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The Use of Dopamine β-Hydroxylase as a Marker for the Central Noradrenergic Nervous System in Rat Brain

(immunofluorescence/microcirculation/norepinephrine)

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ABSTRACT **Improvements in the method for localization of dopamine-β-hydroxylase by immunofluorescence allow the observation of noradrenergic-cell bodies, nonterminal fibers, and axon terminals in the rat brain. The distribution of the hydroxylase correlated well with the results obtained by localization of norepinephrine. Dopamine-β-hydroxylase was not observed in dopaminergic neurons or terminals, indicating that these cells do not have the capacity to synthesize norepinephrine. The use of the hydroxylase as a marker, however, has made it possible to visualize noradrenergic nerve terminals on small arteries in the brain parenchyma that have not been described by catecholamine-fluorescence histochemistry. The source of the terminals on small arteries appears to be central noradrenergic neurons rather than the peripheral sympathetic nervous system. Dopamine-β-hydroxylase generally was not observed in the large arteries of the brain parenchyma. These observations suggest that cerebral microcirculation is regulated by central noradrenergic neurons.**

Dopamine-β-hydroxylase (EC 1.14.2.1; DBOHase) is the enzyme that converts dopamine to norepinephrine. Thus, wherever norepinephrine is synthesized DBOHase must also be present, and could serve as a marker for localization of the noradrenergic nervous system. Immunofluorescent histochemistry has been used to demonstrate the enzyme in both central and peripheral neurons (1–3). Up to the present, however, the sensitivity and resolution of the formaldehyde-induced fluorescence of catecholamines (4) have been superior to the immunofluorescent techniques for demonstrating fine noradrenergic terminals in the central nervous system. We have developed several improvements in the immunofluorescent technique, which now permit the tracing of the central noradrenergic fibers from their cell bodies to their terminals.

MATERIALS AND METHODS

The preparation and criteria for purity of the antibody to DBOHase used in this study was described (2).

Preparation of Fluorescein-Conjugated Antiserum to Rabbit IgG. This step represents the first major improvement over previously reported methodology (2). Purified antiglobulin was prepared by affinity chromatography for conjugation with fluorescein isothiocyanate rather than by conjugation of the entire globulin fraction. The fluorescein isothiocyanate-conjugate produced by this method can be diluted sufficiently so that fluorescence due to nonspecific binding of antiglobulin to tissue sections is negligible, while at the same time the conjugate remains a sufficiently sensitive antibody detector to allow high dilution of the antiserum of DBOHase.

Rabbit antiglobulin of high titer (prepared in goats) was purchased from Gateway Immunosera Co., Cahokia, Ill., (titer 13.5 mg/ml). Purified rabbit IgG was conjugated to Sepharose 4B by the cyanogen bromide linkage (5). Antiglobulin was passed over a column (10 ml of packed Sepharose) of this immunoadsorbant. The specific antibody to rabbit IgG is retained by the rabbit IgG–Sepharose. The column was washed with phosphate-buffered saline [0.14 M NaCl–0.02 M potassium phosphate (pH 7.5)] (PO_4-saline) until no protein was detectable in the eluant. Pure antiglobulin was then eluted from the column with 0.2 M acetic acid. The eluant was immediately neutralized to pH 7.4 with 1.0 M K_2HPO_4. This solution was then dialyzed and concentrated by pressure dialysis against PO_4-saline. The antiglobulin thus prepared was highly purified (94% immunoprecipitable with purified rabbit IgG).

The purified antiglobulin was then conjugated with fluorescein isothiocyanate (Baltimore Biological) by the following method: 5 ml of 0.5 M sodium carbonate buffer (pH 9.5) was added to 5 ml of antiglobulin (20 mg/ml). While stirring at 0°, 1.5 mg of fluorescein isothiocyanate was added (15 μg/mg of antiglobulin), and for the first 2 hr the pH was maintained at 9.5 with 0.1 M NaOH. The container was then tightly stoppered and the reaction was continued at 4° for 20 hr. The reacted fluorescein isothiocyanate was then removed from the antiglobulin solution by Sephadex G-50 chromatography. The molar ratio of fluorescein to antibody was 4 to 1.

Preparation of Tissue Sections. Antiserum to DBOHase prepared in rabbits against beef adrenal DBOHase cross-reacted with rat DBOHase (2). Male Hutchenson rats (250–300 g) were killed by decapitation and their brains were removed. 10-μm frozen sections were cut in a cryostat, melted onto glass slides, dried in air for 3–5 min, fixed for 20 min in 0° chloroform–methanol 2:1, dried in air for 1 hr, and either stained or stored in slide boxes at −90°. Chloroform–methanol fixation precipitates the protein and it also removes lipids that contribute to nonspecific staining and that interfere with the access of antibody to the antigen.

Fluorescent-Antibody Procedure. Sections were incubated for 15 min at 37° in a constant humidity chamber with antiserum to DBOHase (titer 2.6 mg/ml) diluted 1 to 50 with PO_4-saline containing 0.3% Triton X-100 (Shell Chemical Co.). The introduction of Triton X-100 detergent at this stage and in the subsequent washing stage is the second major improvement in the technique. The Triton X-100 reduced nonspecific, low-affinity protein–protein binding, but at these concentra-

Abbreviation: Dopamine-β-hydroxylase, DBOHase.

tions it did not interfere with antigen–antibody binding. The slides were gently rinsed with PO_4-saline and immersed in PO_4-saline containing 1% Triton X-100 for 5 min. The slides were rinsed again with PO_4-saline and immersed in PO_4-saline for 5 min. The sections were then incubated with the purified fluorescein isothiocyanate–antiglobulin and diluted to 0.2 mg/ml globulin with PO_4-saline containing 0.3% Triton X-100 for 15 min. After incubation, the slides were washed as in the first step and mounted in buffered glycerol [0.5 M sodium carbonate (pH 8.6)–glycerol, 1:1]. Diluted antibodies to DBOHase and globulin were always freshly prepared. The slides were examined with a Leitz Orthoplan fluorescence microscope equipped with a Ploem vertical illuminator, under incident blue ultraviolet light (activation filter-BG 12, barrier filter-K 530). Kodak Tri-X-pan film was used. When a single microscopic field was not sufficiently large to demonstrate a particular anatomical struture, photomicrographs of overlapping adjacent fields were taken. The prints of these photographs were then cut and assembled to form a montage that shows the area covered by the several fields. Because the microscopic illumination is not perfectly homogenous, outlines of the separate fields are seen in the completed montage.

Controls consisted of adjacent sections stained as above, except for the substitution of blocked antiserum (antiserum from which the antibodies to DBOHase had been removed by immunoprecipitation with purified DBOHase and replaced by an equivalent amount of normal rabbit IgG or normal rabbit serum diluted to obtain the same concentration of IgG as that in the specific antiserum). Both controls gave very low nonspecific fluorescence. Other sections were stained with Richardson's stain (0.5 g of methylene blue–0.5 g of azure II–0.5 g of sodium borate–100 ml of water) for standard light microscopic observation.

RESULTS

In the central nervous system, DBOHase was localized within cell bodies of neurons, nonterminal nerve fibers, and terminal nerve fibers.

Cell bodies

In the locus coeruleus (Fig. 1*A*), which has the largest concentration of neurons containing DBOHase, the cell bodies were closely packed together. Their nuclei did not fluoresce, and the cytoplasmic fluorescence was granular and of variable intensity. Fluorescent processes could also be seen, but these were more clearly demonstrated in the dispersed cells of the pons and medulla that exhibited specific immunofluorescence (Fig. 1*B*). The distribution of cell bodies containing DBOHase was found to be identical to that described when the histochemical stain was used with norepinephrine (6). Furthermore, no DBOHase was observed in cell bodies in the midbrain raphe or in the substantia nigra, which have been identified by histochemical means to contain serotonin and dopamine neurons, respectively (6). No neuronal cell bodies with DBOHase were observed rostral to the pons.

Nonterminal fibers

The axons from neurons containing DBOHase destined to give rise to noradrenergic terminals were distributed to widespread areas of the brain by systems of fibers that contained enough DBOHase (presumably in transit to the terminals) to make them clearly visible by the immunofluorescent technique (Fig. 2). These fibers coursed through the brain in rather diffuse

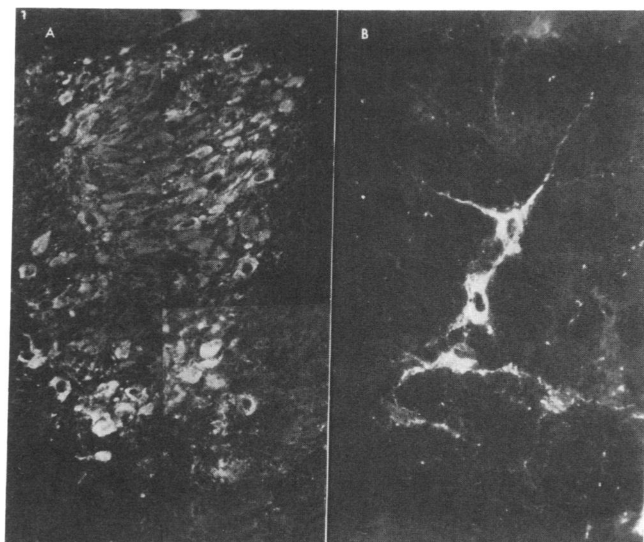

FIG. 1. Cell bodies containing DBOHase. (*A*) Coronal section through the locus coeruleus near its widest point (×140). This nucleus contains the largest concentration of cell bodies containing DBOHase in the brain. (*B*) Several isolated neurons containing DBOHase in the ventral medulla (×220). These cells more clearly show their multipolar nature.

bundles, but followed well-defined pathways to their destinations and were recognized most easily in sections cut parallel to their direction. Many bundles, following the same general course, form the major noradrenergic pathways. The fluorescence in these fibers appeared granular and even beaded but had no regular periodicity. Such nonterminal fibers do not contain sufficient norepinephrine to be visualized in normal rats by the histochemical stain for norepinephrine (7).

Terminal fibers

The fiber tracts described gave rise to many smaller fibers with regularly spaced (periodic) enlargements (varicosities), characteristic of noradrenergic terminals. The size of the varicosities varied from 0.5–5 μm; e.g., the anterior ventral nucleus of the thalamus contained a high proportion of small varicosities (Fig. 3*A*), whereas, the paraventricular nucleus of the hypothalamus (Fig. 4) contained more terminals of a larger size. The terminal areas observed with DBOHase as a marker correlate well with those outlined by Fuxe (8), who used norepinephrine fluorescence histochemistry.

Areas containing a high density of fluorescent axon terminals were especially common in nuclei of the hypothalamus, for example, the paraventricular nucleus (Fig. 4), the supraoptic nucleus, and the interstitial nucleus of the stria terminalis. The anterior ventral nucleus of the thalamus (Fig. 3*A*) and the dentate gyrus of the hippocampus also contained a high density of DBOHase terminals. Several areas had an extremely low density of fibers containing DBOHase. In particular the caudate–putamen appeared to be devoid of any fluorescent fibers or terminals (Fig. 3*B*). No cell bodies containing DBOHase were observed in the substantia nigra. These areas have been associated with the dopamine neuron system (6–8).

Blood vessels

The large arteries at the base of the brain, especially those of the circle of Willis, were innervated by nerve terminals con-

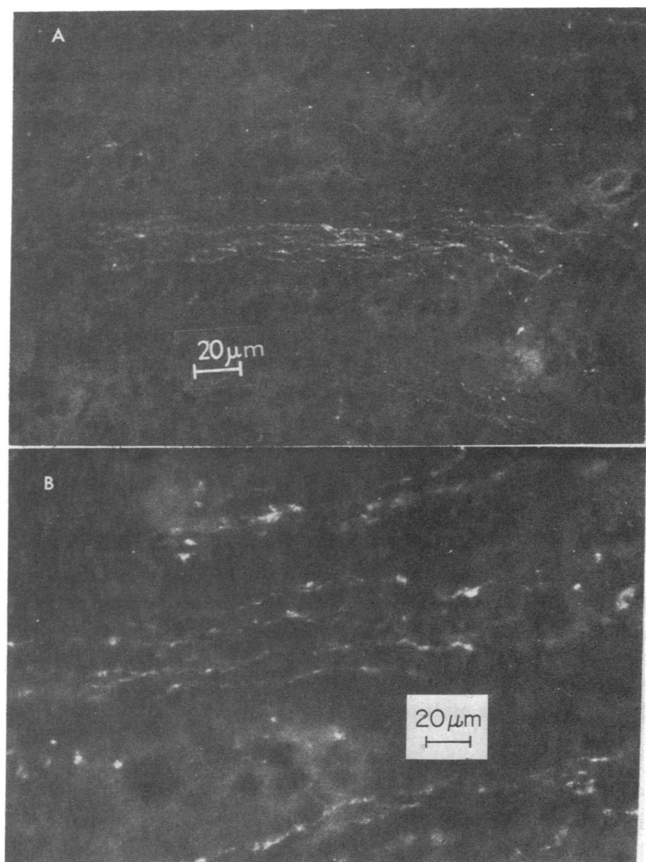

FIG. 2. Nonterminal noradrenergic fibers. (*A*) Field from a sagittal section through the mesencephalon. This group of fibers arose from the rostral locus coeruleus. Note the way in which many fibers are grouped together to form a diffuse bundle. These bundles maintain their integrity over relatively long distances. In cross-sections, groups of fibers such as these appear as random dots of fluorescence. (*B*) Higher power view of a fiber bundle showing the nonperiodic clumps of fluorescence characteristic of these structures. Each fiber probably contains several individual axons.

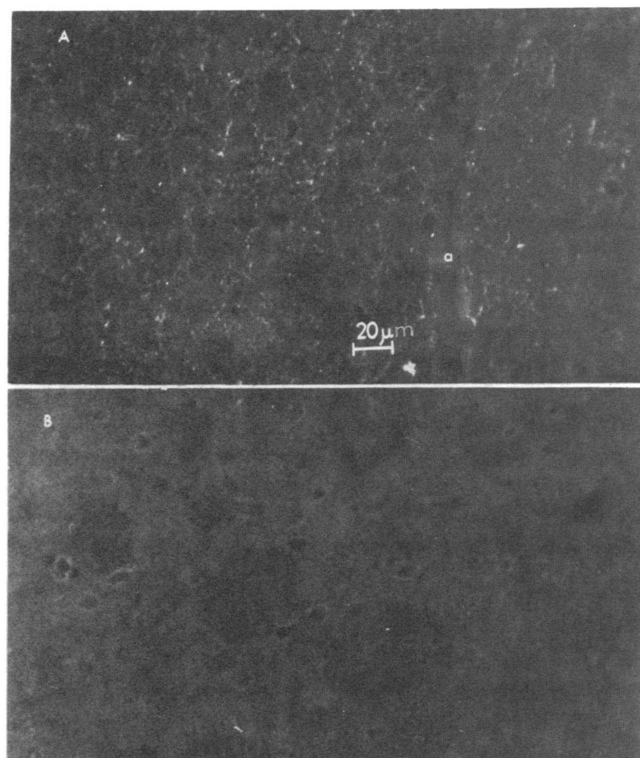

FIG. 3. (*A*) Noradrenergic terminal areas in the anterior ventral nucleus of the thalamus. The fine network of small varicose fibers covers the field. The surface of the small artery (*a*) cut in longitudinal section is sparsely coated with varicosities that are in close contact with its wall. (*B*) A section through the caudate–putamen showing the lack of a terminal network of fibers containing DBOHase (same magnification).

taining DBOHase derived from the superior-cervical ganglion (9, 10). In general, however, large and medium-sized muscular arteries within the brain parenchyma were devoid of this type of sympathetic innervation, although they frequently had nonterminal fibers coursing parallel to them. Such fibers were outside the adventitia and could be traced to fiber tracts originating from cell bodies in the brain stem. In contrast, the small arteries and arterioles branching from these larger arteries were frequently observed to be innervated by terminals containing DBOHase.

The concentration of terminals containing DBOHase on the vessels was highly variable. The most frequently observed pattern of innervation was a sparse scattering of small terminals over the surface of the vessels as seen in Fig. 3*A*. At the other extreme, some arteries had a dense coating of terminals containing DBOHase. Fig. 4 shows a section through a "supply" artery (*arrow*) that has just branched off a larger artery to feed the paraventricular nucleus of the hypothalamus. It should be noted that the DBOHase (fluorescent) terminals form a fine but dense cuff around the "supply" artery and its extensions into the paraventricular nucleus. The major artery itself has no visible terminals with DBOHase. The dense type of noradrenergic innervation has also been observed on the arterial supply of the choroid plexus (to be published). Fig. 5 shows examples of the noradrenergic innervation of both peripheral and cerebral arteries as observed by DBOHase immunofluorescence.

DISCUSSION

Two modifications of our method (2) for localization of DBOHase by fluorescence have permitted the visualization of the entire central noradrenergic neurons in normal untreated rats. With the exception of some minor discrepancies involving the course of several fiber pathways (11), it has been possible to confirm the descriptive anatomy of the central noradrenergic nervous system as determined by the histochemical localization of norepinephrine. In addition, the exclusively dopaminergic nature of the neuron system with cell bodies in the substantia nigra and terminals in the caudate–putamen was confirmed by demonstration of the absence of DBOHase in this system. This observation shows that DBOHase is specific for the noradrenergic system and rules out the possibility that it is present in the dopaminergic system in an inactive or inhibited form (the immunological reaction is not dependent on enzyme activity). The same conclusion was drawn in other studies showing lack of DBOHase immunofluorescence on dopaminergic neurons (12, 13).

In addition to confirming the pattern of localization of norepinephrine, the use of DBOHase as a marker for the central noradrenergic system has demonstrated a well-defined

noradrenergic innervation of small arteries in the brain parenchyma. Our observations further indicate that the terminals on at least many of these small arteries are derived from central noradrenergic cell bodies, rather than from the

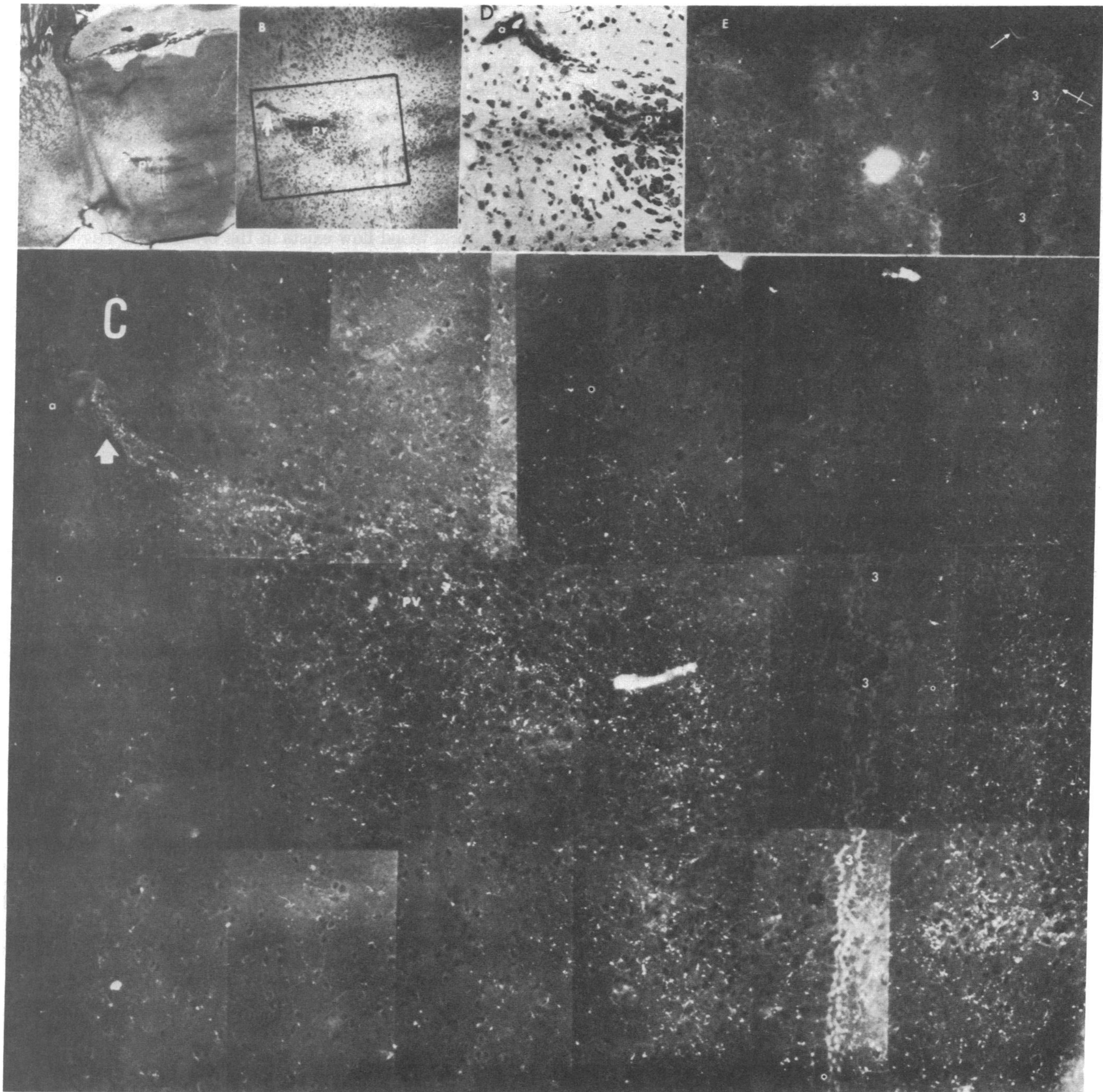

FIG. 4. Montage of the paraventricular nucleus of the hypothalamus. The *arrows* point to a small artery that supplies the paraventricular nucleus (*pv*). (*A*) Richardson's stain of a coronal section through the hypothalamus at the level of the paraventricular nucleus (×6). (*B*) Higher power (×22) of the same section. The *rectangle* represents area covered by montage. (*C*) Montage of the paraventricular nucleus stained by the fluorescent antibody technique for localization of DBOHase (×154). This section is adjacent to that in *B*. A small artery (↑) has just branched from a larger artery (*a*) and is supplying the paraventricular nucleus (*pv*). The third ventricle (*3*) is seen to the right of center. Note that the small supply artery (↑) is covered with a dense "cuff" of fine terminals containing DBOHase. As this branch courses away from its parent artery the noradrenergic terminals follow it into the nucleus. In the other direction, the "cuff" of terminals is seen to stop abruptly at the point of branching with the parent artery (*a*). It should also be noted that this nucleus is richly innervated with terminals containing DBOHase. Many of the neurons are completely encircled by varicose fibers. (*D*) Higher power view (×80) of *A*, showing the parent artery (*a*) and its branch (↑) in greater detail. (*E*) Control (×154) of a segment of the montage from a section adjacent to *C*. The immunofluorescent staining procedure was identical, with the exception that normal rabbit serum replaced the antiserum to DBOHase. The four major types of nonspecific fluorescence represented in the control are fibrous astrocytic processes (↑) staining smoothly and less intensely than DBOHase, ependymal cells (‡) lining the third ventricle, immunoglobulin precipitates such as the large central spot of fluorescence, and neuronal nuclei scattered throughout the field with a dim halo of nonspecific fluorescence.

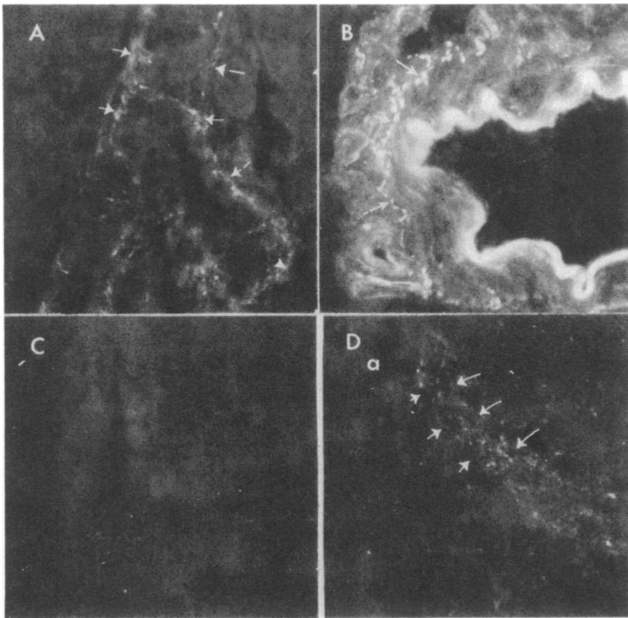

FIG. 5. (*A*) Coronary artery densely innervated with fibers containing DBOHase (↑) (×350). (*B*) Pial artery showing fibers containing DBOHase (↑) that originate from the superior cervical ganglion (×350). The convoluted elastica outlining the arterial lumen is nonspecifically fluorescent. (*C*) Brain parenchymal artery exhibiting typical absence of noradrenergic innervation (×350). (*D*) Higher power view of the dense noradrenergic innervation (↑) of the small brain parenchymal artery shown in Fig. 4 branching from a larger parent artery (*a*) (×350).

peripheral symphathetic nervous system for the following reasons. (*a*) If the noradrenergic supply was carried into the brain along the arterial wall, one would expect to see fibers on the larger arteries, yet they are generally devoid of specific fluorescence. (*b*) Terminal fibers have been observed going to arteries from nonterminal fiber tracts not directly associated with blood vessels. (*c*) Blood vessels in the caudate–putamen do not receive terminal innervation containing DBOHase. If the blood vessels in the caudate–putamen are innervated by terminals containing catocholamines they may be dopaminergic and derived from the nigral–striatal system. (*d*) Preliminary observations (to be published) on superior-cervical ganglionectomized rats have shown that the small parenchymal arteries retain their DBOHase terminals while the terminals on the pial arteries disappear, thus indicating that the terminals are derived from different sources.

The basic difference between the pattern of arterial innervation observed in the brain and in other organs is that, in the brain parenchyma, the noradrenergic fibers and terminals do not begin making contact with the arterial tree until the level of small arteries; whereas, in peripheral blood vessels noradrenergic fibers accompany the large vessels and extend to the smaller ones. One consequence of this innervation pattern would be the possibility of independent regulation of blood flow in different branches of the same artery. For example, in the case of the small arterial branch supplying the paraventricular nucleus shown in Fig. 4, the presence of a "cuff" of noradrenergic terminals starting after the branch point suggests a mechanism for noradrenergic regulation of the blood flow through the branch without altering the flow in the parent artery. Such microregulation of the perfusion of the paraventricular nucleus may be required for its proper functioning in the control of blood volume and osmolarity.

Our observations have thus led us to postulate that one of the functions of the central noradrenergic system is concerned with the microregulation of cerebral blood flow. Several other reports are at least consistent with this concept. First, neurophysiological studies have indicated that a regulating center for cerebral blood flow exists in the brain stem. High medullary, pontine, and mesencephalic lesions in dogs diminish or abolish the responsiveness of cerebral vessels to changes in arterial CO_2 concentration, whereas lesions in the cortex have little effect (14). Electrical stimulation of the mesencephalic tegmentum (one of the major pathways for noradrenergic fibers) in cats resulted in increased cortical blood flow (15). Second, although catecholamines administered intravenously have negligible effect on cerebral blood flow, when these compounds are injected directly into the brain the microcirculation is markedly affected (16). Finally, when lesions are made on the central noradrenergic neurons, they will send terminal fibers into cerebral vessels (although this pattern of innervation was considered abnormal) and transplanted tissues containing smooth muscle (17). Our observations may provide the anatomical explanation for these observations.

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