# Vesicular Neurotransmitter Transporters as Targets for Endogenous and Exogenous Toxic Substances

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## Abstract

Exocytotic release of neurotransmitters requires their accumulation inside preformed secretory vesicles. Distinct vesicular transport activities translocate classical transmitters into synaptic vesicles energized by a $H^+$ electrochemical gradient ($\Delta\mu_{H^+}$), with subtle but important differences in dependence on the electrical and chemical components. The vesicular transporters also interact with toxic compounds and drugs. They mediate neuroprotection by sequestering toxic compounds as well as neurotransmitters into vesicles, reducing their concentration in the cytosol where they may have detrimental effects. Both therapeutic agents and psychostimulants interfering with vesicular transport have yielded insight into the pathogenesis of psychiatric as well as neurodegenerative diseases. Thus, specific inhibitors have helped to characterize both the physiological role and mechanism of vesicular neurotransmitter transport.

# INTRODUCTION

## Accumulation of Classical Neurotransmitters in Synaptic Vesicles

Classical synaptic transmission requires storage of transmitters inside synaptic vesicles and involves $Ca^{2+}$-dependent exocytosis and discharge of the accumulated neurotransmitter (1, 2). The neurotransmitter released activates specific receptors located within or in the vicinity of the synapse (3–5). Precursors for neural peptides and hormones are translocated into the lumen of endoplasmatic reticulum as they are translated into proteins, and thereby reside within the secretory compartment before they are available for release (6). However, classical neurotransmitters are either generated in the cytoplasm or they are recycled by uptake of released neurotransmitter through specific plasma membrane transporters (7). In any case, they require active translocation against their electrochemical gradient across the lipophilic membrane of synaptic vesicles (8).

## V-ATPase Generates a $H^+$ Electrochemical Gradient that Energizes Vesicular Transport

Biochemical studies have identified several vesicular transport activities for the classical neurotransmitters (**Figure 1**). Transport into secretory vesicles of the monoamines dopamine, serotonin, adrenaline, and noradrenaline is mediated by a common carrier, whereas a single carrier mediates translocation of the inhibitory transmitters GABA and glycine (8, 9). Distinct vesicular transport carriers also exist for glutamate and acetylcholine (ACh) (7). Bioenergetically, these activities differ substantially from activities transporting transmitters across the plasma membranes: The vesicular transport activities are energized by a $H^+$ electrochemical gradient ($\Delta\mu_{H^+}$), in contrast to the plasma membrane transport activities, which are mostly coupled to $Na^+$ running down its electrochemical gradient (10, 11). An $Mg^{2+}$-dependent vacuolar-type $H^+$-ATPase (V-ATPase) resides on membranes of the secretory pathway, including secretory vesicles, endosomes and lysosomes (the vacuole in yeast). This pump does not resemble P-type ATPases expressed at the plasma membrane of mammalian cells (e.g., the $Na^+/K^+$-, muscle $Ca^{2+}$-, and gastric $H^+/K^+$-ATPases); rather, it shows structural and functional similarities to the F-type (F0F1) ATPases of bacteria, mitochondria, and chloroplast (12, 13). These proteins make multisubunit complexes consisting of a cytoplasmic complex ($V_1$, $F_1$, or $P_1$) and a membrane-bound complex ($V_0$, $F_0$, or $P_0$), hydrolyzing or synthesizing ATP and translocating $H^+$, respectively (14, 15). Pharmacological disruption of these proteins has provided important insight into molecular mechanisms involved in the function of the V-ATPase. In particular, it has been shown that the macrolide antibiotics bafilomycin and concanamycin intercalate between the helices of subunit c of $V_0$ (16). This subunit consists of six proteolipid subunits that form a $H^+$-binding ring and disruption of its rotation inhibits ATP hydrolysis and $H^+$ pumping. Thus, the vesicular $H^+$-ATPase couples hydrolysis of ATP to the movement of $H^+$ into the vesicle lumen and generates an electrochemical gradient for $H^+$: $\Delta$pH ~1.4 units and an electrical

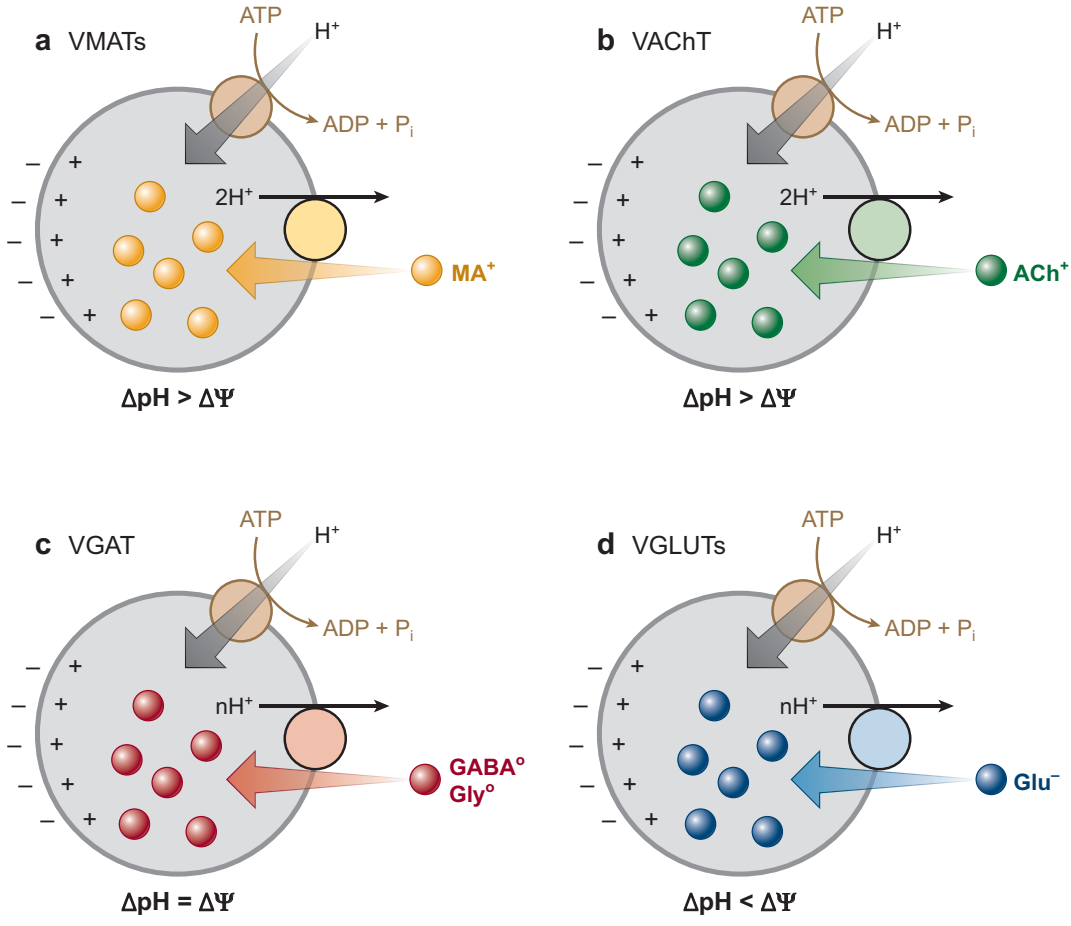


**Figure 1**

Vesicular neurotransmitter transporters depend differentially on the two components of the electrochemical gradient of $H^+$ ($\Delta\mu_{H^+}$). A V-ATPase generates a $\Delta\mu_{H^+}$ across the vesicle membranes. The vesicular transporters use this gradient to drive the transport of transmitters into secretory vesicles by coupling the translocation of transmitter to $H^+$ running down $\Delta\mu_{H^+}$. The different vesicular transporters rely to different extents on the two components ($\Delta$pH and $\Delta\Psi$) of this gradient. (*a*) VMATs and (*b*) VAChT transport their positively charged substrates coupled to the exchange of two $H^+$, and hence rely primarily on $\Delta$pH. (*c*) GABA and glycine are transported as neutral zwitterions by VGAT, which depends equally on both the chemical and the electrical component of $\Delta\mu_{H^+}$. (*d*) VGLUTs transport the negatively charged glutamate and thus rely more on $\Delta\Psi$ than $\Delta$pH.

potential ~+39 mV have been measured in cholinergic and aminergic vesicles (17, 18).

Several ionophores are capable of dissipating the electrochemical gradient for $H^+$ directly. Nigericin dissipates the chemical gradient for $H^+$ ($\Delta$pH) by exchanging $H^+$ for $K^+$, whereas A23187 exchanges $H^+$ for $Ca^{2+}$ or $Mg^{2+}$.

FCCP/CCCP (carbonylcyanide *m*-chlorophenylhydrazone/carbonylcyanide *p*-trifluoromethoxyphenylhydrazone) allows $H^+$ to run down their electrochemical gradient. However, the development of a membrane potential will limit dissipation of the gradient. In contrast, the $K^+$ ionophore valinomycin will specifically disrupt $\Delta\Psi$. Interestingly, although the different vesicular carriers couple neurotransmitter transport to $H^+$ running down its electrochemical gradient, they rely differentially on the two components of the $H^+$ electrochemical gradient. The activities for monoamines and ACh depend to a higher degree on the $\Delta$pH, the vesicular glutamate transport more on the $\Delta\Psi$, whereas GABA and glycine transport relies equally on both gradients (**Figure 1**). As the ionophores may dissipate the two aspects of the $\Delta\mu_{H^+}$ differentially, they also have a differential effect on the accumulation of the different transmitters (19).

### Vesicular Transporters Have a Distinct Pharmacological Profile

Consistent with the differences in bioenergetics, the proteins encoding vesicular activities represent three entirely different families: monoamine and ACh transporters (SLC18), vesicular glutamate transporters (SLC17), and the GABA and glycine transporters (SLC32). However, they all show distant homology to a large number of bacterial resistance genes and vesicular transport resembles the bacterial efflux of many drugs (7), rather than the concentrative uptake by plasma membrane transporters. Accordingly, well-known drugs working on plasma membrane transporters have no effect on vesicular transporters. Rather, the distinct cellular localization of vesicular transporters and their distinct molecular identity suggest that they may be targets of an entirely different set of drugs that includes psychostimulants and environmental toxic substances.

## VESICULAR MONOAMINE TRANSPORT

### VMAT1 and VMAT2

Two monoamine transporters, VMAT1 and VMAT2, have been identified as responsible for the transport of dopamine, serotonin, adrenaline, and noradrenaline into the vesicles (8, 20, 21). Studies on monoamine uptake by chromaffin granules from the adrenal medulla show that the uptake of one cytoplasmic monoamine is coupled to the movement of two $H^+$ in the opposite direction (22). As the activity recognizes the protonated and hence charged form of the substrates, the transport cycle results in net efflux of only one charge despite the two protons. The $H^+$ electrochemical gradient and the coupled stoichiometry favor accumulation of monoamines at molar concentrations in chromaffin granules. Indeed, monoamines interact with other vesicular components to form insoluble aggregates within chromaffin granules (23).

Recent studies have suggested that pharmacological agents increasing the activity of the neuronal isoform VMAT2 are neuroprotective. Pramipexole, a D2/D3 agonist used in the treatment of Parkinson's disease, increases vesicular dopamine uptake and protects against the loss of dopaminergic neurons (24). In addition, apomorphine has been shown to increase VMAT2 function and confer neuroprotection (25). Although

the pathogenesis of Parkinson's disease remains unknown, cytosolic dopamine is toxic owing to its metabolite hydrogen peroxide and to the production of highly reactive quinones (26). The neuroprotective effect of vesicular monoamine transport may be to prevent their metabolism by sequestration of exogenous and endogenous aromatic amines into synaptic vesicles.

Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in rats provides a very useful animal model for idiopathic Parkinson's disease (27). The lipophilic MPTP penetrates the blood-brain barrier relatively easily and is metabolized by glial monoamine oxidase B (MAO-B) to the active metabolite N-methyl-4-phenylpyridinium ($MPP^+$) (28, 29). $MPP^+$ resembles protonated monoamines, and is taken up by plasma membrane monoamine transporters in monoamine cells (30), where it may enter mitochondria, inhibit oxidative phosphorylation, and trigger apoptosis (31–33). However, $MPP^+$ is recognized by the vesicular monoamine transporters, which sequester the toxin inside vesicles and thus protect against its mitochondrial toxicity. Selection in $MPP^+$ was indeed used to isolate the first cDNA encoding a vesicular neurotransmitter transporter (20). Further, VMAT1 and VMAT2 show sequence similarity to bacterial proteins involved in detoxification rather than to plasma membrane monoamine transporters (7, 34–36). Several of the bacterial proteins are inhibited by the same drugs that inhibit VMATs (e.g., reserpine) (37) and transport substrate in exchange for $H^+$ (7). Also, cytotoxic compounds such as doxorubicin, rhodamine, ethidium, isometamidium, and tacrine are recognized by the VMATs as well as bacterial drug-resistance genes, including the toxin-extruding antiporters (TEXANs) (38).

## Substrate Specificity and Turnover Number

VMAT2 is exclusively expressed by monoamine neurons in the central nervous system (e.g., substantia nigra, locus coeruleus, and the raphe nucleus) and selected peripheral endocrine populations (39–41). In contrast, VMAT1 expression occurs in peripheral endocrine tissues (e.g., chromaffin cells in the adrenal medulla) and paracrine cells (e.g., enterochromaffin cells in the stomach and intestines, and small intensely fluorescent cells in sympathetic ganglia) (42).

VMAT2 has a higher affinity for most monoamines (by approximately threefold) than VMAT1 (42, 43), but only VMAT2 appears to recognize histamine, which may bind to a site different from that of other monoamines (44). Both can transport and protect against $MPP^+$ toxicity.

Consistent with differences in the recognition of substrates by the two transporters, reserpine, an alkaloid, competes with monoamines for binding to the VMATs and has threefold higher affinity for VMAT2 ($K_i$ 12 nM) than for VMAT1 ($K_i$ 34 nM) (42, 43). The $H^+$ electrochemical gradient also influences the binding of reserpine, apparently by driving reorientation of the substrate recognition site from the lumen to the cytoplasmic surface of the vesicle (45). Owing to its actions on both VMATs, reserpine is very effective in treating hypertension, but frequently produces the side effect of depression. The former effect is due to its effect on VMAT1 in the peripheral sympathetic nervous system, and the latter on VMAT2 in the brain. Indeed, the

inhibition of VMAT by reserpine gave rise to the monoamine hypothesis of affective disorders (46, 47).

In contrast to reserpine, the binding to VMAT of the other major inhibitor tetrabenazine is affected only by very high concentrations of monoamines, indicating a distinct site of interaction (48). Tetrabenazine shows a high affinity for VMAT2. In contrast, VMAT1 is essentially resistant to inhibition by tetrabenazine both in human and particularly in rat (42, 45, 49). Reserpine administration in vivo does not alter the level of VMATs, but single injections can block the binding site for tetrabenazine (50). The interaction between binding of reserpine and tetrabenazine suggests that the binding sites are either overlapping or that the drugs interact with conformationally distinct stages of the transport cycle (8, 48).

Several other ligands for the tetrabenazine binding site have been identified. Ketanserin, a serotonin receptor antagonist, and a photoactivatable analogue (7-azido-8-iodoketanserin) have been used to demonstrate that the tetrabenazine binding site is located at the N terminus of bovine VMAT2 (51, 52). Lobeline, an alkaloid resembling nicotinic receptor agonists (53), also inhibits VMAT by interacting with the tetrabenazine binding site (54). Thus, VMAT inhibitors appear to fall into two classes: those that bind to the reserpine site and those that bind to the tetrabenazine site. Mutagenesis studies indicate that aspartate-33 and serines-180–182 in VMAT2 play a critical role in substrate recognition, presumably by interacting with the protonated amino group and hydroxyl group on the catechol or the indole rings (44). Tyrosine-434 and aspartate-461 are identified as being responsible for the high-affinity interaction of tetrabenazine, serotonin and histamine in VMAT2 (55).

The turnover number for VMATs depends on the substrate. At 29°C, they transport ~5 molecules of serotonin per second, and four times that number of dopamine molecules (43). This rate has important implications for quantal size. Monoaminergic synaptic vesicles contain 5–20,000 molecules of transmitter and synaptic vesicles can generally recycle within at least 20 s (56, 57). At 5 molecules per second, the vesicle would contain only 100 molecules of transmitter after 20 s—if there were only one transporter per vesicle. Although recent estimates suggest several transporters per vesicle (58), it requires substantially higher turnover at 37°C to fill a rapidly cycling vesicle with monoamine. Indeed, the ability to determine the turnover of VMATs has been made possible by the availability of ligands to quantify the number of transporters and hence provide a denominator for measurements of transport.

## Psychostimulants

Psychostimulants generally act by increasing the extracellular level of monoamines. The mechanism involves an effect on the transport of monoamines, either by inhibition of the transporter responsible for neurotransmitter reuptake or by stimulation of efflux. Both the monoamine plasmalemmal transporter and the vesicular monoamine transporter, VMAT2, are involved. The two most-studied phenylethylamine psychostimulants in this context are amphetamine and the methamphetamines. Amphetamine is a lipophilic weak base that can deplete the monoamines by dissipating the $\Delta\mu_{H^+}$ that drives the dopamine uptake. Amphetamines also inhibit VMAT2 directly. In the

case of VMAT2 KO mice, amphetamine mobilizes the release of the cytoplasmic dopamine and increases the survival of these mice by circumventing the defect in vesicular release.

High-dose administration of D- and D,L-methamphetamine disrupts VMAT2 function and increases the cytoplasmic level of dopamine. This will increase the formation of reactive dopamine species and metabolites, and can lead to the destruction of dopaminergic structures (59). Multiple high doses of methamphetamine decrease VMAT2 levels within one hour and maintain a low level for more than 24 h. At the same time, dihydrotetrabenazine binding sites are removed from vesicles (60). Riddle et al. (62) suggested that methamphetamine displaces VMAT2 away from the vesicles to a site outside the vesicles. Methamphetamine also directly inhibits VMAT2 activity, but in the experiments discussed above methamphetamine was "washed" away from the vesicles and researchers cannot explain the results.

Methylphenidate and lobeline reverse the effect of methamphetamine on VMAT2 (61). Thus, by increasing cytoplasmic dopamine sequestration through redistribution of VMAT2, the toxic effects of cytoplasmic dopamine are decreased. This process seems to take place through dopamine receptors (D1 and D2) because dopamine receptor antagonists abolish its effects (62).

Cocaine is a reuptake blocker, which inhibits the plasma membrane transporters for dopamine (DAT), noradrenaline (NET), and serotonin (SERT) (63). In addition, cocaine mobilizes VMAT2-expressing vesicles and increases the $V_{max}$ of VMAT2 for dopamine and the number of binding sites for dihydrotetrabenazine (64). It also mobilizes a synapsin-dependent reserve pool of dopamine-containing synaptic vesicles, thereby increasing the release of dopamine (65).

Ecstasy, or 3,4-methylenedioxymethamphetamine (MDMA), is a commonly abused drug that mainly affects serotonergic neurons. In vitro, the compound inhibits both the synaptosomal and vesicular uptake of serotonin and dopamine, to roughly the same extent. Ex vivo experiments in rat support dopaminergic inhibition with short-term exposure ($3 \times 15$ mg $kg^{-1}$; 2-h interval) and serotonergic inhibition after long-term exposure ($2 \times 10$ mg $kg^{-1}$ $day^{-1}$ for 4 days) (66). During short-term exposure, there was a reduction in VMAT2 activity, which reversed after 24 h and therefore differs from the effect of methamphetamine (61). The effect of MDMA was also compared with the structurally related antidepressant drug paroxetine. One important difference between paroxetine and MDMA was that paroxetine inhibited plasmalemmal transport of serotonin greater than 100-fold more effectively than vesicular uptake, whereas MDMA inhibited both synaptosomal and vesicular uptake of serotonin at the doses used (66).

## Environmental Toxic Substances

Halogenated aromatic hydrocarbons are chemical compounds that are stable and lipophilic, and can accumulate to high levels through the food chain. They are produced in several millions of tons and spread globally. Almost all humans contain these compounds. Owing to global distillation, a large proportion of these compounds end up in the Arctic areas. The compounds are stored in adipose tissue and redistributed

during starvation (a common occurrence at the poles) to the brain and liver. One such group of environmental toxic substances is the polychlorinated biphenyls (PCBs), of which ~4 million tons have been produced. The PCBs consist of 209 different congeners and are separated into a coplanar group and a noncoplanar group. PCBs are not found as single congeners in nature, but rather in large groups depending on their properties and use. PCBs are used in electrical insulation, paints, building materials, etc. Epidemiologic and behavioral studies have shown that PCBs of the noncoplanar group have an effect on neurological development and impair human cognitive function. Subchronic exposure and several in vitro studies have led to the conclusion that noncoplanar PCBs have an effect on the dopaminergic system (see review in 67). These effects could in part be explained by their inhibition of dopamine transport systems. An effect on the plasma membrane transport system may lead to a reduction in dopamine, whereas an inhibition of VMAT2 will increase the DAT/VMAT2 ratio and thereby the intracellular cytoplasmic level of dopamine. This may favor oxidation of dopamine and the destruction of dopaminergic cells (68, 69). The highest content of PCBs in the brain was found in dead glaucous gulls in the Arctic, reaching concentrations up to 90 μM (70). In vitro studies have shown that PCBs inhibit VMAT2 in the low micromolar concentrations (71). Structure-activity studies have shown that the nonplanar penta- and hexa-PCBs are the best inhibitors. Further, such studies showed that good inhibitors cannot be explained from a few simple factors. The most important parameters are (*a*) that it is nonplanar, although an increased number of orthochlorines were of less importance, and (*b*) the octanol-water coefficient, which indicates the size of the molecule (72). The effect on the dopamine system has been confirmed by long-term administration of PCB in rats, which lowers both VMAT2 protein and activity (73). Further, microdialysis of rat striatum after administration of PCB has suggested that the results could be explained by inhibition of DAT and then inhibition of VMAT2 (74). Treatment of striatal synaptosomes with PCB also showed an increase in cytoplasmic dopamine owing to inhibition of VMAT (75).

Another group of important halogenated hydrocarbons are brominated flame retardants (BFRs), which are produced in several hundred thousands of tons per year. These compounds are widely distributed in clothing, furniture textiles, and electrical devices. The brominated hydrocarbons consist of several groups that, in small doses, have an effect on brain behavior (see review in 67). The BFRs can be separated into three chemical groups: diphenyl ethers, hexabromododecane, and terabromobiphenyls. To date, there have been only a few studies on the molecular effects of BFRs on the nervous system. It has been shown, however, that all three groups inhibit the uptake of dopamine in synaptic vesicles in the low micromolar range ($IC_{50}$: 3–8 μM). They are as efficient as the PCBs as inhibitors of VMAT2 and they may therefore have a toxic effect on dopaminergic structures (72).

Although banned in the 1970s, the organochlorine pesticide heptachlor is still present in high concentrations in the environment, raising concern about human exposure. Studies have shown that exposure to heptachlor during development increases the level of dopamine transporters. Female mice that receive 3 mg $kg^{-1}$ heptachlor every 3 days from two weeks before breeding through pregnancy, lactation, and until the offspring reached 21 days have offspring with altered dopaminergic properties. At

postnatal day 28, DAT increased by 100%, VMAT2 by 70%, and tyrosine hydroxylase by 30%. In addition, the toxic component of heptachlor epoxide inhibits VMAT2 activity (69). The DAT/VMAT2 ratio increased, suggesting that the animals might be more susceptible to Parkinson's disease (76). In addition, the toxic component of heptachlor epoxide inhibits VMAT2 activity (69). Similarly, dieldrin administrated during gestation and lactation led to an increase of both DAT and VMAT2 in striatum and changed the balance between DAT and VMAT2 (77). In this case, the effect on DAT was larger than on VMAT2. The importance of these findings was demonstrated by an increased toxic effect of MPTP in dieldrin-treated mice (77).

One of the problems with the lipophilic environmental toxic substances is the fact that they usually occur together and therefore the final toxic effects are the result of an integrated response of several different toxic substances. We have recently shown that PCB, BFRs, and methyl mercury (MeHg) have an additive effect on the vesicle transport of dopamine and glutamate (78). Their toxic effect may therefore be greater than expected from their effect on a single component.

## THE VESICULAR ACETYLCHOLINE TRANSPORTER

Organophosphorus toxins were used to screen for the gene encoding a vesicular ACh transporter (VAChT). Organophosphorus toxins inhibit acetylcholinesterase (AChE), which terminates cholinergic transmission by degrading ACh in the synaptic cleft. In *Caenorhabditis elegans*, resistance to these toxins suggested a defect in ACh release. Subsequent identification of mutations in a region similar to VMAT2 (*unc-17*) led to the identification of (VAChT) (79). The rat VAChT shows high homology to rat VMATs (40% identity, 65% similarity) and the predicted topology also suggests 12 transmembrane domains with N and C termini in the cytoplasm (80, 81). The biochemical properties of VAChT also resemble those of the VMATs. Transport by VAChT depends more on $\Delta$pH than on $\Delta\Psi$ (82). As ACh is permanently charged and the transport is electrogenic (82), it is believed that more than one proton is coupled to VAChT-mediated transport. However, there are also differences between VAChT and VMATs. Although the coupling of two protons predicts steep concentration gradients, VAChT transport is much less efficient than VMAT transport. Surprisingly, the carrier may leak protons, dissipating the driving force (83). The turnover of VAChT ($\sim$1 $s^{-1}$) is also much slower than the turnover for VMATs (84).

VAChT has a much lower affinity for its substrate (millimolar) compared with VMATs (low micromolar) and also appears to have low substrate specificity (85–87). However, it is not inhibited by the VMAT blocker reserpine or by its own precursor choline (88, 89). Conversely, the best-characterized inhibitor of VAChT, (-)-trans-2-(4-phenylpiperidino)-cyclohexanol (vesamicol) (90), does not inhibit VMATs. VAChT has a distinct binding site for vesamicol but it is also possible that the ACh binding site regulates the access of vesamicol (91). Thus, substrates compete with vesamicol for binding (92), but the inhibition is noncompetitive (93). Several ACh analogues have been characterized (86, 94). Some of them, such as cetiedil, inhibit VAChT competitively by interacting with the ACh binding site (86, 95).

mRNA for VAChT colocalizes with the mRNA for the biosynthetic enzyme choline acetyltransferase (ChAT), and the VAChT protein resides on synaptic vesicles of cholinergic neurons in the central nervous system (80, 96, 97). Drugs that act on proteins involved in the metabolism of ACh may also have effects on VAChT. Rivastigmine is an AChE inhibitor widely used to reduce memory impairment associated with several types of dementia (98). Rivastigmine also increases VAChT immunoreactivity (99). VAChT is not affected by treatment with the Alzheimer drug Aricept (100).

## THE VESICULAR GLUTAMATE TRANSPORTERS

### Molecular Identification of Vesicular Glutamate Transporters

In contrast to the vesicular monoamine and ACh transport, glutamate transport depends primarily on $\Delta\Psi$ rather than $\Delta$pH (101–104). Recently, three closely related proteins, vesicular glutamate transporters (VGLUT)1–3, have been shown capable of translocating glutamate into synaptic vesicles (105–107). They exhibit similar functional characteristics, but the isoforms differ in expression at cellular and subcellular levels, and the isoforms undergo switching during normal development (108–110). In the adult, VGLUT1 and VGLUT2 show a complementary expression pattern, occurring in different subpopulations of glutamatergic nerve terminals (111, 112). In particular, VGLUT1 is pronounced in telencephalic regions and is expressed by glutamatergic cells in the cerebral cortex, hippocampus, and cerebellar cortex. VGLUT2 predominates in glutamatergic cells of diencephalon, including the thalamus, brainstem, and deep cerebellar nuclei. The two homologues are also involved in glial glutamate release (113, 114). In contrast, VGLUT3 is not restricted to glutamatergic neurons, rather it also appears in glial cells and nonglutamatergic neurons, including serotonergic, cholinergic, and GABAergic (109, 115, 116). VGLUT3 is also targeted to the cell bodies and dendrites where it participates in retrograde signaling (117). Finally, transient expression of VGLUT2 and VGLUT3 at high levels in specific cells early in life suggests additional, developmental roles for glutamatergic neurotransmission (109).

### Substrate Specificity and Glutamate Analogues

The apparent substrate affinity of the VGLUTs is low, with $K_m$ ~1–3 mM (101, 102, 118). Surprisingly, the VGLUTs show no affinity for either L-aspartate or D-aspartate, and the aspartates and the sulfur analogue cysteate are not even inhibitors at high concentrations. In contrast D-glutamate, the sulfur analogue homocysteic acid, and L-aminoadipic acid, an amino acid with a higher number of carbon spacing the acidic groups, are inhibitors. Kynuerenate (quinoline-2-hydroxyl,4-carboxylate) is also an inhibitor of vesicular glutamate transport (119). This is surprising because it indicates that a hydroxyl group can substitute for one of the carboxyl groups. The substrate-like compound with the highest affinity is cyclopentane-1,3-dicarboxylate, with a $K_m$ ~40 $\mu$M (120). Hydroxy, methyl, and methylene groups substituted on the carbon

chain maintain the inhibition. A series of quinoline-2,4-dicarboxylic acids, with suitable spacing between carbon atoms, have therefore been investigated as inhibitors of vesicular glutamate transport. Addition of electron donors to the ring tends to reduce the inhibition, but the addition of a 5-, 6-styryl group and diazophenyl analogues conferred particularly good inhibition. 6-substituted aromatic systems bearing alkene and phenyl spacer groups were similarly effective (121). This means that the activity of vesicular glutamate transport is retained with C-C-C, or even an extended carbon chain. Further, substitution of one carboxyl group by -OH, an increase to C4 between the carboxyl groups and D-glutamate allow some recognition at the binding site.

## Chloride Stimulation of Vesicular Glutamate Uptake

The uptake of glutamate, which is an anion, is stimulated three- to sixfold by 1–5 mM chloride, bromide, and to a lesser extent, iodide ions. On the other hand, a low concentration of fluoride inhibited the stimulation (101, 118). The apparent $K_m$ for chloride is 0.2 mM (122). At high chloride, the uptake of glutamate is inhibited owing to the collapse of $\Delta\Psi$ (102). The stimulation by low chloride ions is not completely understood. The ions may activate the transport protein directly either by influx into vesicle lumen, or through binding to an anion-sensitive allosteric site. Different anion channel blockers, such as the stilbene derivatives 4-acetamido-4-isothiocyanostilbene-2,2-sulfonic acids (SITS), 4,4-diisothiocyano-22-stilbene-disulphonic acids (DIDS), and 5-nitro-2-(-3-phenylpropylamino)-benzoic acid (N144), are known to inhibit the vesicular uptake of glutamate (123), and subsequent work showed that SITS and N144 were competitive inhibitors of glutamate and noncompetitive toward chloride (122). DIDS was also later confirmed to be an inhibitor of glutamate uptake (124, 125). Hartinger & Jahn (124) found that the inhibition of glutamate uptake by DIDS was more efficient than its inhibition of $\Delta\Psi$, $\Delta$pH, and the V-ATPase. When the proton pump of the vesicles was substituted by the proton pump of bacteriorhodopsin, the uptake of glutamate was still dependent on chloride ions (126). Consistent with this, Wolosker and coworkers have shown that although chloride flux would influence the driving force for glutamate transport, the regulation by chloride does not depend on $\Delta$pH or $\Delta\Psi$, but rather represents an allosteric form of regulation (127). Recent reconstitution of purified VGLUT2 from a heterologous expression system has demonstrated the same biphasic dependence on chloride observed for the native protein (128), suggesting that the chloride binding site is not encoded by another polypeptide. Further, this hypothesis has recently received some support (129). It was shown that vesicular monoamines were negatively regulated by subunits of trimeric G-proteins, including $G\alpha_{o2}$ and $G_q$. Using a series of mutant mice lacking various G-subunits, it was shown that VGLUTs were exclusively regulated by $G\alpha_{o2}$. Surprisingly, the $G\alpha_{o2}$ seems to affect the regulation by chloride. In contrast to maximal activation of the VGLUTs by 4–10 mM chloride, activation of $G\alpha_{o2}$ with a nonhydrolyzable form of GTP shifts the optimum to lower chloride concentrations. In the absence of $G\alpha_{o2}$ (using $G\alpha_{o2}$ knockout mice), VGLUTs lose their regulation by chloride. Thus, $G\alpha_{o2}$ apparently influences the allosteric regulation of VGLUTs by chloride (129).

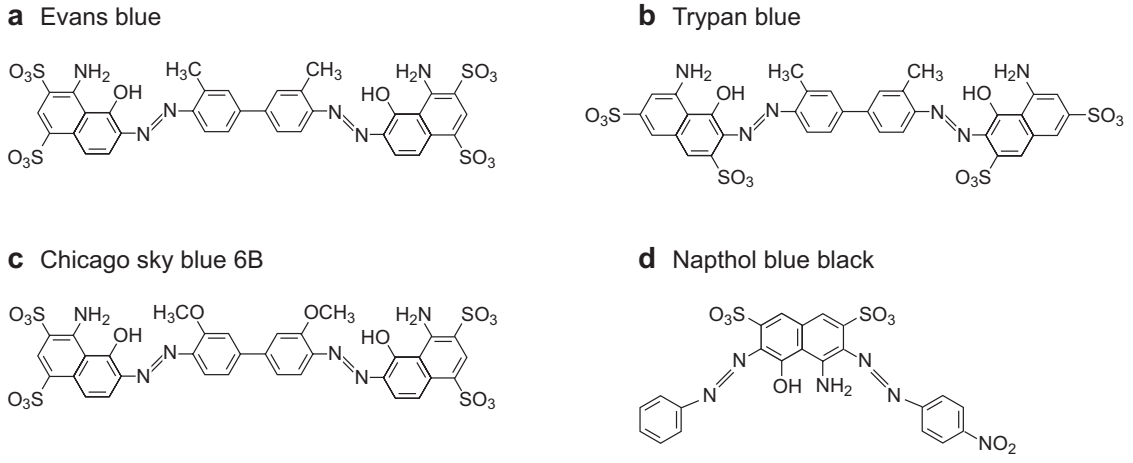


**Figure 2**

Chemical structure of four inhibitors of vesicular glutamate uptake. (*a*) Evans blue, (*b*) Trypan blue, (*c*) Chicago sky blue, and (*d*) Napthol blue black. Note that two methyl groups in Evans blue and Trypan blue have been changed to two methoxy groups in the weaker inhibitor Chicago sky blue 6B. Also note that in the inhibitor Napthol blue black two azo groups are maintained.

## Aromatic Dyes

The best inhibitors of vesicular glutamate uptake are a series of large azo dyes with a biphenyl group and amino and sulfonic acid groups substituted to naphtyl groups on the side chain (**Figure 2**). The most potent compounds in this group are competitive inhibitors of glutamate and they are 100–1000-fold more active toward glutamate than GABA uptake in vesicles. They do not act on the proton pump nor do they inhibit the uptake of glutamate through the plasma membrane. The most potent of these compounds is Trypan blue with an $IC_{50}$ of 49 nM. Many of the aromatic dyes show an affinity for AMPA-type glutamate receptors (130). Interestingly, Evans blue and Chicago sky blue have near identical structures except that Evans blue (and Trypan blue) has two methyl groups sticking out from the diphenyl group, whereas Chicago sky blue has two methoxy groups bending inward. The former could give a better fit to the vesicular transporter and account for the fact that Evans blue is 40-fold more potent as an inhibitor than Chicago sky blue. In addition, the dimethyl group is more hydrophobic than the methoxy groups. The three inhibitors have a near planar form, but are slightly rotated (31°) around the biphenyl bond in the middle. Naphtol blue black, which contains the substituted naphtol group of Trypan blue linked by two azo groups to two phenyl groups, behaves as a strong competitive inhibitor of vesicular uptake ($IC_{50}$ = 330 nM). In contrast, when the substituted naphthol group in Trypan blue is linked by an azo group to one substituted phenyl group only, the inhibition becomes almost 100-fold weaker (131). Altogether, 15 dyes, including Reactive blue, Ponceau SS, benzopurpurin 4B Direct blue 71, and Acid red 114, were found to inhibit vesicular glutamate transport in the nanomolar and low micromolar range (131).

These compounds have been extensively used to identify glutamate vesicular uptake. Thus, the most potent VGLUT inhibitors are hydrophobic and slightly twisted. Further, they contain two azo groups linked to a phenyl group or naphthyl group, and they may contain sulfonic groups instead of carboxylic acid groups.

Interestingly, Evans blue can inhibit VGLUT1 and VGLUT2 better than VGLUT3, and therefore indicate a structural difference around the active site of the three vesicular transporters (115). Subsequently, another dye, Rose Bengal, was reported to be as potent as Trypan blue, but act in a noncompetitive manner. Rose Bengal is heavily chlorinated and iodated, whereas the similar inactive fluorescein has no halogen groups (132). The halogen group will make the molecule more hydrophobic. Rose Bengal does not inhibit vesicular glutamate uptake at the level of the proton pump, but could interact at an allosteric site at the transporter. Because Rose Bengal is membrane permeable, it is much more useful in vivo and in brain slice experiments, where it has been shown to impair glutamate release (133). It is not known whether Rose Bengal has an effect on the AMPA receptor.



## VESICULAR TRANSPORT OF GABA AND GLYCINE

### VGAT Mediates Vesicular GABA and Glycine Transport

GABA was first shown to be taken up into isolated synaptic vesicles from forebrain by an electrochemical proton gradient driven by an Mg-ATPase (134, 135). Similarly, glycine was shown to be taken up through the same mechanism (136, 135). It is more difficult to demonstrate GABA and the glycine transport than glutamate transport, partly because they have a much lower apparent affinity for the transporter ($K_m$ ~4–6 mM and 12–14 mM, respectively), and possibly because they are mechanically less stable (appear elliptical by electron microscopy). Glycine, β-alanine, and vinyl-GABA were competitive inhibitors of vesicular GABA uptake, whereas GABA and β-alanine were competitive inhibitors of glycine uptake, indicating that the two transporters could be similar (137). This view was supported by the finding that the ratio of GABA and glycine uptake was similar in different regions of the brain, particularly in regions where glycine was not regarded as an active neurotransmitter (138). Further, physiological studies documented corelease of GABA and glycine from the same synaptic vesicles (139).

This controversy was finally solved by the characterization of the vesicular GABA transporter (VGAT). Genetic analysis in *C. elegans* identified a protein with multiple transmembrane domains (UNC-47) required for GABAergic transmission (19). The mammalian ortholog VGAT conferred GABA uptake dependent on both $\Delta pH$ and $\Delta\Psi$ when expressed in a heterologous expression system (19). Consistent with the differences in ionic coupling, vesicular GABA transport is encoded by a protein with no discernible sequence similarity to the VMATs or VAChT. The expressed transporter exhibits low-affinity uptake of GABA ($K_m$ ~5 mM), consistent with results from native synaptic vesicles, and glycine inhibits GABA transport ($IC_{50}$ ~25 mM) (19, 52). Furthermore, immunogold electron microscopy demonstrated VGAT expression in GABAergic, glycinergic, as well as mixed terminals (9). Indeed, disruption of VGAT

reduces release of GABA as well as glycine (140), consistent with the evidence for corelease in vivo (139).

### Inhibitors of Inhibitory Transmitter

No drugs acting specifically on VGAT have thus far been identified. The most interesting inhibitor is the anticonvulsant γ-vinyl-GABA, which inhibits GABA-aminotransferase (19, 134). This compound is used at high concentrations as an anticonvulsant, but there is no indication of an effect on vesicular uptake in vivo. Related agents, including allyl-glycine, are poor inhibitors of VGAT. VGAT is inhibited by β-alanine and L-alanine, but not by methyl substitution to the amino group. In addition, the structural analog trans-4-aminocrotonic acid is a better inhibitor than β-alanine. Uptake is not inhibited by the plasma membrane GABA uptake inhibitors nipecotic acid and diaminobutyric acid, nor is it stimulated by chloride (118, 137). The plasma membrane GABA transport inhibitor tiagabine, however, inhibits GABA and glycine uptake into vesicles noncompetitively, in the micromolar range (140a). VGAT is constitutively phosphorylated in the brain on serine or threonine residues, but the phosphorylation has no effect on transport activity (141).

## ENDOGENEOUS INHIBITORS OF VESICULAR TRANSPORT

There are many pathological conditions where vesicular transport may be affected, although it is not necessarily the most important site of action. Conditions such as ischemia, hypoxia, and hypoglycemia lead to loss of ATP production and consequently to the inhibition of energy-driven processes, including maintenance of vesicular transport.

In other conditions, the appearance or release of toxic metabolites may lead to an effect on vesicular transport. It is well known that many newborns have a relatively high level of bilirubin and biliverdin in their bloodstreams, and bilirubin is known to lead to neuronal damage in the cerebellum and in striatum. Both bilirubin and biliverdin inhibit vesicular uptake of dopamine, glutamate, and GABA at physiologically relevant concentrations. Biliverdin is a slightly more potent inhibitor of glutamate uptake than bilirubin (141a), and studies have shown that bilirubin and biliverdin do not have an effect on the vesicular $H^+$-ATPase, but rather an effect directly on the transporter (131).

Fatty acids such as arachidonic acid are released at high concentrations in pathological conditions such as ischemia, hypoxia, and hypoglycemia (142). Polyunsaturated fatty acids including arachidonic acid, icosapentaenoic acid, and linolenic acid inhibit the vesicular uptake of glutamate and GABA to the same extent, whereas the saturated fatty acids, arachidic, stearic, and oleic acid, show no or only poor inhibition (131). In contrast to the saturated acids, polyunsaturated acids inhibit the generation of the proton gradient across the vesicular membrane and collapse a preexisting proton gradient. The inhibitory effect of the polyunsaturated acids is due to their low melting point and subsequently their high fluidity, which allow them to intercalate into membranes. Polyunsaturated fatty acids have a similar inhibitory

effect on plasma membrane neurotransmitter transporters (143). The release of unsaturated fatty acids may therefore release neurotransmitters from nerve terminals to reach toxic extracellular concentrations.

Naturally occurring inhibitors of vesicular transport have also been sought after. Özkan & coworkers (143a) isolated three such proteins that are derived from the cytoskeletal protein fodrin by proteolysis and were found in the brain. The sequence of the inhibitory factor, which has an $IC_{50}$ of 24 nM is identical to the N-terminal sequence in α-fodrin, but fodrin does not inhibit vesicular uptake.

## VESICULAR CONSTITUENTS AFFECTING VESICULAR TRANSPORTERS

### Inhibition of V-ATPase

In addition to the macrolide antibiotics mentioned above, environmental contaminants may also target the V-ATPase. MeHg is an environmental contaminant that continues to cause grave damage to human health. It is found at high concentrations in inland fish and, globally, limits have been placed on exposure to mercury. The toxic effects of MeHg on the CNS implicate the glutamatergic system and inhibition of glutamate transport (144). MeHg inhibits the V-ATPase activity and also dissipates the proton gradient (ΔpH), showing that MeHg also decreases [$^3$H]glutamate uptake in vesicles. Inhibition of glutamate transport would lead to its extracellular accumulation and hence to excitotoxicity (78).

### Chloride Channels

The vesicular $H^+$-ATPase is potently regulated by $Cl^-$. Influx of $Cl^-$ neutralizes the positive charge on luminal $H^+$ and hence dissipates the electrical gradient, allowing the $H^+$ pump to transport more $H^+$ and thereby increase ΔpH. Other mechanisms may serve to increase $\Delta\Psi$ at the expense of ΔpH. The two components of the $H^+$ electrochemical gradient may thus be regulated independently. The expression of vesicular chloride channels or activities that influence $\Delta\mu_{H^+}$ might thus be expected to vary in vesicles that store different neurotransmitters. The intracellular chloride channels (ClC-3, -4, -5, -6, and -7) are widely expressed but ClC-3 has a particularly high level of expression in the brain and is present in both glutamatergic and GABAergic vesicles. Disruption of ClC-3 is accompanied by a 50% loss of VGLUT1, but not VGAT, and a possible loss of excitatory synaptic vesicles (145). In addition, it leads to impaired vesicular acidification and to neural degeneration (145) through mechanisms that remain unclear and may involve expression on the postsynaptic plasma membrane (146). Indeed, it has recently become clear that many of the intracellular ClCs may function as $Cl^-/H^+$ exchangers rather than $Cl^-$ channels (147–149). The known stoichiometry of 2 $Cl^-$: 1 $H^+$ would move relatively large amounts of charge and still enable net acidification by secondary activation of the $H^+$ pump. This may perhaps serve to concentrate chloride inside vesicles, for a function that is not yet understood (150).

## CONCLUSIONS

The general mechanism for uptake of neurotransmitters into synaptic vesicles is established. The use of vesicular transport inhibitors has helped to establish their role in the physiological regulation of transmitter release. The importance of the uptake is demonstrated by the lethal consequences of inactivating VMAT2 and VGLUT1. In addition, vesicles play an important role in protecting neurons and other cells from the cytoplasmic accumulation of neurotransmitter and other putative toxins. Psychostimulants and certain environmental toxic substances exert their effects through an interaction with vesicular uptake. However, there is a great need for better inhibitors of all vesicular neurotransmitter transporters, particularly of the inhibitory neurotransmitters.

## DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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