclear. Possibly, noradrenergic neurons may exhibit some mode of dendro-dendritic transmission.

In the perikarya, tyrosine hydroxylase was less specifically localized than in processes. Some of the tyrosine hydroxylase was associated with organelles, including membranes of the endoplasmic reticulum and Golgi apparatus, possibly reflecting the intracellular sites at which this specific protein is synthesized. However, considerable amounts of reaction product were also observed widely dispersed throughout the neuronal cytoplasm. Whether tyrosine hydroxylase in this form was associated with free ribosomes could not be established. Conceivably, this more diffusely distributed tyrosine hydroxylase label in the cytoplasm may represent the soluble form of the enzyme that is detectable after ultracentrifugation and may account for the higher ratio of soluble to insoluble tyrosine hydroxylase in the cell bodies of central catecholamine neurons (8).

Over the past several years, the application of immunohistochemical methods for the light-microscopic identification of enzymes catalyzing neurotransmitter biosynthesis have afforded a significant advance in the mapping of chemically specific neuronal systems in brain (16, 19, 20). The present finding that it is possible to visualize at least one of these enzymes ultrastructurally further increases the potential scope of the immunohistochemical techniques. It demonstrates that, at least in the area of the nucleus locus coeruleus, the perikarya and processes of catecholaminergic neurons can be identified by their enzyme content and distinguished from other neurons in the neuropil. Conceivably, by suitable labeling of this area with antibodies to other specific neurotransmitter-synthesizing enzymes, such as cholinacetyltransferase (cholinergic neurons) or tryptophan hydroxylase (serotonergic neurons), it should be possible to determine, at the ultrastructural level, the chemical identity of individual synaptic inputs onto specified neurons in brain.

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