Molecular and Functional Analysis of a Novel Neuronal Vesicular Glutamate Transporter*

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Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. Packaging and storage of glutamate into glutamatergic neuronal vesicles requires ATP-dependent vesicular glutamate uptake systems, which utilize the electrochemical proton gradient as a driving force. VGLUT1, the first identified vesicular glutamate transporter, is only expressed in a subset of glutamatergic neurons. We report here the molecular cloning and functional characterization of a novel glutamate transporter, VGLUT2, from mouse brain. VGLUT2 has all major functional characteristics of a synaptic vesicle glutamate transporter, including ATP dependence, chloride stimulation, substrate specificity, and substrate affinity. It has 75 and 79% amino acid identity with human and rat VGLUT1, respectively. However, expression patterns of VGLUT2 in brain are different from that of VGLUT1. In addition, VGLUT2 activity is dependent on both membrane potential and pH gradient of the electrochemical proton gradient, whereas VGLUT1 is primarily dependent on only membrane potential. The presence of VGLUT2 in brain regions lacking VGLUT1 suggests that the two isoforms together play an important role in vesicular glutamate transport in glutamatergic neurons.

Neurotransmission depends on the regulated exocytotic release of vesicular transmitter molecules to the synaptic cleft, where they interact with postsynaptic receptors that subsequently transduce the information. Two types of neurotransmitter transporters have been identified based on membrane localization on plasma membrane or vesicular membrane. Removal of the transmitter from the synaptic cleft results in termination of the signal, and this requires destruction of transmitter or reuptake of transmitter back to the presynaptic terminal or glial cells via a sodium-dependent uptake system on the plasma membrane (1). Packaging and storage of neurotransmitters into specialized secretory vesicles in neurons ensures their regulated release. This storage is also crucial for protecting the neurotransmitter molecules from leakage or intraneuronal metabolism and for protecting the neuron from possible toxic effects. This process is mediated by specific transporters on the vesicular membranes. At least four different types of vesicular transporters have been functionally identified that are specific for transport of classic neurotransmitters: monoamines, acetylcholine, γ-aminobutyric acid (GABA), and glutamate (2, 3). Unlike the plasma membrane transporters, which rely on a sodium gradient across the plasma membrane, all of these vesicular transport processes depend on the proton electrochemical gradient (ΔμH+) generated by a Mg2+-activated vacuolar H+-ATPase (V-ATPase) on the vesicular membrane (4). When protons are pumped into the vesicular lumen, a proton gradient (ΔpH) and a membrane potential (Δψ) occur across the membrane to form ΔμH+, which favors the exchange of luminal protons for cytoplasmic transmitter. The transport of monoamines and acetylcholine rely predominantly on ΔpH (5, 6), whereas the accumulation of GABA depends on both Δψ and ΔpH (7, 8). In the case of glutamate, there is disagreement over whether the transport of glutamate is driven by Δψ only (9, 10, 11) or by both Δψ and ΔpH components of ΔμH+ (12, 13).

In addition to the differences in driving force, kinetic properties, substrate specificity, and ion dependence also clearly distinguish vesicular glutamate transport from high affinity plasma membrane glutamate transport. The vesicular ATP-dependent glutamate transporter is specific for glutamate, is stimulated by millimolar concentrations of chloride, and has a low affinity for uptake (Km about 1–2 mM) (12, 14). These functional characteristics are in contrast to those of the plasma membrane glutamate transporter, which is sodium dependent, accepts aspartate as well as glutamate, and has a high affinity for glutamate with Km in the 3–20 μM range (15, 16).

Although vesicular transporters for other neurotransmitters have been intensively studied in mechanistic, biochemical, and molecular levels, molecular cloning of vesicular glutamate transporters has only occurred recently. BNPI, a brain-specific sodium-dependent phosphate cotransporter originally characterized as a plasma membrane transporter, was recently localized on vesicular membranes of small synaptic vesicles in neurons (17, 18) and further functionally characterized as a vesicular glutamate transporter called VGLUT1 (18, 19). Uptake of glutamate by VGLUT1 has all of the functional characteristics previously reported for vesicular glutamate transporter with Δψ as the predominant driving force. However, only a subset of glutamatergic neurons expresses VGLUT1 (17, 20). Moreover, in Caenorhabditis elegans, loss-of-function muta-

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1 The abbreviations used are: ΔμH+, proton electrochemical gradient; V-ATPase, vacuolar H+-ATPase; VGLUT1, vesicular glutamate transporter isoform 1; VGLUT2, vesicular glutamate transporter isoform 2; Δψ, membrane potential; ΔpH, proton gradient; Km, apparent affinity constant; Vmax, maximal velocity; EST, expressed sequence tag; bp, base pair(s); DCCD, N,N’-dicyclohexylcarbodiimide; DIDS, 4,4’-disothiocyanostilbene-2,2’-disulfonic acid.
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...tions in the VGLUT1 orthologue eat-4, lead to impairment but not to a complete loss of glutamate-mediated neurotransmission (21, 22). These findings imply that an additional vesicular glutamate transporter(s) may exist. To identify new vesicular glutamate transporter isoforms, we searched the GenBank™ EST database using human and rat VGLUT1 sequences (23, 24), and we found a mouse EST clone, which has ~49% homology with human and rat VGLUT1. We report here the molecular cloning and functional characterization of VGLUT2, the second identified vesicular glutamate transporter with different transport characteristics and cellular localization as compared with VGLUT1.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**N,N′-dicyclohexylcarbodiimide (DCCD) was purchased from Fisher Scientific. All other chemicals were obtained from Sigma.

**Isolation of cDNA Encoding Mouse VGLUT2—**The human and rat VGLUT1 sequences were used to search the GenBank™ EST database. One query gene (GenBank™ number AI 841371) was identified from the mouse EST database that has ~49% nucleotide identity with human and rat VGLUT1. This EST clone was obtained from Research Genetics (Huntsville, AL) and sequenced. The sequence was assembled and analyzed. Sequence comparisons were run using O UWware (version 1.1.3) and GenBank™ BLAST searches. The full-length cDNA insert was excised with EcoRI and NorI and subcloned into the same sites of the pSPORT1 vector (LifeTech) and further subcloned into the pIRES2-EGFP vector (CLONTECH, Palo Alto, CA) at EcoRI and BamHI sites, resulting in VGLUT2-pIRES2-EGFP (the name VGLUT2 was given after the determination of transport characteristics).

**Expression of VGLUT2 in PC12 Cells—**A rat pheochromocytoma cell line, PC12 cell (ATCC), which was used to characterize glutamate transport by VGLUT1 (19), was cultured in Dulbecco’s modified Eagle’s F-12 medium, supplemented with 20% fetal bovine serum, and 1% penicillin and streptomycin. VGLUT2-pIRES2-EGFP or pIRES2-EGFP without a cDNA insert (10^5) was transfected into cells by the lipofection method (25). Transfected cells were selected with 800 μg/ml G418, and the most highly expressing cells were identified by fluorescence microscopy and selected for further analysis.

**Preparation of Membrane Fractions from Transfected Cells and Glutamate Uptake—**Stably transfected cells were washed twice with ice-cold phosphate-buffered saline and homogenized in 0.32 M sucrose and 10 mM HEPES-KOH (pH 7.4) containing protease inhibitors. The re-
cold phosphate-buffered saline and homogenized in 0.32 M sucrose and 10 mM HEPES-KOH, pH 7.4 (uptake buffer) at a final concentration of 10 mg of protein/ml. ATP, 4 mM KCl, and 50 mM NaCl/sodium citrate, 0.1% SDS—

**RESULTS**

**Molecular Cloning of Mouse VGLUT2—**We performed a search of the EST database with the nucleotide sequence of human and rat VGLUT1 (BLASTx program). One mouse EST clone from amygdala exhibited significant similarity (~49%) with human and rat VGLUT1. By sequencing the clone from Research Genetics, we obtained a 2528 bp cDNA with an open reading frame of 1746 bp with 638 bp of 5′-untranslated sequence and 134 bp of 3′-untranslated sequence, which we named VGLUT2 (GenBank™ accession number AF324864). The predicted protein has ~75–80% amino acid identity with human and rat VGLUT1 (Fig. 1 and data not shown). Particularly, VGLUT2 showed 97% amino acid identity to a recently cloned human differential sodium-dependent phosphate co-
transporter, called DNPI (28). Hydropathy analysis suggests the presence of 8–10 membrane-spanning domains.

**Functional Characteristics of VGLUT2—**We first determined the transport activity of different membranes from VGLUT2 or vector-only transfected cells. In the presence of 4 mM ATP, chloride and MgSO4, conditions that optimize glutamate accumulation by native synaptic vesicles (12, 29), vesicle membranes from VGLUT2-pIRES2-EGFP-transfected cells exhibited 5- to 6-fold increased uptake. Plasma membrane glutamate uptake from VGLUT2-pIRES2-EGFP-transfected cells did not show any differences from that of IRES2-EGFP-transfected cells (Fig. 2A). The semiquantitative estimates of initial velocities of uptake were plotted in Fig. 2B. Kinetic experiments indicated that VGLUT2-mediated transport is dose-dependent and saturable (Fig. 2B). In one experiment depicted in Fig. 2B, the maximum velocity (V_{max}) was 1405 pmol/min-mg protein,

**FIG. 1. Comparison of amino acid sequences of mouse VGLUT2 and human VGLUT1.** Identical sequences are shown in gray.
and the apparent affinity constant ($K_m$) for glutamate was 1.2 mM. In three separate batches of membranes, the $V_{max}$ was $1219 \pm 109$ pmol/min-mg protein, and the $K_m$ for glutamate was $1.1 \pm 0.2$ mM. The $K_m$ is similar to that of glutamate transport by native synaptic vesicles as well as VGLUT1 (1–2 mM), whereas it is in contrast to that of plasma membrane transporters, which exhibit a $K_m$ for glutamate between 3 and 20 mM (15, 16).

In addition to kinetic properties, vesicular glutamate transport has a number of distinct characteristics from plasma membrane glutamate transport. Accumulation of glutamate into vesicular membranes depends on the vacuolar Mg$^{2+}$-ATPase, which utilizes ATP, whereas plasma membrane glutamate transport depends on energy provided by the sodium gradient. VGLUT2-mediated glutamate transport is ATP-dependent, as the initial rate by VGLUT2 in vesicular membranes at 1.5 min in the presence of various concentrations of glutamate (50 μM to 50 mM). The uptake by vesicular membranes from vector only-transfected cells was subtracted as background. C, glutamate uptake was determined in the presence or absence of 4 mM various nucleotides or ATPase inhibitors for 5 min. Four mM ATP was used for ATPase inhibitor studies. Glutamate uptake by VGLUT2 is inhibited by bafilomycin A1 (Baf A1, 100 nM), DCCD (50 μM), and NEM (200 μM), but not by oligomycin B (5 μM), ouabain (2 mM), or vanadate (50 μM). *, p < 0.01. D, glutamate uptake was measured at various concentrations of chloride for 5 min. *, p < 0.01. E, substrate specificity of VGLUT2 was determined by competition of $[^{3}H]$glutamate uptake with 10 mM unlabeled substrate for 5 min. One μM DIDS inhibits ~70% uptake, *, p < 0.01. F, glutamate uptake was measured at different pH values for 5 min.

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**DISCUSSION**

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. It has been shown that glutamate can be accumulated into synaptic vesicles to concentrations of at least 60 mM through an ATP-dependent mechanism (32, 33). The vesicular transport system, in contrast to the sodium-dependent plasma membrane transport system, is specific for glutamate, has a low affinity for glutamate, and is stimulated by physiologically relevant concentrations of chloride (12, 13, 29, 30). These unique properties of the vesicular glutamate transporter are conserved in such diverse vertebrates as fish, reptiles, amphibians, and mammals (34). Consistent with these observations, glutamate was transported by VGLUT2 in the absence of sodium. Furthermore, the transport required ATP and was inhibited by specific inhibitors of the V-ATPase. Glutamate transported by VGLUT2 is saturable with a $K_m$ of 1.1 mM, which is consistent with characteristics of the vesicular glutamate transporter. In addition, VGLUT2 is specific for glutamate and does not transport aspartate or other amino acids.

The requirement of low Cl$^-$ concentrations is a significant property of vesicular glutamate transport. The presence of chloride at low concentrations (1–5 mM) is essential for the uptake of glutamate, with substantially lower transport activity observed at higher and lower levels (9, 13, 29). This biphasic effect of chloride and the DIDS-sensitive chloride dependence of glutamate uptake, have suggested that the vesicular glutamate transporter possesses an anion binding site, distinct from the substrate binding site, which regulates transporter activity (35). Chloride showed a similar effect on glutamate uptake by VGLUT2. We also observed a 70% inhibition in VGLUT2-mediated glutamate uptake by 1 μM DIDS, which is consistent with previous findings. Taken together, these functional characteristics suggest that VGLUT2 functions as a vesicular glutamate transporter.

Based on the high homology (97% at the amino acid level) between mouse VGLUT2 and human DNPI, we surmised that DNPI is the human homologue of mouse VGLUT2. By using Xenopus oocytes expressing hDNPI, Aihara et al. (28) observed a 75% increase in sodium-dependent phosphate uptake. However, in our substrate specificity experiments, high concentrations of phosphate did not inhibit glutamate uptake by VGLUT2, suggesting that phosphate was not transported by the same mechanism as glutamate. This is a similar situation to VGLUT1, where weak sodium-dependent phosphate uptake was observed in oocyte plasma membranes expressing...
VGLUT1 (23, 24), and relatively strong ATP-dependent glutamate uptake was observed in vesicular membranes expressing VGLUT1 (18, 19). The relationship between glutamate and phosphate transport by VGLUT1 and VGLUT2 remains unclear. As has been proposed for VGLUT1 (19), VGLUT2 may be a bifunctional transporter, which functions as a phosphate transporter at the plasma membrane and a glutamate transporter in synaptic vesicles.

 Whereas it is known that vesicular glutamate transport is driven by an electrochemical proton gradient generated by V-ATPase, the precise manner in which the glutamate transporter and V-ATPase operate is currently under debate. To assess the driving forces of glutamate transport mediated by VGLUT2, we tested the effect of different ionophores on VGLUT2-mediated glutamate uptake. In the presence of 4 mM KCl, the K⁺ ionophore valinomycin, which reduces only Δψ

**Fig. 5. In situ hybridization analysis of VGLUT2 expression in mouse brain.** Mouse brain sections were hybridized with a biotin-labeled specific antisense (panels A–F) and sense (panels G and H; ×8) probes. Mouse VGLUT2 is highly expressed in the cerebral cortex (CTX) (panel A; ×8; panel D × 30), hippocampus (HP) (panel B; ×8; panel E × 30), and thalamus (TH) (panel C; ×8; panel F; ×30).
without changing ΔpH, deceased glutamate uptake by VGLUT2 by 50%. Nigericin, an electroneutral ionophore that exchanges K⁺ for H⁺, eliminates ΔpH whereas allowing Δψ to increase in the presence of 4 mM KCl and subsaturating concentrations of glutamate, inhibited glutamate uptake by VGLUT2 by 40%. This result is consistent with previous findings that both the Δψ and ΔpH components are driving forces for the transport of glutamate into synaptic vesicles (13). However, this functional characteristic is distinct from VGLUT1, because Δψ was suggested as the primary driving force for VGLUT1 (18, 19). Thus, one possible explanation of the previous controversial results observed in synaptic vesicles could be the differing functions of two closely related isoforms. Further elucidation of molecular differences between these two isoforms may answer this question.

Another interesting finding is that the two vesicular glutamate transporters have different expression patterns in brain. VGLUT1 is only expressed in a subset of glutamatergic neurons in the cerebral cortex, hippocampus, and cerebellum (20), whereas VGLUT2 is expressed in cerebral cortex, hippocampus, and the thalamus (Fig. 5). Thalamic nuclei use glutamate whereas VGLUT2 is expressed in cerebral cortex, hippocampus, and cerebellum (20), VGLUT1 is only expressed in a subset of glutamatergic neurons. Thus, VGLUT2 functions as a vesicular glutamate transporter previously characterized from synaptic, neuronal membranes. Identification of two isoforms with different functional characteristics provides molecular information for structure-function studies of vesicular glutamate transporters. In addition, the presence of VGLUT2 in brain regions lacking VGLUT1 suggests that the two isoforms together might account for glutamate transport into synaptic vesicles in glutamatergic neurons.

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