Preface

The synapse is a fascinating structure for many reasons. Biologically, it is an exquisitely organized subcellular compartment that has a remarkable capacity for fidelity and endurance. Computationally, synapses play a central role in signal transmission and processing that represent evolution's solution to learning and memory. Nervous systems, including our own brains, possess an extraordinary capacity for adaptation and memory because the synapse, not the neuron, constitutes the basic unit for information storage.

Because the molecular complexities underlying signal processing and information storage must occur within the tiny space of the synapse, the precise molecular organization of proteins, lipids, and membranes at the synapse is paramount. Given the central role of the synapse in neuronal communication, it comes as no surprise that dysregulation of the synapse accounts for many, if not most, neurological and psychiatric disorders. Clinically, the synapse thus constitutes a prime target for treatments of these diseases.

It is for these reasons that we have chosen to focus our work on deciphering the structural and functional organization of the synapse. We have assembled leaders in the field of synapse biology to describe and distill the wonders and mysteries of the synapse. This book provides a fundamental description of the synapse developed over many decades by numerous investigators, paired with recent insight into new aspects of synapse structure and function that is still in flux and at the cutting edge of research.

This book grew out of a symposium and a research seminar at the University of Iowa that were sponsored, in large part, by the generous support of the Obermann Center for Advanced Studies. Obermann Seminars are specifically designed to gather international scholars and produce interdisciplinary research publications.

We are grateful for the exceptional efforts of our contributing authors, without whom this book would not have been possible. Their willingness to take time from their busy research schedules to share their insight and ideas with the breadth and depth that allow us to compile a collective work that is illuminating and useful, for both the general biologist and specialized neuroscientist, is very much appreciated. We express our gratitude to our assistants Ms. Susan Harward and Ms. Sue Birely for their professionalism and help with the book layout and proof reading. Lastly, we would like to thank our families (Laura and Henrik, Mary, Solon, Anselm, and Hans), who provided the support, encouragement, inspiration and comic relief, that in many ways helped to make this book possible.

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The Role of Glutamate Transporters in Synaptic Transmission

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1 Introduction

Exocytotic release of glutamate from preformed synaptic vesicles confers great speed on synaptic signaling, but also requires mechanisms to maintain the pool of available synaptic vesicles and remove glutamate from the extracellular space. Since the axon terminal is in general located at a great distance from the cell body, synapses rely on the local recycling of both synaptic vesicle membrane and their neurotransmitter contents. In this review, we address recycling of the excitatory transmitter glutamate, focusing on transport activities that operate at the plasma membrane and the synaptic vesicle, and the role that these transporters play in determining the amplitude and time course of synaptic responses.

2 Glutamate Transport into Synaptic Vesicles and the Presynaptic Regulation of Quantal Size

Although analysis of the neuromuscular junction originally suggested that the response to release of a single vesicle filled with neurotransmitter is fixed, and hence represents the elemental "quantum" of synaptic transmission (109), considerable work has now shown that quantal size can change as a function of activity, contributing to such forms of plasticity as long-term potentiation (124). Nonetheless, the locus for this regulation is postsynaptic, and involves changes in receptor number or sensitivity. More recently, it has become clear that changes in quantal size can also reflect presynaptic changes in vesicle filling.

It has long been appreciated that changes in the amount of neuromodulator released per vesicle can have profound consequences for the activation of receptors at a distance from the release site. Many G protein-coupled receptors have a relatively high affinity for their peptide and monoamine ligands, but the small

amounts of ligand that actually impinge on receptors are not likely to saturate binding. As a result, the release of more modulator activates more receptors, and considerable attention has focused on the regulation of quantal size for monoamines, taking advantage of electrochemical detection to measure dopamine release directly and in real time (181).

It has been less clear whether changes in vesicle filling with classical transmitters such as acetylcholine, GABA and glutamate make a difference in the postsynaptic response. These transmitters are generally released in close apposition to postsynaptic receptors, many of which are ionotropic and have a high affinity for ligand (such as NMDA receptors for glutamate). If receptors are normally saturated by the contents of a single vesicle, packaging more transmitter will have no effect on the postsynaptic response.

If receptors are saturated, this will tend to reduce the variation in postsynaptic response. However, quantal size exhibits considerable variation, particularly at central synapses. Although this might result from variation in the distance of different synapses from the recording electrode, due to differences in electrotonic filtering, as well as variation in release probability and the number of receptors at each synapse, a number of observations have demonstrated that variation in quantal size is intrinsic to a single synapse. Focal stimulation of one bouton, or localized dendritic recording, both show variation in quantal size similar to that observed from electrical stimulation of release from multiple boutons (17, 63, 120, 121). Increased cytosolic glutamate in the presynaptic terminal also increases quantal size at the calyx of Held in the auditory pathway (99), providing additional evidence against receptor saturation. Remarkably, a single vesicle filled with glutamate fails to saturate low-affinity AMPA receptors as well as high-affinity NMDA receptors (123, 132). Consistent with this, AMPA and NMDA responses are highly correlated at individual synapses, supporting a presynaptic locus for the variation. GABA receptors at many (but not all) inhibitory synapses also appear not to be saturated by a single vesicle (14, 67, 79).

How can synaptic release fail to saturate receptors? Although the concentration of transmitter achieved in the synaptic cleft is high, the receptors are closely apposed to the release site, and many are of high affinity, the peak concentration of transmitter is very brief, so that only a few receptors become activated. Regardless of the precise explanation, changes in the amount of transmitter per vesicle are thus predicted to have a major influence on the postsynaptic response.

The amount of neurotransmitter released from a synaptic vesicle may be controlled either before or after the fusion event. *After* fusion, premature closure of the pore may interrupt the full release of vesicle contents. Indeed, the exocytosis of large dense core vesicles frequently exhibits "kiss-and-run", but this mechanism remains controversial for small synaptic vesicles, and the topic has recently been reviewed elsewhere (60, 82). This review focuses on changes in quantal size *before* fusion with the plasma membrane, that involve direct changes in vesicle filling.

It is important to note that the mechanism of vesicular release poses several inherent problems. Large amounts of transmitter per vesicle will result in the activation of more receptors, but high rates of firing will also deplete transmitter from the terminal unless it is actively replaced by, for example, recycling or biosynthesis. At the same time, vesicular transport is generally slow, and may limit

refilling if vesicles recycle quickly, even at concentrations of cytosolic transmitter that saturate the transport mechanism. Subsaturating cytosolic concentrations will further slow refilling and release. However, low cytosolic concentrations may be important to prevent the oxidation and toxicity of monoamines such as dopamine (136), and this is compensated for by the ability of the vesicular monoamine transporter to generate an extremely large concentration gradient up to 10^5 higher in the lumen than the cytoplasm. Other classical transmitters including glutamate produce toxicity through a specific interaction with cell surface receptors, and can therefore be tolerated at higher levels in the cytoplasm. This reduces the magnitude of the concentration gradient required to fill vesicles with glutamate, and presumably also speeds filling. We will therefore consider now the factors that influence vesicle filling with glutamate, from its cytosolic concentration to the H^+ electrochemical gradient that drives transport, glutamate transport itself, and finally, the physiological regulation of these mechanisms and their role in synaptic plasticity. The amount of transmitter achieved inside secretory vesicles indeed reflects the cytosolic concentration of transmitter, the driving force, transport into and non-specific leakage across the vesicle membrane.

2.1 Cytosolic Glutamate: Biosynthesis and Recycling

Like any enzymatic reaction, the concentration of lumenal transmitter (product) depends directly on the cytosolic concentration (substrate). In the case of monoamines, electrochemistry has recently made it possible to measure cytosolic concentrations directly, by inserting a small carbon fiber electrode into a patch pipette (136). However, glutamate does not oxidize with the properties required for electrochemical detection, and less direct methods have therefore been used to estimate cytosolic concentrations. Immunocytochemistry using specific antibodies to glutamate followed by electron microscopy originally suggested low millimolar levels in the cytoplasm (173, 179). More recently, dialysis of the calyx of Held with 50 mM glutamate increased the postsynaptic response of immature animals toward that observed by more mature, suggesting that cytosolic glutamate almost certainly exceeds 1 mM and might even approach 50 mM (99, 222).

2.1.1 Glutaminase

The cytosolic concentration of transmitter in turn depends on both biosynthesis and recycling. In the case of glutamate, classical studies have shown that the preponderance of glutamate released as transmitter derives from glutamine (81). The neuronal enzyme glutaminase (also referred to as the phosphate-activated or kidney glutaminase, PAG) converts glutamine to glutamate before transport into synaptic vesicles (42, 114), and a liver isoform may also contribute (45, 113). Interestingly, both isoforms can be regulated by inorganic phosphate (45), which converts the inactive monomer to active tetramer. However, the loss of kidney glutaminase has shown remarkably little defect in excitatory transmission (129). The animals do not survive past birth, but the enzyme also has roles in nitrogen metabolism and pH regulation that may account for the observed lethality. Indeed, excitatory neurotransmission appears grossly normal. The knock-out animals exhibit only a

modest defect in the response to prolonged high frequency stimulation. It is possible that the liver enzyme compensates for loss of kidney PAG, but extracts from the knock-out mice show no residual glutaminase activity. On the other hand, the assay conditions may have precluded measurement of the liver isoform. Despite the early neurochemical and neuroanatomic studies, the physiological role of glutaminase in transmitter release thus remains unknown.

2.1.2 Glutamine-glutamate cycle

In contrast to the direct reuptake of monoamines, GABA, and glycine into the nerve terminals from which they were released, glutamate is cleared primarily by the Na^+-dependent excitatory amino acid transporters EAAT1 and 2 expressed by astrocytes (96, 164, 186, 191). After uptake by astrocytes, glutamate is converted into glutamine by glutamine synthetase (157, 158, 166, 215). The efflux of glutamine by amino acid system N transporters expressed in glia and the uptake of glutamine by closely related system A transporters expressed in neurons suggest a mechanism for glutamine transfer back to neurons, where glutaminase can produce glutamate (reviewed previously (35)).

Despite the circumstantial evidence, does the glutamine-glutamate cycle actually contribute to synaptic transmission? Partial blockade of system A with the high affinity, slowly transported substrate methyl-aminoisobutyric acid has been reported to decrease quantal size in autapses (6). In addition, disrupting glutamine synthesis and transport may reduce epileptic activity (11, 192). However, it has been very difficult to demonstrate a physiological role for glutamine uptake in glutamate release (105). On the other hand, the glutamine-glutamate cycle may have a more clear role at certain inhibitory synapses. Blocking glutamine production by astrocytes and the subsequent transfer to neurons decreases the size of both evoked and miniature inhibitory currents in the hippocampus, although the effect requires moderate synaptic activity (119). The glutamine-glutamate cycle may indeed be particularly important at inhibitory synapses that do not express a plasma membrane GABA transporter, and hence rely on other mechanisms for recycling.

2.2 H^+ Electrochemical Gradient

The release of neurotransmitter by exocytosis requires transport into secretory vesicles, a process that involves the exchange of lumenal H^+ for cytosolic transmitter. The packaging of all transmitters thus depends on a H^+ electrochemical gradient across the vesicle membrane which is generated by the vacuolar-type H^+-ATPase (Fig. 1). Indeed, the vacuolar H^+ pump acidifies endosomes, lysosomes and dense core vesicles as well as synaptic vesicles. (The yeast lysosome is a large membranous compartment called the vacuole). Why does the cell use H^+ rather than other ions such as Na^+, K^+ and Cl^- to drive transport into synaptic vesicles? Along with their small size, the rapid recycling of synaptic vesicles imposes a number of surprising constraints. Unlike the plasma membrane gradients of Na^+, K^+ and Cl^- that are large and stable, synaptic vesicles rely on a H^+ electrochemical gradient that is alternately dissipated and regenerated by exo- and endocytosis. The dependence on H^+ may serve to reduce the inappropriate activation of vesicle transporters on the cell

surface. In addition, a vesicle pH of 6 (only 1 µM) translates into less than one free H^+ per vesicle, although buffering by vesicle proteins requires the entry of more H^+ to reach this lumenal pH. In contrast to the high millimolar concentrations of other ions, fewer H^+ are thus required to generate a gradient of the same magnitude as Na^+, K^+ and Cl^- at the plasma membrane (roughly two log units). The relatively low concentrations of H^+ under physiological conditions appear to confer flexibility, efficiency and the potential for rapid regulation.
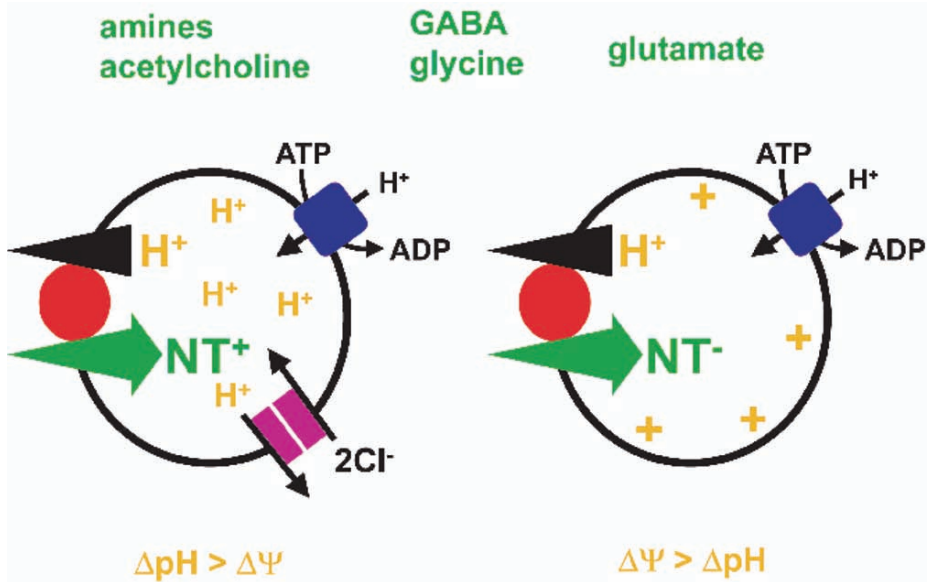

Fig. 1. Bioenergetics of vesicular neurotransmitter transport.
The vacuolar H^+-ATPase generates a H^+ electrochemical gradient that drives the uptake of neurotransmitter into secretory vesicles through a mechanism involving H^+ exchange. However, the transport of different transmitters depends to varying extents on the two components of the H^+ electrochemical gradient, the chemical gradient (ΔpH) and the membrane potential ($\Delta\Psi$). The transport of cationic transmitters such as monoamines and acetylcholinie depends primarily on ΔpH and the transport of anionic glutamate on $\Delta\Psi$, with zwitterionic GABA and glycine transport more equally dependent on both components. Nonetheless, all of the vesicular neurotransmitter transporters depend at least to some extent on both ΔpH and $\Delta\Psi$. The relative expression of ΔpH and $\Delta\Psi$ is in turn influenced by the entry of chloride through Cl^-/H^+ exchangers, which dissipates $\Delta\Psi$ and thus indirectly activates the H^+ pump to make a larger ΔpH.

2.2.1 H^+ Pump

The vacuolar H^+ pump that produces the principal ionic gradient across intracellular membranes resembles the F0/F1-ATPase from mitochondria and bacteria. The vacuolar pump and F0/F1-ATPase both move H^+ into a membranous compartment, but rather than using H^+ flux down its electrochemical gradient to produce ATP as in the case of the F0/F1-ATPase, the vacuolar pump generates a H^+ electrochemical

gradient that depends on ATP hydrolysis. In addition, both proteins comprise two domains, a peripheral V1 domain that catalyzes either ATP production or hydrolysis, and a membrane-embedded V0 domain that moves H^+. Each of these domains in turn contains multiple subunits, whose specific functions remain poorly understood, although structural studies are beginning to provide suggestions (56, 62). Indeed, the v-ATPase uses a rotary mechanism very similar to the F0/F1-ATPase, with the V1 domain rotating relative to V0 during H^+ movement (138). In yeast, the vacuolar H^+ pump undergoes regulation at the level of trafficking and intrinsic transport activity (110, 125). Starvation produces disassembly of the V1 subunit, presumably to conserve ATP (106). However, direct regulation of the H^+ pump in the synaptic vesicle membrane has not yet been shown.

Interestingly, work in yeast and *Drosophila* has suggested a role for the vacuolar H^+ pump and specifically the V0 domain in membrane fusion, independent of its role in vesicle acidification (92, 152). A mutation in the V0 a1 subunit impairs synaptic vesicle exocytosis rather than acidification. Although the mechanism remains unknown, previous work has suggested that another subunit of V0 mediates the non-vesicular release of transmitter (57), and the same subunit contributes to homotypic vacuole fusion in yeast (152).

2.2.2 Chloride flux: pH gradient versus membrane potential

In contrast to the lack of information about direct regulation of the vacuolar H^+ pump, other ions have important roles in the indirect activation of pump activity. Indeed, purified synaptic vesicles (as well as endosomes and lysosomes) acidify very little with the addition of only ATP, and require the subsequent addition of a permeant anion such as chloride for massive acidification (Fig. 1). Presumably, the entry of chloride dissipates the inside positive membrane potential ($\Delta\Psi$) and so reduces one of the gradients against which the pump works, allowing it to generate a larger pH gradient (ΔpH). Consistent with this, chloride increases the ATPase activity of the H^+ pump (62). Chloride flux thus provides a mechanism to regulate independently the electrical ($\Delta\Psi$) and chemical (ΔpH) components of the H^+ electrochemical gradient.

A subset of the ClC channels mediate the flux of chloride across intracellular membranes required for vesicle acidification (102). The ClC-3 isoform is highly expressed in brain, and synaptic vesicles from the ClC-3 knock-out show impaired acidification (178). However, the effect on transmitter release and in particular quantal size was difficult to assess, because the knock-out also shows severe, early degeneration in the hippocampus and retina, with the remaining synapses presumably expressing a different ClC isoform and hence showing no impairment.

Originally considered channels, the intracellular ClCs appear to function as Cl^-/H^+ exchangers (1, 153, 170) (Fig. 1). In light of the H^+ exchange mechanism, how can Cl^- entry promote vesicle acidification? The stoichiometry of ionic coupling apparently provides the answer: the exchange of 2 Cl^- for one H^+ results in the movement of substantially more charge (3+) than H^+ (1). This ionic coupling in turn predicts more dissipation of $\Delta\Psi$ than ΔpH, producing secondary activation of the H^+ pump that exceeds the direct exchange of one lumenal H^+ and hence results in

vesicle acidification. On the other hand, it is not clear why a Cl^-/H^+ exchanger would be selected for over a simple Cl^- channel.

2.2.3 Regulation of the H⁺ electrochemical gradient

Several factors including chloride flux have the potential to regulate the vesicle H^+ electrochemical gradient, but does it undergo physiological regulation? In parafollicular cells of the thyroid, activation of a cell surface calcium receptor promotes vesicle acidification, apparently by increasing the chloride conductance (13, 188). Protracted stimulation of adrenal chromaffin cells also promotes vesicle acidification, increasing the proportion of larger "active" vesicles with a halo around the dense core, and quantal size (155). Indeed, chromaffin cells have been shown to increase quantal size with strong stimulation, and although this has been attributed to changes in the fusion pore, changes in the H^+ electrochemical gradient may in fact be responsible (58). These observations indicate the potential for physiological regulation of H^+ electrochemical gradient, but it is important to note that the mechanisms involved remain poorly understood.

3 Vesicular Glutamate Transport

The transport of all classical transmitters into synaptic vesicles requires a H^+ electrochemical gradient generated by the vacuolar H^+ pump, but transmitters differ in their reliance on the two components of the gradient, ΔpH and $\Delta\Psi$. The vesicular transport of monoamines and acetylcholine depend primarily on ΔpH, while the transport of glutamate depends primarily on $\Delta\Psi$ (33, 103, 131) (Fig. 1). Vesicular GABA transport lies somewhere in between, with more equal reliance on the chemical and electrical components of the H^+ electrochemical gradient (89, 111). Consistent with these differences in ionic coupling, the vesicular neurotransmitter transporters fall into three molecularly distinct families. However, all of the activities rely at least in part on $\Delta\Psi$ and are hence electrogenic. We will now focus on the vesicular glutamate transporters, describing their functional properties, identification, distribution and role in synaptic transmission.

3.1 Functional Characteristics

Vesicular glutamate transport exhibits several distinctive properties. Unlike the plasma membrane transporters, vesicular glutamate transport does not recognize aspartate (137). The primary dependence on $\Delta\Psi$ rather than ΔpH has also suggested that it functions as an ion channel rather than a H^+ exchanger (33, 131). To some extent, the apparent dependence of vesicular glutamate transport on $\Delta\Psi$ and ΔpH will reflect the relative magnitude of the two components under the conditions studied. However, the difference in mechanism between channel and transporter has profound consequences for the amount of glutamate achieved per vesicle. A channel would concentrate glutamate only ~10-fold for a $\Delta\Psi$ ~60 mV, but cytoplasmic glutamate ~10 mM would require a gradient of only ~10 to produce 100 mM

glutamate inside the vesicle. On the other hand, since glutamate is an anion, the exchange of 1 lumenal H^+ would result in the net movement of 2+ charge. With this coupling, ΔpH ~2 and $\Delta\Psi$ ~60 mV would produce a substantially larger glutamate gradient ~10^4. Although high cytosolic concentrations of glutamate indicate that a gradient of this magnitude is not needed to fill the vesicle, most of the evidence supports an H^+ exchange mechanism. Dissipation of ΔpH alone has minimal effect on vesicular glutamate transport, but the residual activity after dissipation of $\Delta\Psi$ is abolished by the simultaneous dissipation of ΔpH (19, 182).

Glutamate transport into synaptic vesicles also exhibits unusual regulation by chloride. 2–10 mM Cl^- confers robust activity, with less but still detectable transport at both lower and higher concentrations (137). High chloride concentrations presumably dissipate the $\Delta\Psi$ necessary for transport. Originally, it was thought that low chloride concentrations produce a small ΔpH that was also necessary for transport. However, the chloride dependence persists even with ΔpH clamped (218), and chloride apparently interacts with an allosteric site on the transporter that regulates both forward and reverse flux (84). Despite repeated demonstration of these findings, physiological analysis has recently shown that intracellular chloride concentration does not affect quantal size (160). At the calyx of Held, where receptors are not saturated by the transmitter in a single synaptic vesicle, the size of spontaneous events does not vary when the presynaptic terminal is dialyzed with a wide range of chloride concentrations from 5–100 mM. How can the physiological and biochemical results be reconciled? Although not proven, it seems most likely that the difference lies in the nature of the measurement. Radiotracer flux assesses only the earliest phase of uptake and is a kinetic measurement. In contrast, quantal size reflects a thermodynamic equilibrium. Thus, chloride concentration may affect only the rate of vesicle filling, not the final gradient achieved. Consistent with this, chloride has much less of an effect on transport assays using high concentrations of glutamate, which may reflect more of a steady-state (218).

3.2 Vesicular Glutamate Transporters

The vesicular glutamate transporters (VGLUTs) were originally identified as type 1 Na^+-dependent inorganic phosphate transporters (140), but it has since become clear that most members of this family perform a function other than phosphate transport. Sialin is a H^+ exchanger that exports sialic acid from lysosomes (135, 203, 219), and the founding member of the type I phosphate transporters NaPi-1 recognizes organic anions (28, 31). In addition, glutamate transport by the VGLUTs does not depend on Na^+, and inorganic phosphate does not inhibit glutamate uptake by vesicles expressing VGLUT1 (19). Rather, heterologous expression of the VGLUTs seems to up-regulate endogenous phosphate transport activities (which can be Na^+-independent or -dependent) (R.H.E., unpublished observations), and NaPi-1 behaves similarly (28). It is therefore remarkable that purified, reconstituted VGLUT2 has recently been shown to catalyze Na^+-dependent phosphate transport as well as glutamate/H^+ exchange (104). Using the reconstituted system, mutations that interfere with glutamate transport do not disrupt phosphate uptake, raising the possibility that the two activities use entirely different mechanisms. What role might

phosphate uptake across the plasma membrane serve at the nerve terminal? Interestingly, phosphate activates both glutaminase isoforms responsible for glutamate production, and since the VGLUTs localize to synaptic vesicles, this might provide a mechanism to link glutamate production to release (18).

Consistent with a primary role in neurotransmitter packaging, heterologous expression of the VGLUTs reproduces all of the functional characteristics previously described using synaptic vesicles from the brain, including selective recognition of glutamate rather than aspartate, and biphasic regulation by chloride (19, 64, 66, 78, 90, 183–185, 201). In addition, the related NaPi-1 shows a large chloride conductance that is inhibited by organic anions (31), and VGLUT1 exhibits a chloride conductance which is inhibited by glutamate (but not aspartate) (19). The chloride conductance may indeed somehow contribute to the allosteric regulation of VGLUTs by chloride, but we still understand very little about the relationship between glutamate transport and chloride. Indeed, the available radiotracer flux assays, particularly after heterologous expression, currently limit our understanding of the VGLUTs. The vesicular monoamine transporters require only transfection into non-neural cells for robust activity, but the VGLUTs require more specialized conditions, and remain difficult to assay.

How do the properties of the VGLUTs contribute to glutamate release evoked by high frequency stimulation? In contrast to the vesicular monoamine transporters, which have an extremely high apparent affinity for their substrates (Km ~1 µM), essentially all of the other vesicular neurotransmitter transporters including the VGLUTs have a low apparent affinity (Km ~1–3 mM in the case of VGLUTs). This low apparent affinity presumably allows the VGLUTs to transport larger amounts of glutamate more rapidly, but we do not know the actual maximal turnover rate per transport protein. The turnover of VMATs for serotonin at $29^{°}$ C is ~5/sec/protein, and presumably higher for the lower affinity substrate dopamine which has a 4-fold higher Vmax (151). The vesicular acetylcholine transporter has an even lower turnover of ~1/sec (200). However, the ability of VGLUTs to acidify synaptic vesicles as glutamate enters suggests that they transport much more rapidly. Indeed, glutamate behaves very much like chloride in promoting vesicle acidification, presumably by dissipating $\Delta\Psi$ and indirectly activating the H^+ pump.

3.2.1 Distribution and Synaptic Role

VGLUT1 and 2. The three characterized VGLUT isoforms show complementary distributions in the mature brain. Excitatory neurons in the cortex, hippocampus and cerebellar cortex (granule cells) express VGLUT1 whereas cells in the thalamus and brainstem express VGLUT2 (19, 64, 66, 78, 90, 184, 185, 201). Considering a possible alternative role in phosphate transport, how does the loss of VGLUT1 influence transmitter release? As predicted, the VGLUT1 knock-out shows no release at mature synapses that normally express VGLUT1 in adulthood (65). However, residual glutamatergic transmission persists at these synapses during early postnatal development, apparently due to the transient expression of VGLUT2. Further, the VGLUT1-independent transmission at these sites appears normal in many respects, suggesting that it originates from distinct synapses. On the other

hand, VGLUT1-independent transmission in the hippocampus does exhibit more rapid synaptic depression than wild type, possibly because the vesicles at these synapses recycle more slowly than the more prevalent VGLUT1 synapses of wild type mice, or because VGLUT1 and 2 themselves differ in the rate of recycling. Consistent with differences in trafficking, recent work has shown that VGLUT1 interacts with endophilin, a protein involved in synaptic vesicle endocytosis, and this interaction influences the rate of recycling (205). Loss of VGLUT1 also produces abnormal endocytic structures (65), suggesting roles for the transporter in aspects of the synaptic vesicle cycle other than vesicle filling. Like vesicular monoamine and acetylcholine transporters (43, 149), however, inhibition of vesicular glutamate transport using the H^+ pump inhibitor bafilomycin does not impair vesicle recycling (227), and hippocampal neurons from the VGLUT1 knock-out can release empty vesicles (217). The VGLUT2 knock-out reported more recently cannot release glutamate at synapses in the brainstem involved in generation of the respiratory rhythm, and hence dies just after birth (134, 211).

VGLUT3. In contrast to the expression of VGLUT1 and 2 by known excitatory neurons, VGLUT3 is expressed by cells not traditionally considered glutamatergic, including serotonergic neurons in the raphe nuclei, cholinergic interneurons in striatum, and a subset of GABAergic interneurons in the cortex and hippocampus (64, 78, 169). VGLUT3 is also expressed outside the nervous system, and in contrast to VGLUT1 and 2, heterologous expression has not yet conferred detectable glutamate release (183), raising questions about the role of this isoform. Indeed, the glutamate packaged by VGLUT3 might simply influence the storage of the other classical transmitter released by VGLUT3$^+$ neurons (e.g., serotonin in the raphe). It is also possible that the glutamate stored by VGLUT3 has a role in metabolism. However, several observations indicate that VGLUT3 does contribute to glutamate release.

In contrast to VGLUT1 and 2, VGLUT3 can localize to the cell body and dendrites as well as the axon terminal. In projection neurons such as serotonergic cells in the raphe nuclei, VGLUT3 localizes almost exclusively to the axon terminal, but in interneurons such as cholinergic interneurons in the striatum and GABAergic interneurons in the cortex and hippocampus, VGLUT3 also localizes to the cell body and dendrites (64). In addition, VGLUT3 appears in the dendrites of pyramidal neurons in layer 2/3 of cortex, where the pharmacology indicates a role for the protein in dendritic release and retrograde synaptic signaling (83). Trafficking of VGLUT3 to different cellular compartments may therefore contribute to novel modes of signaling by glutamate.

VGLUT3 may have a particular role in plasticity. In the brainstem auditory pathway, synapses formed by neurons in the medial nucleus of the trapezoid body (MNTB) onto neurons of the lateral superior olive (LSO) switch from GABA to glycine during the tonotopic refinement and synapse strengthening that occurs in early postnatal development. These changes are presumably important for sound localization. Interestingly, MNTB neurons also transiently release glutamate that activates NMDA receptors, suggesting a role for the glutamate in plasticity, and the brief expression of VGLUT3 coincides temporally with this glutamate release (70). However, it remains unclear whether the early glutamate release actually contributes to synapse development and sound localization.

Other Vesicle Carriers. In addition to the H^+ pump, chloride carriers and neurotransmitter transporters, other factors may influence the contents of synaptic vesicles and hence quantal size. Extracellular ATP activates specific receptors but also promotes the co-storage of cationic transmitters such as serotonin (12) in the cytosol, although the vesicular ATP transporter remains unknown. Similarly, cationic compounds may promote the packaging of an anionic transmitter such as glutamate. A number of polytopic membrane proteins that localize specifically to synaptic vesicles indeed remain poorly understood. The SV2 family shows strong homology to a number of carbohydrate transporters, but its biochemical function also remains unknown. The loss of SV2A alone results in seizures and death shortly after birth, and physiological studies have suggested roles in both calcium regulation and the readily releasable pool of synaptic vesicles (44, 101). Remarkably, the SV2A isoform appears to be the direct target for the major clinical anticonvulsant levetiracetam (Keppra) (122).

4 The Presynaptic Regulation of Quantal Size

The amount of transmitter released per vesicle can be regulated in two distinct ways, by changes in the lumenal concentration, and by changes in vesicle size. We will now present several models for the presynaptic regulation of quantal size, each of which has the potential to influence one or both of these parameters.

4.1 Equilibrium

At thermodynamic equilibrium, the stoichiometry of ionic coupling, the H^+ electrochemical driving force, and the cytosolic concentration of transmitter should determine the concentration of transmitter inside a secretory vesicle. The number of transporters should influence vesicle filling only if there is not enough time to reach equilibrium, which could occur if the rate of vesicle recycling exceeds the rate of filling. The low turnover numbers for vesicular monoamine and ACh transport suggest that this might indeed be the case at aminergic and cholinergic synapses, particularly when stimulated at high frequency. On the other hand, the number of transporters expressed can influence quantal size even under resting conditions, implicating other mechanisms.

4.2 Transporter Expression and Leak

Initial studies of the vesicular monoamine transporter VMAT2 and the vesicular acetylcholine transporter (VAChT) have shown that changes in expression can influence quantal size independent of release rate. The over-expression of VMAT2 in chromaffin cells and neurons increases quantal size measured directly by amperometry (154), and these cells show little spontaneous activity in culture. Over-expression of VAChT at the developing neuromuscular junction also increases quantal size (175). Conversely, the loss of one functional VMAT2 allele reduces monoamine release (61, 196) and a reduction in VAChT by only ~45% both reduces quantal size and impairs cognitive function (159).

If the vesicles have enough time to fill, why does transporter expression make a difference? Multiple observations indicate a nonspecific (i.e., not mediated by the transporter) leak of transmitter across the vesicle membrane. Both native synaptic vesicles and the endosomes used for heterologous expression of the transporters exhibit substantial accumulation of monoamine driven by the H^+ electrochemical gradient, even without a functional transporter. Presumably, the unprotonated, neutral amine diffuses into the vesicle, then undergoes protonation that prevents its efflux. Nonspecific efflux must therefore also exist, and the efflux of monoamine from chromaffin granules triggered by dissipation of ΔpH is not blocked by VMAT inhibitors such as reserpine (128). More vesicle transporters may serve to offset this leak.

VGLUT expression may also influence quantal size. Using purified synaptic vesicles, inhibitors reduce both the steady-state amount and rate of glutamate accumulation (214). Over-expression of VGLUTs in transfected hippocampal neurons also appears to increase quantal size (214, 217). Conversely, the loss of either VGLUT1 or 2 can reduce quantal size (134, 217). The VGLUTs also undergo transcriptional regulation during development (25, 65, 133), and VGLUT1 and 2 were both originally identified in screens for differentially expressed genes (3, 140). Activity reduces VGLUT1 expression at both RNA and protein levels (49), suggesting that it is also under homeostatic control. However, VMAT2 is up-regulated in response to increased activity. Remarkably, the expression of VGLUT1 protein but not mRNA also appears to cycle with a circadian rhythm (223). These changes in transporter expression provide circumstantial evidence for the presynaptic regulation of quantal size.

Despite the evidence for regulation of neurotransmitter transport in vitro and transporter expression in vivo, other work suggests that VGLUT expression does not limit vesicle filling. Heterozygous VGLUT1 knock-out mice carrying a single functional VGLUT1 allele show no difference from wild type in hippocampal field potentials (65). Any change in quantal size should be reflected in this response, so the absence of a difference from wild type effectively rules out a change in quantal size with VGLUT reduction by 50%. Second, quantal size at the *Drosophila* neuromuscular junction does not change as VGLUT expression is reduced (47). Only the frequency of spontaneous events declines as the expression of VGLUT drops below 10–20% of the wild type level. The loss of miniature events presumably reflects the exocytosis of "empty" vesicles. Indeed, a single VGLUT apparently suffices to fill a vesicle, at least at this synapse. Although a modest reduction in VGLUT expression does not affect glutamate release, VGLUT over-expression in *Drosophila* appears to increase quantal size (48).

How can the results *in vivo* be reconciled with the biochemical and physiological studies in vitro? First, it is important to note that radiotracer flux assays measure the kinetics of transport, rather than the thermodynamic equilibrium. Second, the very low levels of residual transporter expression in autaptic cultures made from knock-out mice may well produce smaller quantal sizes than wild type, but the work from *Drosophila* suggests that more physiological reductions in expression might not have produced detectable changes. However, it is more difficult to understand how VGLUT over-expression increases quantal size when even a 50% reduction has no effect. VGLUT expression may influence vesicle size, as suggested by recent work

in *Drosophila* (48). And if VGLUT expression does not influence vesicle filling, why would this differ from vesicular monoamine and acetylcholine transport (61, 154, 175)? One possibility is that cationic molecules may exhibit a greater nonspecific leak across the membrane, possibly due to abundant acidic phospholipid. In contrast, anions would have more difficulty permeating directly through this membrane. In addition, it is important to note that even if VGLUT expression does not affect the size of spontaneous events, it may become limiting with high rates of activity and vesicle recycling, when it is more difficult to measure quantal size directly. Indeed, reduced VGLUT2 expression in retinal ganglion cells of the zebrafish mutant *blumenkohl* has remarkably modest effects on neurotransmission, but dramatic effects on fine behavior guided by vision (174).

Differences in non-specific leak may also account for the distinct effects of vesicular transport inhibitors on vesicle storage. Without stimulation, an inhibitor should deplete stores only in the presence of a leak—in this case, the stores reflect a balance between leak and ongoing transport activity. In contrast, a transmitter that does not leak should require stimulation to deplete the stores. Permanently charged ACh should leak less than reversibly protonated monoamines, and thus require activity to deplete the stores. Indeed, the VAChT inhibitor vesamicol does not affect vesicular stores without stimulation (32), whereas the vesicular monoamine transport inhibitor reserpine rapidly depletes monoamines from resting chromaffin granules, without stimulation (112). Since there are very few if any potent, specific VGLUT inhibitors, it remains unclear how fast the vesicular stores would be depleted after transporter block. The H^+ pump inhibitor bafilomycin depletes stores relatively rapidly, but might simply promote reverse flux through the VGLUTs (227).

4.3 Set Point

Vesicular neurotransmitter transport has been suggested to undergo direct regulation at the level of the protein as well as transcription. A proteolytic fragment of the cytoskeletal protein fodrin was purified as an inhibitor of vesicular GABA and glutamate transport, but the mechanism for this regulation and its physiological role remain unknown (146, 189). In addition, heterotrimeric G proteins inhibit vesicular monoamine and glutamate transport. A variety of G proteins bind to secretory vesicles, and activation of $G\alpha o_2$ inhibits both monoamine and glutamate uptake by 30–50%, affecting Vmax rather than Km (2, 94, 147, 216). The regulation does not apparently involve a change in vesicle acidification, suggesting instead a direct interaction of α subunit with the transporter. In platelet granules that express VMAT2 and store serotonin, α_q has a similar role. Further, inhibition of transport seems to depend on lumenal transmitter: in mice lacking platelet tryptophan hydroxylase and hence serotonin, activated α_q does not inhibit transport, and the addition of monoamine restores inhibition (29). G proteins may thus convey a signal about vesicle filling back to the transporter, and so provide a set point. However, the lumenal sensor for any neurotransmitter remains unknown. Internalized plasma membrane G protein-coupled receptors might subserve this function, but it would require a major decrease in their affinity, which might occur at the low pH of secretory vesicles. In the case of vesicular glutamate transport, $G\alpha o_2$ also changes

the chloride dependence, eliminating the activation by low chloride concentrations in αo_2 knock-out mice and shifting the peak activation to lower concentrations when stimulated by a non-hydrolyzable form of GTP (216). In both cases, the mechanism remains unclear.

4.4 The Relationship Between Neurotransmitter and Synaptic Vesicle Cycles

Is there a relationship between vesicle filling and the synaptic vesicle cycle of exo- and endocytosis? As mentioned above, the inhibition of filling does not appear to influence synaptic vesicle exocytosis or recycling in cholinergic, aminergic or glutamatergic neurons (43, 149, 217, 227), but the converse remains unexplored. Indeed, the allele-specific supression of a point mutation in the acetylcholine transporter by a point mutation in the v-SNARE synaptobrevin supports a connection between the synaptic vesicle and neurotransmitter cycles (167). However, similar mutations in other vesicle proteins partially rescue the transporter mutant, raising the alternative possibility that only the mutant but not wild type proteins interact.

4.5 Changes in Vesicle Volume

Secretory vesicles can vary in size as well as the concentration of lumenal transmitter. Changes in vesicle size were first recognized at the *Torpedo* electric organ, which has two biochemically distinct populations of synaptic vesicles (228). The larger, VP1 vesicles, contain 3-5-fold more acetylcholine than the smaller VP2, but VP2 expresses more acetylcholine transport activity (77). At rest, VP1 vesicles predominate, and the VP2 appear with activity (72). After stimulation, VP1 replaces VP2. VP1 may thus correspond to the reserve pool of vesicles, and VP2 to the recycling (162), although reserve and recycling pools at other synapses are functionally distinct. Considering that VP2 vesicles may simply swell to become VP1, it is interesting that the membrane of VP2 seems twice as thick as VP1 (198).

The analysis of monoamine storage and release has further indicated the potential of filling to influence vesicle size. In PC12 cells, administration of L-Dopa increases the size of dense core vesicles and the dopamine released per vesicle (detected by amperometry) (40, 74). Remarkably, however, the concentration of transmitter released (measured by voltammetry) does not change. Indeed, L-Dopa increases the size of the halo around the dense core, but not the dense core itself (40). Transport can thus regulate vesicle size without changing the concentration of lumenal transmitter. Consistent with the osmotic swelling of preformed vesicles, variation in quantal size in the leech correlates with variation in vesicle volume, and the average concentration of transmitter released per vesicle varies little despite large differences in vesicle size (30).

What accounts for the uniform lumenal concentration of transmitter? The cytosolic concentration of transmitter, the driving force for transport and the number of transporters per vesicle might generate vesicles of uniform concentration in a particular cell, but the development of an osmotic gradient provides an additional constraint that might account for the uniformity between cells. The H^+

electrochemical driving force for transport may thus produce both a concentration gradient of transmitter and an osmotic gradient which will eventually oppose any further increases in concentration. Indeed, assuming the approximate cytosolic concentrations of transmitter (1–10 mM) suggested for glutamate, the driving force and the coupling of VGLUTs to H^+ exchange (which could theoretically produce a concentration gradient of 10^4), the mechanism should produce extremely hyperosmolar lumenal contents. If these contents do not precipitate, they might be expected to distend the vesicle, presumably up to a limit determined by the physical properties of the membrane, and by the H^+ electrochemical driving force.

Changes in vesicle size may also reflect changes in biogenesis and hence intrinsic biochemical differences in the composition of vesicles. A number of *Drosophila* mutants show both an increase in quantal size and increased vesicle volume at the neuromuscular junction (107, 225). One of the mutations affects a *Drosophila* homologue of the clathrin adaptor AP180 (226) and another the lipid phosphatase synaptojanin that mediates clathrin uncoating (54), strongly suggesting that in these cases, the increased size reflects a defect in biogenesis. Chromaffin cells have also been reported to contain two distinct populations of dense core vesicles, and the adaptor AP-3 apparently controls their production (75, 76). 1b and 1s synapses at the wild type *Drosophila* neuromuscular junction also show differences in both quantal size and vesicle volume (107), supporting a role for the regulation of vesicle volume under physiological conditions. A recent study at the calyx of Held has tested the hypothesis that variation in vesicle volume underlies the variation in quantal size. The authors recorded both the capacitance change associated with fusion of a single synaptic vesicle, and the postsynaptic response (220). The results reveal no correlation between capacitance change and quantal size, indicating variation in the lumenal concentration of transmitter rather than vesicle size. However, capacitance detects only the surface area of the vesicle *after* fusion with the plasma membrane, and the role for an osmotic gradient can only operate *before* fusion. With exocytosis, any swollen vesicles presumably return to their original, undistended state.

4.6 Presynaptic Regulation of Quantal Size by Activity

Do changes in vesicle filling actually contribute to synaptic plasticity? At the neuromuscular junction, strong, protracted stimulation reduces quantal size by ~20%, and this change does not reflect alterations in postsynaptic sensitivity, implicating a presynaptic mechanism (55, 139). Inhibition of release delays this decline in quantal size, as if a pool of synaptic vesicles containing more transmitter releases first but is eventually depleted. In addition, a variety of intracellular signaling pathways acting presynaptically can influence quantal size by up to 4-fold (199). Further, blockade of neural activity for one week at the rat neuromuscular junction substantially increases quantal size, apparently through a presynaptic mechanism (212). The lack of correlation with activity in muscle further suggests that this mechanism does not involve retrograde signaling from the postsynaptic cell.

A number of other synapses also exhibit presynaptic regulation of quantal size. Stimulation increases the amount of stored glutamate in synaptosomal preparations from the rat brain (24) and quantal size detected electrochemically with adrenal

chromaffin cells (155). Conversely, suppression of activity reduces the amplitude of spontaneous events through a presynaptic mechanism in hippocampal neurons, perhaps contributing to the homeostatic regulation of brain excitability (85). In *Drosophila*, quantal size fluctuates with the rate of foraging for food. Flies crawl faster just after the shift to a plate that lacks food, and the fastest subgroup exhibits a transient increase in both quantal size and evoked release that appears presynaptic (177). The increase in quantal size appears to reflect an increase in vesicle volume, but it remains unclear whether this reflects swelling of preformed vesicles or the recruitment of distinct vesicles with a different size.

4.7 Conclusions

Previous work has identified many of the proteins required for neurotransmitter uptake by the cell, and for the packaging of secretory vesicles, but we still understand relatively little about their regulation and their role in synaptic transmission. Future work in this area will focus on the physiological role of these mechanisms and their regulation in synaptic plasticity.

5 Glutamate Uptake at the Cell Surface

When a synaptic vesicle fuses with the presynaptic membrane, glutamate rapidly diffuses into the cleft where it has the first opportunity to encounter receptors. Although the peak concentration within the small volume of the cleft is estimated to reach several millimolar (39, 53), glutamate is rapidly diluted in the cleft and surrounding extrasynaptic spaces. The close proximity of low affinity AMPA and kainate receptors to release sites ensures that binding and activation occur reliably despite the rapid exit of glutamate. Although dilution and diffusion contribute substantially to the decline in the concentration of glutamate near receptors, ultimately glutamate must be removed to prevent accumulation and persistent signaling. Unlike the prototypical neurotransmitter acetylcholine, which is hydrolyzed in the extracellular space by acetylcholinesterase, extracellular enzymes capable of catalyzing the conversion or degradation of glutamate have not been identified. Instead, the removal of glutamate is catalyzed by a multigene family of integral membrane proteins termed EAATs (Excitatory Amino Acid Transporters) for the human variants, which transport glutamate from the extracellular space into the cytosol (46).

The EAAT family of transporters consists of five members (EAAT1-5), each a product of a unique gene (46), and for two members (EAAT1 and EAAT2), additional variants are generated through alternative splicing (97, 180). Highly homologous transporters are found in rodents, which have been termed GLAST (EAAT1), GLT-1 (EAAT2), and EAAC1 (EAAT3), EAAT4 and EAAT5. The diversity of glutamate transporters is unparalleled among neurotransmitter transporters (5). Although the precise role of these different transporter isoforms is still uncertain, it is likely that clearance of extracellular glutamate is parsed out among the different variants, with each transporter class responsible for removing glutamate over a distinct time frame or

from a particular volume of extracellular space. By providing multiple routes for the exit of glutamate, EAATs may afford subtle control over glutamate dynamics, and thus receptor activation in and around synapses.

5.1 Clearance of Glutamate from Synapses

The impact of transporter-mediated clearance on glutamate transients and receptor activation at synapses depends on many factors, including the structure of the synapse, the number of release sites, the probability of release and patterns of activity experienced by the synapse, the proximity of neighboring synapses and their release properties, the extent of glial ensheathment, and the properties and locations of receptors. Given the vast structural and functional diversity of synapses in the CNS, it is not surprising that the involvement of transporters in synaptic signaling varies considerably among the population of excitatory synapses. It is also likely that the contribution of EAATs to glutamate clearance is not static, but changes with synaptic reorganization and shifting patterns of activity.

The involvement of EAATs in clearance of glutamate near synaptic receptors can be tested by applying transporter antagonists such as TBOA (threo-beta-benzyloxyaspartate), a substrate analog that is not transported (172), while monitoring spontaneous or evoked synaptic responses. If EAATs curtail synaptic glutamate transients, blocking transporters should increase the peak concentration of glutamate and slow the decay of each glutamate transient. Thus, transporter inhibition should increase the amplitude of synaptic currents and slow their decay; this assumes that under baseline conditions receptors are not saturated and the decay of the current is not already determined by the rate of receptor densensitization. At most excitatory synapses, AMPA receptor responses elicited following fusion of a single vesicle are not affected by transporter antagonists, indicating that quantal responses are determined primarily by the intrinsic properties of the receptors and a passive decrease in glutamate concentration through rapid diffusion and dilution in the extracellular space (91, 168). In contrast, EPSCs elicited in response to stimulation of multiple afferents typically decay more slowly in the presence of transporter antagonists, an effect that becomes more pronounced when trains of EPSCs are elicited at high frequency, or the activation of high affinity NMDA or metabotropic receptors (mGluRs) are monitored (7, 34, 36, 38, 145, 194). Such protocols raise the release probability at individual synapses and increase the likelihood that adjacent synapses will be active. These effects are partially due to the recruitment of receptors located outside the cleft (e.g. NMDA receptors and mGluRs) that are in proximity to transporters (see below). Together, these results suggest that repetitive release of glutamate from individual sites and "pooling" of glutamate from adjacent sites engage EAATs to a greater degree than isolated, univesicular release (16), allowing transporters shape synaptic responses.

In the continued presence of transporter blockers, extracellular glutamate levels rise (98, 100), which can decrease the amplitude of synaptic responses through occlusion and desensitization of receptors, or change frequency the frequency of spontaneous events through tonic activation of presynaptic mGluRs. Thus, EAATs are involved in regulating the ambient level of glutamate surrounding synaptic receptors, as well as the

population of receptors activated following release of glutamate from nerve terminals. To understand how transporters are able to participate in synaptic events lasting only a few milliseconds, some of the characteristics of the proteins that catalyze glutamate translocation need to be considered.

5.1.1 How do transporters accomplish uptake?

The reliance on surface transporters to inactivate extracellular glutamate poses a number of challenges. Foremost among these is that the movement of glutamate into cells is opposed by considerable thermal and electrical barriers. As glutamate is negatively charged, movement through the hydrophobic interior of the membrane must be assisted. However, unlike channels, in which ion flow is dictated by electrochemical gradients, glutamate must be forced to enter the cell against a considerable opposing electrochemical gradient, as cells maintain a negative resting potential and the intracellular concentration of glutamate is likely to be much greater than that typically encountered by transporters (126, 222).

To force the influx of glutamate in the presence of these opposing gradients, EAATs harness the energy stored in Na^+ and K^+ gradients, coupling the movement of glutamate to the inward movement of Na^+ and the outward movement of K^+. Thus, while EAATs do not directly require ATP, they require gradients set up by ATP-dependent pumps. The stoichiometry for EAAT-dependent transport has been measured for GLT-1 (EAAT2) and EAAT3; for every cycle of transport, $3Na^+$, $1H^+$, and 1 glutamate$^-$ move inward, and one K^+ moves outward (118, 224). This stoichiometry predicts that EAATs are capable of establishing a concentration gradient of greater than a million, lowering extracellular glutamate to nanomolar levels at equilibrium when challenged with a single pulse of glutamate (224). Furthermore, due to this unbalanced stoichiometry, completion of a single transport cycle results in the net movement of two positive charges into the cytosol. Thus, EAATs are electrogenic molecules, which can induce macroscopic currents if a sufficient number of transporters are cycling at the same time (9, 15). It has been possible to resolve EAAT-mediated currents in a variety of cell types and even in cell-free, outside-out patches when their density is sufficiently high (10, 20, 22) (Fig. 2), which has given great insight into the capabilities of these transporters.

In addition to the movement of cations that are directly coupled to the movement of glutamate, EAATs also enable anions to move across the membrane. However, reversing the anion gradient, and thus the direction of anion movement, does not alter the next influx of glutamate (206). EAATs therefore contain an anion channel, but they do not require anions to transport their substrate, in contrast to the GABA, glycine and monoamine transporters (195). Under physiological conditions, the flux of Cl^- through all but EAAT5 is minute (8, 143, 206), and the physiological significance of this transporter-mediated anion flux has yet to be determined (see below). Nevertheless, this aspect of EAAT behavior has been exploited for physiological studies. In the presence of anions that are highly permeant through the associated anion conductance (e.g. thiocyanate, nitrate, perchlorate), glutamate-evoked transport currents become much larger, as many more anions flow per cycle than do cations, allowing transporter activity to be monitored when the density or total number of transporters is low (22, 144, 161) (Fig. 2).
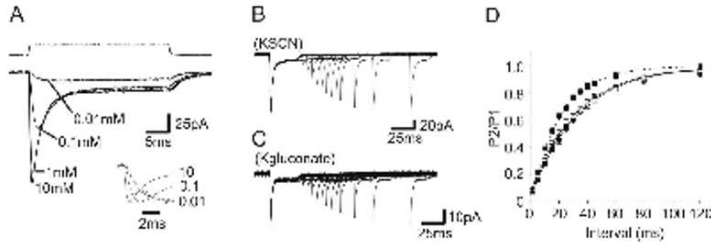
Fig. 2. GLT-1 transporter currents in outside-out patches.
(**a**) Response of GLT-1 transporters to a range of L-glutamate concentrations. The inset shows the peak responses to 0.01 and 0.1 mM L-glutamate, which are scaled to the peak of the response to 10 mM L-glutamate to illustrate the concentration dependence of the rise time of the transporter currents. The rise time to saturating glutamate was 248 μs, indicating that GLT-1 is capable of binding glutamate very rapidly. The trace above is the "open tip" response produced when the patch was removed, which shows the duration of glutamate application (KSCN-based internal solution). (**b**) Response of a patch to pairs of applications of 10 mM L-glutamate (KSCN-based internal solution). The interval between control (30 msec duration) and test (20 msec duration) applications was 1–120 msec. (**c**) Response of a patch to paired applications of 10 mM L-glutamate recorded without permeant anions in the internal solution (K-gluconate-based internal solution). (**d***)* Plot of the ratio of the peak amplitude of the second (P2) to the first (P1) response to paired applications of 10 mM L-glutamate recorded with (filled squares) and without anions (open circles) in the internal solution (solid lines are single exponential fits to the data). Removal of TEA-Cl (10 mM) from the internal solution (open squares) speeded the decay by ~35% (dashed line), suggesting that TEA interacts with the internal K^+ binding site. The time constant of recovery, and thus the turnover rate of these transporters in the absence of TEA was 21.8 ± 0.8 ms (n = 10). All responses were recorded from outside-out patches removed from HEK cells expressing GLT-1. Reproduced from reference (183).

Although equilibrium measurements reveal the extraordinary concentrating power of EAATs, they provide little insight into how transporters will perform in the dynamic environment of the brain. In order to effectively restrict the spread of glutamate, transporters must operate in a concentration range similar to that required for binding to receptors. Measured affinities (K_m values) of the EAATs range from 1–20 μM (9), which are comparable to the affinities of NMDA, high affinity kainate, and mGluRs, as well as the glutamate levels that are able to induce desensitization of AMPA receptors (197). However, because coupled transport is a relatively slow process, glutamate clearance is subject to an additional constraint. To complete a single cycle, multiple binding and unbinding reactions have to occur in precise order to allow the conformational changes required for glutamate translocation; as a result, between 10–70 ms are required before each transporter is available to bind glutamate again (21, 23, 143, 207, 224). Because of this slow turnover rate, EAATs must be present at high densities to ensure that they are not saturated, as synapses can release

at rates greater than 100 Hz and can be challenged by greater quantities of glutamate during episodes of multivesicular release (141, 208). Quantitiative immunogold measurements indicate that the density of EAATs near synapses can be as high as 10,000 per μm^2 (115), comparable to the density of nicotinic acetylcholine receptors at neuromuscular junctions (4), and physiological measurements have revealed that they are typically not saturated, even under high release conditions (52, 96).

The rates of glutamate diffusion within synaptic and extrasynaptic spaces have not been measured directly, but are likely to be rapid at conventional *en passant* synapses containing a single release site, given the speed with which glutamate transients decay (39) and the short delay between release and the onset of transporter currents in perisynaptic astrocytes (20, 22, 51). Although rapid diffusion will help dissipate glutamate transients though dilution, it could also allow glutamate to escape a zone enriched in transporters to reach receptors in extrasynaptic domains and adjacent synapses. However, EAATs seem well-designed to compete with receptors for glutamate. Glutamate-evoked transporter currents in outside-out patches reach a peak in a few hundred microseconds (10, 22, 144, 204, 210), indicating that they are also capable of binding glutamate rapidly (Fig. 2). Chemical-kinetic modeling suggests that binding to transporters (23, 143), like ionotropic receptors (87), is effectively limited only by the rate of glutamate diffusion. This rapid binding of glutamate may allow EAATs to quickly lower the concentration of glutamate below that required for receptor activation simply by binding. Thus, the rapid decline in the glutamate transient may be primarily due to the ability to EAATs to rapidly buffer glutamate as it exits the cleft (194).

The relative efficiency of EAAT-dependent transport can also influence the rate of glutamate clearance. Transport is accomplished when a molecule of glutamate binds to the transporter and is translocated and released into the cytosol; however, glutamate may unbind from the transporter before translocation, or following translocation it may fail to unbind or be replaced by another glutamate molecule and eventually delivered back to the extracellular space as the transporter completes a half cycle in reverse. This transport efficiency is dependent on the intracellular and extracellular concentrations of transported ions and substrate, as well as the transmembrane voltage. Under ideal conditions (0 Na^+, 0 glutamate inside), the efficiency of EAAT2 is estimated to be 65% (23), while EAAT4 has an estimated efficiency of 75% (143). However, under physiological conditions, the efficiency of EAAT4 is only about 50%, due to the high concentration of intracellular glutamate in neurons. By the same means, the cycling rate of these transporters slows down when Na^+ and K^+ gradients collapse. An increase in intracellular Na^+ and a rise in extracellular K^+ can even induce EAATs to cycle in reverse, a phenomenon that is likely to contribute to the prolonged elevation of extracellular glutamate in ischemia (163). Thus, the ability of EAATs to remove glutamate will be highly dependent on their local environment, and transport is likely to be compromised during high rates of activity, when extracellular K^+ increases, intracellular Na^+ and glutamate increase, and cells spend more time at depolarized potentials.

5.2 Localization of Glutamate Transporters

In order to understand the relative contribution of these transporters to the clearance of glutamate from synapses, the distribution of these transporters and their density around synapses must be determined. Anatomical studies have revealed that each EAAT variant exhibits a restricted distribution. EAAT1 is expressed only in glial cells, particularly astroglial cells in the brain and spinal cord (astrocytes, Bergmann glial cells, radial glial cells) and Müller glial cells in the retina (116, 165). Similarly, EAAT2 is, with few exceptions (37, 161), found only in the membranes of astrocytes (Fig. 3). EAAT3 is ubiquitously expressed by neurons throughout the nervous system, while EAAT4 and EAAT5 have a much more restricted distribution. EAAT4 is expressed only by Purkinje neurons in the cerebellum (59, 221), while EAAT5 is expressed by a variety of neurons in the retina (bipolar cells, photoreceptors), but nowhere else (156).

The ability of EAATs to affect the concentration of extracellular glutamate near synapses will depend on their density relative to sites of release. EAAT2 is the most abundant transporter in the forebrain, accounting for > 90% of all activity (164, 190), and the density of EAAT2 in hippocampal astrocytes has been estimated to reach 8,500 per μm^2 using quantitative immunogold analysis (115). EAAT1 reaches 2,300 per μm^2 in the same membranes, but the situation is reversed in the molecular layer of the cerebellum, where EAAT1 is more abundant than EAAT2 (115). Although early reports suggested that EAAC1 (EAAT3) may also be enriched in the postsynaptic membrane (88), recent studies suggest that indicate that it may be
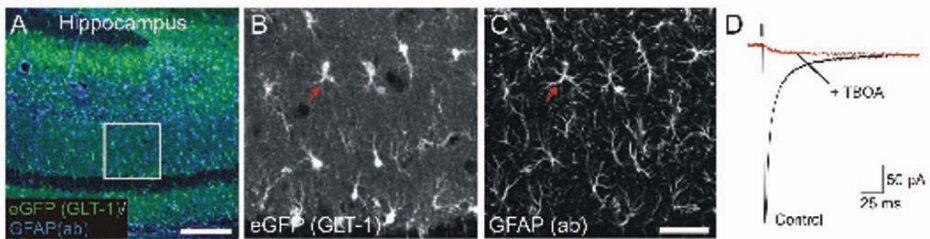

Fig. 3. Restricted expression of GLT-1 by astrocytes.
(**a**) Confocal fluorescence image of the CA1 region of the hippocampus from a GLT-1-eGFP transgenic mouse, in which eGTP is expressed under control of the GLT-1 promoter. This section was also immunolabeled with anti-GFAP antibodies (*blue*), showing that the GLT-1 promoter is only active in GFAP+ astrocytes in this region. Scale = 300 μm. (**b, c**) Higher magnification images of the region delimited by the white box in *A*, showing expression of eGFP (**b**) by GFAP+ astrocytes (**C**). Scale = 50 μm. (**d**) Whole-cell recording of glutamate transporter currents elicited in a stratum radiatum astrocyte (identified expression of eGFP) in response to photolysis of MNI-D-aspartate (125 μM). The red trace shows the response recorded in the presence of the glutamate transporter antagonist TBOA (300 μM). Reproduced from reference (180).

difficult to reliably determine the subcellular distribution of this transporter using immunogold techniques (93), due to the low abundance of this protein. These results suggest that the dominant route for clearance of synaptic glutamate in the forebrain is through uptake into astrocytes. This conclusion is supported by physiological studies which have shown that transporter currents can be recorded in astrocytes in response to photolysis of caged substrates (Fig. 3) and following stimulation of glutamatergic afferents (22), while glutamate transporter currents have not been observed in neurons (21), other than Purkinje cells and some neurons in the retina (see below).

To add to this complexity, the distribution of glutamate transporters is not uniform over the cell surface. High resolution immunogold labeling has revealed that the density of EAAT2 in astrocytes is higher in membranes found next to neuronal membranes than in membranes next to other astrocytes or blood vessels (116). Similarly, the highest density of EAAT4 is reached just outside the cleft in the perisynaptic region of the dendritic membrane (50). Together, these results indicate that there is a broad division of labor at synapses, with some of the glutamate transported into astroglial cells by EAAT2 and EAAT1, and at certain synapses in the cerebellum and retina by EAAT4 and EAAT5, respectively. These differences in transporter abundance suggest that EAATs are positioned near synapses to remove glutamate as it is released and shield receptors as it diffuses from the cleft.

5.3 Contributions of Astroglial Transport

Deciphering the particular roles of the different EAATs in synaptic function has proven difficult, in large part due to the lack of subtype specific antagonists. Nevertheless, comparisons between wild type and transporter-deficient mice have begun to reveal how different EAAT isoforms contribute to uptake at synapses.

The high density of EAAT2 near excitatory synapses in the forebrain indicates that it is likely to serve as the dominant route for glutamate uptake. This hypothesis is corroborated by the phenotype of GLT-1 (EAAT2) knockout mice, which have elevated glutamate in the cerebrospinal fluid, suffer from uncontrolled seizures and die within the first few weeks of life (190). These effects are mimicked by chronic administration of antisense oliginucleotides against GLT-1 to adult animals (164), indicating that this transporter isoform is essential for maintaining a low ambient level of glutamate. GLT-1 deficient mice also show a reduced threshold for the induction of long-term potentiation at Shaffer collateral-commissural synapses (108) and exhibit enhanced activation of mGluRs at recurrent synapses onto CA1 interneurons (96). Furthermore, acute application of dihydrokainate (DHK), a non-substrate antagonist that shows ~100-fold higher affinity for GLT-1, increases the amplitude of NMDA receptor-mediated responses in some CA1 pyramidal neurons (91), indicating that these transporters also shape glutamate transients near synaptic receptors in the hippocampus. However, GLAST (EAAT1) also contributes to uptake at these synapses, as DHK caused a greater enhancement of mGluR currents in EAAT1 (GLAST) knockout mice (96) and GLAST contributes to uptake currents in astrocytes induced following stimulation of Shaffer collateral axons (22). It is not yet clear why both transporters are expressed by astrocytes, as they have comparable affinities and turnover rates (9).

Although GLT-1 is not highly expressed in the molecular layer of the cerebellum, the important role of glial cells in glutamate clearance is conserved. Animals that lack GLAST, the primary transporter expressed by Bergmann glial cells, exhibit coordination deficits and an increased sensitivity to ischemic damage to the cerebellum (213). At the cellular level, slow climbing fiber mediated responses are visible in Purkinje neurons in GLAST knockout mice, which arise from spill over of glutamate from climbing fiber synapses formed onto adjacent Purkinje neurons (187, 213). These aberrant responses also appear in wild type mice in the presence of PMB-TBOA, an antagonist which is selective for GLAST and GLT-1 (187), indicating that GLAST is essential to maintain synapse specificity. Parallel fiber mediated EPSCs evoked by trains of stimuli are also greatly enhanced in GLAST-deficient mice (127), presumably due to enhanced pooling of glutamate from neighboring synapses.

In the mammalian cochlea, GLAST is also expressed by supporting cells (68) that surround synapses formed between inner hair cells and the dendrites of primary afferent neurons, the first synapse in the auditory pathway. Unlike central excitatory synapses, GLAST appears to be the only glutamate transporter present in abundance at these peripheral synapses, as transporter-mediated currents could be elicited in supporting cells, but not inner hair cells or afferent dendrites (73). GLAST knockout mice exhibit an increased sensitivity to acoustic overstimulation and a dramatic slowing of glutamate clearance from the perilymph following exposure to loud sound (80). Thus, even at these ribbon synapses where transmitter is released tonically, glutamate can be cleared effectively by transport into surrounding supporting cells.

The dependence on transporters in glial membranes suggests that the involvement of EAATs in removal of glutamate around synapses will vary depending on the extent to which synapses are ensheathed by the astroglial processes. In the CA1 region of the hippocampus, it has been estimated that only 57% of excitatory synapses are contacted by astrocytes (202), and most are only partially ensheathed, suggesting that transmitter uptake may vary even within a population of synapses formed by similar afferents. In contrast to the hippocampus, parallel and climbing fiber synapses on cerebellar Purkinje cells are tightly ensheathed by the processes of Bergmann glial cells (176), suggesting that these glial cells play an important role in the functional isolation of neighboring synapses in the cerebellar cortex. Recent anatomical studies indicate that in both the hippocampus and cerebellum, the extent of glial coverage is greater on the postsynaptic than presynaptic side (117). A simple prediction from this observation is that glutamate may have the opportunity to diffuse further in the presynaptic direction before being removed by transporters. Weather this anisotropy leads to differences in the spatial dynamics of glutamate diffusion and receptor activation around pre- and postsynaptic membranes remains to be determined.

Recent studies have shown that whisker stimulation enhanced the expression of GLT-1 and GLAST 2- to 4- fold in the corresponding cortical column, an effect that was accompanied by an increase in the extent of glial coverage (69). These provocative findings, and previous results showing that fiber lesions result in a rapid decrease in GLT-1 expression in the target field (71), suggest that the involvement of transporters in glutamate clearance may be highly dynamic. Studies of excitatory

synapses in the hypothalamus have shown that physiological changes in the extent of astrocytic ensheathment can have profound effects on the profile of glutamate and the pattern of receptor activation at excitatory synapses. During lactation, astrocytes in the hypothalamus retract their processes from excitatory synapses (193), which results in a decrease in the density of EAAT2 near presynaptic mGluRs. Correspondingly, the absence of transporters in lactating animals allows enhanced tonic inhibition due to an elevation in ambient glutamate, and greater numbers of mGluR autoreceptors to be activated following activity-dependent glutamate release (142).

5.4 A Role for EAAT3 in Transmitter Clearance?

Simple conservation suggests that there would be advantages for neurons to recapture glutamate by transporting it back into the presynaptic terminal. The best candidate for neuronal uptake is EAAT3 (EAAC1), as it is expressed by neurons throughout the CNS (165). However, this transporter is found mainly in cell bodies and dendrites of principal neurons and occasionally in the terminals of GABAergic interneurons. Furthermore, in contrast to GLAST and GLT-1, which are primarily found at the cell surface, the majority of EAAC1 protein is found in the cytosol (41). In accordance with these findings, EAAT3-mediated transporter currents cannot be resolved in neurons (21), suggesting that it is expressed at a much lower level than GLAST or GLT-1, and CNS deficits have not been observed in EAAT3 knockout mice (150).

It is important to recognize that the threshold for detection of transporters using electrophysiological methods is quite high, even when transport-associated currents are amplified by recording in the presence of permeant anions. Thus, an inability to resolve transporter currents does not necessarily indicate that transporters are not present. Indeed, a density of transporters below the detection limit could likely still exert a significant effect on glutamate dynamics if they are localized near receptors. Although transporter currents have not been observed in CA1 pyramidal neurons, inhibition of postsynaptic uptake by substituting intracellular K^+ with N-methyl-D-glucamine, which does not support the final counter-transport step of the transport cycle, enhanced spillover of glutamate and prolonged NMDA receptor-mediated currents in these neurons. These findings raise the possibility that despite their low density, glutamate transporters in the postsynaptic membrane can influence extracellular glutamate dynamics.

EAAT3 may also be involved in a variety of metabolic processes. Adult animals administered antisense oligonucleotides against EAAT3 exhibited seizures (164). The change in neuronal excitability following acute loss of EAAT3 may result from a decrease in the GABA synthesis, as antisense treated mice exhibited a decrease in GABA levels and a reduction in the amplitude of miniature IPSCs (171), suggesting that less GABA was available for loading into synaptic vesicles. Furthermore, if glutamate transporters are inhibited during bouts of GABA release, the subsequent release of GABA is reduced (130), suggesting that glutamate uptake and GABA synthesis are tightly linked. These findings highlight the fact that glutamate is used in many other cellular processes, including protein synthesis, deamination of

ammonia, and coping with reactive oxygen species through transport of cystine, a precursor of glutathione. It is likely that even high affinity glutamate transporters may play an essential role in providing glutamate for these metabolic processes.

5.5 EAAT4 Shapes Glutamate Transients at Cerebellar Synapses

A prominent role for neuronal uptake has been demonstrated at excitatory synapses in the cerebellum. EAAT4 is abundant in the perisynaptic membrane where mGluR1 densities are highest. If postsynaptic glutamate uptake is inhibited by perfusing Purkinje cells with a $Tris^+$ -based solution, which does not support transporter cycling, parallel fiber evoked mGluR currents are enhanced, and the threshold for the induction of long term depression (LTD) is lowered (27). Furthermore, Purkinje cells that express less EAAT4 show enhanced mGluR-dependent slow inward currents, greater LTD, and greater cannabinoid-mediated inhibition of IPSCs (209), suggesting that EAAT4 transporters normally shield mGluRs from synaptic glutamate. Parallel and climbing fiber mediated EPSCs also decay more slowly in the majority of Purkinje cells in EAAT4 knockout mice, supporting a role for EAAT4 in the rapid clearance of glutamate from these excitatory synapses (186). The dramatic slowing of climbing fiber EPSCs in EAAT4 knockout mice is surprising, as estimates of glutamate translocation based on the size of synaptically evoked transporter currents in Purkinje neurons, currents that are mediated exclusively by EAAT4 (95), indicate that EAAT4 removes less than 20% of the glutamate released from climbing fiber terminals (26, 95, 144).

5.6 EAAT5: A Glutamate-Gated Chloride Channel

The expression of EAAT5 in the terminal membranes of photoreceptors and bipolar cells in the retina (86, 148) suggest that this transporter may perform a unique role in synaptic function. Although all EAATs are permeable to anions (206), EAAT5 allows a much greater flux of Cl^- when glutamate is bound (8). This associated Cl^- conductance is so large that depolarization -induced release of glutamate from a bipolar cell terminal triggers an overt Cl^- current through autoactivation of EAAT5 transporters (148). Furthermore, in the presence of SCN^-, which is highly permeant through the EAAT5-associated anion channel, quantal transporter-mediated anion currents can be resolved in these cells (204). By lowering the membrane resistance (shunting) and by hyperpolarizing the terminal membrane, EAAT5 activation leads to a decrease in the release of glutamate onto amacrine cells. Thus, EAAT5 adds an additional feedback inhibitory influence on bipolar cells.

The presence of EAAT5 in terminal membranes suggests that it may also contribute to clearance. However, the number of glutamate molecules captured by these transporters, which has been estimated by taking into account the size of the EAAT5-mediated Cl^- current, the unitary conductance of the EAAT5 anion channel, and the number of vesicles released (measured through capacitance recordings), indicate that much less than 1% of the glutamate released is captured by EAAT5 (148). Therefore, at bipolar cell terminals, EAAT5 appears to function primarily as a glutamate-gated Cl^- channel rather than a sequestration mechanism. However,

recent studies suggest that presynaptic EAAT5 may contribute significantly to uptake at ribbon synapses between rods and bipolar cells. Recordings from bipolar cells revealed that the prolongation of synaptic currents by TBOA could not be accounted for by clearance through GLT-1, GLAST or EAAC1 (86). In addition, EAAT5 was not found in the postsynaptic membrane of bipolar cells, pointing to a role for EAAT5 in rod terminal membrane in the clearance of glutamate. These results suggest that glutamate clearance mechanisms vary between ribbon synapses in sensory cells of the auditory and visual pathways.

6 Concluding Remarks

Studies of glutamate clearance at excitatory synapses indicate that the involvement of transporters varies considerably in different brain regions, paralleling the striking anatomical and functional diversity of synapses. Although there are risks to generalizing findings from a few model synapses, some general rules have emerged. At the majority of synapses, rapid diffusion and subsequent dilution of glutamate in the extracellular space is sufficient to drop the concentration of glutamate under conditions of isolated release. However, EAAT-dependent uptake plays an important role in restricting the diffusion of glutamate to extrasynaptic receptors and preventing glutamate transients from reaching neighboring synapses. Glutamate transport is accomplished outside the cleft; excluding transporters from the cleft presumably ensures that they do not compete with low affinity receptors for glutamate binding. The dominant route for glutamate transport is through EAAT2 at synapses in the forebrain, and EAAT1 and EAAT4 at synapses in the molecular layer of the cerebellum. Transport of synaptic glutamate into pre- and postsynaptic neurons appears to be restricted to a subset of synapses in the cerebellum and the retina. It is likely that EAAT-dependent uptake is dynamic, and changes in relation to the association of astroglial cells with synapses.

The overwhelming reliance on transporters in glial cells suggests that there is a clear division of labor at excitatory synapses, with neurons involved in packaging and releasing glutamate and astroglial cells involved in removing it from the extracellular space. It is clear that astrocytes provide an environment that is better suited for glutamate uptake. Astrocytes have a higher resting potential than neurons, which fluctuates little during activity. As the ability of EAATs to take up glutamate decreases with depolarization, this ensures that uptake will continue during high rates of activity. Repetitive depolarization, higher intracellular levels of glutamate, and repeated Na^+ influx during activity, would be expected to reduce the capacity of neurons for glutamate uptake. In addition, under extreme conditions, large numbers of glutamate transporters in neurons could be detrimental, due to the ability of EAATs to cycle in reverse. However, these interpretations assume that maximal influx of glutamate under all conditions is the ultimate goal. It is possible that activity dependent reductions in the efficiency or capacity of transport could be used to enhance the activation of perisynaptic receptors during periods of high release, in effect creating a frequency filter by placing transporters and receptors in close proximity.

Acknowledgements

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