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RECEPTORS OF NEUROTRANSMITTERS—V

SIALIC ACID DISTRIBUTION AND CHARACTERIZATION OF THE 5-HYDROXYTRYPTAMINE RECEPTOR IN SYNAPTIC STRUCTURES

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Abstract—Electron microscopy and binding experiments of exogenous 5-hydroxytryptamine-3-^{14}C to sialic acid containing structures of the rat brain were employed to elucidate the chemical nature and the distribution of the 5-hydroxytryptamine receptor in isolated synaptic vesicles and nerve ending membranes. Sialic acid was localized in synaptic structures and differentiated from monoesters of sulfuric acid by electron microscopy after treatment with colloidal iron hydroxide. The distribution of 5-hydroxytryptamine (5-HT*) and sialic acid in isolated synaptic vesicles and nerve ending membranes was analyzed before and after partition between *n*-butanol and water. The sialic acid and 5-HT content was particularly high in the butanol phase of the membrane fractions. This is in accordance with the concept that sialoglycoproteins or sialoglycolipids are components of the 5-HT receptor in the CNS.

EARLIER studies of the 5-HT receptor of the rat stomach fundus[1] and the liver fluke, *Fasciola hepatica*,[2] indicated a correlation between 5-HT binding and sialic acid metabolism. Sialic acid was incorporated into smooth muscle preparations[3] and stimulated the 5-HT induced contraction whereas inhibitors of sialic acid biosynthesis decreased the contraction height. Carroll and Sereda[4] observed that in uterine smooth muscle membranes the sialic acid was chiefly in the form of glycoproteins and not of lipid-soluble gangliosides. The authors suggested that the sialic acid group of sialoglycoproteins is important for the binding of 5-HT to smooth muscle membranes.

In an attempt to compare the chemical nature of 5-HT receptors in different organs we studied in the present paper the 5-HT binding to sialic acid containing synaptic structures of rat brain. Two different binding sites of the CNS for 5-HT were investigated: the synaptic vesicles as the storage organelles and the synaptic membranes where the chemical mediated synaptic transmission takes place and leads to the depolarization of the subsynaptic membranes. As staining procedures have recently been applied to identify acid mucopolysaccharides with colloidal iron hydroxide on the electron microscopic level[5,6] we tried to localize sialic acid as the possible binding group for 5-HT in synaptic vesicles and membranes.

MATERIALS AND METHODS

Isolation of subcellular fractions. Total brain was dissected from male rats, strain Wistar II, 180–200 g. Nerve ending membranes and synaptic vesicles were isolated by

* Abbreviations used: 5-HT = 5-hydroxytryptamine (serotonin); neuraminidase = mucopolysaccharide *N*-acetylneuraminylhydrolase (EC 3.2.1.18); NeuNAc = *N*-acetylneuraminic acid; CNS = central nervous system.

differential and sucrose gradient centrifugation according to methods described before[7] but employing a modified gradient as shown by Fig. 1. This is a variation of the methods first published by Whittaker[8] and De Robertis.[9]

Analytical methods. Protein was assayed in the water phase and in the pellets according to Lowry *et al.*[10] and in the butanol extracts according to Hess and Lewin.[11] 5-HT was estimated fluorimetrically using the reaction with ninhydrin to increase the

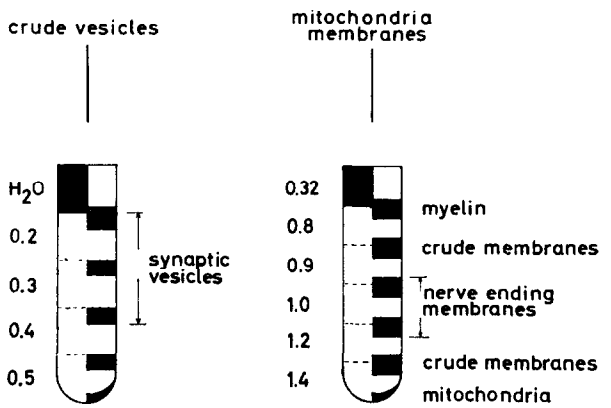

FIG. 1. Sucrose density gradients for the isolation of synaptic vesicles and nerve ending membranes at 51,000 *g*, 2 hr.

fluorescence yield.[12] Sialic acids were determined with thiobarbituric acid[13] as *N*-acetylneuraminic acid after treatment with neuraminidase or 1 hr hydrolysis with 0·1 N H_2SO_4 at 80°.

Enzyme treatment. Purified *Vibrio cholerae* neuraminidase, activity 500 U/ml, was obtained from Behringwerke, Marburg, Germany. Incubation was carried out at 37° and pH 5·5 in 0·05 M Na-acetate buffer containing 0·9% NaCl and 0·1% $CaCl_2$. To 1 ml of each subcellular fraction containing 1·1–1·4 mg protein 0·5 ml of the enzyme preparation were added. After an incubation period of 10 hr another aliquot (0·5 ml) of neuraminidase was added and the incubation continued for a total of 14 hr. These samples along with the controls which had been incubated with buffer only were centrifuged at 150,000 *g*, 30 min. The resulting pellets were washed twice with aqua dest. and used for the thiobarbituric acid assay and for electron microscopy.

Incorporation and extraction procedure. Aliquots of the subcellular fractions were suspended in 4 ml 0·32 M sucrose to give a final concentration of 0·6–1·2 mg protein/ml. The sucrose solution was buffered to pH 7·0 with 0·01 M tris-buffer (pH 7·8). 5-hydroxytryptamine-3-[^{14}C]creatinine sulfate (The Radiochemical Centre, Amersham) was added to give a final concentration of 5×10^{-6} M and a specific activity of 9·1 mc/m-mole. After incubation for 30 min at 37° the samples were centrifuged at 225,000 *g*, 60 min. The pellets were washed twice with a 5×10^{-6} M solution of 5-HT and resuspended in distilled water. An aliquot of each suspension was used to determine the total radioactivity incorporated (control value). The extraction procedure was carried out according to Fiszer and De Robertis[14] with the minor modification that 2 ml of the vesicle or membrane suspension containing 0·9–1·4 mg protein were extracted twice with 2 ml *n*-butanol. The emulsion obtained was separated by centri-

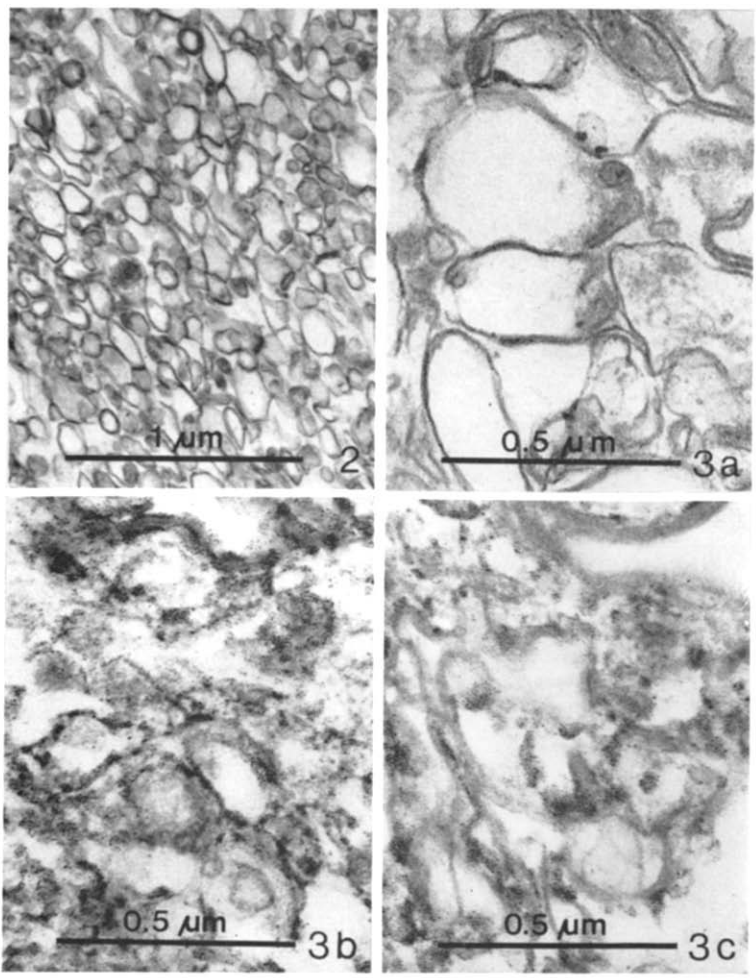

FIG. 2. Synaptic vesicles from rat brain isolated from 0·2 M sucrose (×35,000).

FIG. 3. Nerve ending membranes from rat brain isolated from 1·0 M sucrose (×70,000). (a) Untreated. (b) Treated with colloidal iron hydroxide. (c) Incubated with neuraminidase and treated with colloidal iron hydroxide.

fugation at 11,000 *g*, 15 min, into the water phase, the butanol layer, and a pellet. The radioactively labeled samples were counted in Bray's solution[15] using a liquid scintillation counter (Tri-Carb 3365, Packard) with external standardization.

Electron microscopy. The variously incubated vesicle and membrane fractions were fixed in glutaraldehyde and treated with colloidal iron hydroxide (Hale's stain) according to Benedetti and Emmelot.[5] The pellets were postfixed in 1% osmium tetroxide and embedded in Epon. Electron microscopy of the thin sections was performed after staining with uranyl acetate with a Siemens Elmiskop 101. In control experiments the Hale's stain reaction was omitted and the sections were counterstained with lead citrate.

RESULTS

The vesicles were separated into four main fractions and a precipitate by centrifugation in a discontinuous sucrose gradient (Fig. 1). The small layer on top of the 0·2 M sucrose represented small vesicles with a diameter of 400–500 Å known to be the storage sites for acetylcholine. In the 0·2 and 0·3 M sucrose layer vesicles were concentrated with the high 5-HT content of 0·188 and 0·177 nmoles/mg protein, respectively (Fig. 2). In the 0·4 and 0·5 M sucrose layer and in the precipitate vesicles were found which are heavily contaminated with membranes. The nerve ending membranes isolated from the 1·0 and 1·2 M sucrose layer (Fig. 1) corresponded well to the synaptic membranes described in the literature[8,9] (Fig. 3a). Membranes separated from the 0·9 and 1·4 M sucrose were contaminated with myelin or mitochondria and discarded.

Table 1 summarizes the protein content of the two 5-HT containing vesicle fractions and of the nerve ending membranes. After partition between butanol and water the bulk of the vesicle and membrane protein was found in the butanol–water interface and collected as precipitate by centrifugation while only minor amounts of protein were detected in the water phases. The protein content of the organic extracts of the vesicle fractions was significantly higher than that of the nerve ending membranes.

TABLE 1. DISTRIBUTION OF PROTEIN AFTER EXTRACTION OF SUBCELLULAR BRAIN FRACTIONS WITH BUTANOL AND WATER

Fraction	Ultrastructure*	Total amount (mg/wet weight)	No. exp.	Distribution† (%) between Butanol	H_2O	ppt
0·2 M sucrose	synaptic vesicles	0·24 ± 0·04‡	14	23	7	68
0·3 M sucrose	synaptic vesicles	0·26 ± 0·02	14	17	7	77
1·0 M sucrose	nerve ending membranes	0·74 ± 0·07	16	8	4	85
1·2 M sucrose	nerve ending membranes	1·08 ± 0·11	16	7	4	87

* Examined by electron microscopy.
† The concentration of each subcellular fraction before the extraction procedure was taken as 100%.
‡ Standard deviation.

The results in Table 2 show that only 10–25 per cent of the total amount of *N*-acetylneuraminic acid were extracted by butanol while 70–85 per cent were recovered in the precipitates. Despite of this high sialic acid percentage in the precipitates the relative specific concentration as a measure of the sialic acid content on a protein basis

was lower in the precipitates (0·8) than in the butanol phases (2·5 and 3·2) of the membranes. Thus, related to protein, sialic acid was enriched in the organic layer. The contrary holds true for the butanol extracts of the vesicle fractions with a relative sialic acid concentration of 0·4 and 0·6 indicating a reduction of the sialic acid content.

TABLE 2. ANALYSIS OF *N*-ACETYLNEURAMINIC ACID AFTER EXTRACTION OF SUBCELLULAR BRAIN FRACTIONS WITH BUTANOL–WATER

Fraction	Total amount (nmoles/g wet weight)	No. exp.	Distribution* (%) between Butanol	H_2O	ppt	Relative specific concentration† in Butanol	H_2O	ppt
0·2 M sucrose	5·5 ± 0·6‡	5	10	5	84	0·4	0·7	1·2
0·3 M sucrose	5·7 ± 0·5	5	11	4	83	0·6	0·5	1·1
1·0 M sucrose	10·8 ± 0·3	7	20	9	70	2·5	2·1	0·8
1·2 M sucrose	11·9 ± 0·9	8	24	4	71	3·2	0·8	0·8

* The concentration of each subcellular fraction before the extraction procedure was taken as 100%.

† The relative specific concentration is a measure of the *N*-acetylneuraminic acid content with reference to protein: $\frac{\%\ \text{NeuNAc}}{\%\ \text{protein}}$.

‡ Standard deviation.

The distribution of 5-HT was measured in the same fractions. Radioactively labeled 5-HT was enriched in all butanol phases with respect to the percentage recovery as well as to the relative specific activity (Table 3). Between 70 and 75 per cent of the total radioactivity incorporated by the vesicles was extracted by butanol as compared with about 50 per cent found in the butanol layer of the membranes.

TABLE 3. RELATIVE DISTRIBUTION OF EXOGENOUS 5-HYDROXYTRYPTAMINE-3-^{14}C IN BUTANOL–WATER EXTRACTS OF SUBCELLULAR FRACTIONS OF RAT BRAIN

Fraction	Radioactivity* (%) in Butanol	H_2O	ppt	Relative specific activity† in Butanol	H_2O	ppt
0·2 M sucrose (5)‡	73	4	22	3·2	0·6	0·3
0·3 M sucrose (5)	71	5	23	4·2	0·7	0·3
1·0 M sucrose (7)	54	5	40	6·8	1·2	0·5
1·2 M sucrose (7)	49	4	45	7·0	1·0	0·5

* The subcellular fractions were incubated with 5×10^{-6} M [^{14}C]-5-HT, 9·1 mc/m-mole, and extracted with butanol–water, the total radioactivity before the extraction procedure was taken as 100%.

† The relative specific activity $\left(\frac{\%\ [^{14}\text{C}]\text{-5-HT}}{\%\ \text{protein}}\right)$ before the extraction is by definition 1.

‡ Figures in parentheses refer to numbers of experiments.

As shown by electron microscopy, granular material was deposited on the membranes after treatment with colloidal iron hydroxide, marking the sites of reaction between iron and the acidic groups (Fig. 3b). Incubation with neuraminidase reduced the number of iron granules in the membrane fraction (Fig. 3c). The same results were obtained with the vesicle fractions.

DISCUSSION

The present study was designed in an attempt to extend the chemical characterization of the 5-HT receptors in the CNS. Two morphologically and physiologically different structures were investigated: the synaptic vesicles with the highest 5-HT content of all brain fractions as the storage sites of the transmitter and the nerve ending membranes as the binding sites for 5-HT at the synaptic cleft. Radioactively labeled 5-HT was incorporated by vesicles as well as by nerve ending membranes and was recovered with a high relative specific activity (Table 3) in the butanol extracts. Since this distribution conflicts with the partition of unbound 5-HT[14] our findings could mean that the distribution of 5-HT is mediated by a carrier or receptor substance. The absence of gangliosides in the butanol phase[7,14] and the high relative specific concentration of sialic acid in the membrane extract support the hypothesis that this carrier substance can be classified as sialoglycolipid or sialoglycoprotein. Such an assumption would be in agreement with the chromatographic properties of the "5-HT-complex" described by Fiszer and De Robertis.[14] These authors found that the 5-HT binding of synaptic membranes is related to a proteolipid and cannot be enhanced by the addition of gangliosides. Since sialoglycoproteins have been found in the synaptic region[16] further work has to clarify whether both the storage in vesicles and the binding of 5-HT to nerve ending membranes are mediated by sialoglycoproteins.

Electron micrographs confirmed the analytical results that sialic acid is bound to synaptic vesicles and nerve ending membranes. At the low pH of 1·7 where the Hale stain procedure was carried out only sialic acid and monosulfates were dissociated enough to react with iron hydroxide. Thus the electron dense precipitates in Fig. 3(b) localize the reaction sites of colloidal iron hydroxide with sulfated mucopolysaccharides and sialic acid groups which may be responsible for the binding of 5-HT. After enzymatic release of sialic acid the acidic groups giving a positive Hale stain reaction were diminished. The iron deposits as seen in Fig. 3(c) mark the sulfate esters bound to the membranes. Details on the localization and differentiation of sialic acid from sulfate esters are to be published elsewhere.[17]

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