Molecular, Structural, Functional, and Pharmacological Sites for Vesicular Glutamate Transporter Regulation

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Abstract

Vesicular glutamate transporters (VGLUTs) control quantal size of glutamatergic transmission and have been the center of numerous studies over the past two decades. VGLUTs contain two independent transport modes that facilitate glutamate packaging into synaptic vesicles and phosphate (Pi) ion transport into the synaptic terminal. While a transmembrane proton electrical gradient established by a vacuolar-type ATPase powers vesicular glutamate transport, recent studies indicate that binding sites and flux properties for chloride, potassium, and protons within VGLUTs themselves regulate VGLUT activity as well. These intrinsic ionic binding and flux properties of VGLUTs can therefore be modulated by neurophysiological conditions to affect levels of glutamate available for release from synapses. Despite their extraordinary importance, specific and high-affinity pharmacological compounds that interact with these sites and regulate VGLUT function, distinguish between the various modes of transport, and the different isoforms themselves, are lacking. In this review, we provide an overview of the physiologic sites for VGLUT regulation that could modulate glutamate release in an over-active synapse or in a disease state.

Keywords Vesicular glutamate transporters (VGLUTs) · ATPase · Glutamate (Glu)

Introduction

Glutamate (Glu) is the major excitatory neurotransmitter in the mammalian central nervous system and is involved in all brain functions and in various neurological pathologies [1–5]. Excessive and sustained release of glutamate from synapses triggers glutamate-induced, NMDA receptor-dependent excitotoxicity. The prolonged presence of glutamate in the extrasynaptic space in the hippocampus has been proposed to underlie the onset of a variety of cognitive neurological disorders including those observed after cardiac arrest-induced global or focal brain ischemia, traumatic brain injury, epilepsy, and Alzheimer’s disease (AD) [6–14], among others. Mechanisms to reduce excessive synaptic glutamate release under these conditions could potentially prevent/reduce excitotoxic damage to vulnerable hippocampal neurons. In addition, glutamate is suspected to be at the core of major psychiatric disorders, such as schizophrenia, depression, addiction, and compulsive disorders [15–25]. Treatment options to modulate glutamatergic transmission are limited. Currently, in human studies, most post-synaptic glutamatergic interventions (with the exception of esketamine in major depressive disorders [26–28] and memantine in AD [29–31]) have been disappointing because of poor efficacy or unacceptable side effects [32, 33]. Compounds selectively modulating the presynaptic release of glutamate could constitute a novel pharmacological approach for the prevention of glutamate excitotoxicity and modulation of behavior under various disorders. Possible sites to regulate presynaptic glutamate release are modulation of...
synaptic glutamate synthesis, VGLUT expression level, or sites that mediate the transport of glutamate into synaptic vesicles. Three subtypes of vesicular glutamate transporters (VGLUT1–3) have been identified that package glutamate into vesicles [34–42] reviewed in [43–46]. The three VGLUTs share a high degree of structural homology and, so far, their functional activity cannot be distinguished by their bioenergetic or pharmacological profiles. Nevertheless, identifying the structural and functional sites for VGLUT regulation and understanding the differential molecular and cellular modes of VGLUT regulation themselves is critical to recognize novel potential targets to modulate presynaptic glutamatergic transmission in normal and aberrant states.

VGLUTs: Markers for Glutamatergic Transmission

VGLUT1 and VGLUT2 are expressed in distinct and complementary subsets of neurons in the CNS that display differences in release probability [36, 39]. VGLUT1 is the most abundant subtype in the CNS [47]. Unlike, vesicular transporters for monoamines (VMAT1 and VMAT2) and acetylcholine (VACHT) that are found in both cell bodies and nerve terminals, VGLUT1 and VGLUT2 proteins are restricted to nerve endings where they continuously recycle between the plasma membrane, endosomes, and newly formed synaptic vesicles [48–50]. Thus, VGLUT1 and VGLUT2 likely recycle in synapses for an extended period of time and are therefore unique synaptic markers for select glutamatergic terminals. VGLUT1 is found in asymmetric synapses in the cerebral cortex, the hippocampus, the cerebellum, and the amygdala (for review, see [44]). VGLUT2 is primarily, though not exclusively, used by subcortical excitatory neurons [36, 38, 39, 51–53]. VGLUT1 and VGLUT2 are also co-expressed in some thalamic neurons, layer IV cortical interneurons and pinealocytes [38, 54–57]. Co-expression of VGLUT1 and VGLUT2 in synapses could afford these cells with two distinct modes of release, if they are sorted to different vesicles. VGLUT3, the atypical subtype, is sparingly expressed compared to VGLUT1 and VGLUT2 [44, 58] and is often present in neurons that use other “classic” neurotransmitters, such as serotonin, acetylcholine or GABA [40–42, 59]. Indeed, in some neuronal populations, such as striatal cholinergic interneurons, VGLUT3 is abundantly present in the somatodendritic compartment, although its function there is not yet elucidated [44]. However, VMAT2 sorting to the somatodendritic compartment in neurons confers the activity-dependent release of monoamines as well as multiple retrograde signals involved in synaptic function, growth, and plasticity [60, 61]. VGLUT1–3 are also expressed in sensory nerves from the ventral horn of the spinal cord, suggesting their involvement in nociception [62–68]. The distribution of VGLUT1–3 is conserved between humans and rodents [69].

Mouse lines with deleted VGLUTs demonstrate the importance of VGLUTs for glutamatergic transmission in normal brain function and facilitate recognizing roles VGLUTs could contribute to brain disorders [47, 70–78]. In mice, VGLUT1 or VGLUT2 deletion (VGLUT1-KO mice and VGLUT2-KO mice, respectively) is lethal. VGLUT1-KO mice die 2 to 3 weeks after birth, which is a time that normally follows a strong up-regulation of VGLUT1 [55, 79, 80] and increased synaptic vesicle clustering in VGLUT1 synapses [81, 82]. The post-natal up-regulation of VGLUT1 also replaces the VGLUT2 isoform that is predominant in early cerebellar, hippocampal, and cortical synapses [83]. VGLUT2-KO mice succumb to respiratory failure immediately after birth [71, 72] as VGLUT2 is abundantly expressed in descending and in local brainstem glutamatergic systems that control respiration [39, 52, 84, 85]. Reduced expression of VGLUT2 during neuronal development results in reduced pyramidal neuron plasticity, dendritic refinement, and spatial learning [76]. Unlike VGLUT1-KO mice and VGLUT2-KO mice, VGLUT3-KO mice survive [73, 86]. However, VGLUT3 null mice are deaf, hyperactive, and demonstrate increased anxiety [73–75, 87]. VGLUT3 may also provide protection against neonatal hypoxic stress [88] and be critically involved in reward regulation [78]. Selective modulation of specific VGLUT-encoded systems will be required to repair any alteration in glutamatergic transmission in specific VGLUT pathways that may contribute to excitotoxic or disease pathology.

VGLUT expression levels have been considered as potential pathological or diagnostic markers for impaired or over-active glutamatergic transmission. In humans, altered expression of VGLUT1 is associated with anxiety and mood disorder [15, 17–19], and in neurological conditions, such as Parkinson’s disease, AD, and epilepsy [89–92]. Modulation of VGLUT2 expression levels have been observed in schizophrenia and neuropathic pain [21, 64, 72]. Reduced expression of VGLUT2 is associated with decreased motoneuron degeneration in a mouse model of amyotrophic lateral sclerosis (ALS) [93], yet these mice are more susceptible to clonic seizures [94]. A marked increase in VGLUT1 expression and glutamate release (+40%) has been reported in a tau animal model of AD during the early stages of the pathology [95]. Neuronal hyperactivity and increased functional connectivity have been confirmed in preclinical AD, mild cognitive impairment (MCI), and early AD stages at various levels [96–98]. Later stages of AD in humans and animal models of AD [99] may include outright loss of excitatory synaptic terminals [100–106]. In humans, initial studies pointed to a marked decrease of VGLUT1 expression in the cortex of AD patients [89, 90, 107]. However, recent work suggests that synapse loss is probably not a hallmark specific to AD [108] and only minimal alterations of VGLUT1 are observed in the prefrontal cortex of demented individuals [109]. Instead, Alzheimer’s disease may be a result of presynaptic glutamatergic dysfunction induced by tau and oligomeric β-amyloid [33, 110–112].
Molecular Sites for VGLUT Regulation

VGLUTs are specific molecular and functional markers of glutamatergic transmission as their presence in synaptic vesicles in neurons is sufficient to convey exocytotic glutamate release [35]. Excitatory synaptic vesicles in mammalian synapses are thought to contain between 4 and 14 molecules of VGLUT each [113, 114]. While alterations in levels of VGLUT leads to multiple altered or pathological behaviors in humans and in mouse models, it is not entirely clear how alterations in synaptic VGLUT levels impact glutamate transmission. Primary hippocampal autaptic cultures from VGLUT1- and VGLUT2-KO mice reveal a decrease in quantal size that can be rescued by transgene over-expression of VGLUT1 and VGLUT2, respectively [70, 72]. However, miniature EPSC amplitude, reflecting the amount of glutamate released per vesicle (as well as the postsynaptic response) does not differ in acute hippocampal slices from VGLUT1-KO mice relative to wild-type littermates [115].

Likewise, severe reduction of VGLUT3 (up to 80%) does not alter glutamatergic signaling [116]. Liu and colleagues verified biophysically that increasing the number of VGLUT1 molecules at hippocampal excitatory synapses in dissociated neuronal cultures results in an increase in the amount of glutamate released per vesicle into the synaptic cleft [117]. Control of the neurotransmitter content by transporter copy number has been interpreted as a result of an equilibrium between glutamate uptake and leakage. The modulation of synaptic strength by VGLUT1 expression is endogenously regulated, both across development to coincide with a maturational increase in vesicle cycling and quantal amplitude and by excitatory and inhibitory receptor activation in mature neurons to provide an activity-dependent scaling of quantal size via a presynaptic mechanism [117–119]. Indeed, presynaptic scaling of VGLUT1 and VGLUT2 levels in synapses is observed at the molecular and synaptic level [55, 120]. Presynaptic scaling also occurs with the vesicular GABA transporter (VIAAT/VGAT) [55, 121]. Work in Drosophila suggests that a single copy of VGLUT on a vesicle is sufficient to load a vesicle [122]. While increasing VGLUT levels in Drosophila also results in increased quantal size (and synaptic vesicle volume) a compensatory decrease is observed in the number of synaptic vesicles released that maintains normal levels of synaptic excitation [123]. Molecular mechanisms of VGLUT regulation for homeostasis may differ in Drosophila, which only express a single VGLUT type, and higher organisms that express 3 VGLUTs in the brain.

Original findings revealed that a reduction of VGLUT1 expression results in the loss of synaptic vesicles in nerve terminals [115]. More recent studies indicate that synaptic vesicle clustering in VGLUT1 terminals is mediated through a tripartite interaction of VGLUT1, endophilinA1, and intersectin1 resulting in a combined reduction of axonal synaptic vesicle super-pool size and miniature excitatory events frequency [124, 125]. Indeed, low glutamate release probability is a characteristic feature of VGLUT1-encoded synaptic terminals [126–129]. Similarly, using high-resolution stimulated emission depletion (STED) microscopy, decreased VGLUT3 protein levels seems to be accompanied by a reduction in the number of VGLUT3-positive vesicles in varicosities [116]. VGLUT expression in mammalian synapses may therefore not only contribute to quantal size, but also to the availability of vesicles for release, which could explain the different release properties of VGLUT1- and VGLUT2-encoded synapses.

Molecular regulation of VGLUT synthesis and degradation represent powerful targets to control glutamate availability at the glutamate site on VGLUTs for transport into vesicles and subsequent exocytotic release at synapses. Regulation of VGLUT expression is used endogenously to provide resistance against glutamate-induced neurodegeneration. For instance, ischemic tolerance is a well-known phenomenon in which brief ischemic insults (ischemic preconditioning) confer robust neuroprotection to hippocampal CA1 neurons against a subsequent severe ischemic challenge [130–133]. Similarly, one or more brief seizures can serve to activate endogenous protective programs which render brain regions temporarily less susceptible to damage following an otherwise harmful episode of status epilepticus (i.e., a prolonged seizure) [78, 134, 135]. Furthermore, ischemic/hypoxic preconditioning can protect the brain from seizure-induced damage while epileptic preconditioning can protect vulnerable neurons to ischemia-induced injury [136, 137] suggesting some common molecular mechanisms for neuroprotection.

Although the molecular mechanisms underlying ischemic/epileptic tolerance are not yet fully delineated, the considerable delay (~ 24 h) from the preconditioning stimulus until onset of tolerance is consistent with a role for transcriptional changes in such neuroprotection. In neuronal cortical and hippocampal culture models, preconditioning induces tolerance to exocytotic injury by suppressing vesicular glutamate release and increasing vesicular release of GABA [138–141]. Accordingly, preconditioning stimuli result in the presynaptic down-regulation of VGLUT1 expression in excitatory neurons and up-regulation of VIAAT and the GABA synthesizing enzymes GAD65 and GAD67 in inhibitory neurons [55, 118, 120, 121, 142, 143]. Interestingly, preconditioning stimuli also up-regulate VGLUT2 in select VGLUT1-encoded synapses in cortical neurons that synapse onto GABAergic neurons [120], suggesting that selective trafficking of VGLUT2 in these neurons to synapses that target GABAergic inhibitory neurons could promote glutamate-induced feed-forward inhibitory transmission as neuroprotective strategy for neural circuit stability.

Molecular sites for selective VGLUT regulation are not yet well defined. A critical challenge moving forward is to be able...
to selectively modulate discrete VGLUT-driven pathways in the brain. Understanding the genetic controls and physiologic factors that regulate VGLUT expression is therefore critical [120, 144–148]. In addition, the development of viral vectors that allow efficient glutamatergic-selective gene expression or knockdown would permit the selective modification of VGLUT levels in defined neuronal cell populations [149–151]. Indeed, restoration of hearing in the VGLUT3 knockout mouse has been accomplished using virally mediated gene therapy, which is an important step towards gene therapy of human deafness [152].

**VGLUT Structural Sites**

The superfamily of solute carrier transmembrane transporters (SLC) is encoded by more than 300 genes organized into 52 families. The substrates used by these carriers are very diverse, including charged or neutral organic molecules and various ions. Currently, in the SLC superfamily, nine genes divided into three families (SLC17, SLC18, SLC32) have been identified as coding for vesicular transporters and are divided according to their natural substrates (Fig. 1) [43]. These three SLC family members predominantly use the transmembrane proton electrochemical gradient ($\Delta\mu H^+$) generated by a vacuolar-type H+ pump (V-ATPase) to translocate substrates. The SLC17 family includes (i) VGLUT1–3 (substrate: glutamate, $K_m \sim 1$ mM) [153], (ii) Sialin (substrate: sialic acid, $K_m \sim 0.2$ mM) [154], and (iii) a vesicular nucleotide transporter VNUT (substrate: ATP, $K_m \sim 1$ mM) [155]. The SLC17 family also includes the Na+-dependent inorganic phosphate (Pi) transporters NPT-1, NPT-3, NPT-4, and NPT-5 (substrate: Pi, $K_m \sim 3$–6 mM) [156]. The SLC18 family includes the vesicular polycation transporter VPAT, (SLC18B1, substrate: spermine and spermidine, $K_m \sim 100$ $\mu$M) [157], vesicular amine transporters for adrenaline, dopamine, norepinephrine, histamine, and serotonin (VMAT1 and VMAT2, SLC18A1 and SLC18A2, $K_m \sim 1$ $\mu$M) [158–161] and acetylcholine (VACHT, $K_m \sim 1$ mM) [162, 163]. SLC32 includes a single member, the vesicular inhibitory amino acid transporter (VIAAT or VGAT) [164, 165] that can transport GABA or glycine ($K_m \sim 5$–10 mM) [166, 167].

VGLUTs are composed of 560 to 582 amino acids with 12-membrane spanning segments and with the N- and C-termini in the cytoplasm. No crystal structures for these proteins are currently available, but technical progress in transporter crystallography of distantly related bacterial transporters provides some clues to the resolution of VGLUT structure. VGLUTs show primary sequence homology with the major facilitator superfamily (MFS), the second major family of transmembrane transporters involved in the translocation of small solutes using the driving force of an electrochemical gradient [168]. The crystallographic 3D structures of the lactose bacterial permease, also known as glycerol-3-phosphate transporter (GlpT), as well as D-galactonate transporter (DgoT) led to the conclusion that these transporters consist of 12 $\alpha$-helices organized into two groups of 6 (two halves) [169, 170]. The two groups of 6 $\alpha$-helices are connected by a cytoplasmic flexible loop, forming a hydrophilic cavity at their center deep in the transporter for translocation of hydrophilic substrates. The amino acid residues responsible for the specificity of the transporter are located on the walls of this polar pocket [170–172]. Because of the distant, yet distinct, homology between GlpT, DgoT, and VGLUTs, a putative 3D homology model of VGLUTs can be postulated (Fig. 2).

Site-directed mutagenesis of VGLUTs has identified several transmembrane charged residues important for the recognition and translocation of substrates (i.e., Arg184, His128, and Glu191) [173]. Arg184 is located on transmembrane domain 4 (H4). This residue is conserved in all the members of the SLC17 family, suggesting a common and essential role, such as recognition of anionic substrates (Glu, Asp, ATP), and $\text{Cl}^-$ ions. Its mutation into a neutral residue (e.g., alanine), or a

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**Fig. 1** H+-dependent vesicular neurotransmitter transport. Specific H+-dependent transporters are responsible for neurotransmitter vesicular uptake and belong to different families depending on the global charge of their respective substrates: SLC17 for glutamate and ATP, SLC18 for monoamines and acetylcholine, and SLC32 for GABA and glycine. The driving force for the vesicular accumulation of all neurotransmitters is an H+ electrochemical gradient ($\Delta\mu H^+$) generated by the vacuolar V-ATPase.
positively or negatively charged amino acid (e.g., glutamate, or even lysine), leads to complete inhibition of transport [173]. Interestingly, Na\(^+\)-dependent Pi transport of this mutant was normal [173]. More recent studies show that the arginine residue Arg184 in TMD4 selectively controls the allosteric binding of Cl\(^-\) in the vesicle lumen, which might be important to VGLUT activation [174] (see below). His128 and Glu191 reside on transmembrane domain 2 and 4 (TMD2 and TMD4), respectively and are conserved in the 3 subtypes of VGLUTs—mutation of these residues also inhibit glutamate transport [173]. However, His128 is not conserved in the anionic transporter sialin [175], suggesting an important role for His128 in the selectivity of substrate transport. In this regard, sialin transports both aspartate and glutamate, while VGLUTs only recognize glutamate. In the VGLUT 3D putative model, these three charged residues are located deep in the hydrophilic binding pocket created by \(\alpha\) helices [173, 176]. In alternate access model of transport, the interaction of Cl\(^-\) ions with a basic residue may induce a conformational change that facilitates the translocation of glutamate (Fig. 3).

The molecular and functional methods available today will enable the identification of additional structural sites important for VGLUT-specific transport function. A clever idea to understand basic mechanisms of \(\Delta\mu\text{H}^+\)-driven substrate translocation across intracellular brain vesicles is to understand these mechanisms in simpler organisms, such as their bacterial homologs. For example, Shuldiner and colleagues examined SARs between various transmembrane embedded charged amino acids in the bacterial multiresistant transporters for the study of their mammalian counterparts, such as VMATs [178–181]. Recently, Edwards and colleagues used a similar approach with DgoT showing an interesting cooperation of H\(^+\) transfer between transmembrane embedded glutamate (Glu133) and arginine (Arg47) residues that suggest a mechanism that couples H\(^+\) flux to substrate recognition [170]. That is, protonation of this bacterial transmembrane glutamate residue effectively releases a transmembrane arginine residue to bind and translocate substrate. If there is no substrate bound, the glutamate residue must give up its H\(^+\) so that it can form a charge pair with the arginine residue and reorient empty to complete the transport cycle [170]. Genetic tools may also be of great value to identify important structural residues involved in vesicular transporter function. Rand and colleagues identified numerous point mutants in Caenorhabditis elegans that map to highly conserved regions of the VACHT gene and exhibit behavioral phenotypes consistent with a reduction in vesicular transport activity and neurosecretion [182]. These mutants display selective defects in initial acetylcholine transport velocity with \(K_m\) values ranging from 2- to 8-fold lower than that of wild-type. This indicates that specific structural changes in VACHT translate into specific alterations in the intrinsic parameters of transport and in the storage and synaptic release of acetylcholine in vivo [182]. Similar work in Drosophila [183] or other organisms [184] where genetic manipulation can be performed with relative ease could identify additional important structural sites in VGLUT important for transport function and synaptic release of glutamate.

**VGLUT Functional Sites**

Glutamate accumulates in synaptic vesicles by virtue of one of the three VGLUT subtypes and considerable efforts have been made to understand how VGLUTs operate compared to the
Fig. 3 Model of transport and ion binding of VGLUTs. One model that has been proposed to describe the uptake of glutamate by VGLUTs is based on the alternate access model [177].

a. VGLUTs have several binding sites for transported ions: glutamate, Cl⁻, K⁺ and H⁺ (and Na⁺ and Pi).

b. In high chloride concentrations and in the cytosol upon open conformation, VGLUT is loaded with one glutamate, one Cl⁻, and partially by one K⁺. Glutamate and K⁺ are released into the lumen when VGLUT shifts to a lumen open conformation (step 1). At this stage, the anionic binding site is filled with another Cl⁻ while the cationic site loads one proton (step 2). Finally, VGLUT reopens in the cytosol where it releases one Cl⁻ and one proton (step 3).

c. In the presence of low luminal concentrations of chloride, VGLUT follows the same steps as in b but during step 2, the partially filled cationic site is fully occupied by a proton, while only a few chloride ions are released into the cytosol in step 3.
other vesicular neurotransmitter transporters in the brain. In the early 1980s, two independent groups showed, using purified synaptic vesicles from rat or bovine brain, that vesicular glutamate transport is dependent on a transmembrane H⁺ gradient generated by the vacuolar type (V-ATPase) proton pump [185, 186]. Shortly, thereafter it was discovered that Cl⁻ ions greatly stimulates glutamate uptake into synaptic vesicles in vitro [187]. Several teams rapidly confirmed these initial findings [188–190]. VGLUTs have relatively low affinity for glutamate (Kₘ ~ 1–2 mM) but are highly selective for glutamate compared to other structurally similar amino acids, such as aspartate or glutamine. Estimates of glutamate levels in synaptic vesicles suggest between 60 and 120 mM concentrations [191, 192].

Inorganic Phosphate Site for VGLUT Regulation

VGLUT1–3 belong to the family of Na⁺-dependent inorganic phosphate transporters (NPTs) forming the SLC17 subfamily and were initially shown to transport inorganic phosphate (Pi) [193, 194]. Interestingly, upon originally cloning of this brain-specific inorganic Pi transporter (formerly called BNPI), it was revealed that it has strong sequence similarity to EAT-4, a *Caenorhabditis elegans* protein implicated in glutamatergic transmission and localized almost exclusively to mammalian brain terminals forming asymmetric excitatory-type synapses [195]. Although BNPI (now called VGLUT1) [34, 35] depends on a Na⁺ gradient for Pi transport across the plasma membrane, surprisingly BNPI associated preferentially with the membranes of small synaptic vesicles [195]. Since phosphate-activated glutaminase (PAG) in nerve terminals produces glutamate from glutamine for release as a neurotransmitter [196], it was proposed that BNPI (VGLUT1) may augment excitatory transmission following vesicle exocytosis by increasing its expression at the plasma membrane and thereby increase cytoplasmic Pi concentrations within the nerve terminal to activate PAG and hence replenish glutamate synthesis lost by neurotransmission [195]. Such intrasynaptic sequestration of transport proteins involved in the Ca²⁺-dependent expression on the plasma membrane, and in replenishment of transmitter synthesis and/or vesicle sequestration for release, has previously been reported in cholinergic and GABAergic neurons [197–199]. When expressed in artificial liposomes VGLUTs transport Pi in a Na⁺-coupled manner with glutamate competing for binding, but at lower affinity [173, 200]. Pi transport did not require Cl⁻ and was not inhibited by Evans blue, a competitive inhibitor of VGLUTs [173]. Pi transport into vesicles via a transmembrane ΔμH⁺ has also been reported [200], but also with lower affinity than glutamate. This Pi may also be transported out of the vesicle in a Na⁺-coupled manner, which may be involved in Pi homeostasis within glutamatergic neurons (Fig. 4) [200]. That VGLUTs possess intrinsic transport machineries that are independent of each other: Na⁺-dependent Pi flux and H⁺-dependent vesicular glutamate uptake, would suggest dual mechanisms to support glutamatergic neurotransmission, especially under high activity. However, other transporters have been proposed as the major Pi transporters in the brain [201]. The identification of activity-regulated transport of Pi across the synaptic membrane following exocytosis and glutamate release could reveal a specialized role for VGLUTs in regulating Pi transport and synaptic glutamate synthesis.

Proton Site for VGLUT Regulation

Classic studies have concluded that cytosolic ATP activates the vacuolar-type ATPase in the membrane of intracellular storage organelles to transport H⁺ ions into the vesicle lumen creating a transmembrane electrochemical gradient (ΔμH⁺) (Fig. 4) [202, 203]. Compounds that dissipate the transmembrane ΔμH⁺, such as FCCP (H⁺ ionophore), were originally used to show H⁺-dependent glutamate transport by synaptic vesicles [185, 186] to differentiate this activity from Na⁺-coupled glutamate transport across the plasma membrane [204]. The electrical component (Δψ) of the total ΔμH⁺ generated by the V-ATPase is the primary driving force for the transport of glutamic acid and subsequent acidification of excitatory vesicles by glutamate itself [188]. Indeed, selective inhibition of the Δψ component of the transmembrane ΔμH⁺ gradient (thus, favoring Δφ) by A23187 (a divalent cation ionophore) [205], nigericin (an electroneutral cation that exchanges H⁺ against K⁺) [206], or ammonium ion (NH₄⁺) [205, 207] increases vesicular glutamate uptake at low (4 mM) cytoplasmic chloride concentrations. Inhibition of the V-ATPase with bafilomycin A1 reduces the amplitude of glutamatergic miniature excitatory postsynaptic currents (mEPSCs) in vitro, indicating that decreasing the amount of glutamate in synaptic vesicles reduces quantal size [208]. However, direct inhibition of the V-ATPase or the dissipation of the transmembrane Δψ would inhibit transport of all neurotransmitters into synaptic vesicles. While blocking the transmembrane Δψ component may be selective to VGLUT stimulation in synaptic vesicles, this too would abolish transport by vesicular amine transporters that rely on protons for H⁺ antiport (e.g., VMAT1 and 2, VACHT).

Whether VGLUTs operate as glutamate/H⁺ exchangers [192, 209], glutamate/C¹⁺ exchangers [205, 209], or as electrogenic uniporters [210] remains controversial [174, 177, 211]. However, the luminal H⁺ contribution to Δψ activates VGLUTs to drive the filling of synaptic vesicles with glutamate. For an electrogenic transport mechanism, the presence of a chloride-H⁺ exchanger (e.g., CIC3) [212] and a cation-H⁺ exchanger (e.g., NHE) [213] in the synaptic vesicle membrane could also contribute to charge and H⁺ balance. For example, K⁺/H⁺ exchange could play a role in sustaining glutamate uptake as it would maintain Δψ and decrease Δψ [213].
Recent evidence using reconstituted systems indicate that VGLUTs have cation (K⁺) and H⁺ binding sites on the cytoplasmic and vesicle lumen sides, respectively [177, 211]. The localization of the H⁺ site on the luminal side of VGLUT required for VGLUT activation is not known. Whether this proton binding site allows for H⁺ antiport that is coupled to electrogentic glutamate transport into vesicles as suggested [192] is not entirely clear [177, 211]. Nevertheless, VGLUTs themselves can exchange K⁺ for H⁺ (Fig. 4) and this may only loosely couple to glutamate transport [177], this property could be influenced by physiologic conditions in the brain to modulate vesicular glutamate storage.

Following exocytosis of synaptic vesicles VGLUTs are positioned in the plasma membrane such that glutamate could leak out of the cytoplasm into the synaptic cleft. It is not clear how much or how long VGLUTs could reside on plasma membranes following exocytosis in normal or disease conditions before they are endocytosed [48], although kiss-and-run exocytosis is obviously short-lived [214]. But, under conditions of massive depolarization (high extracellular K⁺) or energy failure such as that observed following transient cerebral ischemia and traumatic brain injury, the extent and duration of VGLUT expression on the plasma membrane may increase. Extracellular H⁺ (ΔpH) can increase the release of glutamate from cytoplasm through the plasma membrane via VGLUTs [211, 215] and this could occur with ischemic/seizure insults in vivo [172, 216–220]. Whether this H⁺ binding site on the extracellular facing VGLUTs is the same site as the internal H⁺ binding site in the vesicle lumen exposed following activation of the V-ATPase (and controlling glutamate transport into vesicles) is not presently known. However, such a H⁺ binding site could restrict glutamate flux to synaptic vesicles under normal conditions [211]. Drugs that selectively interfere with H⁺ binding to this site may therefore have importance to inhibit the leakage of glutamate from synaptic terminals under conditions of acidic extracellular pH such as following ischemia or traumatic brain injury [172, 219, 220] where extracellular glutamate levels dramatically increase [216–218]. Drug binding to this H⁺ site on VGLUTs before engulfing by endocytosis and reformation of vesicles may also serve to limit H⁺ activation of VGLUTs in the vesicle lumen and thus affect vesicular glutamate sequestration and release.

**Chloride Site for VGLUT Regulation**

Early work had established a biphasic dependence of glutamate transport on extravesicular Cl⁻ ion concentrations with low levels (4 mM) greatly stimulating and higher (> 20 mM) levels inhibiting transport [187, 188]. In the absence of a permeable anion, the V-ATPase generates only a transmembrane electrical gradient (i.e., Δψ). However, in the presence of Cl⁻ (> 20 mM), a measurable ΔpH is generated by the luminal accumulation of H⁺ and Cl⁻ (i.e., HCl), which limits vesicular glutamate transport [188, 206]. Jahn and colleagues also described the presence of a Cl⁻ binding site on the cytoplasmic side of the transporter, distinct from the substrate binding site, and that low Cl⁻ concentrations (e.g., 4 mM) stimulate transport activity directly through this site [221]. Wolosker’s work confirmed that Cl⁻ affects glutamate transport by two different mechanisms: one is related to a change of the proportions between the transmembrane Δψ and ΔpH components of the total ΔμH⁺: and the other involves a direct stimulatory interaction of Cl⁻ with the glutamate transporter [205].

The molecular mechanism by which chloride ions could differentially affect transvesicular Δψ and · pH gradients was thought to occur by Cl⁻ influx (at higher > 20 mM cytoplasmic levels) through exogenous Cl⁻ channels (e.g., CIC-3) that are present on synaptic vesicles [212]. However, upon
molecular identification of VGLUT1, Edwards and colleagues demonstrated that the transporter itself displays a $\text{Cl}^-$ conductance (Fig. 4) and that glutamate and $\text{Cl}^-$ ion could compete for the same transport site [34]. Thus, the lumen of glutamatergic vesicles will acidify by both glutamate and $\text{Cl}^-$ flux [188, 205, 206]. Indeed, glutamate or $\text{Cl}^-$ transported into the vesicle may further stimulate the V-ATPase to maintain a positive $\Delta\psi$ [222]. Glutamate-induced acidification of vesicles also enables the $\Delta\phi$-dependent uptake of monoamines, acetylcholine (ACh) or GABA by $H^+$ antiport in neurons that co-express co-express VGLUT3 and VMAT2, VACHT or VGAT/ VIAAT (see [223] for review).

To clarify the role of $\text{Cl}^-$ in VGLUT regulation, simplified in vitro transport flux studies were developed with proteoliposomes containing purified recombinant VGLUT and the $H^+$-ATPase of Bacillus stearothermophilus (TF0F1 = F-ATPase) [173]. Using this system, contributions of any endogenous vesicular components that would affect transmembrane $\Delta\phi$ and $\Delta\phi$ gradients and various ion fluxes (e.g., $K^+$, $\text{Cl}^-$, $Pi$) due to other proteins are eliminated. These studies clearly show that VGLUT-mediated glutamate influx into vesicles exhibits a biphasic dependence on $\text{Cl}^-$ ion [177, 209]. Thus, VGLUTs (and not the chloride channel CIC-3 [212]) may represent the major $\text{Cl}^-$ permeation pathway in glutamatergic synaptic vesicles. Importantly, high luminal $\text{Cl}^-$ concentrations markedly enhance loading of glutamate by $\Delta\phi$-driven uptake [209]. This is consistent with the idea that upon glutamate release into the synaptic cleft, vesicles are regenerated by endocytosis where they may contain high levels of Na$^+$ and $\text{Cl}^-$ ions (~130 mM) [190]. Using live-cell imaging with pH- and $\text{Cl}^-$-sensitive fluorescent probes in cultured hippocampal neurons or wild-type and VGLUT1-deficient mice, Martineau and colleagues confirmed that export of $\text{Cl}^-$ ion from the vesicle lumen into the cytoplasm can drive vesicular glutamate transport [192]. It is not clear what happens to luminal Na$^+$ following endocytosis, but it is possible that an increase in $\Delta\phi$ following $\text{Cl}^-$ efflux could also be a result of residual Na$^+$ ions left in the vesicle lumen, or maybe they just leak out of the vesicle into the cytoplasm (Fig. 4).

A novel approach to study VGLUT without the activity of an endogenous $H^+$ pump, and its link to $\Delta\phi$ and various ion gradients associated with it, is by the electrophysiological analysis of VGLUT-associated currents. Recently, Edwards and colleagues used patch clamp recording from enlarged endosomes of VGLUT-transfected cells and confirmed once again that $\text{Cl}^-$ interacts with VGLUTs as both a permeant ion and allosteric activator [174]. Remarkably, in addition to the allosteric activation of VGLUTs by low cytosolic $\text{Cl}^-$ as described above, endosome recording revealed allosteric regulation of glutamate transport by luminal $\text{Cl}^-$ as well [174]. Interestingly, neutralization of the highly conserved arginine residue in TM4 is sufficient to confer the activation normally provided by $\text{Cl}^-$ [191]. This basic residue is predicted to face the vesicle lumen and that in the absence of luminal $\text{Cl}^-$, it prevents both vesicular glutamate transport and the associated $\text{Cl}^-$ conductance [174]. Thus, in the presence of luminal $\text{Cl}^-$, this intravesicular $\text{Cl}^-$ binding site activates glutamate transport into vesicles and $\text{Cl}^-$ efflux from vesicles into the cytoplasm (Fig. 4). The $\text{Cl}^-$ trapped in recycling vesicles by endocytosis (or transported into the vesicle from the cytoplasm) by VGLUTs, could therefore confer additional allosteric activation required for glutamate transport into vesicles. As glutamate entry into the vesicle dissipates $\Delta\psi$ the resulting drop in luminal $pH$ would activate $\text{Cl}^-$ efflux that would support the maintenance of $\Delta\phi$, and so maintain the driving force for glutamate uptake. These results support the idea that $\text{Cl}^-$ permeates through a low affinity conduction pathway of a channel, and glutamate through the alternating access mechanisms of a transporter [174, 224], similar to the $Na^+$-coupled excitatory amino acid transporters (EAATs) [225, 226].

Understanding the physiological role of the $\text{Cl}^-$ allosteric binding sites and the $\text{Cl}^-$ conductance’s among all VGLUT isoforms and the structural and functional sites involved will require more specific tools to inhibit $\text{Cl}^-$ binding/permeation by the VGLUTs. Inhibitors, such as DIDS (EC$_{50}$ = 0.7 $\mu$M) and SITS (EC$_{50}$ = 0.2 $\mu$M) inhibit glutamate uptake by competing with their $\text{Cl}^-$ binding site [227]. DIDS also potently inhibits $\text{Cl}^-$ dependent vesicular ATP uptake with an IC$_{50}$ of 1.5 $\mu$M [155]. On the other hand, DIDS and SITS are also potent $\text{Cl}^-$-HCO$_3$-exchange blockers [228] and also block monoamine transport into synaptic vesicles [229] (Scheme 1).

Ketone bodies (acetoacetate and 3-hydroxybutyrate) and $\alpha$-keto acids have been proposed to compete with $\text{Cl}^-$ for VGLUT activation at a cytoplasmic-exposed site and therefore inhibit glutamate uptake into vesicles and release from synapses [227, 230, 231]. Metabolic derivatives such as acetoacetate, pyruvate, phenylpyruvate, kynurenate, $\alpha$-keto-$\beta$-methyl valerate, and $\alpha$-keto-isovalerate ketones bind to this $\text{Cl}^-$ site to modulate the activity of VGLUTs by negative cooperation. One explanation for the beneficial effect of ketogenic diets for the treatment of young patients with epilepsy then could be that these compounds modulate glutamatergic neurotransmission by reducing vesicular glutamate accumulation and quantal size by an allosteric mechanism [228289]. Whether VGLUTs can affect metabolism (or at least metabolic pools of glutamate in synapses) in addition to their synaptic function is not known. Further exploration of endogenous and dietary compounds that could modulate $\text{Cl}^-$ binding in VGLUTs and whether these compounds interfere with $\text{Cl}^-$ flux properties, as well, could lead to the discovery of novel pharmacological tools to treat neurological disorders caused by excessive synaptic release of glutamate.

$\text{Cl}^-$ fluxes across the plasma membranes of glutamatergic synapses can be mediated by axo-axonal GABAergic synapses [232], neuron-specific cation-$\text{Cl}^-$ co-transporters [233], or other modulators [234]. Thus, modulation of cytoplasmic $\text{Cl}^-$
concentrations could be important effectors of the cytoplasmic Cl\(^-\) binding and permeation sites of VGLUTs, in some way. However, varying intracellular levels of Cl\(^-\) in the ‘giant’ synapses from the calyx of Held in the auditory brainstem has little bearing on quantal size of glutamate released [235]. However, more recent studies have shown that presynaptic Cl\(^-\) levels play a biphasic regulatory role in the process of glutamate refilling into those vesicles via VGLUTs [236], similar to that observed with in vitro transport experiments. Excitability of hippocampal neurons could be greatly affected by intracellular Cl\(^-\) levels, especially under potential excitotoxic conditions [237, 238]. Since extracellular Cl\(^-\) levels (or various drugs) that are present in the extrasynaptic space are endocytosed into newly formed vesicles they could also potentially modulate intravesicular Cl\(^-\), glutamate, and H\(^+\) binding sites and fluxes across vesicles and thus, intracellular storage of vesicular glutamate available for synaptic release.

**Glutamate Site for VGLUT Regulation**

Under normal conditions, cytoplasmic glutamate levels for vesicular loading are likely mostly derived from glucose metabolism, pyruvate carboxylation and tricarboxylic acid (TCA) intermediates such as α-ketoglutarate [239–242]. Neuronal glutamate reuptake after release may also occur to some extent in forebrain regions [243–245]. Whole-cell recording of the presynaptic terminals in the calyx of Held reveal that the content of a single vesicle is insufficient to saturate AMPA receptors such that the release of several vesicles is required [246–249]. Indeed, direct injection of glutamate into this synapse increases quantal size and thereby postsynaptic receptor saturation by single vesicle release of glutamate [246, 249]. Single vesicle release also may not saturate post-synaptic glutamate receptors at hippocampal synapses either [250] and since vesicle release probability is very low in hippocampal synapses [126, 129] single vesicle release and nonsaturation of post-synaptic glutamate receptors likely predominates there too [251, 252].

Regulation of vesicle filling levels can be mediated by the cytoplasmic glutamate concentration that is available to the glutamate site on VGLUTs for uptake into synaptic vesicles (Fig. 4). The cytoplasmic concentration of glutamate in glutamatergic synaptic terminals is not precisely known. Semiquantitative electron microscopic analysis of the distribution of glutamate-like immunoreactivity in excitatory hippocampal pathways have estimated the glutamate concentration in synapses to range from 1 to 10 mM [253–257]. Transporter flux experiments using brain synaptic vesicles in vitro indicate that maximal vesicle filling in vitro saturates at ~ 4 mM external glutamate concentrations [117], which is similar to cholinergic synaptic vesicles that also exhibit low affinity for acetylcholine [162, 258]. Thus, if vesicular glutamate storage capacity is not maximal under normal conditions [246, 247, 249, 250] then endogenous cytoplasmic glutamate concentrations may lie somewhere below 4 mM; likely around the Km of VGLUTs for glutamate (1–2 mM) to enable activity-stimulated regulation. Sub-saturating levels of glutamate at the glutamate binding site on VGLUTs in the cytoplasm under normal conditions suggests that regulation of vesicle filling by modulating biosynthesis or degradation of glutamate in the terminal would affect vesicular glutamate filling. That the vesicle fill level is dependent on the cytoplasmic neurotransmitter levels available for filling in synaptic terminals may be a common feature of vesicular transmitter storage, in general, as the extrasynaptic level of all other transmitters such as acetylcholine [162, 259], GABA [142], and the monoamines [260] in synaptic vesicles can also be scaled pharmacologically by interfering with the metabolism or by providing precursors for transmitter synthesis.
Rapid replenishment of neurotransmitter glutamate synthesis under conditions of high neural activity in various neurological conditions (e.g., epileptic seizures, traumatic brain injury, cerebral ischemia, and others) would be required to maintain synaptic cytoplasmic glutamate levels at levels optimal for continued release [261]. Original suggested that glutamine transport into synapses (derived from surrounding astroglial cells [262]) is a preferred precursor for transmitter glutamate released following K⁺-depolarization (56 mM KCl) of slices of the molecular layer of the dentate gyrus [263–265]. Thus, the availability of glutamine to nerve terminals could affect synaptic glutamate synthesis and vesicular glutamate storage for release [266–269], especially under conditions of excessive synaptic glutamate release. However, Kam and Nicoll reported that excitatory transmission can persist for hours without glutamine in young neuronal cultures and slices [270]. Yet, synaptic transmission is impaired under intense stimulation by preventing the conversion of glutamine to glutamate in phosphate-activated glutaminase (PAG) knockout mice [271]. Indeed, the enzymatic activity of PAG has been detected in neurons and in nerve endings, suggesting local glutamate biosynthesis in axon terminals [272–274]. Whether Na⁺-dependent glutamine import into neuronal synapses from astroglial synapses to replenish synaptic cytoplasmic glutamate stores under high synaptic activity is a potential novel target to prevent excessive glutamate release under conditions of excitotoxicity is currently not known. While glutamine synthetase (GS) is a critical component of the glutamate/glutamine cycle, GS-KO mice develop epilepsy [275], likely because GS must inactivate synaptically released glutamate that is taken up in astrocytes, and cleared from the synaptic cleft, by the EAATs [276, 277] as well. However, activity-induced modulation of synaptic glutamate efficacy [278] and glutamate epileptiform activity [279–281] are significantly reduced by acute inhibition of glutamine synthesis in astrocytes with methionine sulfoximine or by application of 2-(methylamino-isobutyrate) (MeAIB), a competitive and reversible inhibitor of the neuronal Na⁺-coupled glutamine transporters (SNAT; system A) subtypes 1 and 2 [282]. However, SNAT1 and SNAT2 are confined to cell soma and proximal dendritic regions of neurons [283] and are excluded from axon terminals supporting the notion that an unidentified synaptic glutamine/MeAIB transporter could support activity-stimulated/excitotoxic glutamate release from synapses [284].

**VGLUT Pharmacologic Sites**

Despite their importance, the pharmacology of VGLUTs remains remarkably underdeveloped. The evaluation of VGLUT inhibitors has generally been performed using in vitro transport assays measuring the flux of radiolabeled glutamate across the membranes of whole brain synaptic vesicles and more recently using liposomes with engineered VGLUTs and transmembrane ΔμH⁺ gradients. Only a few VGLUT inhibitors have been shown to affect glutamatergic transmission in electrophysiologic recordings of rodent hippocampus and cerebral cortex [285, 286].

**Competitive Inhibitors**

**Substrate Analogs**

Among competitive modulators of glutamate uptake into synaptic vesicles, cyclic and noncyclic glutamate analogues, such as (2S, 4R)-4-methyl-glutamate (IC₅₀ = 0.7 mM), L-trans-ACP (IC₅₀ = 0.8 mM), and DL-trans-ACHD (IC₅₀ = 0.23 mM), are the most potent [287–289]. These compounds also modulate the activity of mGluR [290] (Scheme 2).
Quinolines and Quinoxaline Analogs

The capacity of quinoline and quinoxaline to inhibit glutamate uptake was reported 20 years ago [291]. Compounds such as kynurenate, xanthurenate, 7-chloro-kynurenate, and 2-quinoxaline carboxylate are competitive modulators of glutamate transport. However, these compounds also act at the postsynaptic level on ionotropic receptors [187, 287, 291, 292] (Scheme 3).

Kynurenate is a neuroactive endogenous compound (antiexcitotoxic and anticonvulsant) derived from tryptophan metabolism. Carrigan and coworkers developed a

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Scheme 4: Structures of QDCs analogs as VGLUTs inhibitors (Carrigan et al., 2002)

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Scheme 5: Azo dyes (Favre-Besse et al., 2014; Omote et al., 2016; Tamura et al., 2013)
series of ligands from this motif [293] and SAR studies highlight conserved and crucial chemical functions to ensure potent inhibitory activity. This includes the importance of negative charges of the QDC motif, as well as the size of the hydrophobic aryl unit. Substitution of the phenyl moiety by biphenyl induces an affinity gain of one log [293–295]. The best modulators are 6-biphenyl-QDC and 6-(4-phenylstyril)-QDC (affinities of 41 μM and 64 μM, respectively) (Scheme 4).

Azoic Dyes

Azoic dyes are formed by a polar region connected to a hydrophobic body via an azo bond. These molecules have at least one negative charge provided by a sulfonate group. The following has been surmised of these molecules: (1) the negative charge is important for ligand activity and plays the same role as the COO proximal glutamate moiety; (2) the NH2 group, present on the polar part of some inhibitors, plays the same role as the amino group of glutamate. This NH2 does not seem to be essential for glutamate affinity [296]. Currently, azo dyes that inhibit VGLUTs with the best affinity are as follows: Bright Yellow (BY, IC50 = 15 nM); Chrysophenine (CP, IC50 = 27 nM), which corresponds to the methylated analog of Bright Yellow; trypan blue (TB, IC50 = 50 nM); Evans blue (EB, IC50 = 90 nM); and Violet Direct 51 (VD51, IC50 = 148 nM) [224, 286, 296, 297] (Scheme 5).

SAR studies have identified important pharmacophoric sites for the affinity of azo dyes (e.g., TB) [296, 297]. These studies highlighted the importance of the hydrophobic aryl motif. In particular, increased size (changing phenyl for a biphenyl, for example) or incorporation of halogen groups induced a significant increase in affinity to the VGLUT (Scheme 6).

An SAR study was also conducted using BY and its derivatives [296]. These investigations confirmed the importance of the negative charge of the sulfonate group, the importance of the hydrophobic aryl body, the azo bond, and the H bond acceptor moiety (this time, in para position to the azo link). The OH group in BY or the OMe group in Chrysophenine (Scheme 5) establishes a key hydrogen bond with Tyr195 of VGLUTs, which prevents the binding of glutamate to charged residues (Arg184, His128, and, in particular, Glu191) in the VGLUT hydrophilic pocket [296]. The distance between the nitrogen atom of the azo bond and the negatively charged oxygen atom of the sulfonate group (distance N-O) is also an important factor regarding the affinity of azo dyes. This distance is similar in Brilliant Yellow (d (NO-) = 9.324 Å), trypan blue (d (NO-) = 9.799 Å) and Violet Direct 51 (d (NO-) = 8.274 Å, from (NO) = 10.354 Å) [296]. The negative charges of the naphthyl-disulfonic polar motif also play an important role in the inhibition of VGLUTs [297] (Scheme 7).

Evans blue was one of the first compounds found to inhibit vesicular transport of glutamate [298]. Evans blue also potently inhibits the Cl− dependent vesicular transport of ATP with an IC50 of 40 nM [155]. Recent studies indicate that Evans blue reduces both Cl− and glutamate currents in enlarged endosomes with a similar IC50 (~0.2 μM) [174]. Evans blue therefore appears to bind to the glutamate site on VGLUTs but it is not yet known if it (or other similar compounds) require a H+ gradient across the membrane, similar to reserpine for VMATs [299]. It should be noted that azo dyes do not remain...
Scheme 7  Pharmacophore of azo dyes and various potent inhibitors superposed to pharmacophore model of VGLUTs. The pharmacophore model allows the 3D representation of key features for potent VGLUT inhibitory activity. This model was generated based on the activity of various ligands (trypan blue (EC50 = 50 nM), Evans blue (EC50 = 90 nM), Brilliant Yellow (EC50 = 15 nM), Chicago Blue Sky (EC50 = 330 nM), Direct violet 51 (EC50 = 148 nM), Chrysophenin (EC50 = 27 nM), four 2,4-dicarboxy-quinolines (EC50 = 41 μM; 64 μM; 167 μM; 288 μM), four monoaзоic dyes (EC50 = 1.6 μM; 3.3 μM; 25 μM; 40 μM)). Structural key requirements to obtain a potent VGLUT inhibitor are represented by color spheres (green = H bond donor, slight blue = aromatic moiety, dark blue = negative charge); the putative ligand/VGLUT steric bump are represented by exclusion gray spheres.

Scheme 8  Fluorescein analog inhibitor of VGLUTs
VGLUT-specific at high concentrations [298] as high doses of Evans blue (>20 μM) inhibit GABAergic vesicular transport [298, 300] and lysosomal ATP transport [301], but not vesicular transport of dopamine [298]. Evans blue is used as a means to assess the permeability of the blood-brain barrier (BBB) [302] and therefore cannot access VGLUTs in vivo. Comparable nontransported inhibitors of VGLUTs that can cross the BBB are not available.

Noncompetitive Inhibitors

Noncompetitive modulators of VGLUTs include polyhalogenated fluorescein analogs, among which Rose Bengal has the highest affinity (IC\(_{50}\) = 25 nM) [303–305] (Scheme ). SAR studies of Rose Bengal and its derivatives facilitated identification of the bioactive form of Rose Bengal derivatives (acid form of the molecule, see Scheme 8) [303]. The lactone form of Rose Bengal exhibits reduced affinity. The halogen substituents on xanthene and phenyl are particularly important in generating high affinity for VGLUTs (25 nM). However, the binding site for these types of ligands has not yet been identified. Some studies have suggested allosteric modulation of Rose Bengal on VGLUT [304], while others think indirect modulation occurs by blocking the V-ATPase [303]; although Rose Bengal strongly and specifically affects glutamatergic but not GABAergic boutons [192]. A recent study showed that Rose Bengal is a specific inhibitor of glutamate transport but not the VGLUT-associated Cl\(^{-}\) conductance [192], so it could be an allosteric modulator of VGLUTs, like tetrabenazine for VMATs or vesamicol for VAChT. On the other hand, Evans blue inhibits both Cl\(^{-}\) and glutamate currents with submicromolar potency [174]. A 50% inhibition of maximal uptake levels by submaximal concentrations of the competitive inhibitor trypan blue (like Evans blue), is eventually overwhelmed by glutamate competition (i.e., 4 mM) where vesicular levels reach control values [117]. On the other hand, a 50% knockdown of functional transporters by the noncompetitive inhibitor Rose Bengal produces a steady-state accumulation of glutamate that is approximately 50% less than control values at all external glutamate concentrations [117], yet maximal accumulation still occurs at the same external (4 mM) value. These data support evidence that the level of functional VGLUTs in vesicles is a critical determinant in vesicle filling.

Conclusions

Multiple isoforms of VGLUT in mammals provide diversity in vesicular glutamate storage and release in discrete synapses as the three subtypes are differentially expressed in glutamatergic neurons in the brain and are likely subject to differential regulation at the level of biosynthesis and degradation. Indeed, the level of expression of VGLUTs in synapses could be a critical determinant that regulates both quantal size and the availability of vesicles for glutamate release. Changes in VGLUT expression levels in synapses are seen in a variety of neurological disorders and disease states. Adaptive changes in VGLUT expression levels may even be used as a neuroprotective strategy to support neural circuit stability to avoid excessive glutamate release from synapses and excitotoxicity. While we are not yet able to pharmacologically modify VGLUT biosynthetic pathways, molecular approaches to modify VGLUT expression in synapses using viral-mediated strategies are clearly a major avenue being explored to selectively target distinct glutamatergic systems that may affect aberrant synapses in neurologic disorders and disease.

The three subtypes of VGLUTs are so closely structurally related that none of the currently available inhibitors may be able to discriminate between them functionally. An inability to selectively interfere with vesicular glutamate storage and release in distinct glutamatergic neurons would render these compounds ineffective in any clinical setting. However, the inhibitory effects of these compounds have largely been performed with brain synaptic vesicle preparations that contain a mixed population of synaptic vesicles or in cortical/hippocampal neurons where VGLUT1 predominates. The evaluation of current inhibitors, and new pharmacological compounds, in recombinant VGLUT systems are therefore essential to determine if any are specific for VGLUT isoforms and could help distinguish between the sites in VGLUTs that are important for glutamate transport to better understand the mechanisms involved.

Biochemical transport flux experiments in vitro using radiolabeled tracers has long been instrumental in our understanding of vesicular neurotransmitter transport in brain synaptic vesicles for all of the classic neurotransmitters. Recent studies have shown important contributions are being made following the incorporation of recombinantly expressed transporters into artificial vesicles (liposomes) equipped with an H\(^{+}\)-ATPase and individual VGLUTs without any interfering synaptic vesicle components. Indeed, these recent flux studies have provided important information to the existence of critical binding/flux sites for H\(^{+}\), monovalent cations, and Cl\(^{-}\) on both sides of VGLUTs. Such assays could therefore also be used to screen established and novel VGLUT inhibitors to determine whether any of them are isoform-specific, or not. Surprisingly, the ionic binding/flux properties of VGLUTs that have recently been described may help generate transmembrane vesicular H\(^{+}\) gradients (i.e., \(\Delta\psi\)) under physiological conditions to facilitate glutamate transport into vesicles and maintain vesicular glutamate storage levels. In this regard, a unique feature of vesicle exocytosis is that endocytosed vesicles contain the content of the extracellular milieu (esp., Cl\(^{-}\)) that can increase \(\Delta\psi\) within vesicles (positive inside) during
In vivo studies.

The unique ionic features of VGLUTs make them susceptible to regulation by physiologic factors such as changes in extracellular Cl\(^{-}\), K\(^{+}\), or H\(^{+}\) that occur under seizure, ischemic, and other acute and chronic excitotoxic conditions. Exposure of internal regulatory sites on VGLUTs to the extrasympathetic space following exocytosis allows for a potential means to introduce drugs to the vesicle interior, to modulate glutamate filling for synaptic release.

Glutamate-induced excitotoxicity is an important concern in a variety of detrimental brain injuries and disease. A powerful means to control vesicular glutamate filling and synaptic glutamate release is by regulating the availability of glutamate to the glutamate site on VGLUTs. However, the glutamate sites in the three VGLUT isoforms are likely highly conserved. Since, neurons expressing the different VGLUT isoforms display inherent differences in activity (esp., VGLUT1 and VGLUT2), it is likely that they also express differential requirements to supply and replenish cytoplasmic transmitter glutamate levels in synapses to sustain glutamatergic neurotransmission in an activity-dependent manner. It is not clear if differences in metabolic precursors (e.g., glucose, \(\alpha\)-ketoglutarate, lactate, alanine, glutamine, etc.) and other potential pathways used for glutamate synthesis and neurotransmission exist in the different VGLUT-operated synapses. However, activity-dependent regulation of synaptic glutamate synthesis or release from cerebrocortical slice cultures. Astron Res 50:179-187

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