VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate

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Exocytotic release of the excitatory neurotransmitter glutamate depends on transport of this amino acid into synaptic vesicles. Recent work has identified a distinct family of proteins responsible for vesicular glutamate transport (VGLUTs) that show no sequence similarity to the other two families of vesicular neurotransmitter transporters. The distribution of VGLUT1 and VGLUT2 accounts for the ability of most established excitatory neurons to release glutamate by exocytosis. Surprisingly, they show a striking complementary pattern of expression in adult brain that might reflect differences in membrane trafficking. By contrast, VGLUT3 is expressed by many cells traditionally considered to release a different classical transmitter, suggesting novel roles for glutamate as an extracellular signal. VGLUT3 also differs from VGLUT1 and VGLUT2 in its subcellular location, with somatodendritic as well as axonal expression.

Glutamate is a ubiquitous amino acid that is required by all cells for its role in protein synthesis and intermediary metabolism. Cells such as neurons that release glutamate as an external signal have, however, evolved specialized mechanisms for its regulated secretion. The bulk of glutamate that is released as a neurotransmitter derives from glutamine [1,2], and excitatory synapses appear to recycle released glutamate in the form of glutamine. Astrocytes take up the released glutamate by the action of excitatory amino acid transporters (EAAT1 and EAAT2, in particular) [3,4] and in these cells glutamate is converted to glutamine by glutamine synthetase [5]. Recent work has also suggested that a system N transporter on the astrocyte and a closely related system A transporter on the neuron mediate the transfer of glutamine back to the neuron [6,7], where glutaminase converts glutamine to glutamate. Transport into secretory vesicles then enables the regulated exocytotic release of glutamate from the nerve terminal.

Previous studies have shown that native synaptic vesicles accumulate radiolabeled glutamate through an energy-dependent mechanism. ATP provides the energy for glutamate uptake by activating the vacuolar H⁺-ATPase that generates a H⁺ electrochemical gradient across the vesicle membrane [8]. The transport of all classical transmitters into synaptic vesicles depends on this gradient and involves exchange for lumenal H⁺[9]. However, the transport of different transmitters relies on different components of this gradient. Cationic transmitters such as monoamines (which are recognized in their protonated state) and ACh depend principally on the chemical component of this gradient (ΔpH), whereas glutamate uses primarily the electrical component (ΔΨ) [10,11]. Indeed, it has been suggested that vesicular glutamate transport exhibits channel-like properties [12,13].

The differences in ionic coupling suggest that vesicular glutamate transporters might reside on synaptic vesicles different from those storing other classical transmitters. In particular, the primary dependence of vesicular glutamate transport on ΔΨ would suggest that this activity is expressed on vesicles with ΔΨ greater than ΔpH, whereas vesicular monoamine and ACh transporters might be expected to reside on vesicles with ΔpH larger than ΔΨ. How do secretory vesicles regulate these two components of the H⁺ electrochemical gradient? Chloride appears to serve an important role in determining the relative magnitude of ΔpH and ΔΨ. Synaptic vesicles, in addition to endosomes and lysosomes, acidify to a much greater extent in the presence of Cl⁻ than in its absence [12,13]. Presumably, Cl⁻ entry dissipates the luminal positive charge and so enables the H⁺ pump to generate a larger ΔpH [8]. The analysis of knockout mice has indeed demonstrated an important role for intracellular Cl⁻ channels in the diverse functions of endosomes and lysosomes [14,15]. However, Cl⁻ channels of synaptic vesicles should dissipate the ΔΨ that drives glutamate uptake. Interestingly, loss of the intracellular Cl⁻ channel CIC-3 produces massive retinal and hippocampal degeneration, perhaps because increased vesicle filling with glutamate (due to increased ΔΨ) results in excitotoxicity [16]. Chloride also exerts an allosteric effect on vesicular glutamate transport that is independent of its role in establishing ΔpH and ΔΨ [17,18]. Synaptic vesicles exhibit maximal glutamate uptake at 2–10 mM Cl⁻, with less activity at lower and higher concentrations.

Despite the dependence of vesicular monoamine and glutamate transport on different components of the H⁺ electrochemical gradient, several neurons have been...
reported to release both classes of transmitter, raising the possibility of release from different populations of secretory vesicles. 5-Hydroxytryptamine (5-HT) and dopamine-containing neurons in culture form excitatory, glutamatergic synapses [19,20]. In addition, cultured glial cells appear to release glutamate by exocytosis [21,22]. However, it has been difficult to show glutamate release in vivo, raising the possibility that these cells de-differentiate in vitro. Identification of the proteins responsible for vesicular glutamate transport would help to explore these questions.

Isolated by expression cloning, the type I inorganic phosphate transporters were originally considered to mediate the cotransport of inorganic phosphate with Na⁺ across the plasma membrane [23]. However, subsequent work revealed a variety of alternative, organic anion substrates for several members of the family [24,25]. In addition, one member uses a H⁺ rather than a Na⁺ electrochemical gradient to transport substrate across intracellular membranes [25,26], and another has a large associated Cl⁻ conductance [24,27]. The brain-specific Na⁺-dependent phosphate transporter (BNPI), which is expressed by a subset of glutamate neurons, belongs to this family. Genetic studies of eat-4, the BNPI ortholog in C. elegans, demonstrated a specific role for the protein in glutamate release [28]. The recognition of organic anions by other members of the family and dependence on H⁺ as the driving force by one of them [26] raised the possibility that BNPI mediates vesicular glutamate transport. Indeed, heterologous expression of BNPI confers vesicular glutamate transport with all of the properties previously described for native synaptic vesicles, including selective recognition of glutamate rather than of aspartate, biphasic dependence on Cl⁻ concentration, and reliance primarily on ΔVₚ [29,30]. However, the analysis also demonstrated a clear role for ΔpH [29], supporting the function of BNPI as a transporter rather than a channel. A second, closely related type I inorganic phosphate transporter, DNPI, was reported around the time that BNPI was found to be a vesicular glutamate transporter [31] and also confers vesicular glutamate transport on heterologous expression [32–35]. Interestingly, both VGLUT1 and VGLUT2 were originally identified as cDNAs upregulated in response to specific extracellular signaling molecules; to subtoxic levels of the glutamate receptor ligand NMDA in the case of VGLUT1 [23], and to growth factors (activin A and betacellulin) in the case of VGLUT2 [31]. The extent of their transcriptional regulation in vivo under physiological or pathological conditions remains poorly understood.

**Distribution of VGLUT1 and VGLUT2 correlates with properties of release**

VGLUT1 and VGLUT2 exhibit an essentially complementary pattern of expression in the adult brain [32,34–37]. Cerebral cortex, hippocampus and cerebellar cortex (but not deep nuclei) express predominantly mRNA for VGLUT1 (Figure 1). The only exceptions in the neocortex are cells in layer 4 that express VGLUT2 transcripts. By contrast, the brainstem and deep cerebellar nuclei (but not cerebellar cortex) express almost exclusively VGLUT2. In the thalamus, different nuclei express mainly VGLUT1 or VGLUT2 but VGLUT2 predominates. Remarkably, even within one brain structure, basolateral nuclei of the amygdala label for VGLUT1 and more medial nuclei for VGLUT2.

VGLUT1 and VGLUT2 also segregate at the level of the protein. In the cortex, terminals in all layers label for VGLUT1, but with slightly lower density in layer 4, the principal site of labeling for VGLUT2 [32,35]. In the dentate gyrus and hippocampus, essentially all terminals in the dendritic fields stain for VGLUT1 [38] but the granule and pyramidal cell layers label selectively for VGLUT2 [32]. Even in the ventral striatum, which receives input from both VGLUT1-expressing and VGLUT2-expressing neurons, the projections segregate [39]. In the thalamus and hypothalamus, different nuclei receive either VGLUT1-positive input (posterior and ventromedial thalamic nuclei) or VGLUT2-positive input (paraventricular, anterodorsal and reticular thalamic nuclei) [35]. In the cerebellum, parallel fibers express only VGLUT1 whereas climbing fibers, which synapse onto the same Purkinje cell dendrites [32], express only VGLUT2. Nonetheless, pinealocytes [40], primary sensory neurons [41,42] and certain pancreatic islet cells [43,44] express both VGLUT1 and VGLUT2, and the two isoforms reside on the same populations of secretory vesicles. In the brain, thalamic neurons express the mRNA for both VGLUT1 and VGLUT2, but the proteins were not observed to colocalize [34].

The division of central excitatory neurons into VGLUT1-positive or VGLUT2-positive suggests that these cells might differ in some aspect of the packaging and regulated release of glutamate. For example, the two isoforms could exhibit intrinsic differences in their transport properties such as the speed of transport, with the
faster isoform more appropriate for synapses with high firing rates. The expression of VGLUT1 and VGLUT2 mRNA does not, however, correlate with the rates of neuronal firing. VGLUT2 is expressed both at climbing fiber synapses that release glutamate infrequently and in sensory relay pathways that are active at extremely high rates. Indeed, in at least four studies, no consistent difference in the transport activity of VGLUT1 and VGLUT2 has been detected [29,30,32,33]. Rather, the expression of VGLUT isoforms appears to correlate more consistently with the probability of transmitter release, which is generally low at synapses in the hippocampus and parallel fiber synapses in the cerebellum (which use VGLUT1) [45,46] and high at climbing fiber synapses in the cerebellum (which use VGLUT2) [47]. Interestingly, synapses with a low probability of release such as those in the hippocampus have a higher potential for plasticity than those with a high release probability such as climbing fibers. The expression of VGLUT isoform could thus correlate with the potential for synaptic plasticity. At the calyx of Held, more glutamate filling increases the postsynaptic response [48], illustrating the potential for presynaptic mechanisms to alter quantal size. Indeed, several synapses do not saturate with a single vesicle of transmitter.

Why would the expression of VGLUT isoforms correlate with the probability of transmitter release? Perhaps because VGLUT1 and VGLUT2 differ in their membrane trafficking. Subtle but consistent differences have been observed in their localization upon heterologous expression in PC12 cells [32]. In neurons, however, VGLUT1 and VGLUT2 localize only to nerve terminals, not to cell bodies or dendrites, and the difference between localization of the two isoforms is less obvious. Nonetheless, the cytoplasmic C terminus of VGLUT1 contains two polyproline motifs not found in VGLUT2, which presumably interact with other proteins and might influence membrane trafficking. It is possible that different protein–protein interactions simply affect the recycling of VGLUT isoforms at the nerve terminal, with consequences for the rate of vesicle filling. Alternatively, the VGLUTs could influence the recycling of synaptic vesicle membrane, with consequences for other aspects of presynaptic function.

VGLUT1 and VGLUT2 also undergo transcriptional regulation during development. In rodents, VGLUT1 transcription increases during the weeks after birth, whereas that of VGLUT2 declines [49]. Although expressed by different neuronal populations in the adult, this inverse relationship raises the possibility that individual cells might switch expression of VGLUT isoforms. Indeed, parallel fibers in the cerebellum switch from VGLUT2 to VGLUT1 in the weeks after birth [50]. During the transition, the deep molecular layer labels for VGLUT1 and the more superficial for VGLUT2, until the upward migration of VGLUT1 immunoreactivity replaces VGLUT2 by one month after birth. As observed using immunoelectron microscopy, individual neurons thus express both isoforms transiently, although it remains unclear whether the isoforms colocalize on the same vesicles [50]. The functional significance of the switch also remains unclear, although vesicular glutamate content also increases with age at the calyx of Held [51]. In addition, the organization of docked and reserve synaptic vesicle pools [52] and the probability of transmitter release [53] change dramatically during the postnatal period when VGLUT1 expression is upregulated.

Location of VGLUT3 suggests novel modes for glutamate signaling

The distribution of VGLUT1 and VGLUT2 in essentially all neurons accepted to be glutamatergic accounts for their ability to release glutamate by exocytosis. However, neither isoform is expressed by several cell populations previously shown to release glutamate in vitro. Whereas catecholamine cells in the pons and medulla (C1–C3 and A2 cells, and cells in the area postrema) express VGLUT2 as well as tyrosine hydroxylase [54,55], midbrain dopamine- and 5-HT-containing neurons in raphe nuclei that form excitatory glutamatergic synapses in vitro [19,20] do not express VGLUT1 or VGLUT2. Initial studies also failed to detect VGLUT1 or VGLUT2 in glia, although astrocytes might express lower levels of these isoforms that are difficult to discern in the background of strong neuronal labeling. Nonetheless, the apparent absence of VGLUT1 and VGLUT2 from midbrain dopamine- and 5-HT-containing neurons as well as astrocytes suggested the possibility of a third VGLUT isoform. A search of the available cDNA databases failed to identify the novel sequence. Human and mouse genomic sequence did, however, reveal a novel type I phosphate transporter more closely related to VGLUT1 and VGLUT2 than to other members of the family. Consistent with a low level of expression by the brain as a whole, VGLUT3 occurs at very low frequency in brain cDNA and is difficult to detect by northern analysis [92,56]. Unlike VGLUT1 and VGLUT2, VGLUT3 is also expressed outside the nervous system, in the liver and kidney [57,58].

Within the brain, VGLUT3 mRNA appears in a specific subset of cell populations, accounting for the low overall abundance. In cortex and hippocampus, scattered cells express VGLUT3 mRNA and protein [56,57], suggesting expression by a population of excitatory interneurons. Immunostaining confirms the expression by interneurons and most, if not all, VGLUT3-positive interneurons in the hippocampus double label for glutamic acid decarboxylase, the enzyme that synthesizes GABA [57]. Although double staining for the vesicular GABA transporter has not yet been reported, labeled terminals can be seen to form symmetric synapses onto the cell bodies and proximal dendritic shafts of pyramidal cells by immunoelectron microscopy, raising the possibility of glutamate co-release with GABA at inhibitory synapses [57,58]. The expression of VGLUT3 in cell bodies also differs from the strictly presynaptic location of VGLUT1 and VGLUT2. In the striatum, cholinergic interneurons express VGLUT3, again in their cell bodies as well as their terminals [57,58]. Furthermore, cells in the substantia nigra pars compacta and ventral tegmental area express low levels, and cells in the raphe nuclei express high levels, of VGLUT3 mRNA. Unfortunately, the VGLUT3 protein expressed by these brainstem cell groups does not localize.
to cell bodies, and it was initially difficult to confirm whether the expression was by monoaminergic neurons, rather than by non-monoaminergic neurons in the same regions. In addition, double staining for several monoamine markers including tyrosine hydroxylase, the 5-HT transporter and 5-HT failed to reveal colocalization with VGLUT3 in dopamine- and 5-HT-containing projections [57]. To address the possibility of expression by non-monoaminergic cells in the raphe, where VGLUT3 mRNA is expressed at higher levels than in dopaminergic neurons, Fremeau et al. examined autaptic cultures and detected VGLUT3 in ~70% of the 5-HT-positive neurons. Interestingly, the VGLUT3 protein in these single cell cultures does not always colocalize with 5-HT [57], suggesting segregation of the monoamine and glutamate phenotypes to distinct processes, as previously suggested for dopaminergic neurons [20], which could account for the lack of colocalization with most monoamine markers in vivo. However, the vesicular monoamine transporter VMAT2 does show some colocalization with VGLUT3 in brain sections [56], and the apparent difference from other monoamine markers remains unresolved. In addition to neurons, a subpopulation of astrocytes expresses VGLUT3 [57]. By light and electron microscopy, glial VGLUT3 localizes both to glial processes in neuropil and to astrocytic endfeet on brain capillaries, where VGLUT3 could contribute to the blood–brain barrier.

The expression of VGLUT3 in vivo by multiple cell populations that form excitatory synapses in vitro supports a physiological role for glutamate release by cells traditionally considered to release a different classical transmitter. However, several alternative possibilities remain. VGLUT3 might simply provide an intracellular storage pool of glutamate that can be used for other purposes, such as conversion to GABA in interneurons. Heterologous expression of VGLUT1 and VGLUT2 suffices to make inhibitory neurons glutamatergic [30,33] but this has not been reported for VGLUT3 [58]. Expression by non-neural cells in liver and kidney might also suggest a role distinct from exocytotic glutamate release. However, the role of an intracellular glutamate depot in cholinergic and monoaminergic neurons remains unclear. Further, VGLUT3 expression by liver and kidney might reflect a role in exocytosis, even if the released glutamate has a role in epithelial transport or metabolism rather than signaling. The plasma membrane excitatory amino acid carrier EAAC1 presumably mediates uptake across the apical membrane [60] but the mechanism underlying basolateral glutamate efflux has remained unclear. It will thus be interesting to determine whether VGLUT3 contributes to the reabsorption of glutamate in the kidney by enabling exocytotic release across the basolateral membrane. Similarly, in nitrogen metabolism by the liver, the glutamate produced from glutamine in periporal hepatocytes is released through an unknown mechanism for uptake by perivenous hepatocytes, where it reduces levels of circulating ammonia by reforming glutamine [61]. Finally, it is possible that VGLUT3 recognizes substrates other than glutamate. High concentrations of aspartate have been reported by two groups to inhibit the uptake of 3H-glutamate by VGLUT3 [58,59], but one of the groups also observed inhibition of VGLUT2 and others have not observed any consistent effect of aspartate on VGLUT3 [56,57]. Further, it seems that 3H-aspartate is not taken up by VGLUT3 (Robert T. Fremeau, Jr and Robert H. Edwards, unpublished), and that the Km for glutamate is in the same range as that for VGLUT1 and VGLUT2, strongly suggesting that VGLUT3 also transports primarily glutamate. Nonetheless, it remains possible that VGLUT3 recognizes substrates in addition to glutamate. The role of any VGLUT isoform in phosphate transport remains particularly unclear.

If VGLUT3 does promote the exocytotic release of glutamate, a fundamental question that needs to be addressed is whether GABAergic interneurons, cholinergic interneurons in the striatum and monoaminergic neurons store, and hence release, glutamate from the same vesicles as the transmitters with which they are usually associated. Double labeling of cholinergic interneurons in the striatum for VGLUT3 and the vesicular ACh transporter suggested expression of the two transport proteins in the same processes, but the use of immunoperoxidase labeling for the ACh transporter precluded a definitive conclusion about colocalization on vesicles [58]. In addition, the expression of VGLUT3 by GABAergic, and particularly by cholinergic and monoaminergic, neurons raises the possibility of multiple, distinct vesicle populations with properties appropriate for storage of the different transmitters. If these neurons contain distinct vesicle populations, they might also form different synapses with a wider range of target cells than previously anticipated. The well-established potential for modification of glutamatergic synapses might further increase the plasticity of the circuit. The localization of VGLUT3 to cell bodies and dendrites indeed already suggests variation in the site, mechanism and physiological role of glutamate release.

Previous work has indicated the possibility of glutamate release from dendrites that is presumably exocytotic. It is well established that cells in the olfactory bulb form dendrodendritic synapses in which one cell releases glutamate and the other either GABA or glutamate, resulting in either recurrent inhibition (and synchronization) or excitation [62,63]. More recently, interneurons in the cortex have been shown to release glutamate from dendrites [64] and immunoelectron microscopy for VGLUT3 now confirms the localization to a pool of subsynaptic vesicles that could mediate retrograde synaptic signaling [57].

Concluding remarks
Identification of the VGLUTs raises many questions about excitatory neurotransmission. First, how do VGLUT1 and VGLUT2 differ, and how might this difference contribute to or reflect differences in the probability of transmitter release? Second, how much isoform switching occurs during development, and how does this influence synaptic function? Also, how much regulation of the VGLUTs occurs in adulthood? Third, is VGLUT3 expressed on the same vesicles that store other classical transmitters, and how does this influence filling? Fourth, what is the role of glutamate release by astrocytes and by cells traditionally
considered to release another transmitter? The answers will help us to understand the cellular organization of neurons, synaptic transmission and the processing of information by neural circuits.

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