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E. ADRENERGIC ENDINGS AND VESICLES ISOLATED FROM BRAIN[1]

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Catecholamines are widely distributed in different tissues. In most cases, cells that synthesize these monoamines are of neuronal nature or related to neurons, *e.g.*, the adrenomedullary cells and the adrenergic chromaffin cells, diffusely distributed in tissues, embryologically derived from the neural crests. Of the various catecholamines norepinephrine (NE) is the postganglionic transmitter in the sympathetic system. It is concentrated in the adrenal medulla of certain species and in the sympathetic system, reaching a maximum in the splenic nerve (54). Catecholamines are also present in brain with a preferential localization in certain anatomical regions. The highest concentration of NE is in the hypothalamus, central gray matter of the mesencephalon, and area postrema (4, 9, 27, 53), while dopamine is localized mainly in the corpus striatum and associated with basal ganglia (see 8, 38).

The subcellular distribution of catecholamines has been studied mainly in the adrenomedullary cells, from which special granules containing these monoamines were isolated (see 3, 7, 26, 32, 48). Von Euler and Hillarp (55) separated a granular fraction rich in NE from homogenates of the spleen and splenic nerves. Weil-Malherbe and Bone (56) isolated a particulate NE from brain homogenates, and similar findings were obtained by Bertler *et al.* (6). Chruściel (10) found NE concentrated in the vesicular fraction of Whittaker (57), which contains nerve endings, and this was confirmed by Inouye *et al.* (33).

Electron microscopic studies on catecholamine deposits were initially done on cells of the adrenal medulla, and special membrane-bound granules or vesicles which intensely reduce osmium tetroxide were recognized (35, 50). The formation of these catechol-containing vesicles in relation to the Golgi complex and their secretion after stimulation of the splanchnic nerve were studied with the electron microscope (24, 25).

GRANULATED VESICLES IN SYMPATHETIC AXONS AND ENDINGS.

In adrenergic axons and endings innervating the pineal gland and in the splenic nerve a plurivesicular material was described by De Robertis and Pellegrino de Iraldi (11, 16, 17). This consists of clear homogeneous vesicles similar to the synaptic vesicles of De Robertis and Bennett (14, 15), intermingled with granulated vesicles containing a dense granule of reduced osmium. Similar components have been observed in various parts of the sympathetic system (31, 44, 52). In 1961 Pellegrino de Iraldi and De Robertis (39) showed that the granulated vesicles almost completely disappear a few minutes after a single injection of reserpine.

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Restoration of the normal amount of granulated vesicles occurred after 6 to 8 days. The experimental work carried out in this laboratory with denervation of the pineal gland, the action of drugs releasing catecholamines, and the injection of various catecholamine precursors and monoamine oxidase (MAO) inhibitors on the ratio of granulated to nongranulated vesicles in the adrenergic endings, suggests that the granulated vesicles are of adrenergic nature (13, 40, 42). Similarly the findings of Wolfe *et al.* (58), that by radioautography H^3-NE is localized in the region of granulated vesicles also suggests that the contents of these vesicles are adrenergic.

GRANULATED VESICLES IN THE HYPOTHALAMUS

In the brain the cellular localization of catecholamines has been studied with the light microscope by a special fluorescense method (see 9, 28, and Sections III F and G, VIIIC). Fine varicose nerve fibers were found in the preoptic region and in the supra- ,para- and periventricular nuclei of the anterior hypothalamus. They were interpreted as terminal adrenergic axons making synaptic contacts. In our laboratory the same hypothalamic region of the rat was studied with the electron microscope. After a special technique involving perfusion of formalin followed by osmium tetroxide (29), a small piece of the anterior hypothalamus containing the *nucleus hypothalamicus anterior*, the *nucleus periventricularis inferior*, and the *anterior hypothalamic* area was dissected and observed with the electron microscope (41). In the neuropile of this region numerous varicose axons containing granular vesicles are present. In the nerve terminals there is a wide variety of vesicles. In addition to round or oval synaptic vesicles there are others having an elliptical shape (fig. 1). These clear vesicles constitute about 80 % of the total, whereas the other 20 % correspond to granulated vesicles of different size and shape containing a dense deposit of reduced osmium. The clear vesicles range between 200 and 800 Å with a mean diameter of 510 Å, and the granulated vesicles range between 700 and 1700 Å with a mean of about 1300 Å. These special granulated vesicles of the anterior hypothalamus were tentatively interpreted as representing catecholamine stores, and this has now been more directly supported by chemical analysis of the isolated fractions (21). In addition Shimizu and Ishii (49) with reserpine and Matsuoka *et al.* (36), with the NE releaser Win 18501-2 have shown that these granulated vesicles tend to disappear from the hypothalamus, as they do in the adrenergic endings of the pineal body.

SUBCELLULAR LOCALIZATION OF NE AND DOPAMINE AND OF RELATED ENZYMES IN THE BRAIN

The discovery of synaptic vesicles as the most characteristic morphological component of nerve endings (14, 15) permitted the identification of these structures in brain fractions (18, 19, 30). The technique of cell fractionation employed in our laboratory involves a mild homogenization and the separation of the four primary fractions: nuclear, mitochondrial, microsomal, and soluble (table 1a). The "mitochondrial" fraction contains numerous intact nerve endings in addition to free mitochondria and myelin. On a sucrose-density gradient, five sub-

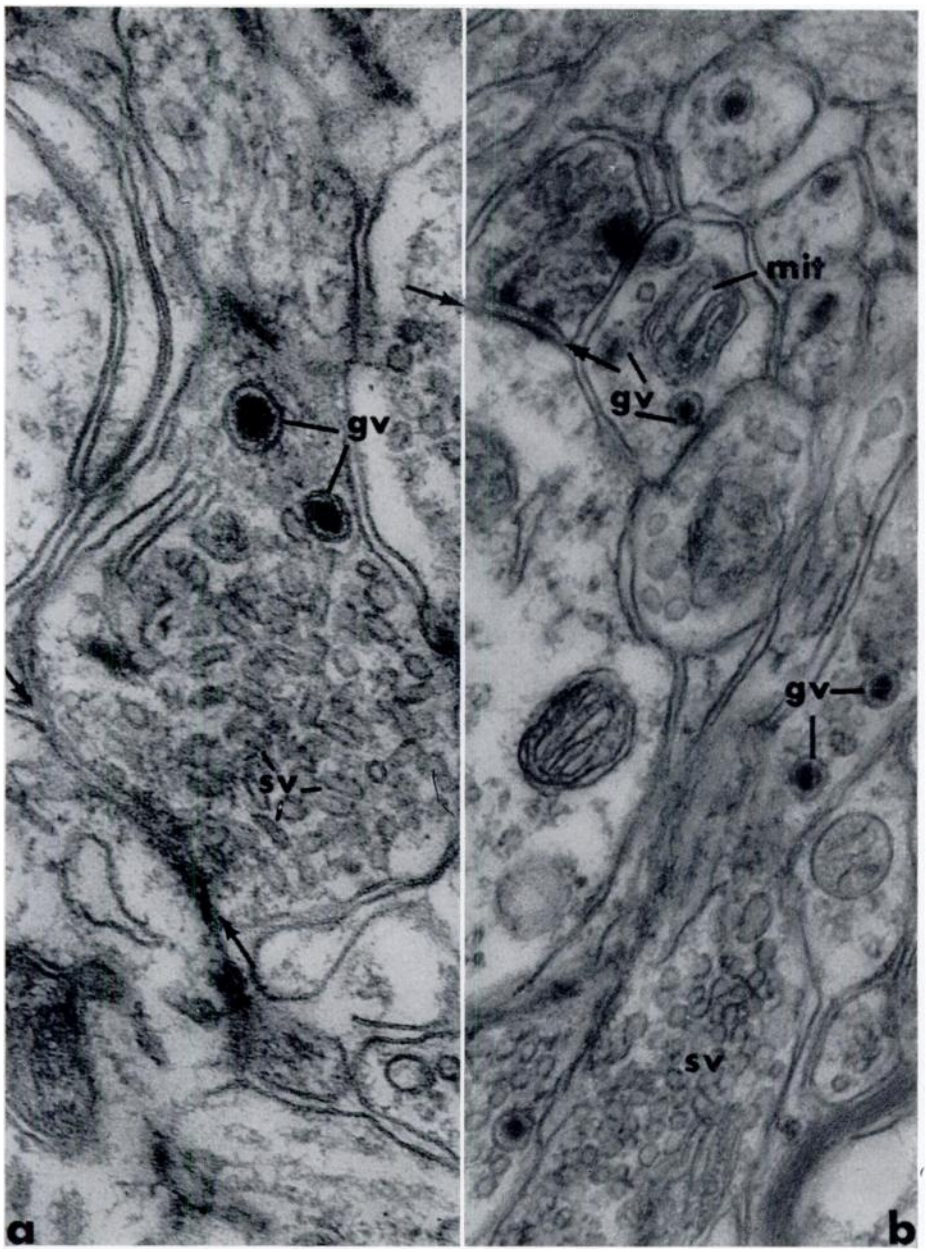

FIG. 1. Electron micrographs of the anterior hypothalamus of the rat showing: a, A typical synaptic ending on a dendrite with elliptical clear vesicles and two larger granulated vesicles (gv). b, Axons and endings containing several granulated vesicles (gv) and smaller clear synaptic vesicles (sv). mit, Mitochondria. Arrows indicate synaptic clefts. a, × 70,000; b, × 80,000.

TABLE 1

Subcellular distribution of NE, dopamine, and three related enzymes in rat brain by centrifugation (a) and by density-gradient separation of the mitochondrial fraction, either without (b) or with (c) previous osmotic disruption. (For techniques see 20, 23)

Fractions	Ultrastructure	Protein	NE	Dopamine	5-Hydroxytryptophan Decarboxylase	MAO	COMT
		%	Relative specific concentration		Relative specific activity		
			Table 1a				
	References		60		46	45	*
Nuclear	Nuclei, capillaries	14	0.72	0.90	0.49	1.10	0.42
Mitochondrial	Myelin, mitochondria, nerve endings	49	1.04	0.91	1.00	1.80	0.72
Microsomal	Microsomes	12	1.42	0.93	0.68	0.35	0.59
Supernatant	Soluble	25	0.88	1.25	2.05	0.00	2.03
			Table 1b				
			Submitochondrial fractions on gradient				
A	Myelin	20.0	0.32	0.79	0.05	0.00	0.54
B	Synaptic debris, membranes	9.6	2.05	1.85	1.05	0.00	0.94
C	Nerve endings	23.0	1.66	1.13	2.05	0.17	1.02
D	Nerve endings	32.0	0.77	0.91	1.22	1.16	1.42
E	Free mitochondria	15.4	0.72	0.71	0.26	2.28	1.01
			Table 1c				
			Submitochondrial fractions after osmotic shock				
M_1	Myelin, mitochondria, nerve endings, ghosts	65.2	0.40	0.49	0.33	1.41	0.39
M_2	Synaptic vesicles, membranes	10.8	2.56	2.46	0.51	0.00	1.04
M_3	Soluble	24.0	1.93	1.72	3.05	0.00	2.46

* Unpublished results.

fractions (A to E) with the morphological composition shown in table 1b can be isolated for study under the electron microscope and by chemical analysis (20). Another technique, based on the hypotonic treatment of the mitochondrial fraction, results in the swelling and bursting of the nerve endings with release of the synaptic vesicles and other components. By differential centrifugation, three fractions can be separated: M_1 contains the nerve ending-ghosts, myelin, and mitochondria; M_2 is mainly composed of synaptic vesicles; and M_3 represents the soluble axoplasm (table 1c) (22, 23).

The use of these two techniques has given interesting information about the subcellular localization of the acetylcholine and 5-hydroxytryptamine (5-HT) systems (see 13, 59) and of the enzymes related to the glutamic acid, glutamine, and γ-aminobutyric acid cycles (47). Thus acetylcholine, acetylcholinesterase,

and cholinacetylase are concentrated in the C fraction of nerve endings and acetylcholine and cholinacetylase in the synaptic vesicles isolated from the rat brain (20, 23). On the other hand glutamic decarboxylase, the enzyme that synthesizes γ-aminobutyric acid, is concentrated in the nerve endings of fraction D.

Here the results obtained on the localization of NE and dopamine, assayed by the method of Bertler *et al.* (5), and of some of the enzymes related to the metabolism of these amines will be briefly summarized. NE and dopamine are concentrated in the mitochondrial and microsomal fractions; about 50 % of NE and 45 % of dopamine are in the mitrochondrial fraction. Dopamine is more soluble than NE (table 1a). 5-Hydroxytryptophan decarboxylase is mainly soluble, but a large proportion of the bound enzyme is present in the mitochondrial fraction. MAO is mainly bound to mitochondria, and catechol-O-methyltransferase (COMT), although mainly soluble, is also present there. Among the subfractions of the mitochondrial fraction, NE and dopamine are concentrated in fractions B and C, which contain synaptic debris and nerve endings, respectively; NE shows a higher concentration there than dopamine (table 1b). 5-Hydroxytryptophan decarboxylase and COMT are localized in fractions C and D, the nerve ending fractions; while MAO is a mitochondrial enzyme.

Laverty *et al.* (34) observed that most of the dopamine in the caudate nucleus of the dog is soluble and the remainder is associated with nerve endings. Also related to the localization of NE is the work of Potter and Axelrod (43), who demonstrated that radioactive-NE is taken up by the layer of the gradient containing nerve endings. More recently Snyder *et al.* (51), after intraventricular injection of H^3-NE, H^3-E and H^3-dopamine, found them concentrated in the nerve ending fraction. After hyposmotic shock NE and dopamine are concentrated in M_2, MAO remains with the mitochondria in fraction M_1 and 5-hydroxtryptophan decarboxylase and COMT are solubilized into M_3 (table 1c). The first report of NE in synaptic vesicles was presented at the Galesburg Meeting on Biogenic Amines in January 1963 (12). Further studies on the localization of NE and dopamine in synaptic vesicles were presented by Zieher and De Robertis (60) and De Robertis *et al.* (21). Maynert *et al.* (37) reported NE and 5-HT concentration in vesicles from nerve endings disrupted by ultrasound and hyposmotic shock.

ISOLATION OF NERVE ENDINGS AND SYNAPTIC VESICLES FROM THE ANTERIOR HYPOTHALAMUS.

The finding of special axons and nerve endings containing granulated vesicles in the anterior hypothalamus of the rat led us to attempt the isolation of nerve endings and synaptic vesicles from this region of the brain, which is specially rich in NE (21). The anterior and intermediary hypothalamus were homogenized and fractionated. Homogenates from total brain were prepared and compared with the above. In the mitochondrial and the nerve ending fractions of the hypothalamus, numerous isolated nerve endings are found (fig. 2). They are identical with those observed in the intact hypothalamus (fig. 1) and filled with vesicles of different size and shape. The largest ones are empty or contain a dense deposit of osmium separated by a clear rim from the enveloping membrane. Some vesicles

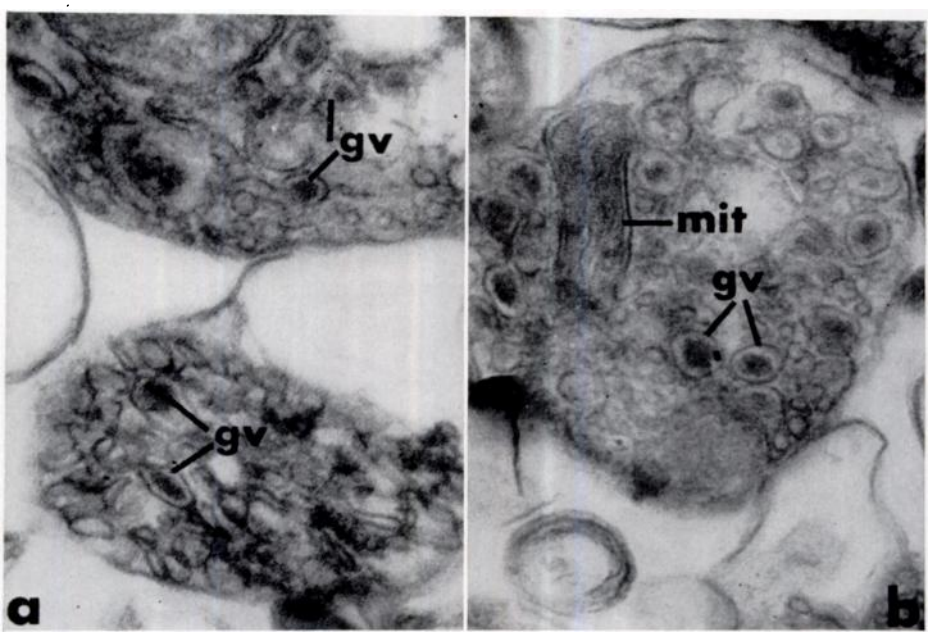

FIG. 2. Electron micrograph of isolated nerve endings from the anterior hypothalamus of the rat. The three endings in this figure contain granulated vesicles (gv) and in one there are elliptical clear vesicles. mit, Mitochondria. a and b, × 80,000. (From De Robertis *et al.*, Life Sci. **4**: 193, 1965.)

are elliptical and may represent stages in the development of the granulated vesicles (fig. 2). The electronmicrographs from M_2 show the polymorphism of the vesicular material (fig. 3). Small synaptic vesicles identical to those found in the corresponding fraction of total brain (22, 23) are intermingled with elongated ones and much larger elements. In spite of the osmotic shock, which probably has released part of their content, many vesicles show a small deposit of osmium. The number of granulated vesicles may vary between 10 and 20% while in M_2 from total brain they are found only exceptionally (23). The remarkable similarity of the histograms of isolated synaptic vesicles to those *in situ* suggests their identity (21).

In table 2 the content of NE of the hypothalamus is compared to that of total brain. In both cases the highest relative specific concentration is in M_2. In absolute values there is about 10 times more NE per gram in the vesicular fraction of the hypothalamus than in whole brain.

In figure 4 the highest NE content is in the synaptic vesicle fraction with 38 ng NE per mg protein in hypothalamus as against 7.1 ng NE per mg protein in brain; in other words, hypothalamus has 5.3 times more NE in this fraction than does brain. In the hypothalamus, the synaptic vesicle fraction has 2.5 times more NE (ng/mg protein) than does the total homogenate.

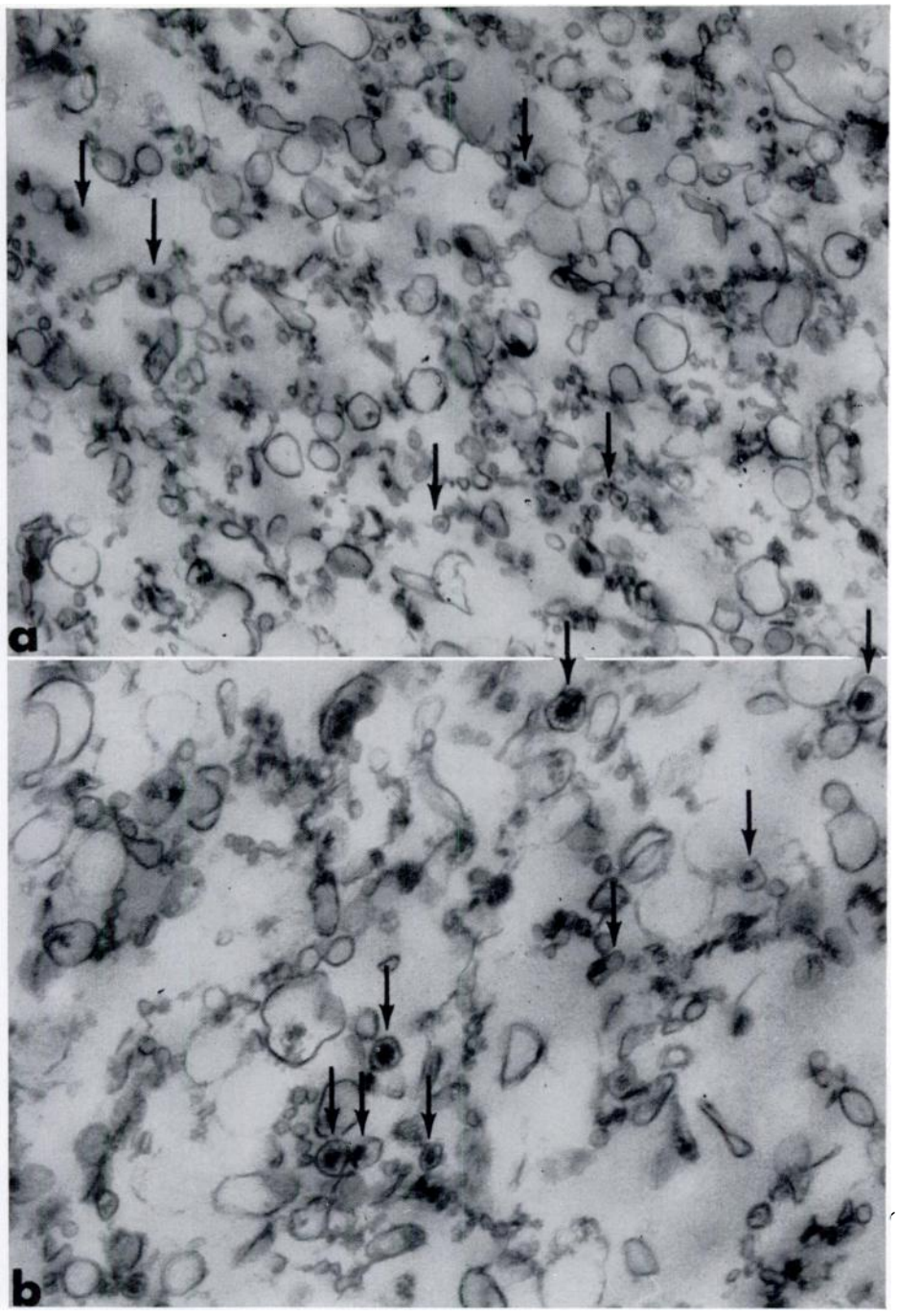

FIG. 3. Electron micrographs of the isolated vesicular fraction from the rat hypothalamus See the variety of sizes and shapes of the isolated vesicles and the presence of numerous granulated ones (arrows). a, × 45,000; b, × 70,000. (From De Robertis *et al.*, Life Sci. **4:** 193, 1965.)

TABLE 2

Comparison of whole brain and hypothalamus with respect to NE in subfractions of the crude mitochondrial fraction after osmotic disruption

Fraction	Ultrastructure	Norepinephrine			
		Total brain		Hypothalamus	
		ng/g	Relative specific concentration	ng/g	Relative specific concentration
M_1	Synaptic ghosts Mitochondria Myelin	32.4	0.40	730	0.67
M_2	Synaptic vesicles	34.2	2.56	300	1.94
M_3	Soluble	57.5	1.93	550	1.58

NE is expressed in ng (10^{-9} g) per g wet tissue and in relative specific concentration, *i.e.*, percent recovered NE divided by percent recovered protein. Absolute values of NE in mitochondrial fraction for total brain: 111 ng/g; for hypothalamus: 1100 ng/g. Recovered NE for total brain: 111%; for hypothalamus: 148%. Absolute values of protein in mitochondrial fraction for total brain: 33.0 mg/g; for hypothalamus: 57.6 mg/g. Recovered protein for total brain: 90%; for hypothalamus: 126% (from ref. 21).

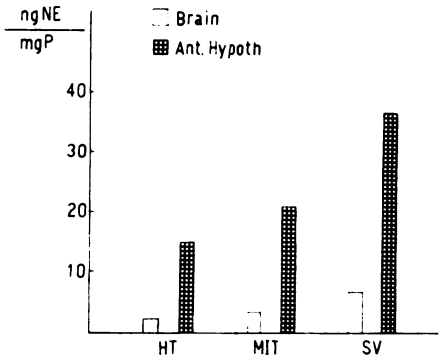

FIG. 4. Diagram showing the concentration of NE in ng/mg protein in total homogenate (HT), crude mitochondrial fraction (MIT), and synaptic vesicle fraction (SV) of both of the total brain (exclusive of the cerebellum) and the anterior and intermediary hypothalamus of the rat. (From De Robertis *et al.*, Life Sci. **4**: 193, 1965.)

BIOCHEMICAL ORGANIZATION OF THE ADRENERGIC SYNAPTIC COMPLEX

The correlations summarized above suggest a tentative scheme of spatial organization of the catecholamines of the brain and some of the key enzymes involved in their metabolism in relation to the synaptic complex.

There is considerable evidence that bound NE and dopamine are present in the nerve ending fraction from total brain (10, 12, 13, 33, 34, 43, 51, 60) and from the anterior hypothalamus (21). The disruption of the nerve ending by osmotic shock demonstrates that the fraction containing the synaptic vesicles has the highest relative specific concentration of both NE (12, 37) and dopamine (60). This

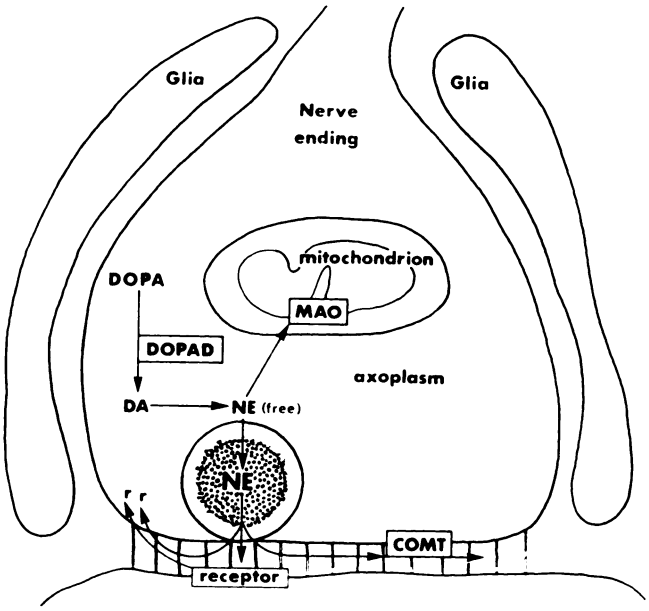

FIG. 5. Diagram of a central noradrenergic synapse in which the main data from electron microscopic and cell fractionation studies are indicated. The nerve ending with the mitochondrial, axoplasmic, and synaptic vesicle compartments makes synaptic contact with the second neuron. See the intersynaptic filaments attaching the two synaptic membranes (23). MAO localized in the mitochondria of the ending (and in others) may inactivate free NE and dopamine. Dopa decarboxylase is in the axoplasm of the ending and may produce dopamine from dopa. Dopamine is transformed into NE, which can be free or become trapped and bound within the synaptic vesicle. A similar mechanism of binding may take place in dopaminergic synapses. Granulated vesicles are thought to be the quantal units of NE ready to be released upon arrival of the nerve impulse. The NE released reacts with the receptor and may be inactivated by refixation (r) on the ending or by the action of COMT.

indicates that the synaptic vesicle is the main store for NE and dopamine as was previously demonstrated for acetylcholine (23). For the three transmitters, the synaptic vesicle represents a multimolecular or quantal unit of storage and release. The results on NE in the anterior hypothalamus, in which a large proportion of granulated vesicles is found both in the axons and terminals *in situ* and in the vesicular fraction, as well as the pharmacological investigations on the adrenergic nerve endings in the pineal gland (39, 40) and hypothalamus (36, 49), suggest that the granulated vesicles are the containers of the adrenergic transmitter. The presence of a membrane in these granulated vesicles would protect the NE stored from the action of the inactivating enzymes (fig. 5).

The results on the localization of 5-hydroxytryptophan decarboxylase, and by extension of dopa decarboxylase, indicate that this enzyme that synthesizes both 5-HT and dopamine, in spite of its solubility, is contained within the nerve ending (46, fig. 5). It may be postulated that dopa decarboxylase is probably in the axo-

plasm of the ending not bound to any special intrasynaptic structure. The dopamine synthesized may remain free in the axoplasm or may be stored within the synaptic vesicles of dopaminergic synapses. The finding of a larger proportion of free dopamine than NE is in accordance with this line of reasoning. In noradrenergic synapses it may become hydroxylated and transformed into NE. Unfortunately the localization of dopamine-β-hydroxylase within the synaptic complex has yet not been determined.

Cell fractionation methods give information also about the subcellular distribution of MAO and COMT, the two enzymes that inactivate catecholamines. The mitochondrial localization of MAO indicates that it could act intracellularly within the nerve ending, controlling the level of free amine, whereas a postsynaptic action of this enzyme, after release of the transmitter at the synaptic cleft, would be more difficult to explain on these structural bases (fig. 5). COMT (2) is now definitely located in nerve endings, but its high degree of solubility and lack of structural binding makes very difficult its fine localization within the synaptic complex. This enzyme may have a synaptic action, as postulated by Axelrod (1), but in adrenergic synapses the refixation or uptake of the NE liberated at the ending may be another important mechanism of physiological inactivation.

Figure 5 should be considered as a tentative diagram of a noradrenergic synapse of the brain in which the main data from the electron microscope and cell fractionation studies are put together in a manner susceptible of physiological interpretation. It differs from other models in the use of these data to reveal the true structure of the synapse and thus introduces more facts and less fancy to explain the function of an adrenergic synapse.

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