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COMMENTARY

RADIOLIGANDS OF THE VESICULAR MONOAMINE TRANSPORTER AND THEIR USE AS MARKERS OF MONOAMINE STORAGE VESICLES

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Radioligands have been widely used in the study of receptors, but less in enzymological studies, in which the enzymatic activity is generally a sensitive detection technique. However, for membrane-bound transporters, the activity is more difficult to follow since it is vectorial, requiring the assay of substrate accumulation in a closed compartment. In this case, the labeling by specific radioligands is a convenient scalar assay of the transporter. This approach has been used for the monoamine transporter of chromaffin granules, the catecholamine storage organelles of adrenal medulla chromaffin cells. The radioligands thus developed are valuable tools for the study of the monoamine uptake system of all monoaminergic vesicles. They also allow a reliable labeling of these vesicles.

THE VESICULAR MONOAMINE UPTAKE SYSTEM

Catecholamine uptake by chromaffin granules. The catecholamines adrenaline and noradrenaline are released into the bloodstream by adrenal glands following stimulation of the splanchnic nerve. Release occurs by exocytosis of the chromaffin granules and, since their content is diluted in the systemic circulation, it is not surprising that the catecholamine concentration in the granules is very high (about 0.5 M). Studies performed with "ghosts" obtained by osmotic lysis of purified chromaffin granules have indicated that this high concentration results from an active ATP-dependent uptake of catecholamines by the granule membrane.

The mechanism of this active transport has been unravelled in the last 10 years by several laboratories (see Refs. 1–4 for a review). Catecholamine uptake is a secondary transport coupled to and driven by the transmembrane proton electrochemical gradient ($\Delta\mu H^+$) generated by an inwardly directed proton pump ATPase. The pump uses cytosolic ATP, and it is related to the proton pump of plant vacuoles, lysosomes and coated-pit vesicles but is different from that of mitochondria (see Refs. 1–3 and 5 for a review). In chromaffin granules, the $\Delta\mu H^+$ drives catecholamine uptake by activating a monoamine transporter sensitive to both the transmembrane pH gradient ΔpH and the transmembrane potential gradient $\Delta\Psi$ [1–3, 6, 7]. The transporter catalyzes an electrodissipative catecholamine/proton antiport, and generates a monoamine gradient proportional to $\Delta\Psi + 2Z\,\Delta pH$ (where $Z = RT/F$).

The kinetic parameters of the uptake process have been analyzed in detail [1, 2]. The transporter has a low specificity, since it translocates with similar efficiency not only dopamine, noradrenaline and adrenaline, but also monoamines that are not present in the adrenal medulla, such as serotonin and tyramine. Moreover, non-physiological compounds structurally unrelated to monoamines, such as *m*-iodobenzylguanidine [8], a radiopharmaceutical used for pheochromocytoma imaging, and the quaternary ammonium 1-methyl-4-phenylpyridinium, the active metabolite of the Parkinson's syndrome-inducing drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [9, 10], are also substrates of the transporter.

Three different classes of agents block vesicular monoamine uptake: inhibitors of the H^+-pump, H^+ ionophores, and specific inhibitors of the monoamine transporter. The last compounds do not affect the ATP-induced $\Delta\mu H^+$, and they inhibit the catecholamine uptake driven by a pH gradient artificially imposed on chromaffin granule membrane vesicles to bypass the proton-pump [11]. The alkaloid reserpine and the quinolizine tetrabenazine are representatives of this class of inhibitors (Fig. 1). Both drugs are monoamine depleting agents *in vivo* [12]. The tetrabenazine depleting effect is of short duration, whereas that of reserpine has a long lasting action. Reserpine, which is an analog of serotonin, is the most powerful inhibitor, characterized by a subnanomolar value of the *in vitro* inhibition constant K_i (see Table 1, Ref. 13). At a micromolar concentration, reserpine has also additional effects on $\Delta\mu H^+$ generation by the proton-pump [14]. Tetrabenazine, which is an analog of dopamine, inhibits catecholamine uptake with a K_i of 3 nM [15]. It has no effect on $\Delta\mu H^+$ generation at concentrations up to 20 μM [11]. Ketanserin (Fig. 1), a well-known antagonist of serotoninergic S_2 receptors [16], is also an inhibitor of catecholamine uptake by chromaffin granules (K_i = 70 nM, Ref. 17). Finally, drugs such as phenothiazines, haloperidol or spiperone are inhibitors of catecholamine uptake in the 1–100 μM concentration range, but these compounds simultaneously affect the transporter and the ATP-

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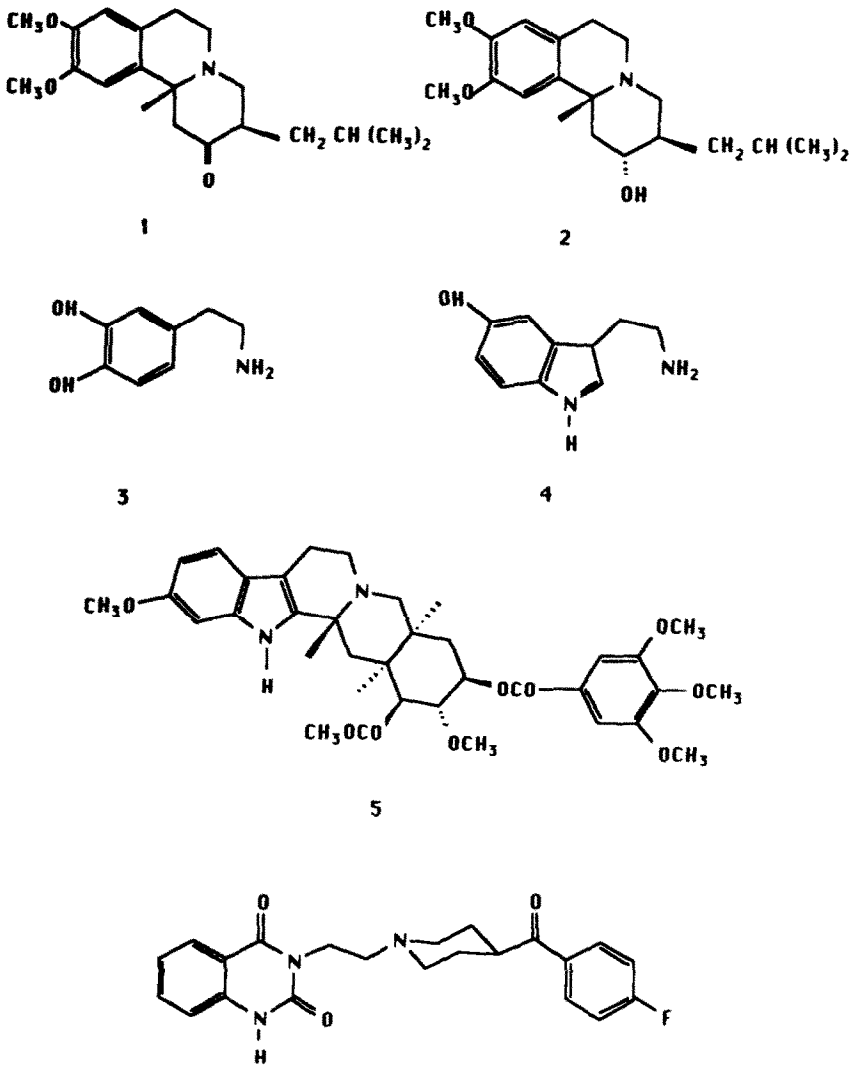

Fig. 1. Chemical structures of: (1) tetrabenazine; (2) dihydrotetrabenazine; (3) dopamine; (4) serotonin; (5) reserpine; and (6) ketanserin.

induced $\Delta\mu H^+$ ([11] and unpublished data).

Monoamine uptake in other storage vesicles. Numerous secretory cells produce and store in granules (or vesicles) the biogenic monoamines serotonin, dopamine, noradrenaline and adrenaline. Serotonin-containing vesicles have been described in brain neurons, platelets, enterochromaffin cells, mast cells, basophils and some peptidic hormone secreting cells such as pancreatic B-cells. Catecholamine vesicles have been observed in central (dopamine, noradrenaline, adrenaline) and peripheral (noradrenaline) neurons, and in the adrenal medulla (noradrenaline, adrenaline). In spite of important differences in their composition, all storage vesicles are likely to accumulate monoamines by an identical mechanism. This hypothesis initially arose from the general depleting effect of reserpine [12]. It was subsequently confirmed by *in vitro* studies on isolated vesicles since: (i) monoamine uptake is in all systems inhibited by reserpine and dependent upon $\Delta\mu H^+$ generation by a proton pump ATPase [18–30], and (ii) the same monoamine specificity is observed, characterized by a K_m value for serotonin of about 1 μM at pH 7.5, and by 2- to 8-fold higher K_m values for catecholamines [19, 24, 27–29, 31–33].

Histamine is a biogenic amine present in storage vesicles from central neurons, mast cells and platelets from various species such as rabbit and pig, and it seems to be taken up by the same ATP-dependent reserpine-sensitive process as the other monoamines, as suggested by *in vivo* [34] and *in vitro* [23, 35] data. The rate of histamine uptake however, is more than 20-fold slower than that of serotonin and catecholamines [35–37], presumably due to a lower affinity of uptake [23, 38]. On the other hand, the quaternary ammonium acetylcholine is taken up into

storage vesicles by an active ATP-dependent process which is unrelated to that of monoamines [39].

Comparison between vesicular and plasma membrane monoamine transporters. Monoaminergic cells, and especially monoaminergic neurons, possess a second monoamine uptake system located on the plasma membrane. Monoamine uptake by this system is also an active secondary transport. However, plasma membrane uptake differs from the vesicular one by three main points: (i) the former is coupled to Na^+ influx, and not to an H^+ efflux [3]; (ii) in contrast with the vesicular uptake, serotonin, dopamine and noradrenaline are taken up by different plasma membrane transporters characterized by distinct pharmacologies; and (iii) plasma membrane and vesicular uptakes have different inhibitors: for instance, serotonin plasmatic uptake is inhibited by imipramine and paroxetine, and is insensitive to reserpine and tetrabenazine [40]. Thus, the two classes of transporter have different pharmacologies, and radioligand binding does not indicate any cross-reactivity.

LIGANDS OF THE CHROMAFFIN GRANULE MONOAMINE TRANSPORTER

Radioligands of receptors may be chosen among agonists or antagonists. In the case of a transporter, the choice is limited to inhibitors. Radioligands derived from three inhibitors of vesicular monoamine uptake, tetrabenazine, reserpine and ketanserin, have been studied in our laboratory.

Ligands derived from tetrabenazine. Because of its high specificity, tetrabenazine was the first molecule tested to label the monoamine transporter [15]. This compound was radiolabeled by reduction of its keto-group by tritiated borohydride [41]. Of the two diastereoisomers thus obtained and which can now be purchased from CEA, Gif sur Yvette, France, the most abundant, α-[2-^{3}H]dihydrotetrabenazine ([^{3}H]TBZOH), has the highest affinity for chromaffin granule membranes [42]. Binding can be analyzed by filtration on cellulose or glass fiber filters, by centrifugation or by gel filtration, and is characterized by low nonspecific binding [15]. Specific binding is restricted to chromaffin granule membranes and occurs on one homogeneous class of binding sites, the characteristics of which are summarized in Table 1. The excellent correlation existing between the occupancy of these sites and the inhibition of monoamine uptake indicates that the sites are associated with the monoamine transporter.

[^{3}H]TBZOH is displaced from its binding sites by various substrates and inhibitors of uptake. For inhibitors, the concentration displacing 50% of the ligand (EC_{50}) is identical to the IC_{50} for inhibition of uptake. However, it should be stressed that substrates displace [^{3}H]TBZOH very poorly. In a Michaëlian system, a substrate displaces theoretically 50% of a competitive inhibitor at a concentration equivalent to its K_m. Such a result is not observed with [^{3}H]TBZOH; the EC_{50} and K_m values differ by two orders of magnitude.

Another characteristic of [^{3}H]TBZOH binding is that it is independent of the ATP-induced $\Delta\mu\tilde{H}^+$, which affects neither the binding kinetics nor the equilibrium values [13]. This characteristic, added to the fact that monoamines have a low affinity for the tetrabenazine binding site, makes [^{3}H]TBZOH a valuable radioligand of the monoamine transporter in crude homogenates (see below). It may be noted that [^{3}H]TBZOH binds also to the transporter solubilized by detergents such as cholate, digitonin, or octyl β-glucoside [43].

Various derivatives of tetrabenazine have been tested as ligands of the monoamine transporter. These compounds were all derivatized by reduction of the keto group of tetrabenazine, leading to unsubstituted and substituted amines and to various esters of dihydrotetrabenazine. The affinity of these compounds is proportional to their hydrophobicity, measured by their octanol–water partition coefficient [42].

Arylazido derivatives of tetrabenazine have also been prepared [4, 44]. These compounds are useful for photolabeling the monoamine transporter. In most of the molecules synthesized, γ-aminobutyric acid (GABA) was used to link the arylazido to the tetrabenazine moiety, giving an equilibrium dissociation constant of about 50 nM. These compounds were tritiated in different ways, and it was observed that the higher the specific radioactivity was, the more specific was the covalent labeling. Electrophoresis analysis of the photolabeled material indicated a molecular weight of 70 kD. This value was confirmed by target size analysis, utilizing the technique of inactivation by ionizing radiation which gave a figure of 68 kD for the functional molecular weight of the [^{3}H]TBZOH binding site [45].

Ligands derived from ketanserin. Ketanserin is a classical ligand of the serotonergic S_2 receptor [16], though binding to the α_1 adrenoceptor and the histamine H_1 receptor have also been reported [46]. In addition, [^{3}H]ketanserin binds in the brain, especially in the striatum, to "nonspecific displaceable sites", from which it is displaced by an excess of ketanserin, but not by S_2, α_1 and H_1 antagonists [47]. Serotonin is efficient on these sites only in the millimolar concentration range. Recently, these striatal "nonspecific displaceable sites" have been identified as the [^{3}H]TBZOH binding site of the monoamine transporter of dopaminergic synaptic vesicles, for the following reasons [17]: (i) [^{3}H]ketanserin specifically binds to purified chromaffin granule membranes on the [^{3}H]TBZOH binding site, as shown by similar density and pharmacology (Table 1); (ii) ketanserin is a competitive inhibitor of [^{3}H]TBZOH binding, and it inhibits noradrenaline uptake with a K_i value identical to its K_d of binding; and (iii) ketanserin is displaced from "nonspecific displaceable sites" of the striatum by tetrabenazine.

The equilibrium dissociation constant is 45 nM at 30° and 6 nM at 0°. The half-life of dissociation is too short to be measured at 30°, and it is 40 sec at 0°. This rapid dissociation is likely to be responsible for the lack of a monoamine depleting effect of ketanserin. The binding assay procedure had to be adapted to this fast dissociation.

In addition to [^{3}H]ketanserin, several iodinated derivatives of ketanserin are commercially available

Table 1. Characteristics of reserpine, TBZOH and ketanserin binding to chromaffin granule membranes and inhibition of catecholamine uptake

	[^{3}H]Reserpine*	[^{3}H]TBZOH†	[^{3}H]ketanserin‡
(A) Binding characteristics:			
ATP dependence	+	−	−
Binding at equilibrium			
K_d (nM)	0.030	3.0	45
B_{max} (pmol/mg protein)	13	60	65
Kinetic constants			
k_{+1} (/sec/M)	4.0×10^5	2.2×10^5	
k_{-1} (/sec)	1.2×10^{-5}§	1.8×10^{-3}	
$T_{\frac{1}{2}}$ (hr)	16	0.11	$< 10^{-3}$
Displacement by substrates (EC_{50}, μM)			
Noradrenaline	20	1200	800
Serotonin	−	240	250
(B) Inhibition of noradrenaline uptake:			
(IC_{50}, nM)	< 0.5	3	70

Binding data are for tritiated analogs of reserpine, TBZOH and ketanserin. K_d: equilibrium dissociation constant. k_{+1}: association rate constant; k_{-1}: dissociation rate constant. Values were obtained at 25° for reserpine and TBZOH, and at 30° for ketanserin.

* From Refs. 13 and 53.

† From Ref. 15.

‡ From Ref. 17.

§ Calculated as $k_{-1} = K_d \cdot k_{+1}$.

from New England Nuclear (Boston, MA). Because of the high specific activity of ^{125}I-labeled molecules, [^{125}I]iodoaminoketanserin and [^{125}I]iodoazidoketanserin may be specially useful to label the vesicular monoamine transporter ([48], and Darchen and Scherman, unpublished data). The limitations raised by the rapid dissociation of the ligands have been circumvented by operating at 0°. The equilibrium dissociation constants have been estimated as 5 and 10 nM respectively (unpublished data). [^{125}I]Iodoazidoketanserin has been used to photolabel chromaffin granule membranes [48]. The results are identical to those obtained with tritiated arylazido derivatives of tetrabenazine, but the experiment is easier to perform, because of the higher specific activity of the iodinated ligand and the possibility of rapid autoradiographic detection.

Reserpine. Though reserpine is the oldest and the most potent of the inhibitors of catecholamine uptake, it is only recently that *in vitro* reserpine binding to chromaffin granules has been investigated satisfactorily. The first *in vitro* binding experiments were unsuccessful, and the only way to label rat chromaffin granules was to administer the drug *in vivo* and to isolate the granules after killing the animal [49]. This result and the low turn-over of the radioactivity bound to the granules suggested a covalent binding of reserpine [50]. However, Schuldiner and his colleagues showed that the inhibitory effect of reserpine on catecholamine uptake could be reversed *in vitro* by washing with liposomes [51]. *In vitro* [^{3}H]reserpine binding was reported some years later, when it was shown to be greatly accelerated in the presence of the proton gradient generated by the ATPase pump [13, 52]; this observation may explain the lack of binding in the previous experiments, which had been performed in the absence of ATP and at 0° [50]. Extraction of bound [^{3}H]reserpine by organic solvents indicated that binding was not covalent [13].

Saturation isotherms in the presence of ATP indicated two different sites: a low affinity site, identified as the tetrabenazine binding site, and a high affinity site, described in Table 1 [13, 52]. The equilibrium dissociation constant of this high affinity site was first estimated as 0.4 to 10 nM; however, recent work from our laboratory has indicated that this value was overestimated, and suggested a figure of 30 pM, obtained by working at very low membrane concentrations [53]. The rate of dissociation of reserpine from these sites is difficult to measure experimentally, but it may be calculated from the equilibrium constant and the association rate constant. The half-life of 16 hr thus obtained [53] is consistent with the long lasting *in vivo* amine depleting effect [12]. Another feature of this high affinity binding site is that substrates of uptake are potent displacers of [^{3}H]reserpine, with an EC_{50} equivalent to the K_m of uptake [13, 52].

In chromaffin granule membranes, the density of the high affinity binding site for reserpine represents only 10–20% of that of the [^{3}H]TBZOH binding site (Table 1). This low density is an artifact of the "ghost" preparation, since in intact chromaffin granules both ligands have the same site density [13, 54]. In our hands, the resealing step required for the formation of "ghosts" after osmotic lysis of chromaffin granules has a low yield, as indicated by a decrease in the maximal velocity of uptake [54]. The relative loss of [^{3}H]reserpine sites in "ghosts" reflects the fact that ATP-dependent binding on these sites requires resealed functional vesicles.

The synthesis of reserpine photoactivable derivatives has not been reported. However, target size analysis experiments indicated for the high affinity reserpine binding site a molecular weight of 40 kD

[45], significantly lower than that of the tetrabenazine binding site. This polypeptide chain may be identical to that labeled with an azido derivative of serotonin [55].

Relationship between tetrabenazine and reserpine binding sites. The study of [^{3}H]reserpine and [^{3}H]TBZOH binding to chromaffin granule membranes has revealed two high affinity binding sites, that of tetrabenazine and ketanserin (site T), and that of reserpine (site R). The two sites are associated with the monoamine transporter and are involved in monoamine uptake. The existence of two distinct sites is supported by the fact that they are associated with polypeptide chains of different molecular weights [45]. The sites differ mainly by their sensitivity to the $\Delta\mu H^+$ and their affinity for substrates (Table 1).

The existence of two distinct sites may seem paradoxical, in view of *in vivo* experiments suggesting a competitive action of reserpine and tetrabenazine [12, 50]. In these experiments, advantage was taken of the difference in duration of the monoamine depletion induced by the two drugs: administration of tetrabenazine before reserpine resulted in a short depletion characteristic of tetrabenazine, thus indicating that this drug had protected against reserpine. This point has been investigated recently by analyzing *in vitro* the effect of tetrabenazine on [^{3}H]reserpine binding: tetrabenazine initially inhibits [^{3}H]reserpine binding, but is inefficient after a long incubation period [53]. This experiment confirms the existence of two distinct sites and shows that binding at either of these two sites excludes binding at the other.

Though a discussion of the mechanism of the monoamine transporter is out of the scope of this commentary, it may be indicated that the latter result may reflect the existence of two conformations of the transporter expressing exclusively site T or R. This hypothesis suggests for the monoamine transporter a mechanism of the alternate gated type [4], similar, for instance, to that proposed for the mitochondrial adenine nucleotide transporter. In this mechanism, conformation R is assumed to be favored in the presence of a proton gradient. To be consistent with experiments on the asymmetry of reserpine activity [56], site R should be on the external cytoplasmic side of the membrane. Because of its high affinity for the substrates, this site would bind monoamines and would thus act as a charge site. Substrate binding or transporter deprotonation would result in a conformational change to conformation T. The substrate, now bound to site T thought to be located on the internal face of the membrane vesicle, would dissociate from this site because of its low affinity for monoamines, and site T would thus act as a discharge site.

RADIOLIGAND BINDING IN OTHER MONOAMINERGIC SYSTEMS

Binding to isolated vesicles. As indicated before, the general *in vivo* depleting effect of reserpine and the similar *in vitro* properties of monoamine uptake suggest the involvement of a common transporter molecule in the various monoamine storing vesicles.

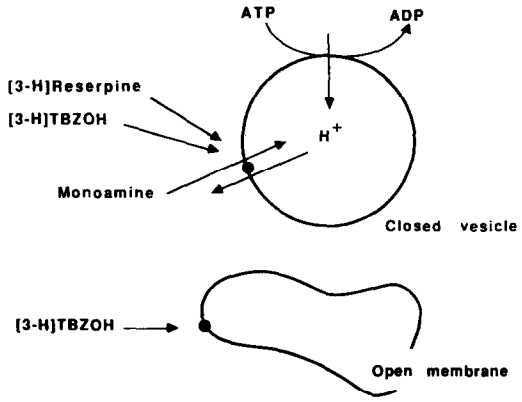

Fig. 2. Selective use of [^{3}H]reserpine and [^{3}H]TBZOH to assay the vesicular monoamine transporter. [^{3}H]Reserpine labels the transporter on sealed functional vesicles, whereas [^{3}H]TBZOH binds to the transporter indifferently on either functional or unsealed non-energized vesicles.

This hypothesis has been partly confirmed with the ligands of the chromaffin granule monoamine transporter. Thus, [^{3}H]TBZOH was shown to bind to a single class of sites on isolated synaptic vesicles from bovine or mouse corpus striatum, with an apparent dissociation constant (K_d) of 2.3 to 2.7 nM, similar to that found with bovine chromaffin granules [29, 57, 58]. The striatum receives predominantly dopaminergic nerve endings. Identical [^{3}H]TBZOH binding properties were observed on isolated vesicles from various mouse brain regions containing different relative amounts of serotonin (5-HT) and noradrenaline, and in 5-HT rat platelets [33], implying that the binding site was identical in all monoaminergic systems. Moreover, in these vesicle fractions, the K_d value was similar to the TBZOH inhibition constant of catecholamine uptake, and the density of binding sites was correlated to the monoamine uptake activity [29, 58].

Comparison of the properties of [^{3}H]reserpine binding among monoaminergic systems is difficult, because of the very high affinity of the ligand which complicates the experimental determination of the K_d value. As for chromaffin granules, binding to striatal vesicles was dependent upon ATP-induced energization [31], with K_d values in the nanomolar [31] or subnanomolar [33] concentration range.

Photoaffinity ligands have been used in an attempt to identify and compare the vesicular monoamine transporter in various systems. With iodoazidoketanserin, the apparent molecular weight varied from 65 to 85 kD [48], and with the azidoderivative of 5-HT, it varied from 34 to 52 kD [3], depending on the source of the vesicles. These variations of the molecular weight may reflect proteolysis or post-translational processing and/or species differences. Thus, in spite of the similar pharmacological and functional properties of the vesicular monoamine transporter in various systems and species, structural differences cannot be ruled out at the moment.

Assay of the transporter in tissue homogenates. The different characteristics of [^{3}H]TBZOH and [^{3}H]reserpine binding suggest different uses of these two ligands (Fig. 2). Both ligands are liposoluble

compounds which freely permeate biological membranes and which readily reach their intracellular target. However, [^{3}H]reserpine, the binding of which is dependent upon vesicle energization by the proton pump ATPase, labels selectively the monoamine transporter of sealed functional vesicles [54]. After homogenization, these vesicles may represent only a small population, as observed in crude rat brain homogenates, where specific [^{3}H]reserpine binding could not be detected [59]. Nevertheless, [^{3}H]reserpine should be useful for *in vivo* experiments because of its low dissociation kinetics, which allows non-specific binding to be washed out *in vivo* and specific binding to dissociate only slowly during the homogenization step [60].

On the other hand, [^{3}H]TBZOH binds to the transporter independently of vesicle energization and it should be preferred for *in vitro* labeling. It has been used to assay the vesicular monoamine transporter in crude tissue homogenates [33, 57, 59, 61–64], in addition to vesicles and vesicle fragments or solubilized fractions. The specificity of the labeling in tissue homogenates has been established by the following results:

(1) Binding occurs on a homogeneous class of sites with a K_d value identical to that found in isolated synaptic vesicles [57, 64]. A low affinity binding site (K_d = 100–400 nM) has been described recently, in addition to the high affinity binding site [64]; this site, which was more abundant in a striatum crude microsomal fraction and in liver homogenates, does not seem to be related to the weak dopamine receptor antagonist property of tetrabenazine [65, 66], and it has not been identified. It is clear nevertheless from the difference in K_d values that, in assays performed at nanomolar ligand concentration, [^{3}H]TBZOH selectively labels the high affinity binding site.

(2) Synaptic vesicle fractions are enriched 4- to 6-fold in [^{3}H]TBZOH binding sites, when compared to homogenates [29, 58, 64].

(3) The anatomical distribution in the brain of this site parallels that of catecholamines and serotonin (Table 2), the site density being proportional to the total amount of biogenic amines present in each structure [57]. No binding sites are detectable in cholinergic tissues [61].

(4) The density of binding sites in peripheral noradrenergic neurons decreases following 6-hydroxydopamine treatment [63], and it is nearly completely lost in the striatum following 6-hydroxydopamine injection in the substantia nigra (Darchen, Masuo, Vidal, Rostene and Scherman, unpublished result).

[^{3}H]TBZOH has been used for subcellular distribution studies in human, bovine or rodent brain since the monoamine transporter is a membrane bound marker of synaptic vesicles, more reliable than the rapidly diffusing amines [67]. These experiments indicated that the yield of synaptic vesicles by classical procedures was low, since only 10–20% of the [^{3}H]TBZOH binding sites from the starting homogenate were recovered in the fraction enriched in synaptic vesicles [29]. This low and poorly reproducible preparation yield should be considered when an accurate determination of the vesicular monoamine transporter level is required, for instance for regulation studies. In this case, [^{3}H]TBZOH binding on crude homogenates should be preferred to the assay of monoamine uptake activity, which can only be determined on isolated vesicles. Thus, it has been shown that nervous stimulation of rat adrenal medulla produced a "trans-synaptic" induction of the monoamine transporter concomitant with that of tyrosine hydroxylase, dopamine β-hydroxylase and enkephalins [62]. On the other hand, the monoamine transporter and catecholamine biosynthesis enzymes do not seem to be under strict coregulation, since the expression of the transporter is persistent in newborn rat sympathetic neurons cultured in a "cholinergic" medium which represses tyrosine hydroxylase and dopamine β-hydroxylase [61].

Table 2. Regional distribution of specific [^{3}H]TBZOH binding in mouse, rat and human brain

Structure	Specific [^{3}H]TBZOH binding (fmol/mg protein)		
	Mouse*	Rat†	Human‡
Cerebellum	56		
Pons-medulla	167		
Midbrain	240	154	
Substantia nigra			
Reticulata			141
Compacta			465
Hippocampus	141		83
Thalamus	111		
Hypothalamus	411	280	245
Striatum	1345	1195	
Caudate			766
Putamen			742
Accumbens			751
Pallidum			
Internal			115
External			128
Cortex			
Cingular			91
Temporal			52
Frontal	115	101	57 (Area 10)

[^{3}H]TBZOH concentration was 15 nM.
* Data are from Ref. 57.
† Scherman and Boschi, unpublished results.
‡ From Ref. 59.

Use of [^{3}H]TBZOH binding site as a monoaminergic marker. As a marker of monoamine storage vesicles, [^{3}H]TBZOH binding presents several drawbacks and advantages over classical markers such as the monoamines themselves, their metabolites and their biosynthesis enzymes. The major drawback is the lack of discrimination between the various monoamines. This is of minor importance in tissues which are homogeneous with regard to their monoamine content, such as peripheral sympathetic neurons (noradrenergic), brain striatum (predominantly dopaminergic), or platelets (which in most species contain mainly 5-HT). The major advantage resides in the stability of the [^{3}H]TBZOH protein binding site, which is not affected by freezing or lyophilization and shows little variations after 72 hr at 22° [59]. In contrast, the other monoaminergic markers are sensitive to post-mortem

Table 3. Prediction of the time required for monoamine accumulation inside storage vesicles

Tissue	Molecular turnover number (/min)	Monoamine per transporter ratio	Time required for monoamine accumulation (min)	Noradrenaline turnover (% per hr)
Midbrain	128	510	3.8	30
Hypothalamus		580	4.3	32
Striatum	146	330	2.5	
Maxillary glands		550	4.0	12–22
Heart		2,250	16.5	5–11
Brown adipose tissue		3,450	25.6	
Vas deferens		6,950	51.5	0.5–2
Adrenals	133	281,000	2,086.0	<0.3
Platelets		⩾6,250	⩾46.2	

Data are from Ref. 33, except for noradrenaline turnover values which are from Refs. 81 and 82. The molecular turnover number was determined on isolated synaptic vesicles and chromaffin granule membranes. It was calculated as the ratio of V_{max} of 5-HT uptake divided by the number of functional monoamine transporter molecules (assayed by the binding of [^{3}H]reserpine). The monoamine per transporter ratio was obtained by adding all biogenic monoamines and dividing by the B_{max} of [^{3}H]TBZOH binding in the same homogenate fraction.

delay [68–72]. This is important for human brain studies. For instance, in a study on 18 striata, variance among individuals was two times smaller for [^{3}H]TBZOH binding than for dopamine level [59]. Moreover, while no correlation was found between age and dopamine, a negative correlation was observed between age and [^{3}H]TBZOH binding, in agreement with previous observations based on amine and metabolite determination [70, 73]. [^{3}H]TBZOH is presently used for the evaluation of monoaminergic innervation in neurodegenerative diseases. In patients with Parkinson's disease and progressive supranuclear palsy, the binding in striatum is decreased by 70–90% [74].

The use of specific ligands of the vesicular monoamine transporter in autoradiographic studies for the mapping of monoaminergic pathways has so far been limited. After i.v. injection of high amounts of [^{3}H]reserpine, the tritium distribution corresponded to that of monoaminergic cell bodies and nerve endings [75]. Promising results have been obtained by autoradiography of frozen slices treated with [^{3}H]TBZOH (Darchen, Masuo, Vidal, Rostene and Scherman, unpublished result).

An additional property which distinguishes the vesicular monoamine transporter from the other monoaminergic markers is its localization in the membrane of the storage vesicles, which is useful for axonal transport studies. In an early work, when [^{3}H]reserpine of high specific activity was injected in rat substantia nigra, some of the radioactivity was detected subsequently in the ipsilateral striatum, probably due to fast anterograde axonal transport [75]. Evidence for axonal transport of vesicles has also been observed in the ligated rat sciatic nerve, by using [^{3}H]TBZOH binding to nerve fragment homogenates [63]. Fast anterograde and retrograde transports of [^{3}H]TBZOH binding sites were detected. In both cases, the binding affinity constants were the same, suggesting the retrograde transport of the undegraded protein. Moreover, anterograde and retrograde transports were of similar amplitude, indicating nearly complete retrieval of the vesicular transporter after exocytosis, at variance with noradrenaline or dopamine β-hydroxylase which are totally or partly secreted. The intrinsic monoamine carrier thus appears to be a marker adapted to retrograde transport studies.

DYNAMIC PROPERTIES OF MONOAMINE STORAGE VESICLES

Although the chemical composition of monoamine storage vesicles is well documented [67, 76–79], their biogenesis has not been studied as extensively [80, 81]. Little quantitative data have been gathered on dynamic processes such as vesicle loading with transmitter or vesicle recycling after exocytosis. The use of ligands of the monoamine transporter has provided insights on the physiology of vesicle loading. For instance, since vesicle biogenesis and vesicular monoamine uptake are distinct processes, and since monoamines are almost exclusively present in the intravesicular compartment, the ratio of monoamines per transporter molecule reflects the level at which vesicles are loaded with transmitters. Similar values of the monoamine/transporter ratio were found in eight mouse brain regions containing various relative amounts of monoamine [57]. This result stresses the similarity existing between the various brain monoamine storage vesicles. Moreover, the monoamine turnover rate (defined as the metabolite/monoamine ratio) was highly variable in the brain areas studied. Since this rate reflects synaptic activity, this result suggests that the level at which brain vesicles are loaded with monoamines is optimum under basal conditions and does not appear to vary with neuronal activity.

In the experiment presented in Table 3, a second dynamic parameter of monoamine storage vesicles was investigated: the time required for their loading [33]. Both [^{3}H]reserpine and [^{3}H]TBZOH were used for this purpose. The molecular turnover number of the transporter was determined on isolated vesicles

and deduced from the V_{max} of [^{3}H]5-HT uptake and from the number of functional transporter molecules assayed by [^{3}H]reserpine. The molecular turnover number was identical in central or peripheral tissues containing different monoamines, thus confirming the existence of a common transporter in the various monoamine storage vesicles. [^{3}H]Reserpine had to be used in this case instead of [^{3}H]TBZOH, since preparations of isolated vesicles or chromaffin granule membranes contain an important proportion of open vesicles which do not take up monoamines, but still contain transporter molecules binding [^{3}H]TBZOH [54].

Inversely, [^{3}H]TBZOH binding was used to determine the monoamine/transporter ratio in crude homogenates. Combined with the catecholamine content of individual storage vesicles, it was calculated that each chromaffin granule was equipped with about 10–20 molecules of transporter (i.e. 10–20 times less than membrane-bound dopamine β-hydroxylase which is the major protein component of chromaffin granule membranes [79]). Lower values were obtained for synaptic vesicles. Dividing the monoamine/transporter ratio by the molecular turnover rate of the transporter gave the minimal time necessary for loading the storage vesicles with monoamines, at saturating substrate concentrations. In rat adrenal medulla, the calculated value of Table 3 is consistent with experimental determinations [81, 83]. This agreement suggests that, in adrenal medulla, monoamine uptake, instead of tyrosine hydroxylation, may be rate-limiting in the formation of fully loaded mature granules, as already proposed [7, 62]. The values of Table 3 for central or peripheral synaptic vesicles reflect much shorter maturation times than in endocrine systems. It may be relevant that the predicted monoamine accumulation times in Table 3 were inversely correlated to the transmitter turnover rate, suggesting that rapid recycling and reuse of vesicles after exocytosis may play a more important role in central synapses than in peripheral tissues.

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