

## From Multidrug Resistance to Vesicular Neurotransmitter Transport

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## 1. General introduction

Multidrug resistance is a major concern in medical and agricultural diseases. In medicine, the emergence of resistance to multiple drugs is a significant obstacle in the treatment of several tumors as well as many infectious diseases. In agriculture, the control of resistance of plant pathogens is of major economic importance. However, these traits existed for aeons long before the drugs and antibiotics were discovered by humans. Resistance to a wide range of cytotoxic compounds is a common phenomenon observed in many organisms throughout the evolutionary scale and probably developed in order to cope with the variety of toxic compounds which are part of the natural environment in which living cells dwell. Only those organisms which have developed through evolution the ability to cope with a wide variety of compounds have been able to survive.

One of the strategies which evolved is removal of toxic substances by transport. Multidrug transporters are the proteins usually responsible for performing this task and have been found in many organisms, from bacteria to man. They can actively remove a wide variety of substrates in an energy dependent process and decrease the concentration of the offending compounds near their target. A great diversity of multidrug transporters are known to us today and we can group them in five different families (Fig. 1) based on structure similarities. In all cases, two basic energy coupling mechanisms have been characterized: those proteins which utilize the hydrolysis of ATP to actively remove toxic compounds and those which utilize the proton electrochemical gradient ( $\Delta\tilde{\mu}_{H^+}$ ) generated by primary pumps. One family (ABC type) is best known for the P-glyco-protein or MDR in its various forms, which confers multidrug resistance to cancerous cells [1–5]. This family includes also many bacterial transport proteins (see Chapter 8). The ABC transporters utilize ATP to actively transport a wide variety of compounds.

All the other families which have been studied at a mechanistic level are antiporters. They exchange various drugs with one or more protons and utilize in this way the proton electrochemical gradient to actively transport a variety of substrates. The TEXANs (Toxin EXtruding ANtiporters) include proteins which render fungi and bacteria resistant to antibiotic treatment [6–9] and mammalian proteins located in intracellular organelles which transport neurotransmitters such as dopamine, adrenaline, serotonin and Acetylcholine [10,11]. A third family (Mini-TEXANs) is represented by very small proteins, about 100 amino acids long which render bacteria resistant to a variety of toxic cations [7,12,13]. In another family (RND) it is not clear that all the proteins are transporters and their energy coupling mode has not been studied although they do not have nucleotide binding domains [14]. The last ‘family’ has a single protein: the organic cation antiporter from

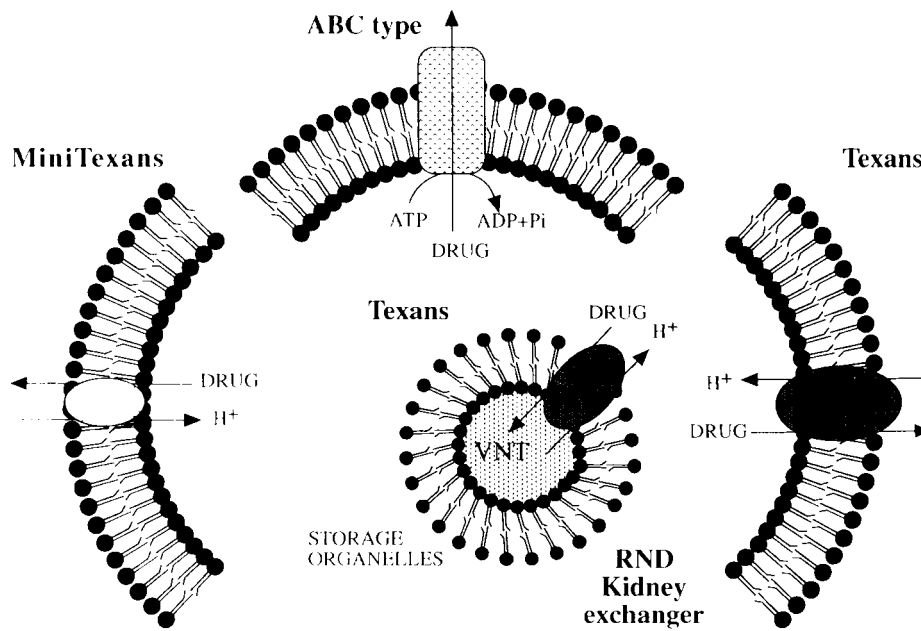


Fig. 1. Families of multidrug transporters. For explanation, see text.

kidney, unrelated to the others in its primary sequence but capable, as all the others, to remove a wide variety of toxic compounds against concentration gradients [15].

A question commonly asked about multidrug transporters is what is their 'real' function. Are these proteins functioning solely for protection of the organism against toxic compounds or do they have a very specific function and just by chance they happen to be also polyspecific. As in many other cases, the answer seems to be a complex one: thus, clearly in proteins functioning in the blood brain barrier [16] or in the kidney [15] there is little doubt that they are protecting the organism against toxic compounds by removing them from the organism or by preventing their passage to the brain. In the case of bacterial proteins such as the *Bacillus* BmrI and *E. coli* EmrA or Mar proteins, whose expression is regulated by multiple xenobiotics [17,18], it also seems reasonable that they have still a major role in protection of the cell as judged from their regulation. Yet, in other cases, it seems that given proteins, have evolved to perform specific roles other than multidrug resistance such as is the case of vesicular neurotransmitter transporters (VNTs), lipid translocators [19] and bacterial amino acid and sugar transporters from the ABC family [5].

This review will focus mainly in novel findings in the study of the vesicular neurotransmitter transporters, proteins which play a central role in neurotransmission. The topic seems to us very timely because of the realization of their functional and structural similarity with bacterial multidrug transporters. Also, their study

hints about the possible new modes of protection from toxic substrates in mammalian cells by compartmentalization of the drugs. Their study should link together many different fields, such as pharmacology, microbiology and neurobiology.

The study of VMAT has also led us to search for other, simpler model systems of multidrug transporters, the Mini TEXANs, which we will very briefly describe at the end of this review. The Mini TEXANs turn out to display unique properties and may serve as models for understanding the molecular basis of high-affinity recognition of multiple substrates and the mechanism of ion-coupled transport.

## 2. TEXANs

The VNTs are part of a family which includes at least 40 proteins from prokaryotes and eukaryotes and has been surveyed in several monographs [7,10,20]. Based on an analysis of the evolutionary relationships four subgroups have been identified. All the proteins present in microorganisms are presumed to be exporters located in the cytoplasmic membrane of these cells which confer resistance to a large list of compounds due to their ability to actively remove them from the cell (Fig. 1). The VNTs, on the other hand, are located in intracellular vesicles. While the bacterial transporters extrude the toxic compounds to the medium, those presently known in mammals, remove neurotransmitters from the cytoplasm into intracellular storage compartments. In both cases, as a result of their functioning, the concentration of the substrates in the cytoplasm is reduced. When the substrate of the VNTs is cytotoxic, such as is the case with MPP<sup>+</sup>, a substrate of the vesicular monoamine transporter (VMAT), the removal of the toxic compound from the cytoplasm, away from its presumed target will ameliorate the toxicity of the compound. Indeed, CHO cells expressing VMAT are more resistant to MPP<sup>+</sup> ([21] and see below). As mentioned above, these findings suggest a novel type of drug resistance in eukaryotic cells: protection of the life essential devices by compartmentalization (Fig. 1). Similar strategies have previously been suggested to explain tolerance to high salt and toxic compounds in plants and yeasts [22–24], however no molecular description of these phenomena has yet been proposed. One orphan mammalian protein, more similar in sequence to the bacterial Tet A-Bmr cluster than to the VNTs, has been described thus far [25].

Most transporters of the family have a very broad specificity for substrates. All of the substrates are aromatic compounds, usually bearing an ionizable or permanently charged nitrogen moiety. In some substrates, however, carboxylic groups are also present (i.e. norfloxacin); in others, a phosphonium moiety is present (i.e. TPP<sup>+</sup>), and yet in others, no positive charge is present at all (i.e. actinorhodin, uncouplers). Many of the substrates are common to many multidrug transporters, as can be seen for example in Fig. 2A and B in which structural formulas of some of VMAT and EmrE (a MiniTEXAN) inhibitors and substrates are presented. Although the proteins are unrelated in their sequence, there is a large overlap in substrates, albeit they recognize them with different affinities (Fig. 2). All of the compounds described in Fig. 2 also interact with Mdr1. This large overlap in substrate recognition may hint about common solutions to the problem.

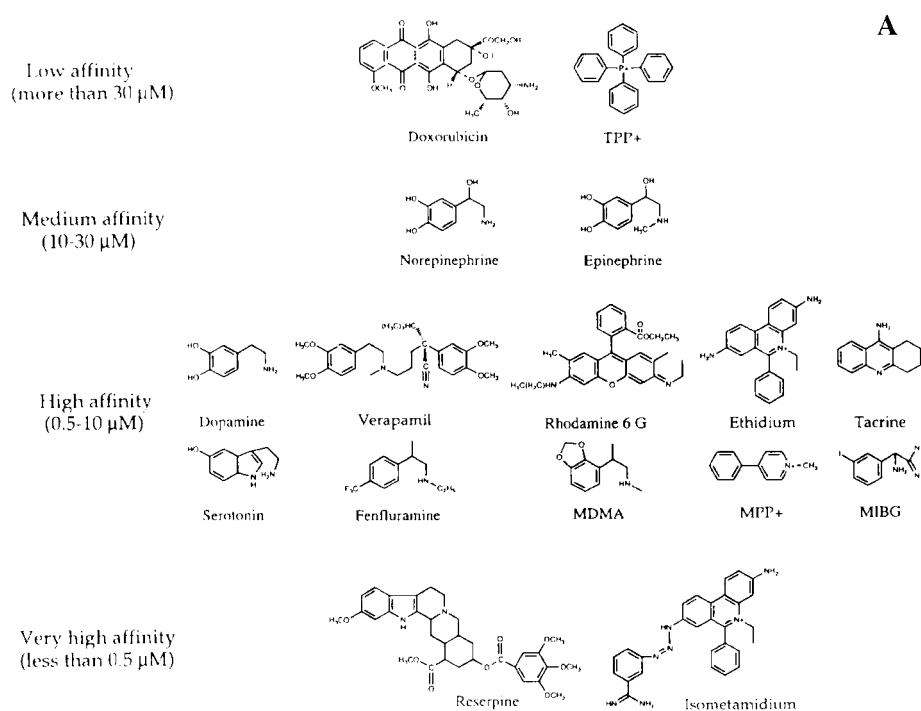


Fig. 2. Substrates and inhibitors of two multidrug transporters. (A) VMAT; *opposite*: (B) EmrE .

All the transporters of the TEXAN family are located in membranes across which  $\text{H}^+$  electrochemical gradients exist. The gradients are generated by primary pumps, such as the bacterial respiratory chain or the  $\text{H}^+$ -translocating ATPases of both bacteria and intracellular storage organelles. The gradient is utilized by the protein through the exchange of a substrate molecule with one or more hydrogen ions. All the neurotransmitter storage vesicles studied thus far, in brain, platelets, mast cells and adrenal medulla, contain a vacuolar type  $\text{H}^+$  pumping ATPase, similar in composition to the ATPase of lysosomes, endosomes, Golgi membranes and clathrin coated vesicles [26–28]. In all these organelles, the activity of this proton pump generates an  $\text{H}^+$  electrochemical gradient ( $\Delta\tilde{\mu}_{\text{H}^+}$  acid and positive inside). In synaptic vesicles and neurotransmitter storage organelles the proton electrochemical gradient is utilized by the VNTs, which couple efflux of  $\text{H}^+$  ions to the uptake of a neurotransmitter molecule (for review see Ref. [10]). In the case of Tet proteins, it has been suggested that the exchange is between a metal–tetracycline complex and one proton in an electroneutral process. Also BMR mediated drug efflux is apparently driven by a transmembrane pH gradient. While very little is known about the other transporters the fact that they all display sequence similarities, and none of them show any ATP binding domains, in addition to the fact that they are all found in membranes with  $\text{H}^+$  gradients suggests that they all are antiporters which exchange one or more  $\text{H}^+$  ions with a substrate molecule.

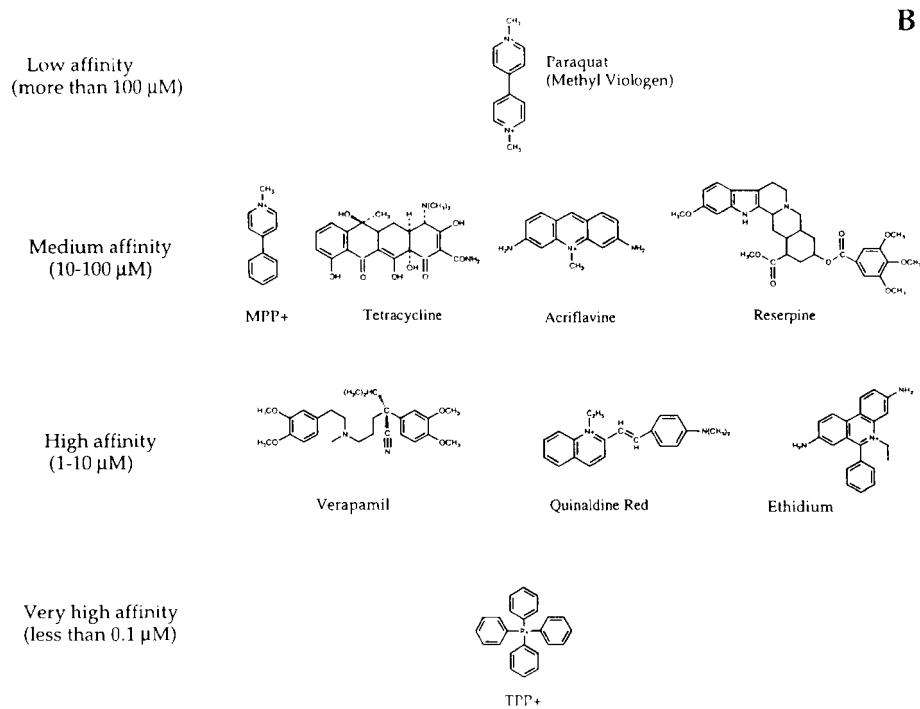


Fig. 2 (B). Caption opposite.

### 3. Transporters and neurotransmission

Synaptic transmission involves the regulated release of transmitter molecules to the synaptic cleft whereby they interact with postsynaptic receptors which subsequently transduce the information. Removal of the transmitter from the cleft enables termination of the signal, it usually occurs by its reuptake back to the presynaptic terminal or into glial elements in a sodium dependent process. This process assures constant and high levels of neurotransmitters in the neuron and low concentrations in the cleft.

Storage of neurotransmitters in subcellular organelles ensures their regulated release, and is crucial for protecting the accumulated molecules from leakage or intraneuronal metabolism and the neuron from possible toxic effects of the transmitters. In addition, the removal of intraneuronal molecules into the storage system effectively lowers the concentration gradient across the neuronal membrane and thus acts as an amplification stage for the overall process of uptake. Drugs which interact with either transport systems have profound pharmacological effects as they modify the levels of neurotransmitter in the cleft. Drugs that interfere with these activities include the tricyclic antidepressants, stimulants such as amphetamines and cocaine, antihypertensives such as reserpine and neurotoxins such as MPP<sup>+</sup>.

Plasma membrane transporters have been intensively studied at a mechanistic, biochemical and molecular level. The molecular characterization began with the purification, amino acid sequencing and cloning of the  $\gamma$ -aminobutyric acid (GABA) transporter [29]. Since, it has become clear that these  $\text{Na}^+$  and  $\text{Cl}^-$ -coupled transporters represent a group of integral membrane proteins encoded by a closely related family of genes which include the transporters for monoamines, GABA, glycine, proline, choline and taurine [30,31]. A different class of plasma membrane transporters is represented by the  $\text{Na}^+$  and  $\text{K}^+$ -coupled glutamate transporter [32,33, 34,35] (see also Chapter 19).

Vesicular transport has been observed for several classical transmitters, including acetylcholine (ACh), the monoamines, glutamate, GABA and glycine (reviewed in Ref. [10]). The vesicular monoamine transporter (VMAT) and ACh (VACHT) have been the most intensively studied and are the ones for which most molecular information have been obtained and we will review the most salient features of both. In both cases, the key for this knowledge resides in the availability of excellent experimental paradigms for their study and potent and specific inhibitors.

#### 4. Pharmacology of VNTs

The best characterized inhibitors of VNTs are reserpine and tetrabenazine, the two principal agents that inhibit vesicular monoamine transport [36,37]. In recent years, vesamicol, a novel inhibitor of VACHT has been introduced and studied in detail [38–40].

##### 4.1. Molecular mechanism of reserpine action

Reserpine presumably binds at the site of amine recognition as judged by the fact that it inhibits transport in an apparent competitive way, with  $K_i$ s in the subnanomolar range [41,42], that it binds to the transporter with a  $K_D$  similar to its  $K_i$  [41,42] and that transport substrates prevent its association in a concentration range similar to the range of their apparent  $K_m$ s [41]. Its effect *in vitro* is practically irreversible [43], in line with the *in vivo* effect of the drug which is extremely long lasting and is relieved only when new vesicles replace the ones that were hit [44]. As a result of this action, it depletes monoamine stores, providing considerable information on the physiological role of biogenic amines in the nervous system [45]. Reserpine has been used because it potently reduces blood pressure. However, it frequently produces a disabling effect of lethargy that resembles depression and has limited its clinical utility [46]. This observation has given rise to the amine hypothesis of affective disorders which in modified form still produces a useful framework for considering this group of major psychiatric disorders.

The time course of reserpine binding is relatively slow. This low rate of association is consistent with a similar time course for inhibition of monoamine transport [43]. Reserpine binding is accelerated by  $\Delta\tilde{\mu}_{\text{H}^+}$  whether generated by the  $\text{H}^+$ -ATPase [41,47] or artificially imposed [43]. This acceleration is observed also in proteoliposomes reconstituted with the purified protein [48].

In all cases, in the presence or absence of  $\Delta\tilde{\mu}_{\text{H}^+}$  and in the native as well in the purified protein, two distinct populations of sites have been detected [41,48]. A high affinity site,  $K_{\text{D}} = 0.5$  nM,  $B_{\text{max}} = 7\text{--}10$  pmol/mg protein in the native chromaffin granule membrane vesicle preparation (0.3 and 310, respectively for the purified protein) and a low affinity site,  $K_{\text{D}} = 20$  nM,  $B_{\text{max}} = 60$  pmol/mg protein in the native system (30 and 4200, respectively for the purified preparation). Surprisingly the apparent  $K_{\text{D}}$  does not change with an imposition of a  $\Delta\tilde{\mu}_{\text{H}^+}$  even though the on rate increases several fold [41]. It has to be assumed that the off rate changes also accordingly, although the off rate is so slow that it is impossible to measure. In this context, it is important to note that the  $K_{\text{D}}$  values obtained under conditions where the concentration of ligand binding sites does not exceed the value of the dissociation constant, is 30 pM, i.e. about ten times higher affinity than previously estimated [42].

The reserpine binding rate is less sensitive than transport to changes in the  $\Delta\text{pH}$ , and is stimulated equally efficiently by  $\Delta\text{pH}$  and  $\Delta\psi$  [43]. These findings suggest that fewer protons are translocated in the step which generates the high affinity binding site than in the overall transport cycle. Changes in binding rate probably reflect changes in the availability of reserpine binding sites and translocation of a single  $\text{H}^+$  generates the binding form of the transporter. Figure 3 schematically demonstrates a model to explain transport, reserpine binding, and reserpine occlusion by the vesicular monoamine transporter. In this model, the high affinity form of the transporter ( $\text{TH}^+$ ) is apparently achieved by either protonation of VMAT or by  $\text{H}^+$  translocation. The energy invested in the transporter may be released by ligand binding ( $\text{THS}$ ) and converted into vectorial movement of a substrate molecule across the membrane or directly into binding energy in the case of reserpine ( $\text{THR}$ ). In the case of a substrate a second conformational change results in the ligand binding site being exposed to the vesicle interior, where the substrate can dissociate ( $\text{TH}_2\text{S}$ ). The second  $\text{H}^+$  in the cycle, may be required to facilitate the conformational change or to allow for release of the positively charged substrate from the protein. In the model this second  $\text{H}^+$  binding and release is arbitrarily located, since there is no information about the order of the reactions. Interestingly, in this respect, are the findings that the apparent affinity of the transporter for substrates drops when the pH decreases [49,50]. This could reflect a mechanism for releasing the substrate in the acidic luminal milieu. Substrate and  $\text{H}^+$  release regenerates the transporter ( $\text{T}$ ) which can now start a new cycle. In the case of reserpine, however, its structure (bulk of its side chain?) restricts the conformational change so that instead of releasing the ligand on the interior, the complex becomes trapped in a state from which reserpine cannot readily dissociate and which cannot translocate another  $\text{H}^+$  to regenerate the high affinity form. It is not known whether the slow binding of reserpine requires also protonation of VMAT. If this is the case, protonation in the absence of  $\Delta\tilde{\mu}_{\text{H}^+}$  would be the rate limiting step.

Tetrabenazine (TBZ) is another potent inhibitor of the transporter. Radiolabeled dihydrotetrabenazine (TBZOH) has been used in binding studies to characterize the protein [41,51], to study the regulation of its synthesis [52–54] and its distribution in various tissues [55]. Binding of TBZOH to the transporter is not modified by the imposition of a  $\Delta\tilde{\mu}_{\text{H}^+}$  as shown for reserpine. In addition, binding is not inhibited by

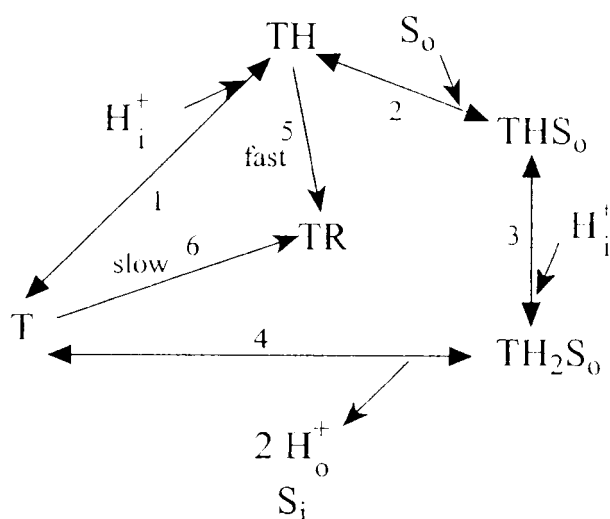


Fig. 3. Model for substrate transport and reserpine binding. See explanations in text. T, transporter; S, substrate; R, reserpine;  $H^+$ , hydrogen ion. Subscripts denote whether the substrate is inside the vesicle (i) or outside (o).

reserpine at concentrations which fully inhibit transport. Moreover, transport substrates block binding only at concentrations 100 fold higher than their apparent  $K_m$  values. These findings have led Henry and collaborators to suggest that TBZOH binds to a site on the protein which is different from the reserpine and substrate binding site [42,51,56]. It has been suggested that both sites are mutually exclusive, i.e., VMAT exists in two different conformations, each conformation binding only one type of ligand, TBZ or reserpine. According to this interpretation, addition of TBZ would pull the conformational equilibrium toward the TBZ binding conformation, which is unable to bind reserpine. Indeed, under proper conditions (low protein concentration and short incubation times) 50 nM TBZOH inhibits reserpine binding by 70%. ATP, through the generation of  $\Delta\tilde{\mu}_{H^+}$ , would pull the conformational equilibrium towards the RES binding site [42]. Although elegant and attractive, this model has yet to explain the lack of effect of  $\Delta\tilde{\mu}_{H^+}$  on TBZ binding, which should be inhibited, if the two forms were mutually exclusive. Also, the concentrations of reserpine required to inhibit TBZ binding are higher than those required for site occupancy (binding and inhibition of transport).

#### 4.2. Acetylcholine transporter

An important development in the study of VAcHT was the discovery of a specific inhibitor, *trans*-2-(4-phenylpiperidino)cyclohexanol, code named AH5183 and now called vesamicol, which blocks neuromuscular transmission and shows unusual characteristics of action. Marshall [38] hypothesized that AH5183 blocks storage by synaptic vesicles and indeed it inhibits ACh storage by purified *Torpedo*

synaptic vesicles with an  $IC_{50}$  of 40 nM. The drug was the most potent inhibitor found among at least 80 compounds initially screened ([57,58] and see Ref. [40] for review). [ $^3H$ ]vesamicol binding showed an apparent  $K_D$  of 34 nM [59–63]. ACh inhibits vesamicol binding only at very high concentrations (20–50 mM) and other high affinity analogues were shown to competitively inhibit binding. However, in all cases where the analogues are transported the inhibition constant is about 20 fold higher than the apparent constants for transport. Non transported analogues show the same efficiency for inhibition of both ACh transport and [ $^3H$ ]vesamicol binding [64]. A kinetic model has been suggested in which it is assumed that vesamicol binds to an allosteric site to form a dead end complex when ACh is not bound. As described above, the existence of two sites with similar properties has been observed also for VMAT. In the latter, TBZ is a potent inhibitor of monoamine accumulation but its binding is inhibited by monoamines only at very high concentrations (see above for detailed discussion and references). In the case of VMAT, both binding sites are present in one protein, since the purified and the recombinant proteins show high sensitivity to TBZ. In the case of VAcHT, the existence of a 'receptor' has been postulated which could lie on the same protein or in a separate one. The vesamicol 'receptor' has been extensively studied by Parsons and collaborators, it has been purified [65] and labelled with photoaffinity labels [40,66,67]. The 'receptor' solubilized in cholate and stabilized with glycerol and a phospholipid mixture was purified to yield a specific binding of 4400 pmol/mg protein, a purification factor of about 15. Unfortunately, the purified receptor exhibits very heterogeneous electrophoretic mobility in SDS PAGE with very diffuse stain at about 240 kDa. This is the typical behavior of membrane glycoproteins which are not fully monodispersed because of the detergent used and in addition boiled, treatments known to induce aggregation of membrane proteins.

Recently, glycoproteins from various species which bind vesamicol with high affinity have been expressed in CV1-fibroblasts ([68–70] and see Section 6). In addition the rat VAcHT expressed in CV1-fibroblasts catalyzes vesamicol-sensitive ACh accumulation [68]. As will be seen below, the evidence available now clearly demonstrates that vesamicol binds to the vesicular ACh transporter itself.

## 5. Identification of functional transporters

The only VNT purified in a functional form is the bovine VMAT. The high stability of the complex [ $^3H$ ]reserpine–transporter has been used to label the transporter and follow its separation through a variety of procedures. In these experiments a small amount of Triton X100-extracts from prelabeled membranes were mixed with a four to five-fold higher amount of extract from unlabelled membranes. Purification of the material labeled in this way has revealed the presence of two proteins that differ in pI, a very acidic one (3.5) and a moderately acidic one (5.0) [48]. Reconstitution in proteoliposomes has shown that both catalyze monoamine transport with the expected properties. The more acidic isoform is a glycoprotein of 80 kDa, which has been purified and reconstituted in proteoliposomes. It catalyzes transport of serotonin with an apparent  $K_m$  of 2  $\mu$ M and a  $V_{max}$  of 140 nmol/mg/min,

about 200-fold higher than the one determined in the native system. Transport is inhibited by reserpine and tetrabenazine, ligands which bind to two distinct sites on the transporter. In addition, the reconstituted purified transporter binds reserpine with a biphasic kinetic behavior, typical of the native system. The results demonstrated that a single polypeptide is required for all the activities displayed by the transporter, i.e. reserpine- and tetrabenazine-sensitive,  $\Delta$ pH-driven serotonin accumulation, and binding of reserpine in an energy-dependent and independent way. Based on these and additional findings it was estimated that the transporter represents about 0.2–0.5% of the chromaffin granule membrane vesicle and that it has a turnover of about  $30 \text{ min}^{-1}$ .

The assignment of the activity to the 80 kDa polypeptide and the localization of the tetrabenazine and reserpine binding sites in the same polypeptide is confirmed by several independent approaches, including direct sequencing of the purified protein [71,72] and cloning and analysis of the recombinant protein [21,73,74]. Vincent and Near purified a TBZOH-binding glycoprotein from bovine adrenal medulla using a protocol identical to the one used to purify the functional transporter. The TBZOH-binding protein displays an apparent Mr of 85 kDa [75]. In addition, Henry and collaborators, labelled bovine VMAT with 7-azido-8- $^{125}\text{I}$  iodoketanserin, a photoactive derivative of ketanserin, which is thought to interact with the TBZ binding site. The labelled polypeptide displayed an apparent Mr of 70 kDa and a pI ranging from 3.8 to 4.6 [76]. In all the cases broad diffuse bands, characteristic of membrane proteins, were detected so that the differences in the Mr's reported (70, 80 and 85 kDa) are probably due to a different analysis of the results and not to innate variations. Sequencing of the labelled protein confirmed that it is identical with the functional transporter [72].

The basis of the difference between the two isoforms has not yet been studied and could be due to either covalent modification (i.e phosphorylation or different glycosylation levels) of the same polypeptide backbone, to limited proteolysis during preparation or to a different polypeptide backbone. Since we know now (see Section 6) that there are at least two types of VMATs: VMAT2, which is sensitive to TBZ, and VMAT1, which is less sensitive, it should be determined whether the activity of the high pI form is less sensitive to TBZ. The sequence of 26 N-terminal amino acids of the purified protein is practically identical to the predicted sequence of the bovine adrenal VMAT2 ([71,72,77] and see below). Antibodies raised against a synthetic peptide based on the described sequences specifically recognize the pure protein on Western blots and immunoprecipitate reserpine binding activity under conditions where the 80-kDa protein alone is precipitated [71].

## 6. Cloning and functional expression

### 6.1. VMATs

Several sequences of VMAT and VACHT from various species are now available. Rat VMAT was cloned by Edwards et al. [21] and Erickson et al. [74] practically at the same time using different strategies (reviewed in Refs. [10,78]). Erickson and

coworkers [74] used expression cloning in CV-1 cells transfected with c-DNA prepared from rat basophilic leukemia (RBL) cells mRNA. Edwards et al. took advantage of the ability of VMAT1 to render CHO cells resistant to MPP<sup>+</sup> by means of its ability to transport the neurotoxin into intracellular acidic compartments thereby lowering its effective concentration in the cytoplasm [21].

Sequence analysis of the cDNA conferring MPP<sup>+</sup> resistance (VMAT1) shows a single large open reading frame which predicts a 521 amino acid protein. Analysis by the method of hydrophobic moments predicts 12 putative transmembrane segments (TMS). A large hydrophilic loop occurs between membrane domains 1 and 2 and contains three potential sites for N-linked glycosylation. According to the 12 TMS model [21] this loop faces the lumen of the vesicle, and both termini face the cytoplasm.

Biochemical and quantitative evidence for the identity of the cloned cDNA was provided by developing a cell free system in which membranes were assayed for dopamine transport and reserpine binding [21,73].

VMAT1 expressed in MPP<sup>+</sup>-resistant CHO cells accounts for about 0.1% of the total cell membrane protein [73] while the bovine vesicular transporter for 0.2–0.5% of the chromaffin granule membrane [48].

A transporter distinct from VMAT1 has been identified in rat brain (VMAT2) and in RBL cells [74]. The predicted protein shows an overall identity of 62% and a similarity of 78% to VMAT1. The major sequence divergences occur at the large luminal loop located between the first and the second transmembrane domains and at the N- and C-termini [72,77,79,80].

### *6.2. The two VMAT subtypes differ also in some functional properties*

A comprehensive comparison between the functions of rat VMAT1 and VMAT2 has been performed by Edwards and collaborators in membrane vesicles prepared from CHO stable transformed cells lines in which the respective proteins are expressed [81]. According to these studies VMAT2 has a consistently higher affinity for all the monoamine substrates tested. In the case of serotonin, dopamine and epinephrine the apparent  $K_m$  of VMAT1 for ATP dependent transport is 0.85, 1.56 and 1.86  $\mu\text{M}$  respectively, while the corresponding  $K_m$ s measured for VMAT2 are 4- to 5-fold lower, 0.19, 0.32 and 0.47  $\mu\text{M}$  correspondingly. Although the affinities are slightly different, the rank order for the various monoamines is similar. Also other substrates such as MPP<sup>+</sup> (1.6 vs 2.8  $\mu\text{M}$ , respectively) and methamphetamine (2.7 vs 5.5  $\mu\text{M}$  respectively as estimated from measurements of the ability to inhibit reserpine binding) display a similar pattern. A most striking difference is detected for histamine: 3  $\mu\text{M}$  for VMAT2 and 436  $\mu\text{M}$  for VMAT1. Also, VMAT1 is significantly less sensitive to tetrabenazine,  $\text{IC}_{50} = 3\text{--}4 \mu\text{M}$  [21,81], than either VMAT2 ( $\text{IC}_{50} = 0.3\text{--}0.6 \mu\text{M}$ ) [74,81] or the native [37,42] and the purified transporter ( $\text{IC}_{50} = 25 \text{ nM}$ ) [48] from bovine adrenal medulla. Interestingly, the apparent affinities determined in heterologous expression systems or in proteoliposomes reconstituted with the purified transporter are higher than those determined in chromaffin granules membrane vesicles (see Ref. [82] for references). The turnover number (TO) of the

recombinant protein has been calculated based on the  $V_{\max}$  for serotonin transport and the number of reserpine binding sites. The TO for VMAT1 is  $10 \text{ min}^{-1}$ , while that for VMAT2 is 40 [81]. A similar analysis of the purified bovine transporter (VMAT2 type) showed a TO of  $30 \text{ min}^{-1}$  (Section 5 and Ref. [48]). These values are lower than the  $135 \text{ min}^{-1}$  estimated for intact bovine chromaffin granules [83] but coincide well with the values obtained from brain regions ( $10\text{--}35 \text{ min}^{-1}$ ) [55] and other estimates (15 and 35) in chromaffin granules [56].

### 6.3. Tissue distribution

Tissue distribution of rat VMAT subtypes has been studied very intensively using a variety of techniques: Northern analysis [21] [74], *in situ* hybridization [21] and immunohistochemistry [84,85]. From these studies it is concluded that expression of VMAT1 and VMAT2 is mutually exclusive: VMAT1 is restricted to non neuronal cells and VMAT2 to neuronal cells. VMAT1 is expressed in endocrine/paracrine cells: in the adrenal medulla chromaffin cells, in the intestine and gastric mucosa in serotonin and histamine-containing endocrine and paracrine cells and in SIF cells of sympathetic ganglia. VMAT2 is expressed in neuronal cells throughout, including in the intestine and stomach. There are two exceptions for this restriction: a subpopulation of VMAT2-expressing chromaffin cells in the adrenal medulla and a population of VMAT2, chromogranin A-positive endocrine cells of the oxyntic mucosa of the rat stomach [84].

While the studies in rat are very definitive, the situation is very different in other species. In human pheochromocytoma mRNA for both subtypes is found [84]. The bovine adrenal medulla expresses a VMAT2 type transporter whose message has also been detected in brain [72,77]. VMAT2 is the main adrenal medulla transporter as judged from direct protein purification studies [48,71,72,77]. The purified transporter from bovine adrenal is VMAT2 and accounts for at least 60% of the activity in the gland.

### 6.4. Subcellular targeting of VMAT

Storage of monoamines differs from that of other classical neurotransmitters. Thus, while the latter are stored in small synaptic vesicles, monoamines in the adrenal medulla are stored with neural peptides in large dense core chromaffin granules. In the CNS, neurons store monoamines in small vesicles that may contain a dense core. The difference in the storage of the monoamines as compared to that of classical transmitters may reflect differential sorting of the VNTs. Sorting of VMAT was studied with immunohistochemical and biochemical tools [86,87].

In heterologous expression of VMAT1 in CHO cells, the transporter is targeted to a population of recycling vesicles and co-localizes with the transferrin receptor. Thus, localization in CHO cells is similar to that of other neuronal vesicle proteins such as synaptophysin and SV2 [86].

In PC12 cells, endogenous VMAT1, occurs principally in large dense core granule (LDCV). In synaptic like microvesicles (SLMVs) and in endosomes only small amounts are found [86].

In the rat adrenal medulla, immunoreactivity for VMAT1 occurs at several sites in the secretory pathway but most prominently in the chromaffin granules, supporting the results in PC12 cells [86].

In central neurons r-VMAT2s localization was studied in the nuclei of solitary tract, a region known to contain a dense and heterogenous population of monoaminergic neurons [87]. VMAT2 localizes primarily to LDCVs in axon terminals. It is also detected in less-prominent amounts in small synaptic vesicles, the trans-Golgi network and other sites of vesicles transport and recycling. Thus, both VMAT1 and VMAT2 are primarily sorted to LDCVs in all the cell types studied.

#### 6.5. Regulation of expression of VMAT

Evidence for regulation of expression of VMAT was first obtained in insulin shocked rats in which an increase in the number of [<sup>3</sup>H]TBZOH binding sites in the adrenal medulla was detected. The increase was maximal after 4–6 days [52]. A similar increase was observed *in vitro* in bovine chromaffin cells in culture in the presence of carbamylcholine or depolarizing concentrations of potassium ions [54]. The response was mimicked by forskolin and by phorbol esters and was blocked by actinomycin and cycloheximide, suggesting involvement of transcriptional activation.

This suggestion was supported by the detection of an increase in message for VMAT2 after 6 h depolarization [72]. After 5 days the cells contained fewer secretory granules and those left had a higher density, suggesting that they are newly synthesized and immature. While the catecholamine, chromogranin A and cytochrome b561 content decreased, [<sup>3</sup>H]TBZOH binding sites increased about 1.5-fold. The physiological significance of these findings is not obvious. It has been speculated that this phenomenon may reflect the fact that vesicular uptake might be rate limiting and that in order to accelerate refilling an increase in VMAT is needed. Mahata et al., have suggested, however, that there is no increase in other membrane proteins in the rat granule [88,89]. They have reported no changes in the level of VMAT1 message (the main subtype in rat adrenal) under conditions at which mRNA for the matrix peptide NPY increased [88,89]. Since in the same system the [<sup>3</sup>H] TBZOH binding sites increased, the latter findings may suggest a novel mode of regulation of activity of preexisting protein.

### 7. Vesicular acetylcholine Transporters

The powerful genetics of the nematode *Caenorhabditis elegans* has provided important information regarding VAcHT. The elegant approach used by Rand and collaborators [90] was based on the analysis of one of the mutants described by Brenner twenty years ago [91]. Mutations in the *unc-17* gene of the nematode result in impaired neuromuscular function, which suggest that cholinergic processes might be defective in the mutant. In addition, *unc-17* mutants were resistant to cholinesterase inhibitors [91], a resistance which may result from decreased synthesis or release of the transmitter. Moreover, *unc-17* was found closely linked to *cha-1* gene, which encodes choline acetyl-transferase [92]. The genomic region of

*unc-17* was cloned by walking from the *cha-1* gene and thereafter cDNA was isolated from a library [90]. Injection of a cosmid containing the complete coding sequence of the isolated cDNA rescues the mutant phenotypes of *unc-17* animals. A protein with 532 amino acids is predicted from the isolated DNA sequences. This protein (UNC-17=VACHT) is 37% identical to VMAT1 and 39% identical to VMAT2. The findings strongly suggested that UNC-17 is a vesicular acetylcholine transporter. This was supported by the fact that antibodies against specific peptides stain most regions of the nervous system. Within individual cells, staining was punctate and concentrated near synaptic regions. Double labeling with anti-synaptotagmin showed colocalization of the two antigens. In addition, in *unc-104* mutants, a mutation in a kinesin related protein required for the axonal transport of synaptic vesicles, synaptic vesicles accumulate in cell bodies. In these animals, anti-UNC-17 staining was restricted to neuronal cell bodies. More than 20 alleles, viable as homozygotes have now been identified. Their phenotypes vary from mild to severe. In two of these mutants, staining was dramatically decreased throughout the nervous system. Two other alleles were isolated which are lethal as homozygotes and they seem to represent the null *unc-17* phenotype. This is the first demonstration that the function encoded by a VNT is essential for survival.

Homology screening with a probe from *unc17* allowed for the isolation of DNA clones from *Torpedo marmorata* and *Torpedo ocellata* [70]. The *Torpedo* proteins display approximately 50% identity to UNC17 and 43% identity to VMAT1 and VMAT2. Message is specifically expressed in the brain and the electric lobe. The *Torpedo* protein, expressed in CV-1 fibroblast cells, binds vesamicol with high affinity ( $K_D = 6$  nM). Interestingly, the UNC17 protein expressed in the same cells binds also vesamicol, albeit with a lower affinity (124 nM) [70]. Mammalian VACHTs (human and rat) have been identified [68,69]. The predicted sequences of both proteins are highly similar to those of the *Torpedo* and *C. elegans* counterparts. The rat VACHT has been shown to bind vesamicol with high affinity ( $K_D = 6$  nM). It also catalyzes proton-dependent, vesamicol sensitive, ACh accumulation in transfected CV1 cells [68]. The distribution of rat VACHT mRNA coincides with that reported for choline acetyltransferase (ChAT), the enzyme required for ACh biosynthesis, in the peripheral and central cholinergic nervous systems. The human VACHT gene localizes to chromosome 10q11.2 which is also the location of the ChAT gene. The entire sequence of the human VACHT coding area is contained uninterrupted within the first intron of the ChAT gene locus [68]. Transcription of both genes from the same or contiguous promoters provides a novel mechanism for coordinate regulation of two proteins whose expression is required to establish a phenotype.

#### **8. Structure–function studies: identification of residues/domains with putative roles in structure and function**

The only topological information about the VNTs thus far is that obtained from an hydropathic analysis of the protein sequence. The results predict 12 putative transmembrane segments and a large hydrophilic loop between transmembrane domains

1 and 2. The loop contains three potential sites for N-linked glycosylation. Previous studies have demonstrated that all the glycan moieties in glycoproteins of chromaffin granules and AcCh storage face the lumen. Therefore, according to the latter finding and the model, the loop faces the lumen and both termini face the cytoplasm.

Identification of functional residues in VNTs is based on studies using site directed mutagenesis and relatively specific chemical modifiers. These studies are facilitated by the availability of sequences from different species and subtypes. It is usually assumed that residues which play central roles in catalysis are conserved throughout species. The degree of conservation in the group of VNTs is rather high. The members which are farther away in the group (human VMAT2 and the *C. elegans* VACHT) are still 38% identical and 63% similar. In the schematic representation in Fig. 4, an alignment of the nine known sequences of the VNTs is presented. The highest divergence is detected in the N- and C-termini and in the glycosylation loop between putative TMS I and II. The highest identity is observed

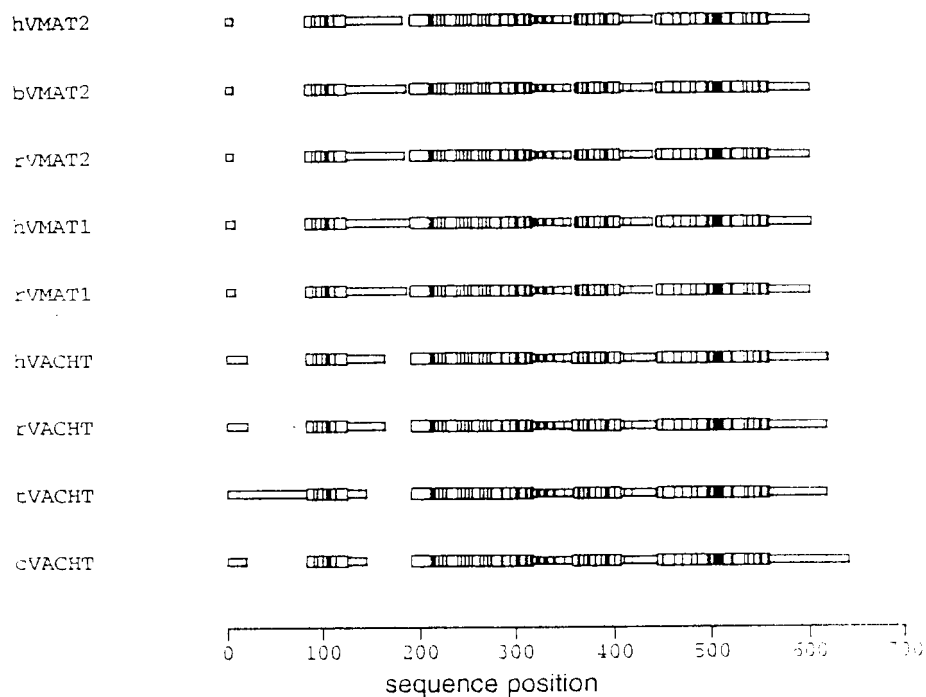


Fig. 4. Schematic representation of a multiple alignment of vesicular neurotransmitter transporters. Multiple alignment was first performed using Pileup (Wisconsin Package, 1994) and then blocks of similarity were identified using MACAW [118]. The best blocks are shown, a vertical line indicates fully conserved residues. The width of the line is proportional to the number of conserved residues. The sequences aligned are as described in Table 1 of Schuldiner, 1995. The sequence of hVMAT1 was kindly made available before publication by J. Erickson (NIH). The least conserved domains are the N- and C-termini and the large glycosylation loop. TM VIII is conserved within the VMATs and the VACHTs but there are only four residues identical in both.

Table 1  
Summary of mutants in VMAT

Mutation	Transport	Reserpine binding	Coupling to $\Delta\mu\text{H}^+$	Remarks	Subtype and Reference
D33E D33N	Normal Undetectable	Normal Normal	Normal Normal	Impaired Substrate recognition	VMAT2 [96]
G151L T154A N155Q N155D G158L	Normal Normal Normal Normal Normal	Normal Normal Normal Normal Normal	Normal Normal Normal Normal Normal		VMAT2
(Stmd3A) S180-182A	Undetectable	Normal	Normal	Impaired substrate recognition	VMAT2
S197, 198, 200, 201A (Stmd4A)	Normal	Normal	Normal		VMAT2
H384C H384R	Normal	Normal	Normal		VMAT1 [103]
D404E D404C D404S	Shifted to acidic pH Undetectable Undetectable	Normal Undetectable Undetectable	Normal – –		VMAT1 (Mordoch et al., unpublished)
H419C H419R	Undetectable Undetectable	Normal Normal	None None		VMAT1 [103]
D431E D431S D431C	Undetectable Undetectable Undetectable	Normal Normal Undetectable	Normal Normal No expression		VMAT1 (Mordoch et al., unpublished)

in TM I, II and XI where at least 11 amino acids are fully conserved. In TMS XI the conservation is particularly striking since practically all the amino acids conserved are in a contiguous stretch 'SVYGSVYAIAD'.

### 8.1. Carboxyl residues

Particularly striking are four conserved Asp residues D34, D267, D404 and D431 (numbers of rVMAT1) in the middle of TMS I, VI, X and XI. In addition to their being conserved charged residues in the membrane, biochemical evidence was available that N,N'-dicyclohexylcarbodiimide (DCC) inhibits VMAT mediated

transport [93–95]. DCC reacts with a carboxyl residue whose availability is influenced by the occupancy of the tetrabenazine binding site [94]. Reaction with the above carboxyl residue inhibits not only overall transport activity but also TBZ and reserpine binding, suggesting that the residue plays a role in one of the first steps of the transport cycle. As with all chemical modifiers, indirect effects such as steric hindrance by the DCC moiety or indirect effect on the structure of the protein cannot be ruled out at present. Therefore, mutagenesis studies of the roles of these four Asp residues on VMAT should be instructive.

Conservative replacement of Asp33 with Glu in rVMAT2 reduced activity but did not abolish it [96]. Replacement with Asn abolished transport indicating the crucial role of a negative charge at this position. However, VMAT could still bind [<sup>3</sup>H] reserpine and binding was accelerated by  $\Delta\tilde{\mu}_{\text{H}^+}$  [96]. Inhibition of reserpine binding in D33N by serotonin differs dramatically from the wild type suggesting interference with substrate recognition. Similarly, replacements in positions 404, 431 located in TMS X and XI had dramatic effects on activity [96a]. In the case of 431, even a conservative replacement with Glu led to transport inhibition. Replacement of Asp 404 with Glu generated a very interesting protein; the pH optimum of transport was shifted by about 1 pH unit to the acidic side. Replacement with either Cys or Ser on both positions yielded proteins which displayed no transport at all. The Asp431 replacements, D431E and D431S, but not D431C which was not expressed at detectable levels, bound [<sup>3</sup>H] reserpine normally and binding was accelerated by  $\Delta\tilde{\mu}_{\text{H}^+}$ . The Asp404 replacements D404S and D404C showed no binding at all [96a]

An important conclusion from these studies is that replacements of negative charges in the middle of putative transmembrane segments, while having dramatic effects on transport, do not necessarily perturb the protein structure since some of the mutants still bind a high affinity ligand and respond to  $\Delta\tilde{\mu}_{\text{H}^+}$  (see Table 1). The ability to measure partial reactions in VMAT allows for a more sophisticated analysis of mutagenized proteins than previously possible. Thus, although it does not transport, it was shown that the D33N mutant protein has a lower affinity to serotonin than the wild type. Also, the mutant D404E, shows an acid shift on the overall cycle but not on partial reactions suggesting an effect on a pKa important for the final steps of transport (Fig. 3). It is tempting to speculate a direct role of D404 in the translocation of the second H<sup>+</sup> needed for the overall cycle. Direct proof of this contention will need further experimentation.

In this context, it is interesting to point out that only two of the 19 mutants described in Table 1 are completely inactive. One of them, is not expressed to detectable levels.

## 8.2. His and Arg residues

Also His residues have been suggested to play a role in H<sup>+</sup> translocation and sensing in other H<sup>+</sup>-coupled transporters [97,98]. In VMATs, there is only one His conserved (His419) in loop 10, between TMS IX and X. This His is immediately behind an Arg residue conserved throughout the whole VNT family and very close to the

longest conserved sequence stretch (see above). Although present also in the rat, human and *C. elegans* VACHT, it is replaced by a Phe in the Torpedo VACHT. Biochemical evidence suggested a role for His also in VMAT. Phenylglyoxal (PG) and diethyl pyrocarbonate (DEPC) are reagents relatively specific for Arg and His residues, respectively. They both inhibit serotonin accumulation in chromaffin granule membrane vesicles in a dose dependent manner ( $IC_{50}$  of 8 and 1 mM, respectively) [99,100]. The inhibition by DEPC was specific for His groups since transport could be restored by hydroxylamine [99]. Neither PG nor DEPC inhibited binding of either reserpine or tetrabenazine, indicating that the inhibition of transport is not due to a direct interaction with either of the known binding sites. Interestingly, however, the acceleration of reserpine binding by a transmembrane  $H^+$  gradient was inhibited by both reagents [99]. The results suggest that either proton transport or a conformational change induced by proton transport is inhibited by both types of reagents. Several other transport systems are sensitive to DEPC and phenylglyoxal (review in [99,101,102]).

A more direct analysis of the role of histidines in VMAT has been carried out by site directed mutagenesis of rVMAT1 [103]. Replacement of His419, the only His conserved in VMAT1, with either Cys (H419C) or Arg (H419R) completely abolishes transport as measured in permeabilized CV1 cells transiently transformed with the mutants DNA. In the absence of  $\Delta\tilde{\mu}_{H^+}$ , reserpine binding to the mutant proteins is at levels comparable to those detected in the wild type. However, acceleration of binding in the presence of  $\Delta\tilde{\mu}_{H^+}$  is not observed in either H419C or H419R. These results suggest that His419 plays a role in a step other than binding and may be associated directly with  $H^+$  translocation or in conformational changes occurring after substrate binding.

### 8.3. Serine residues

In the  $\beta$ -adrenergic receptor and in the dopamine plasma membrane transporter [104] it has been suggested that serines play a role in ligand recognition. Two groups of serine residues occur in VMAT2: in TM III and TM IV. Simultaneous replacement of 4 serines in TM IV (S197, 198, 200 and 201) with alanine does not affect transport activity. On the other hand, mutant VMAT2 in which serines 180, 181 and 182 (in TM III) were replaced with alanine, showed no transport activity. Moreover, binding of [ $^3H$ ] reserpine was at normal levels and it was accelerated by  $\Delta\tilde{\mu}_{H^+}$ . However, in contrast to wild type, and similar to D33N, binding was not inhibited by serotonin even at concentrations of 500  $\mu M$ . The results suggest a possible role of Ser 180-182 in substrate recognition [96].

### 8.4. Mutations in TetA and BMR proteins

Interesting information can also be inferred from studies in homologous proteins performing similar or identical functions. TEXANs have evolved in many living organisms as transport proteins which play central roles in survival. Because of the overall similarities in mechanism ( $H^+$ /substrate antiporters), secondary structure

and sharing of some of the substrates, the information obtained in studies with other TEXANs deserve some attention. Two proteins, TetA and Bmr, have been studied in detail and a large number of mutants were characterized [105–108].

Comparison to VMAT has suggested a number of residues that may be involved in catalysis. Replacement of N155 in VMAT2 with the Asp found in related bacterial protein does not affect transport activity. Also, replacement of the adjacent potential phosphorylation site (T154) with alanine does not impair function, indicating that phosphorylation at this site is not required for activity. Replacement of Gly 151 and 158 in the conserved motif GXXXXRXG in loop 3 has no effect on transport.

Random mutagenesis of BMR gave rise to four independent mutants exhibiting altered spectra of cross-resistance to various drugs (A. Neyfakh, personal communication). All these mutants were located within the region of transmembrane domains IX–XI. In addition, a reserpine-insensitive mutant located in the same region was identified [108]. Four more site-directed mutations in this region were engineered and all of them changed quantitatively the cross-resistance profile of bacteria expressing Bmr (A. Neyfakh, personal communication). These site-directed mutations were designed based on the following principle: the *S. aureus* homolog NorA, is very similar to Bmr in the cross-resistance profile it confers to *B. subtilis* [109], but different from their homologous Tet transporters. Amino acids conserved between Bmr and NorA were converted into the Tet-specific amino acids. For example, if a certain residue in Bmr and NorA is M, but in TetA, B and C the corresponding residue is G, M was converted in Bmr into G. Using the latter principle control mutations in some other regions of the transporter were made, one in the VIIth TM domain, another in the IVth TM domain. Both these control mutations gave changes in the resistance profile as large as the ones observed with the mutations in the IX–XI region indicating that other residues in different areas of the protein are needed for substrate specificity. It is not clear therefore why all the random mutations showing altered cross-resistance profile, were clustered in the IX–XI region (the probability of such a clustering by pure chance is just 1.2%).

An extensive mutagenesis study has been performed also in the *E. coli* TetA protein [105–107]. These studies highlight residues with putative roles in catalysis in TM domains VIII–IX, as well as in I–III. Other transport proteins, like the *lac* permease from *E. coli*, a 12 TM protein which catalyzes symport of H<sup>+</sup> and  $\beta$ -galactosides but does not belong to the TEXAN superfamily imply a similar conclusion. In addition to important residues in TM IX and X, it was found by Kaback and coworkers that very few of the residues in the other TM areas are essential [101]. In fact, TM II–VII can be completely deleted and the protein still catalyzes facilitated diffusion of lactose [110]. Studies by Wilson and collaborators on the melibiose symporter suggest that the above suggestions may be an oversimplification since more than one domain is involved in substrate recognition [111].

## 9. The MiniTEXANs, a family with unique properties

It was only during recent years that it became evident that there are more multidrug transporters than previously thought [11,21]. As described in the introduction, we

Table 2  
Members of the MiniTEXAN family

Name	Organism	Other names	Length	Known function	Acc. no.
EmrE	<i>Escherichia coli</i>	Ebr, Mvrc	110	Multidrug resistance	M62732
Smr	<i>Staphylococcus aureus</i>	QacC, Ebr	107	Multidrug resistance	M33479
QacE	<i>Klebsiella aerogenes</i>	–	110	Multidrug resistance	X68232
SugE	<i>Escherichia coli</i>	–	105	Suppression of GroE mutation	X69949

believe that this diversity is the result of a continuous selection that favors those organisms which have developed one or more strategies to cope with as many toxic compounds as possible.

One of the most interesting families of multidrug transporters, the MiniTEXANs, has received quite a lot of attention during the past year. This family includes only a few proteins (Table 2). The genes coding for most of them were identified on the basis of the resistance conferred. One protein in the family (SugE) has no known transport activity.

A unique feature of the proteins in this family is their size: 100–110 amino acids proteins with four putative transmembrane segments catalyze the same activities which have previously been observed in proteins three to nine times larger. The fact that the small proteins of this family are transporters has been previously hypothesized before [12,112,113]. Recently, EmrE and Smr were purified and reconstituted in a functional form and it was clearly demonstrated that a single polypeptide catalyzes the exchange of a toxic cation with protons, in an electrogenic exchange which suggests a possible stoichiometry of  $2\text{H}^+$ /cation [13,114]. The question has been raised then why are the other transporters so large when the minimal subunit necessary is at the most 110 amino acids. One possibility is that the Mini TEXANs function as a homo-oligomer to form at least the twelve transmembrane segments usually observed in other transporters. On the other hand, it is also possible that only some domains of the larger proteins are needed for transport and the rest functions for regulation of the activity and/or interaction with other proteins.

Another very unique feature of EmrE, is its solubility in chloroform:methanol which provides us with a powerful and unique tool for purification and characterization [13]. Since only a handful of minor proteins is soluble in these organic solvents, after overproduction of EmrE it was possible to extract it highly purified. Most importantly, EmrE was active after this treatment suggesting that the extraction did not induce major irreversible changes in its structure. The reconstituted protein has an apparent  $K_m$  for methyl viologen of  $247 \mu\text{M}$  and a  $V_{\max}$  of  $1572 \text{ nmol/min/mg protein}$ . The latter represents a turnover number of  $14 \text{ min}^{-1}$ , which is in the same order of magnitude of many ion-coupled transporters [115]. No quantitative data is available on the native protein but the specificity range of the purified protein is practically identical to the range of resistance reported. The solubility of EmrE in organic solvents does not reside in a specially high proportion

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EmrE      m n P Y I Y L G G A I L A E V I G T T L M K F S E G F T R L W P S V G T I I C Y
QacE      m k G W L F L V I A I V G E V I A T S A L K S S E G F T K L A P S A V V I I G Y
Smr       m - P Y I Y L L I A I S T E V I G S A F L K S S E G F S K F I P S L G T I I S F
SugE      m - S W I I L V I A G L L E V V W A V G L K Y T H G F S R L T P S V I T V T A M

EmrE      C A S F W L L A Q T L A Y I P T G I A Y A I W S G V G I V L I S L L S W G F F G
QacE      G I A F Y F L S L V L K S I P V G V A Y A V W S G L G V V I I T A I A W L L H G
Smr       G I C F Y F L S K T M Q H L P L N I T Y A T W A G L G L V L T T V W S I I I F K
SugE      I V S M A L L A W A M K S L P V G T A Y A V W T G I G A V G A A I T G I V L L G

EmrE      Q R L D L P A I I G M M L I C A G V L I I N L l s r s t p h
QacE      Q K L D A W G F V G M G L I V S G V V V L N L l s k a s a h
Smr       E Q I N L I T I V S I V L I I V G V V S L N I f g t s r --
SugE      E S A N P M R L A S L A L I V L G I I G L K L s t h ----

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Fig. 5. Alignment of MiniTEXANs. Multiple alignment was first performed using Pileup (Wisconsin Package, 1994) and then blocks of similarity were identified using MACAW [118].

of hydrophobic amino acids: the percentage in proteins is 49%, which is not different from other classical ion-coupled transporters, which is between 41 to 49.6% in seven randomly chosen  $H^+$ -coupled bacterial transporters analyzed based in the sequences available from the databank [13]. On the other hand the percent of charged amino acids is 7.2 while in the twelve transmembrane helix transporters scanned as above it is between 11 and 14.8%. Another important difference is the number of net charges which is only +1 in EmrE. The range in the other transporters is between -7 and +7 with the lowest absolute number of net charges in the group analyzed being 4. It is therefore suggested that the existence of unpaired charges on the protein represent the main energy barrier for solubilization in the highly hydrophobic milieu of the organic solvents. This has been extensively discussed in the case of charge insertion in membranes [116] and has also received some experimental support in experiments in which neutralization of charges of membrane proteins with  $H^+$  or  $Ca^{++}$  rendered them soluble in organic solvents [117].

The range of substrates recognized by EmrE has been summarized in Fig. 2. As already discussed there is a large overlap with the specificities displayed by other multidrug transporters.

An alignment of the four proteins is shown in Fig. 5. TMS I and TMS II show the highest conservation. Several of the conserved residues have been mutagenized and activity is lost or modified [113,114].

Although there is no sequence similarity with other multidrug transporters, the MiniTEXANs share many of the properties of their larger analogs: they confer resistance to a variety of toxicants thanks to their ability to actively remove them away from their target. Because of their size and unique properties, the MiniTEXANs may provide a very useful model to understand the molecular basis of recognition of multiple substrates with high affinity as displayed by the other multidrug transporters. It also should be a good model to understand structure-function aspects of transport reactions in ion-coupled processes in general.

## Abbreviations

DCC, , N,N'-dicyclohexylcarbodiimide  
 DEPC, diethylpyrocarbonate  
 LDCV, Large dense core granules  
 MPP<sup>+</sup>, N-methyl-4-phenylpyridinium  
 SLMV, Synaptic like microvesicles  
 TBZ, , Tetrabenazine  
 TBZOH, Dihydro-tetrabenazine  
 VACHT, Vesicular acetylcholine transporter  
 VMAT, Vesicular monoamine transporter  
 VNT, , Vesicular neurotransmitter transporter  
 $\Delta\tilde{\mu}_{H^+}$ , Proton electrochemical gradient

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