Molecular cloning has identified two vesicular monoamine transporters (VMATs), one expressed in non-neuronal cells of the periphery (VMAT1) and the other by multiple monoamine cell populations in the brain (VMAT2). Functional analysis has previously shown that VMAT2 has a higher affinity than VMAT1 for monoamine neurotransmitters as well as the inhibitor tetrabenazine. The analysis of chimeric transporters has also identified two major regions required for the high affinity interactions of VMAT2 with these ligands. We have now used site-directed mutagenesis to identify the individual residues responsible for these differences. Focusing on the region that spans transmembrane domains 9 through 12, we have replaced VMAT2 residues with the corresponding residues from VMAT1. Many residues in this region had no effect on the recognition of these ligands, but substitution of Tyr-434 with Phe and Asp-461 with Asn reduced the affinity for tetrabenazine, histamine, and serotonin. Although the ability to affect recognition of multiple ligands suggests a general structural role for these residues, the mutations did not affect dopamine recognition, indicating a more specific role, possibly in recognition of the ring nitrogen that occurs in tetrabenazine, histamine, and serotonin but not dopamine. The mutation K446Q reduced the affinity of VMAT2 for tetrabenazine and serotonin but not histamine, whereas F464Y reduced serotonin affinity and perhaps histamine recognition but not tetrabenazine sensitivity, providing more evidence for specificity. Interestingly, the $V_{\text{max}}$ of both VMATs for dopamine exceeded that for serotonin by 3–5-fold, indicating a difference in the speed of packaging of these two neurotransmitters. We also found that VMAT1 has a higher affinity for tryptamine than VMAT2. This mutually exclusive interaction with serotonin and tryptamine also suggests a physiological rationale for the existence of two VMATs. Surprisingly, the residue responsible for this difference, Tyr-434, also accounts for the higher affinity interaction of VMAT2 with tetrabenazine, histamine, and serotonin. Interestingly, replacement of Tyr-434 with alanine increases the affinity of VMAT2 for both serotonin and dopamine and reduces the rate of dopamine transport.

Previous studies have identified several distinct vesicular transport activities that use a proton electrochemical gradient as the driving force for active transport (1, 2). In the case of monoamines, the activity involves exchange of two luminal protons for one cytoplasmic monoamine and generates large concentration gradients of $10^4$–$10^5$ (3, 4). The inhibitors reserpine and tetrabenazine have helped to elucidate the mechanism of active transport as well as its biological role. Monoamines compete with reserpine for binding to the vesicular transporters, suggesting that reserpine binds at the site of substrate recognition (5, 6). In contrast, monoamines inhibit tetrabenazine binding very poorly, suggesting that this drug binds elsewhere (7). The presence of the driving force, $\Delta \text{pH}$, stimulates reserpine binding but not tetrabenazine binding, further suggesting that the two drugs interact at different sites (8). However, tetrabenazine can inhibit reserpine binding, raising the possibility of interaction between the two sites (7).

Plasma membrane neurotransmitter transport differs from vesicular transport in many respects. Plasma membrane transport involves cotransport of Na$^+$ rather than proton exchange, and in the case of monoamines transport is inhibited by cocaine and antidepressants rather than reserpine and tetrabenazine (9, 10). In addition, the plasma membrane transport of monoamines involves distinct transporters for dopamine, norepinephrine, and serotonin (9). In contrast, a single activity appears to mediate vesicular monoamine transport by all monoamine populations in the brain (11). Thus, vesicular monoamine transport has a broad substrate specificity. In addition, vesicular monoamine transport recognizes toxins as substrates.

Selection of transfected cells using the neurotoxin MPP$^+$ has led to the identification of a novel gene family that includes two vesicular monoamine transporters (VMATs)$^1$ (12–14). The VMATs apparently sequester the toxin inside secretory vesicles and hence protect against its toxicity to mitochondria, further implicating the VMATs in neural degeneration. In rat, VMAT1 occurs in the adrenal medulla, whereas VMAT2 occurs in multiple monoamine populations of the central nervous system and sympathetic ganglia as well as mast cells and other histamine-containing cells in the gut (15, 16). Functional analysis showed that the two VMATs also differ in substrate recognition and inhibition by drugs. VMAT2 has a higher apparent affinity for monoamine substrates than VMAT1 and transports histamine, whereas VMAT1 does not transport histamine (17, 18). Although reserpine inhibits both VMATs, tetrabenazine potently inhibits VMAT2 but not VMAT1, raising the possibility that histamine and tetrabenazine interact at the same site, distinct from the site for recognition of other substrates and reserpine (17, 19).

To determine the structural basis for recognition of monoamines...
mine substrates and sensitivity to drugs, we have previously taken advantage of the high sequence similarity between the two VMATs and constructed a series of chimeric proteins that retain transport function. The analysis of these chimeras identified multiple regions that confer differences between VMAT1 and VMAT2 in affinity for substrates and sensitivity to tetrabenazine (20). Interestingly, the high affinity interactions with serotonin, histamine, and tetrabenazine characteristic of VMAT2 all appear to require the simultaneous presence of two major regions from VMAT2, one spanning transmembrane domains 5 through 8 (TMD5–8) and the other TMD9–12. This was surprising in light of previous work suggesting that histamine and tetrabenazine interact at a site distinct from serotonin. However, several other regions affect serotonin affinity but not histamine or tetrabenazine affinity, presumably accounting for the difference observed between serotonin recognition and recognition of both histamine and tetrabenazine.

The identification of two major regions both required for the interactions with serotonin, histamine, and tetrabenazine suggested that individual residues may interact with multiple ligands. In the present study, we have focused on the region encompassing TMD9–12. Introducing mutations that convert the VMAT2 sequence to VMAT1, we have identified specific residues that influence the interaction with serotonin, histamine, and tetrabenazine. In combination, they account for essentially all of the functional differences between the two VMATs attributed to TMD9–12. Although several residues influence the interaction with multiple ligands, others affect only specific interactions. Three residues identified in this way account for the higher affinity of VMAT2 for serotonin than dopamine. We have also found that VMAT1 interacts at higher affinity with tryptamine than VMAT2, and the residue responsible for this difference contributes to the higher affinity of VMAT2 for serotonin. This mutually exclusive recognition of two ligands supports the direct nature of the interaction and suggests a physiological rationale for the existence of two VMATs.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—Wild-type VMAT2 was subcloned into the EcoRI site of the expression vector pcDNA 1 Amp (Invitrogen). Mutagenic oligonucleotides were constructed which contain 1–3 base mismatches with wild-type VMAT2. Site-directed mutagenesis was performed using single-stranded DNA containing uracil from the CJ236 strain of Escherichia coli (21). Using this DNA template, a single mutagenic oligonucleotide was annealed and extended to synthesize the complementary strand, and the product was transformed into the XL-1 Blue strain of E. coli. To verify that the desired mutation and no other was introduced, a restriction fragment containing the mutation was sequenced in its entirety by the dideoxy method (22) and subcloned back into the wild-type VMAT2 cDNA. Double mutants were made by two successive mutagenesis reactions, whereas the triple mutant was made by subcloning the Y434F single mutant into the K460Q/D461N double mutant. These mutations were also verified by sequence analysis.

**Transient Expression**—Plasmid DNA was prepared by alkaline lysis and transfected into COS1 cells by electroporation. COS1 cells were grown to confluence in Dulbecco’s modified Eagle’s medium containing 10% Cosmic calf serum (Hyclone Laboratories) at 37 °C in 5% CO2. For electroporation, the cells from each 15-cm plate were detached, pelleted in calcium/magnesium-free phosphate-buffered saline, detached from the plate with trypsin, collected by centrifugation, and resuspended in 380 μl of cold 0.32 M sucrose, 10 mM HEPPS-KOH, pH 7.4 (SH buffer) containing 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml E-64, 1 μg/ml pepstatin A, and 0.2 mM diisopropyl fluorophosphate. The cell suspension was then sonicated in an ice-cooled bath sonicator (Branson) at medium intensity for 20 s and the cell debris removed by sedimentation at 16,000 × g for 5 min. The supernatant was divided into aliquots and stored at −80 °C. All membranes from 30 to 60 dishes were frozen and used within 60 days with no significant reduction in transport activity.

**Transport Assay**—For each experiment, a frozen aliquot of membranes was thawed on ice and 10 μl (50–100 μg of protein) added to 200 μl of SH buffer at 29 °C containing 4 mM KCl, 2 mM MgSO4, 2.5 mM ATP, and either 20 nM [1,2-3H]serotonin (NEN Life Science Products), 20 nM [2,5,6-3H]dopamine (NEN Life Science Products), or 29 nM [2,5,6-3H]histamine (Amersham Corp.). To determine the Km for serotonin, nonradioactive serotonin was added to the reaction solution at concentrations ranging from 80 to 980 nM and transport measured after incubation for 1 min. To determine Km for dopamine, nonradioactive dopamine dissolved in 1 mM ascorbate was added to the reaction solution at concentrations ranging from 0.13 to 2.98 μM and transport measured after incubation for 30 s. To determine the concentration of compounds needed to inhibit serotonin transport by 50% (IC50), tetrabenazine (Fluka) dissolved in 4 mM HCl was added to the reaction solution at concentrations ranging from 0.2 to 3 μM, histamine (Sigma) was added at concentrations ranging from 5 to 300 μM, and tryptamine (Sigma) dissolved in ethanol was added at concentrations ranging from 0.5 to 50 μM. A stock solution of 5-methyltryptamine was dissolved in water, 7-methyltryptamine in 1 M HCl and N-methyltryptamine in ethanol. After incubation for 1 min at 29 °C, the transport reaction was terminated by rapid dilution with 3 ml of cold SH buffer and filtration through 0.2-μm Supor 200 membranes (Gelman). The filters were then dried and the bound radioactivity measured by scintillation counting in Cytoscint (ICN). Each mutant was transfected at least twice and the transport activity determined in duplicate on a total of three separate occasions. The protein concentration of each membrane sample was measured using a Bradford assay (Bio-Rad). The unpaired two-tailed t-test was used for all statistical comparisons.

**RESULTS**

Analysis of chimeric transporters identified two major regions of VMAT2 responsible for its high affinity interactions with serotonin, histamine, and tetrabenazine (20). One region extends from TMD5 to the beginning of TMD8 and the other from the end of TMD9 through TMD12. Inclusion of TMD5–8 from VMAT2 in a series of chimeras with VMAT1 at the NH2 terminus and VMAT2 at the COOH terminus (VMAT1-VMAT2 series) was required for the high affinity interactions characteristic of VMAT2. In the series of chimeras with VMAT2 at the NH2 terminus and VMAT1 at the COOH terminus (VMAT2-VMAT1 series), however, TMD5–8 of VMAT2 did not suffice to confer the high affinity interactions. In this series, inclusion of TMD9–12 of VMAT2 was required for these interactions. Thus, TMD5–8 of VMAT2 appears to require COOH-terminal VMAT2 sequences, presumably TMD9–12, for the high affinity interactions and TMD9–12 appears to require NH2-terminal VMAT2 sequences, presumably TMD5–8. To determine if specific residues account for the high affinity of VMAT2 relative to VMAT1, we have focused on TMD9–12 since this region contains the smallest number of divergent residues between VMAT1 and VMAT2. In addition, we have introduced the VMAT1 residues into VMAT2 rather than vice versa because the chimeric analysis indicates that a single change in TMD9–12 should suffice to reduce the affinity of VMAT2 interactions, whereas multiple replacements in both TMD5–8 and TMD9–12 would be required to increase the affinity of VMAT1. Of the 23 residues in TMD9–12 which differ between VMAT1 and VMAT2, we have focused on 10 of the most nonconservative differences (Fig. 1).

**Tetrabenazine Sensitivity**—Previous work has shown that the sensitivity of VMAT2 to tetrabenazine is 10-fold greater than VMAT1 (17). Focusing on the contribution of TMD9–12 to the greater sensitivity of VMAT2, we have replaced residues in VMAT2 with the corresponding residues from VMAT1. Substitution of Tyr-383 with Phe (Y383F), Val-416 with Thr (V416T),...
Ligand Recognition by VMAT2

Membranes were prepared from COS1 cells transfected with the cDNAs indicated and incubated at 29 °C for 1 min in 3H]serotonin. The reaction was terminated by rapid dilution followed by filtration, and the bound radioactivity was measured by scintillation counting. To assess tetrabenazine sensitivity and histamine recognition, the amount of ligand required to inhibit serotonin transport by 50% (IC50) was determined. The IC50 values for serotonin were determined from Lineweaver-Burk analysis using a range of serotonin concentrations. Uptake for zero time at 0 °C was subtracted as background. The values shown derive from three separate experiments and are expressed as mean ± S.D. Each value was compared with the value obtained from VMAT2 using the unpaired two-tailed t test, with * indicating \( p < 0.05 \) and ** indicating \( p < 0.01 \).

TABLE I

| Tetrabenazine IC50 | Histamine IC50 | Serotonin \( K_{in} \) |
|--------------------|---------------|-----------------------|
| VMAT1 | 4.17 ± 0.56** | 378 ± 30** | 0.59 ± 0.12* |
| VMAT2 | 0.46 ± 0.09 | 42 ± 15 | 0.29 ± 0.01 |
| Y383F | 0.60 + 0.19 | 52 ± 14 | 0.29 ± 0.05 |
| Y416T | 0.64 ± 0.19 | 53 ± 9 | 0.26 ± 0.04 |
| Y434F | 0.82 ± 0.11** | 122 ± 25** | 0.38 ± 0.02** |
| A440T | 0.50 ± 0.07 | 62 ± 12 | 0.17 ± 0.02** |
| K446Q | 0.85 ± 0.09** | 48 ± 11 | 0.34 ± 0.04 |
| T455V | 0.38 ± 0.06 | 45 ± 9 | 0.24 ± 0.04 |
| I459T | 0.39 ± 0.03 | 55 ± 5 | 0.20 ± 0.01** |
| D461N | 0.78 ± 0.27 | 62 ± 24 | 0.37 ± 0.03* |
| F464Y | 0.56 ± 0.06 | 59 ± 15 | 0.45 ± 0.08* |
| F469C | 0.40 ± 0.03 | 52 ± 10 | 0.29 ± 0.03 |
| Y434F/K446Q | 0.87 ± 0.31 | 145 ± 21** | 0.41 ± 0.15 |
| Y434F/D461N | 1.58 ± 0.32** | 193 ± 48** | 0.36 ± 0.08 |
| K446Q/D461N | 0.96 ± 0.09* | 70 ± 31 | 0.26 ± 0.06 |
| Y434F/K446Q/D461N | 2.29 ± 0.16** | 159 ± 9** | 0.67 ± 0.12** |
| Y434A | 11.78 ± 0.40** | 238 ± 59** | 0.07 ± 0.02** |
| S372C | 3.36 ± 0.97 | 130 ± 51* | 1.10 ± 0.40* |
| S468C | 0.41 ± 0.01 | 26 ± 2* | 0.17 ± 0.04* |

* Ref. 20.

Since the introduction of multiple VMAT1 residues into VMAT2 reduced tetrabenazine sensitivity and none of these alone accounted for all of the differences between VMAT1 and VMAT2 attributed to TMD9–12, double and triple mutants were constructed and analyzed, focusing on the three most influential residues. Table I shows that the combination of Y434F and K446Q (Y434F/K446Q) did not reduce the sensitivity more than the individual mutation of these residues. However, the combination of Y434F with D461N (Y434F/D461N) and K446Q with D461N (K446Q/D461N) significantly reduced tetrabenazine sensitivity to an extent significantly greater than any of the double mutants (\( p < 0.05 \)). Supporting a role for Asp-461 suggested by analysis of D461N alone, the combination of all three influential residues. Table I shows that the combination of Y434F, Lys-446, and Asp-461 interact independently with the inhibitor or, in an additive manner, suggesting that Tyr-434, Lys-446, and Asp-461

Thr-455 with Val (T455V), Ile-459 with Thr (I459T), Phe 464 with Tyr (F464Y), and Phe 469 with Cys (F469C) did not significantly change tetrabenazine sensitivity as measured by the IC50, for inhibition of serotonin transport (Table I). However, replacement of Tyr-434 with Phe (Y434F) and Lys-446 with Gln (K446Q) significantly reduced tetrabenazine sensitivity and substitution of Asp-461 with Asn (D461N) appeared to reduce tetrabenazine sensitivity.
Ligand Recognition by VMAT2

**Fig. 2.** Transport of[^3]H]histamine by VMAT1, VMAT2, and the triple VMAT2 mutant Y434F/K446Q/D461N. Membranes were prepared from COS1 cells transfected with the cDNAs indicated and incubated at 29 °C in[^3]H]histamine for the times indicated. The reaction was terminated by dilution followed by filtration, and the bound radioactivity was measured by scintillation counting. Uptake for zero time at 0 °C was subtracted as background. The amount of total protein used was measured using the Bradford assay. The results show increasing accumulation of[^3]H]histamine with time for VMAT2 and no accumulation for VMAT1. The triple mutant Y434F/K446Q/D461N accumulates[^3]H]histamine but substantially less than that of VMAT2. The 5-, 10-, and 30-min time points show a significant difference in the transport of[^3]H]histamine between VMAT2 and the triple mutant (p < 0.01). The values are derived from three separate experiments and expressed as means ± S.D. using the unpaired two-tailed t test to assess the significance of the comparisons.

The analysis of chimeric transporters indicated that the same regions of VMAT2 are responsible for the high affinity interactions of the transporter with both tetrabenazine and histamine (20). Similar to the analysis of tetrabenazine sensitivity, the Y383F, V416T, T455V, I459T, and F469C mutations had no effect on the ability of histamine to inhibit transport of serotonin by VMAT2 (Table I). Of the three mutations found to influence tetrabenazine sensitivity (Y434F, K446Q, and D461N), only the Y434F mutation significantly reduced histamine recognition. Mutations D461N, A440T, and F464Y appeared to reduce histamine recognition, although to a lesser extent than Y434F, and K446Q had no effect. Indeed, the combination of K446Q with either Y434F (Y434F/K446Q) or D461N (K446Q/D461N) caused no further reduction in histamine recognition from Y434F or D461N alone. Similarly, combination of K446Q with both Y434F and D461N (Y434F/K446Q/D461N) caused no further reduction in histamine recognition from Y434F/D461N alone. In contrast, the addition of Y434F to D461N in Y434F/D461N and to K446Q/D461N in the triple mutant reduced histamine recognition. The addition of D461N to K446Q in K446Q/D461N, to Y434F in Y434F/D461N and to Y434F/K446Q in the triple mutant tended to reduce sensitivity to histamine, but the results did not reach statistical significance. Thus, Tyr-434 and to a lesser extent Lys-446 appear to contribute to histamine recognition. Replacement of both Tyr-434 and Lys-446 of VMAT2 with the corresponding VMAT1 residues also accounts for all of the difference in histamine recognition attributed to TMD9–12 by the analysis of VMAT2-VMAT1 chimeras (Table I) (20).

To confirm our results using histamine to inhibit[^3]H]serotonin transport, we have also measured the transport of[^3]H]histamine. Fig. 2 shows that VMAT2 but not VMAT1 transports histamine. The Y434F/K446Q/D461N triple mutant in VMAT2 remained able to transport histamine but at a level significantly reduced from VMAT2. Thus, the same residues involved in the inhibition of serotonin transport by histamine, Tyr-434 and Asp-461, also contribute to histamine transport.

*Affinity for Serotonin—*Previous work had suggested that histamine and tetrabenazine might interact at a site distinct from serotonin and reserpine (7, 8, 18). However, the analysis of chimeric transporters indicated that the same region (TMD9–12) contributes to the high affinity interactions of VMAT2 with tetrabenazine, histamine, and serotonin (20). To determine if the same residues that influence tetrabenazine recognition also influence serotonin affinity, we have measured the Kₘ for serotonin. Similar to the results for tetrabenazine sensitivity and histamine recognition, the Y383F, V416T, T455V, and F469C mutations each had no effect on serotonin affinity (Table I). Of the two mutations that influence tetrabenazine and histamine recognition, the Y434F and D461N, both significantly reduced serotonin affinity. The K446Q mutation, which influences tetrabenazine but not histamine recognition, tended to reduce serotonin affinity although to a lesser extent. However, analysis of Y434F/K446Q/D461N confirmed the role of Lys-446 in serotonin recognition because this triple mutant had a significantly lower affinity than the Y434F/D461N double mutant (p < 0.01). Therefore, the same three residues that influence tetrabenazine sensitivity, Tyr-434, Lys-446, and Asp-461, also influence serotonin affinity. Interestingly, the F464Y mutation, which had no effect on tetrabenazine sensitivity but may influence histamine recognition, significantly reduced serotonin affinity. In addition, the A440T and I459T mutations significantly increased serotonin affinity. Thus, serotonin appears to interact with more sites on VMAT2 than the lower affinity substrate histamine or the inhibitor tetrabenazine. To determine if Tyr-434, Lys-446, and Asp-461 contribute independently to the high affinity interaction of VMAT2 with serotonin, we also examined double and triple mutants. Although Y434F/K446Q, Y434F/D461N, and K446Q/D461N did not show a lower affinity than the single mutations, the triple mutant Y434F/K446Q/D461N did show significantly reduced affinity compared with these double mutants (p < 0.05), suggesting that each of the three residues interacts independently with serotonin.

*Structurally Related Ligands—*To identify features of the ligands whose recognition is influenced by Tyr-434, Lys-446, and Asp-461, we have compared the interactions of VMAT2...
mutants with structurally related compounds. Tetrabenazine, histamine, and serotonin all have a nitrogen within a ring structure (Fig. 3). Since Tyr-434 and Asp-461 both influence the interaction with all three of these ligands, we examined the effect of mutations at these sites on transport of dopamine that lacks a ring nitrogen. Indeed, Y434F and D461N did not affect the apparent affinity of VMAT2 for dopamine either individually or in combination (Table II), consistent with the influence of Tyr-434 and Asp-461 on recognition of the ring nitrogen in tetrabenazine, histamine, and serotonin.

Lys-446 influences tetrabenazine sensitivity and serotonin affinity but not recognition of histamine. Since tetrabenazine and serotonin both have oxygen-containing groups (a methoxy and a carbonyl group in tetrabenazine and an hydroxyl group in serotonin), we examined the effects of the K446Q mutation on the interaction with dopamine, which has two hydroxyl groups (Fig. 3). However, K446Q did not influence dopamine affinity (Table II), suggesting that Lys-446 does not influence the interaction with oxygen-containing groups.

Since tryptamine has no oxygen-containing groups, we also examined the interaction of VMAT2 mutants with this ligand. Surprisingly, tryptamine inhibited serotonin transport by VMAT1 much more potently than by VMAT2 (Table II). As such, tryptamine is the first monoamine demonstrated to interact at substantially higher affinity with VMAT1 than with VMAT2. However, the K446Q mutation did not affect tryptamine recognition. Rather, the Y434F mutation significantly increased tryptamine recognition by VMAT2 to the level of wild-type VMAT1. Further, the Y434F/K446Q/D461N triple mutation increased tryptamine recognition to the same extent as Y434F alone, confirming that Y434F accounts for all of the difference observed between VMAT1 and VMAT2. Thus, the same residue responsible for the high affinity interactions of VMAT2 with tetrabenazine, histamine, and serotonin also accounts for its low affinity interaction with tryptamine.

To determine whether VMAT2 specifically requires the hydroxyl group at the 5 position of serotonin for high affinity recognition, we have examined the inhibition of VMAT2 by 5-methyltryptamine (Table III). 5-Methyltryptamine inhibits both VMAT1 and 2 with a potency similar to unsubstituted tryptamine, strongly suggesting that the simple presence of any substituent at the 5 position does not confer the properties of the native ligand serotonin. Rather, the higher affinity interaction of serotonin with VMAT2 appears to derive specifically from the presence of a hydroxyl group. Interestingly, methyl substitution at the 7 position increases the affinity of tryptamine for both VMAT1 and 2 (Table III), possibly by promoting hydrophobic interactions with the transport protein. The Y434F mutant shows a similar affinity for both serotonin \( K_m; 0.38 \mu M \) and tryptamine \( IC_{50}; 0.35 \mu M \), indicating that this mutation eliminates selective recognition of the hydroxyl group at the 5 position on the ligand and making predictions about other amino acid substitutions at this residue. As expected from Y434F, Y434A shows dramatically reduced sensitivity to histamine and tetrabenazine and an increased sensitivity to tryptamine. However, Y434A shows a substantially higher apparent affinity for serotonin than the wild-type protein (Table I). Although this result suggests that a hydroxyl at residue 434 is not required for high affinity recognition of serotonin, kinetic analysis suggests a defect in turnover by Y434A.

The kinetic analysis of wild-type VMAT1 and VMAT2 shows that the \( V_{max} \) for dopamine transport generally exceeds that for serotonin transport by 3–5-fold (Table IV). Thus, although the VMATs have a higher apparent affinity for serotonin than dopamine, they both transport dopamine at a higher rate. Interestingly, Y434A shows a reduced \( V_{max} \) for dopamine to the same level observed for serotonin with both wild-type VMAT2

**Table II**

| Ligand Recognition by VMAT2 | \( K_m \) \( \mu M \) | \( IC_{50} \) \( \mu M \) |
|-----------------------------|----------------|-----------------|
| Dopamine                    | 0.86 ± 0.09    | 2.19 ± 0.94     |
| Tryptamine                  | 0.94 ± 0.25    | 1.59 ± 0.42     |
| Y434F                       | 0.99 ± 0.25    | 1.94 ± 0.63     |
| K446Q                       | 0.94 ± 0.25    | 1.59 ± 0.42     |
| D461N                       | 0.94 ± 0.25    | 1.59 ± 0.42     |
| Y434F/K446Q/D461N           | 0.99 ± 0.25    | 1.94 ± 0.63     |

**FIG. 3.** Structure of substrates and inhibitors of vesicular monoamine transport.
and Y434A (Table IV). Although differences in expression may complicate the analysis, the increased apparent affinity of this mutant thus appears to involve a reduction in the rate of transport of one substrate relative to another, further implicating the hydroxyl on Tyr-434 in the transport cycle.

**DISCUSSION**

Previous studies have suggested that the site on VMAT2 involved in recognition of serotonin and reserpine differs from the site involved in recognition of either tetrabenazine or histamine. First, monoamine substrates inhibit reserpine binding to the transporter at concentrations close to their \( K_m \) for transport, whereas much higher concentrations are required to inhibit tetrabenazine binding (8). Second, histamine does not inhibit reserpine binding, and reserpine inhibits histamine transport only weakly, suggesting that histamine interacts at a site distinct from that involved in recognition of serotonin and reserpine (18). Third, tetrabenazine potently inhibits histamine transport, suggesting that both ligands may interact at the same site (18). However, the analysis of chimeras showed that the combination of TMD5–8 and TMD9–12 is required to increase the affinity of VMAT2 for tetrabenazine, histamine, and serotonin, suggesting that the three ligands act at a similar site (20). Further supporting this possibility, we now find that Tyr-434 and Asp-461 influence the interaction with tetrabenazine, histamine, and serotonin. However, our results are subject to alternate interpretation. In particular, Tyr-434 and Asp-461 might only influence serotonin affinity. Since the assays for tetrabenazine sensitivity and histamine recognition involve inhibition of \( ^3\text{H} \)serotonin transport, a reduction in the potency of inhibition may only reflect the lower affinity for serotonin rather than a distinct role in the interaction with tetrabenazine and histamine. However, the P464Y mutation substantially reduces serotonin affinity without affecting tetrabenazine sensitivity, indicating a dissociation between recognition of serotonin and the other ligands at this site. In addition, the K446Q mutation reduces serotonin affinity without influencing histamine recognition. Further, the Y434F and D461N mutations substantially reduce the transport of \( ^3\text{H} \)histamine, indicating a specific influence of these two residues on the interaction with this substrate. Taken together, the results indicate a specific influence of these residues on recognition of tetrabenazine, histamine, and serotonin. Thus, the recognition sites for these three ligands overlap.

Additional residues in TMD9–12 influence the interaction of VMAT2 with only selected ligands. Lys-446 influences the recognition of tetrabenazine but not histamine. Thus, although the analysis of histamine transport and VMAT2 chimeras has suggested similar recognition sites for both tetrabenazine and histamine (18, 20), they are not identical. Lys-446 also influences serotonin affinity, indicating involvement of this residue in transport of a high affinity substrate (serotonin) but not a low affinity substrate (histamine). In addition, Phe-464 influences serotonin affinity and perhaps histamine recognition but not tetrabenazine sensitivity. Since histamine and serotonin but not tetrabenazine are substrates for transport, this suggests a role for Phe-464 in substrate translocation as well as ligand recognition.

To characterize the influence of residues in TMD9–12 on interactions with the ligands, we have compared the interactions of VMAT2 mutants with several structurally related compounds. Since Tyr-434 and Asp-461 influence the interaction with tetrabenazine, histamine, and serotonin and these three ligands have a ring nitrogen in common, we studied the influence of mutations at these sites on the recognition of dopamine, a substrate that lacks a ring nitrogen. Indeed, Y434F and D461N mutations do not impair recognition of dopamine, consistent with a role for Tyr-434 and Asp-461 in recognition of the ring nitrogen in tetrabenazine, histamine, and serotonin. However, a clear role for the ring nitrogen remains uncertain since only histamine and serotonin are available for hydrogen bonding at this site; tetrabenazine is a tertiary amine (Fig. 3).

Since the K446Q mutation interferes selectively with tetrabenazine and serotonin but not histamine recognition, and histamine lacks the oxygen-containing groups present on tetrabenazine and serotonin, we also studied the influence of this residue using dopamine. Dopamine has two hydroxyl groups, but K446Q does not reduce the apparent affinity of VMAT2 for dopamine, mitigating against a role for Lys-446 in the recognition of oxygen-containing groups.

Previous work has indicated that the recognition of dopamine by VMATs differs in several ways from that of serotonin (4, 17). Both VMATs have a higher apparent affinity for serotonin than dopamine (17). If the VMATs govern the cytoplasmic concentration of neurotransmitter, this difference will result in higher cytoplasmic levels of dopamine than serotonin. Since monoamines oxidize easily to produce considerable toxicity, this may contribute to the selective vulnerability of dopamine neurons. However, both VMAT1 and VMAT2 show a \( V_{\text{max}} \) for dopamine 3–5-fold greater than that for serotonin, as suggested by previous work using chromaffin granules (4). This property should accelerate vesicle filling by dopamine relative to serotonin. Thus, the kinetic analysis of VMATs suggests a balance between apparent affinity and transport rate. Since VMAT2 occurs in multiple central monoamine populations, the possible need for more rapid dopamine packaging may therefore involve a reduced affinity for dopamine relative to serotonin. The reduced affinity may in turn result in higher cytoplasmic concentrations of dopamine and so contribute to the preferential vulnerability of dopaminergic neurons. We have now found that Tyr-434, Lys-446, and Asp-461 contribute to the recognition of serotonin but not dopamine, providing a molecular basis for the observed differences in recognition of these two monoamine substrates by VMAT2.

The residues identified here as responsible for the differ-
ences between VMAT1 and VMAT2 may either interact directly with the ligand or contribute indirectly to the conformation of a recognition site. Tyr-434 and Asp-461 influence interactions with multiple ligands, suggesting an indirect role. However, Tyr-434 and Asp-461 do not influence dopamine affinity, supporting a more specific influence of Tyr-434 and Asp-461 on the recognition of tetrabenazine, histamine, and serotonin. The lack of influence on dopamine further suggests that these residues may interact directly with the ring nitrogen on the other ligands, but the marked chemical differences between tyrosine and aspartate make it unlikely that the two residues interact in the same way. The occurrence of Tyr-434 in TMD11 and Asp-461 in TMD12 of VMAT2 also makes it difficult to predict their proximity in the tertiary structure of the protein. Supporting distinct roles for the two residues, the comparison of single and multiple mutants indicates that they act independently to increase the affinity of VMAT2. Thus, Tyr-434 and Asp-461 may either interact directly but in different ways with the ring nitrogen or contribute to the conformation of a domain that specifically recognizes this feature of the ligand. In the case of Lys-446 and Phe-464, the specific influence on a subset of ligands suggests a direct interaction, but an indirect effect on the position of other residues certainly remains possible.

To assess further the influence of specific residues in the VMATs on the recognition of hydroxyl groups, we have used tryptamine. Tryptamine resembles serotonin but lacks the hydroxyl group. Surprisingly, tryptamine inhibits serotonin transport by VMAT1 much more potently than by VMAT2. As such, tryptamine is the first monoamine ligand found to interact at higher affinity with VMAT1 than with VMAT2. Tryptamine is synthesized from tryptophan by the same aromatic amino acid decarboxylase that synthesizes serotonin from 5-hydroxytryptophan, suggesting that the lower affinity of VMAT2 for tryptamine may provide a mechanism to prevent the interference of tryptamine with serotonin storage in central serotonergic neurons. Although the physiological significance of the interaction with tryptamine remains unclear, the mutually exclusive nature of the interactions with tryptamine and serotonin suggests a biological rationale for the existence of two VMATs, each with a finely tuned substrate preference that corresponds to substrate usage and availability in the tissues in which they are expressed.

Tyr-434 accounts for all of the difference in tryptamine recognition between VMAT1 and VMAT2. Thus, the same residue that contributes to the high affinity interaction of VMAT2 with serotonin also accounts for the low affinity interaction of VMAT2 with tryptamine. This suggests that Tyr-434 interacts directly with the ligand. However, the feature of the ligand recognized by Tyr-434 remains unclear. Since Y434F recognizes serotonin and tryptamine with similar affinity as a result of reduced apparent affinity for serotonin and increased sensitivity to tryptamine, the hydroxyl at Tyr-434 appears to discriminate between these two compounds. The compounds themselves differ only in the presence of a hydroxyl group at the 5 position, further suggesting that Tyr-434 hydrogen bonds to serotonin. In addition, 5-methyltryptamine interacts with the two VMATs in a very similar way to tryptamine, indicating that the mere presence of a substituent at this position does not suffice to confer the properties of serotonin. Rather, a hydroxyl appears required for the higher affinity interaction with VMAT2 than with VMAT1. However, Tyr-434 also influences histamine recognition, and histamine has no hydroxyl. Further, although Y434A shows many properties similar to Y434F such as dramatically reduced sensitivity to tetrabenazine and histamine, it shows an apparent affinity for serotonin higher than that of wild-type VMAT2. These observations mitigate against the formation of a direct hydrogen bond between Tyr-434 and serotonin. Since Y434A increases dopamine as well as serotonin affinity, this substitution differs dramatically from Y434F, which reduces serotonin but not dopamine affinity. Thus, Tyr-434 may influence the interaction with the ring nitrogen that differs between serotonin and dopamine but presumably acts indirectly. Interestingly, Y434A also appears to reduce the turnover of dopamine but not serotonin, suggesting that the increased apparent affinity may occur at the expense of the transport rate.

In summary, we have identified several residues in TMD9–12 which promote the high affinity interactions of VMAT2 with multiple ligands. These residues also account for the preferential recognition of serotonin over dopamine. Although the specificity of their influence on recognition of specific ligands and the mutually exclusive recognition of serotonin and tryptamine by Tyr-434 in particular suggest direct interactions with the ligands, the ability of these residues to influence recognition of multiple ligands, their different chemical properties, and the divergent and selective effects of different amino acid substitutions at the same site raise the possibility that the residues identified here act primarily to influence the direct interaction of other, as yet unidentified, residues with particular features of the ligands.

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REFERENCES

1. Edwards, R. H. (1992) Curr. Opin. Neurobiol. 2, 586–594
2. Schuldiner, S., Shirvan, A., and Linial, M. (1985) Physiol. Rev. 75, 369–392
3. Knoth, J., Zallakian, M., and Njus, D. (1981) Biochemistry 20, 6625–6629
4. Johnson, R. G. (1988) Physiol. Rev. 68, 232–307
5. Schuldiner, S., Shirvan, A., and Linial, M. (1995) Biochemistry 34, 1–9
6. Schuldiner, S., Liu, Y., and Edwards, R. H. (1995) J. Biol. Chem. 270, 1–9
7. Darchen, F., Scherman, E., and Henry, J. P. (1989) Biochemistry 28, 1692–1697
8. Scherman, D., and Henry, J. P. (1984) Mol. Pharmacol. 35, 1–9
9. Amara, S. G., and Kuhar, M. J. (1993) Annu. Rev. Neurosci. 16, 73–93
10. Kanner, B. I. (1994) J. Exp. Biol. 196, 237–249
11. Scherman, D. (1986) J. Neurochem. 47, 331–339
12. Liu, Y., Roghani, A., and Edwards, R. H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9674–9678
13. Liu, Y., Peter, D., Roghani, S., Prive, G. G., Eisenberg, D., Brecha, N., and Edwards, R. H. (1992) Cell 70, 393–395
14. Erickson, J. D., Eiden, L. E., and Hoffman, B. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10993–10997
15. Peter, D., Liu, Y., Sternini, C., de Giorgio, R., Brecha, N., and Edwards, R. H. (1995) J. Neurosci. 15, 6179–6188
16. Weihe, E., Schafer, M. K., Erickson, J. D., and Eiden, L. E. (1994) J. Mol. Neurosci. 5, 149–164
17. Peter, D., Jimenez, J., Liu, Y., Kim, J., and Edwards, R. H. (1994) J. Biol. Chem. 269, 7231–7237
18. Merickel, A., and Edwards, R. H. (1995) Neuropharmacology 34, 1543–1547
19. Erickson, J. D., Schafer, M. K. H., Bonner, T. I., Eiden, L. E., and Weihe, E. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5166–5171
20. Peter, D., Yu, T., and Edwards, R. H. (1996) J. Biol. Chem. 271, 2979–2986
21. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) Methods Enzymol. 204, 125–139
22. Sanger, F., Nicklen, S., and Coulson, A. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
23. Cohen, G. (1980) J. Neurotransm. Suppl. 22, 229–238
24. Darchen, F., and Henry, J. P. (1989) Annu. Rev. Neurosci. 12, 1543–1547
25. Conner, R., and Edwards, R. H. (1993) Genomics 18, 720–723
26. Surratt, C. K., Persico, A. M., Yang, X., Edgar, S. R., Bird, G. S., Hawkins, A. L., Griffin, C. A., Li, X., Jabs, E. W., and Uhl, G. R. (1993) FEBS Lett. 318, 325–330
27. Howell, M., Shirvan, A., Stern-Bach, Y., Steiner-Mordoch, S., Strasser, J. E., Dean, G. E., and Schuldiner, S. (1994) FEBS Lett. 338, 16–22