Chapter 12
Transport of Amino Acid Neurotransmitters into Synaptic Vesicles

Shigeo Takamori

Abstract Fast chemical neurotransmission at the synapse is mediated by the release of neurotransmitters from synaptic vesicles (SVs) by exocytosis. In the mammalian central nervous system, the majority of neurons utilize amino acids such as glutamate, γ-aminobutyric acid (GABA), and glycine. Glutamate is the major excitatory neurotransmitter, whereas GABA and glycine are inhibitory. These amino acids are present at relatively high levels in the cytoplasm of presynaptic terminals and are accumulated into SVs for their exocytotic release. Over the past several decades, this essential process has been biochemically characterized and proteins responsible for neurotransmitter loading have been molecularly identified. Analysis of knockout animals has elucidated physiological significance of this process and moreover has deepened our understanding of glutamatergic and GABAergic neural circuits. However, the precise mechanism of the transport system remains largely unknown. In this chapter, I overview advances in the vesicular loading process and discuss some controversial concepts that may have important consequences for synaptic transmission.

Keywords Synaptic vesicles • Vesicular glutamate transporter • Vesicular GABA transporter • Vacuolar-type H^+ ATPase • Cl^- channels

12.1 V-ATPase Drives Neurotransmitter Uptake

Unlike plasma membrane neurotransmitter transporters that utilize the Na^+ gradient across the membrane as a driving force, transporters responsible for neurotransmitter refilling into SVs rely on a proton electrochemical gradient across the vesicle membrane, generated by the vacuolar-type H^+ ATPase (V-ATPase) (Moriyama et al. 1992). The V-ATPase consists of at least 13 subunits, comprising the largest protein complex (~800 kDa complex) present on SVs (Takamori et al. 2006) (see Chap. 7). It is divided into two functional domains: V1 is a peripheral sector and V0 is a membrane sector. The overall structure resembles the mitochondrial F_0F_1-ATP

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S. Mochida (ed.), *Presynaptic Terminals*, DOI 10.1007/978-4-431-55166-9_12

synthase which catalyzes ATP synthesis from ADP by using the H^+ gradient (Nelson et al. 2002). V-ATPase performs the exact opposite task. It uses the hydrolyzing energy of ATP and translocates H^+ from the cytoplasm into the vesicle lumen. As a result, the lumen becomes more acidic and the voltage more positive, both of which act to drive neurotransmitter transport into SVs. While the potential across the SV membrane has not been experimentally determined, the pH gradient across SV membrane has been estimated from vesicular pH measurements (Maycox et al. 1988; Moriyama and Nelson 1987). By using pH-sensitive GFP variants, which are targeted to the luminal region of SVs in neuronal culture preparations, vesicular pH was determined as 5.6–5.8 (Miesenbock et al. 1998). Assuming that the cytoplasmic pH is close to neutral (7.0–7.4), ΔpH was estimated to be approximately ~1.5 pH unit (~30-fold gradient). It should be noted that because of the small size of SVs (diameter of 40 nm), a pH of 5.5 ($[H^+] = 10^{-5.5}$) corresponds to less than one free H^+ in the lumen (Takamori et al. 2006). Although little is known about the intrinsic buffering of the vesicle lumen, this simple calculation indicates that few H^+ ions are required to establish the proton electrochemical gradient. This markedly contrasts to Na^+, or other ion gradients such as K^+ or Cl^-, across the plasma membrane. Moreover, in contrast to the relatively stable ion gradient of Na^+ and K^+ across the plasma membrane, the proton electrochemical gradient across the SV membrane is made and dissipated during repeated exocytosis and endocytosis. Given the continuous activity at synapses, it is probably more economical for SVs to utilize minor ion species such as H^+, which involves less ion movement than major ions such as Na^+.

12.2 The Regulation of $\Delta\mu H^+$

The proton electrochemical gradient ($\Delta\mu H^+$) generated by V-ATPase consists of an electrical gradient (ΔΨ, inside positive) and a chemical gradient (ΔpH, inside acidic) (Fig. 12.1). Their relative influence on the $\Delta\mu H^+$ can be modulated by other ion channels or transporters present on SVs. The extent of both components has been assessed in isolated SVs by fluorometric assays using either voltage-sensitive dyes (i.e., oxanol) for ΔΨ or membrane-permeable weak-base dyes such as acridine orange for ΔpH (Maycox et al. 1988; Tabb et al. 1992). V-ATPase is an electrogenic pump; thus, in the absence of counterion movements, H^+ influx is restricted by the voltage developed as a result of H^+ entry, and thereby a large membrane potential (ΔΨ) is established. On the other hand, when counterions are present, their movement across the SV membrane compensates for charges associated with H^+ influx. While this facilitates greater H^+ influx into the lumen to establish a greater pH gradient (ΔpH), ΔΨ is attenuated (Fig. 12.1a, b). The physiological counterion that affects $\Delta\mu H^+$ is chloride. It has been shown that ΔΨ is maximal in the absence of Cl^- (and other haloids), while ΔpH is very small. As extravesicular Cl^- increases, ΔΨ is attenuated, whereas a larger pH

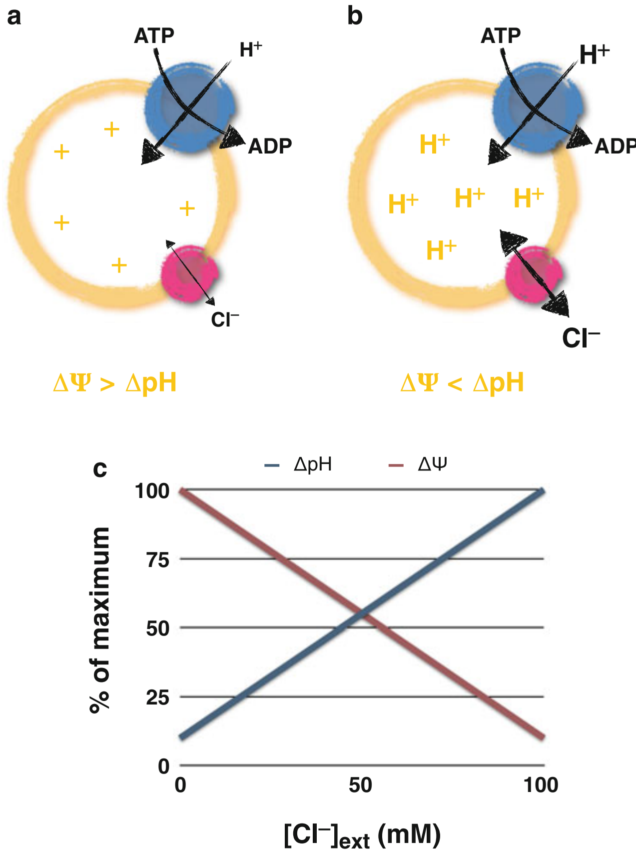

Fig. 12.1 Electrogenic property of the vacuolar-type H^+ ATPase. Vacuolar-type H^+ ATPase (V-ATPase) (*blue*) utilizes an energy produced by ATP hydrolysis and generates a proton electrochemical gradient that consists of two components, ΔΨ and ΔpH. Since the V-ATPase is electrogenic, H^+ influx is critically restricted by ΔΨ, and either accompanying anon influx or efflux of cation is necessary to facilitate proton transport into the lumen. SVs contain a putative Cl^- channel (*pink*) that affects the balance of ΔΨ and ΔpH. (**a**) In the presence of low Cl^-, H^+ influx is small and more ΔΨ is generated. (**b**) In the presence of high Cl^-, Cl^- influx would dissipate ΔΨ; therefore, more H^+ can flow into SVs. This facilitates ΔpH buildup and attenuates ΔΨ. (**c**) The relationship between ΔΨ and ΔpH as a function of external Cl^- concentrations. Both components have been monitored by using fluorescent indicators for the voltage and ΔpH

gradient is generated (Fig. 12.1c). The molecular identity of this Cl^- pathway has been elusive (discussed below).

Experimentally, it is possible to manipulate the two components of $\Delta\mu H^+$ in isolation. For instance, ammonia (NH_3) is a proton acceptor and can penetrate into the SV lumen where it attenuates ΔpH in a concentration-dependent manner (Hell et al. 1988). Likewise, the K^+/H^+ exchanger, nigericin, produces proton efflux in concert with K^+ influx, to selectively dissipate ΔpH yet facilitate ΔΨ (Tabb

et al. 1992). The Mg^{2+}/H^+ exchanger, A23187, has also been used as a substitute for nigericin (Wolosker et al. 1996). In contrast, the K^+ ionophore, valinomycin, eliminates the formation of $\Delta\Psi$ and therefore strongly augments ΔpH (Moriyama and Nelson 1987). Given that changes in the composition of $\Delta\mu H^+$ have been indirectly monitored, the absolute values of intravesicular pH and transmembrane voltage in any conditions are unknown. Yet, it is generally believed that for cationic neurotransmitters such as acetylcholine and monoamines, uptake depends largely on ΔpH. By contrast, the uptake of anionic neurotransmitter such as glutamate depends predominantly on $\Delta\Psi$. The zwitterionic transmitters GABA and glycine both depend on ΔpH and $\Delta\Psi$ for efficient uptake (Edwards 2007). As will be discussed in the following sections, the precise mechanism of how H^+ drives neurotransmitter transport is not completely understood.

12.3 Mechanisms of Neurotransmitter Uptake into Synaptic Vesicles

The biochemical mechanism underlying the uptake of amino acid neurotransmitters into SVs has been characterized using isolated SVs from mammalian brains. Glutamate uptake into SV preparations was shown to be dependent on ATP hydrolysis, because proton ionophores such as CCCP or FCCP abolished ATP-dependent glutamate accumulation, demonstrating that the process is dependent on a proton gradient (Naito and Ueda 1983, 1985). A prominent feature of glutamate uptake into SV preparations is an unusual biphasic dependence on chloride concentrations in the assay medium (Naito and Ueda 1985). Glutamate uptake activity was found to be maximal in the presence of ~mM of Cl^-, yet relatively depressed at lower or higher Cl^- concentrations. The extent of depression depended on glutamate concentrations used for the assays. In terms of the composition of $\Delta\mu H^+$, at low mM Cl^-, $\Delta\Psi$ predominates over ΔpH. Manipulations to selectively dissipate ΔpH, such as nigericin and NH_4^+ applications, had no overwhelming effect on glutamate uptake, whereas those that abolished $\Delta\Psi$ such as valinomycin strongly reduced it (Hell et al. 1988; Moriyama et al. 1990; Naito and Ueda 1985). Given that the majority of glutamate at neutral pH exists in the negatively charged form, these results led to a long-standing belief that glutamate uptake is predominantly dependent on $\Delta\Psi$ across the SV membrane. In fact, when $\Delta\Psi$ was artificially generated by VAL and Rb^+, significant glutamate transport was detected, albeit to a lesser extent (~50-fold or more) than that achieved by the activation of V-ATPase (Shioi and Ueda 1990). This led to the extreme hypothesis that $\Delta\Psi$ alone is sufficient to drive glutamate transport. Furthermore, the nonselective anion channel blocker, DIDS, was found to attenuate V-ATPase activity and glutamate transport with different IC_{50} values, leading to another hypothesis that Cl^- directly binds to the glutamate transporter and allosterically activates it (Hartinger and Jahn 1993). This theory was also able to explain why low

transport activity was observed in the absence of Cl^-. In this context, the attenuation of glutamate transport in high Cl^- conditions can be explained by the attenuation of $\Delta\Psi$ as Cl^- increases. On the other hand, there is substantial evidence to suggest the pH gradient is required for glutamate transport. First, 20 mM NH_4^+ dramatically reduced uptake, a concentration that is thought to completely abolish ΔpH yet facilitate $\Delta\Psi$ (Carlson et al. 1989). Second, application of either NH_4^+ or A23187 completely dissipated ΔpH, but effects on glutamate uptake were dependent on the Cl^- and glutamate concentrations used to measure glutamate transport activity. Both treatments facilitated glutamate transport at low Cl^- concentrations but attenuated it at higher Cl^- concentrations (Wolosker et al. 1996), indicating a complex regulation of glutamate transport by both Cl^- and $\Delta\mu H^+$. Third, when intravesicular pH was clamped with external solution by using nigericin, glutamate transport was sensitive to pH, reaching a maximum at pH ~6.8 and thus indicating that not only ΔpH but also optimal pH is required to drive glutamate transport (Tabb et al. 1992). These results suggest that pH is an essential factor for glutamate transport and optimal protonation of amino acid side chains of the transporter is crucial for its function. Investigations to clarify the mechanisms involved have largely used reconstitution systems (see below).

There has been some evidence that outward leak of neurotransmitter from the synaptic vesicle may affect transport kinetics (Wolosker et al. 1996). In vitro radiotracer flux assays have demonstrated that this dissipation of ΔpH promotes glutamate efflux. Furthermore, glutamate efflux was promoted by the application of Cl^- at relatively high concentrations similar to that attained at steady state following glutamate transport. Collectively, such evidence indicates that changes in intravesicular pH affect glutamate storage. Although the mechanism and pathway for this glutamate leak has not been established, these results suggest that the combination of glutamate efflux and influx determine the rate of glutamate transport. Despite these biochemical observations, whether $\Delta\mu H^+$ is essential for neurotransmitter maintenance in the SVs remains controversial. Application of the V-ATPase inhibitor, Bafilomycin A_1 ($BafA_1$), in hippocampal autaptic cultures resulted in activity-dependent attenuation of EPSCs supporting the idea that there is almost no glutamate leak from already filled SVs (Ikeda and Bekkers 2009). In contrast, glutamate transported into isolated SVs in vitro was found to be somewhat more leaky. Acidification of SVs by glutamate can be monitored by acridine orange, thus providing a proxy measure for glutamate transport into SVs (Maycox et al. 1988). Upon application of $BafA_1$ or FCCP, such acidification was rapidly reversed, indicating that glutamate as well as H^+ leaks out by disrupting $\Delta\mu H^+$ (Hnasko et al. 2010; Maycox et al. 1988). Consistent with these observations, glutamate leak was also observed in an assay of radio-labeled glutamate flux, although the rate and extent of the efflux therein seemed to be dependent on $BafA_1$ in a concentration-dependent manner (our unpublished observations). In addition to glutamate, proton leakage exhibited different properties both in vitro when monitored by acridine orange and also in vivo measured with a pH-sensitive luminal probe (i.e., pHluorin). As mentioned, $BafA_1$ rapidly reversed acridine orange quenching, while $BafA_1$ application did not dramatically alter the acidity

of the SV lumen, measured as fluorescent changes from pHluorin expressing SVs (Sankaranarayanan and Ryan 2001). Therefore, it is unclear if glutamate efflux, as well as H^+ leak, is relevant in physiological conditions.

In contrast to glutamate transport, transport of inhibitory neurotransmitters such as GABA and glycine into SVs is less well characterized. Due to the low affinity of the carrier (>5 mM) and the meager amount of GABA-containing SVs isolated from whole brain tissues, the signal-to-noise ratio of GABA/glycine uptake assays is much lower than that for glutamate (Burger et al. 1991; Takamori et al. 2000b). Nevertheless, GABA/glycine uptake also depends on a proton electrochemical gradient generated by the V-ATPase (Fykse et al. 1989; Hell et al. 1991; Kish et al. 1989). Selective dissipation of $\Delta\Psi$ and ΔpH revealed that GABA and glycine transport into SVs was moderately inhibited by both modifications. As with glutamate transport, the uptake of GABA and glycine was dependent on Cl^-, albeit to a lesser degree (Hell et al. 1990; Kish et al. 1989). Collectively, GABA and glycine transport has been proposed to be dependent equally on $\Delta\Psi$ and ΔpH. How these gradients drive GABA and glycine transport is uncertain, though a recent study that reconstituted vesicular GABA transporter protein has proposed a novel model in which GABA transport is driven solely by $\Delta\Psi$ and is stoichiometrically coupled to Cl^- transport (Juge et al. 2009) (see more details below in Sect. 12.6).

12.4 Molecular Identities of Vesicular Glutamate and GABA Transporters

12.4.1 VGLUTs

The vesicular glutamate transporters were originally cloned as Na^+-dependent inorganic phosphate transporters and categorized into the group of type I phosphate transporters (Takamori 2006). The gene encoding one of the phosphate transporter family members has been identified to be upregulated in cortical neuron preparations following exposure to sub-toxic levels of a glutamate receptor agonist. The gene was highly homologous to the founding member of the phosphate transporter family, NaPi-1, and highly expressed in the central nervous system. When mRNA of the gene was heterologously expressed in *Xenopus oocytes*, substantial Pi uptake was observed in a Na^+-dependent manner. This evidence led the gene responsible to be named brain-specific Na^+-dependent inorganic phosphate transporter 1 (BNP1). However, subsequent studies on BNP1 protein suggested it was localized to SVs rather than the plasma membrane (Bellocchio et al. 1998; Takamori et al. 2000a). Moreover, BNP1 was not ubiquitously expressed in all neurons in the CNS, but restricted to those containing glutamate (Bellocchio et al. 1998). When BNP1 was heterologously expressed in neuroendocrine cell lines, vesicles isolated from these cell lines were capable of transporting glutamate. Intriguingly, this process was akin to that observed in a biochemical transport assay of isolated SVs, suggesting

that BNP1 indeed functions as a vesicular glutamate transporter (Bellocchio et al. 2000; Takamori et al. 2000a). Notably, by expressing BNP1 in non-glutamatergic neurons in culture, the cells were subsequently capable of quantal glutamate release. Such data clearly supports a primary role of BNP1 for glutamate transport into SVs, and consequentially, BNP1 was renamed as vesicular glutamate transporter 1 (VGLUT1).

VGLUT1 expression was not detected in all brain regions where glutamatergic synaptic transmission is thought to occur. In areas such as the hypothalamus and striatum, the absence of VGLUT1 expression promoted the hypothesis that other VGLUT isoforms may be responsible for glutamate uptake. Indeed, two more VGLUT isoforms were identified and named: VGLUT2 and VGLUT3 (Takamori 2006 and references therein). The genes for VGLUT1, VGLUT2, and VGLUT3 are now categorized into the solute carrier protein (SLC17) family and coded as SLC17A7, SLC17A6, and SLC17A8, respectively. The amino acid sequences of VGLUT1~3 share nearly 80 % identities. Specifically, their transmembrane regions are almost identical to one another (>95 % identities), yet their N- and C-terminal cytoplasmic tails are highly variable, indicating that all three share a common mechanism in glutamate transport and diversities among VGLUTs may exist in their trafficking that requires distinct protein-protein interactions with cytosolic components. Indeed, the glutamate transport activity of VGLUT isoforms did not significantly vary in measurements from heterologous systems, whereas the subcellular distributions of individual VGLUTs in neuroendocrine cell lines were somewhat different. When VGLUT1 and VGLUT2 were expressed in PC12 cells, a greater quantity of VGLUT2 was localized at the cell surface compared to VGLUT1 (Fremeau et al. 2001). In addition, throughout the CNS, VGLUT1 and VGLUT2 are almost exclusively expressed at the presynaptic terminals of glutamatergic neurons, whereas VGLUT3 is expressed not only at presynaptic terminals but, in some interneurons, is also present at somatodendritic locations (Fremeau et al. 2002). Some of the key factors that determine cellular localization of VGLUTs will be discussed separately in Sect. 12.7.

In addition to the distinct subcellular localization of VGLUT isoforms within neurons, the three isoforms show complementary expression patterns in the mammalian CNS with very little overlap. In the adult brain, excitatory neurons in the cortex, hippocampus, and cerebellar cortex (granule cells) mainly express VGLUT1, whereas excitatory neurons in the thalamus and brainstem predominantly express VGLUT2 (Fremeau et al. 2001; Fujiyama et al. 2001). Consistent with their expression patterns, glutamatergic transmission in the hippocampus was largely shut down in VGLUT1 knockout mice (Fremeau et al. 2004; Wojcik et al. 2004), whereas in the thalamus, transmission was lost in VGLUT2 knockout mice (Moechars et al. 2006). Some residual glutamatergic transmission was observed in hippocampal preparations (both cultured hippocampal neurons and hippocampal slices) to suggest that VGLUT2 is transiently expressed during early postnatal development. Indeed, the developmental regulation of VGLUT1 and VGLUT2 expression occurs in many brain regions (Miyazaki et al. 2003; Nakamura et al. 2005). Overall, the expression of VGLUT1 is very low at birth

and increases during postnatal development up to 3 weeks, whereas the expression of VGLUT2 is detectable already at birth and stays relatively constant throughout postnatal development. Consistent with the low expression of VGLUT1 in early postnatal development, VGLUT1 knockout mice survive up to 3 weeks after birth without any apparent deficiency, suggesting its limited importance for glutamatergic transmission during development. VGLUT1 knockout mice begin to suffer from feeding and die, in most cases, during postnatal 4th week (Fremeau et al. 2004; Wojcik et al. 2004). In contrast, VGLUT2 knockout mice die immediately after birth presumably due to the lack of glutamatergic transmission in the brainstem involved in the generation of respiratory rhythm (Moechars et al. 2006; Wallen-Mackenzie et al. 2006). In contrast to the expression of VGLUT1 and VGLUT2, VGLUT3 is expressed in a small population of neurons not traditionally considered to be glutamatergic neurons, including cholinergic neurons in the striatum, a small set of GABAergic interneurons in the cortex and the hippocampus, and dopaminergic neurons in the raphe (Fremeau et al. 2002). VGLUT3 knockout mice exhibit non-convulsant seizures and deafness (Ruel et al. 2008; Seal et al. 2008). The latter is likely caused by a loss of glutamate release from inner hair cells in the cochlea where VGLUT3 is highly expressed (Akil et al. 2012). Furthermore, VGLUT3 knockout mice exhibited an unexpected hypercholinergic phenotype, suggesting that, together with the fact that VGLUT3 is coexpressed with vesicular acetylcholine transporter, glutamate transport by VGLUT3 affected the amount of acetylcholine in the same vesicles (Gras et al. 2008).

12.4.2 VGAT

Vesicular GABA transporter was identified as a rat orthologue of unc-47, one of the *C. elegans* genes responsible for the GABAergic transmission (McIntire et al. 1997; Sagne et al. 1997). In the worm, the 26 GABA-expressing neurons are required to inhibit contractions of the head muscles during foraging, to inhibit contractions of the body muscles during locomotion, and to stimulate contraction of the enteric muscles that mediate the defecation cycles. The unc-47 mutant showed impairments of all behaviors and, importantly, responded normally to GABA receptor agonists, indicating the role of unc-47 gene product at the presynaptic site. Furthermore, the unc-47 gene product was selectively localized to SVs. When a rat orthologue of unc-47 was transfected into PC12 cells, the isolated intracellular membrane acquired the ability to transport GABA, in a similar manner to that observed in isolated synaptic vesicles, adding weight to the idea that the unc-47 gene product functions as a vesicular GABA transporter (VGAT). Similar to synaptic vesicles, GABA uptake by VGAT was competitively inhibited by glycine, albeit with lower efficiency (Burger et al. 1991), supporting the notion that these inhibitory neurotransmitters in the CNS share a common transporter (thereby, VGAT is also referred to as vesicular inhibitory amino acid transporter, VIAAT) (Sagne et al. 1997). In fact, VGAT is not only localized at glutamic acid

dehydrogenase (GAD)-positive GABAergic terminals but also at putative glycinergic terminals (Chaudhry et al. 1998; Wang et al. 2009). Furthermore, loss of VGAT caused a drastic reduction of neurotransmitter release in both GABAergic and glycinergic neurons, further suggesting that GABA and glycine are transported by the same transporter (Wojcik et al. 2006). Recent studies revealed that the plasma membrane glycine transporters (GlyT2) ensured high levels of cytoplasmic glycine at glycinergic terminals (Rousseau et al. 2008), whereas the presence of GAD was associated with high levels of cytoplasmic GABA at GABAergic terminals. In this respect, although they share the same vesicular transporter to accumulate the respective neurotransmitters, the type of neurotransmitter is defined by the relative concentration of transmitter in the cytoplasm. Little is known about how VGAT is regulated and the subsequent effects on inhibitory neurotransmission. Although VGAT is constitutively phosphorylated in vivo, dephosphorylation of VGAT did not alter its transport activity (Bedet et al. 2000).

Inactivation of VGAT led to embryonic lethality as a result of an abdominal defect known as omphalocele (Saito et al. 2010; Wojcik et al. 2006), which was also observed in GAD67 knockout mice. Both effects indicate a crucial role for GABAergic transmission during prenatal development. As expected, the loss of VGAT caused a drastic reduction in both GABAergic and glycinergic neurotransmission. However, as a reduced number of IPSCs remained, it is possible that GABA release could have occurred independently of VGAT (Wojcik et al. 2006). Whether another VGAT isoform was responsible or the residual IPSCs were caused by non-vesicular GABA release remains uncertain.

12.5 Channels and Transporters: $\Delta\mu H^+$ Modulators?

Since vesicle refilling is a secondary active transport process, modulating the driving force would have profound consequences. Little is known on the regulation of V-ATPase function; however, it is thought that the proportion of ΔpH and $\Delta\Psi$ is affected by the permeability of counterions that accompany H^+ flux (Grabe and Oster 2001). As described above, one of the factors that potentially affect $\Delta\mu H^+$ is a chloride channel that supports vesicle acidification. From earlier evidence it was proposed that the ClC-3 chloride channel was partly responsible for the acidification of SVs (Stobrawa et al. 2001). This was supported by observations from crude SV fractions isolated from ClC-3 knockout mouse brains, which exhibited significantly lower Cl^--induced acidification. However, this conclusion was complicated by the fact that ClC-3 knockout mice underwent severe neuronal degeneration after 2 weeks, with complete loss of hippocampal structures in adulthood. Moreover, expression of VGLUT1, glutamate-induced acidification, and glutamate uptake were all reduced in the SV fraction. Thus, the alternative hypothesis is that the impairment of Cl^--induced SV acidification resulted from a reduction in VGLUT1-laden SVs, rather than the loss of ClC-3. Indeed, the amplitude of miniature EPSCs recorded from CA1 hippocampal pyramidal cells did not differ between wild-type

and ClC-3 knockout brains, further casting doubt that ClC-3 was responsible for SV acidification and, by association, Cl^--dependent glutamate uptake. This hypothesis was supported by subsequent evidence from ClC-3-deficient SVs isolated prior to the onset of neural degeneration, which showed normal Cl^--dependent acidification, glutamate uptake, and VGLUT1 expression (Schenck et al. 2009). In contrast, vesicles isolated from VGLUT1-overexpressing PC12 cells exhibited an enhanced Cl^--induced acidification, raising the possibility that VGLUT1 itself has a Cl^- conductance (Bellocchio et al. 2000). Indeed, SVs isolated from VGLUT1 knockout mice showed a marked reduction in Cl^--induced SV acidification, suggesting that VGLUT1 has a chloride conductance responsible for SV acidification (Schenck et al. 2009). However, this model has been recently challenged (Juge et al. 2010) (see below). In any case, the Cl^- influx into SVs has only been monitored indirectly using acridine dye measurements of Cl^--induced acidification. A direct demonstration of Cl^- transport via VGLUT1 will be necessary to clarify the model.

As with glutamate transport, a role for ClC-3 in GABA loading into SVs has been proposed (Riazanski et al. 2011). When miniature IPSCs in the hippocampal CA1 region of ClC-3 KO mice were analyzed, there was a clear reduction in both their amplitude and frequency. Furthermore, the SV fraction devoid of ClC-3 showed deficient Cl^--induced acidification as revealed by an acridine orange assay. Collectively, it was suggested that the additional acidification of GABA vesicles resulting from ClC-3 activity would facilitate proton-dependent GABA loading into SVs. As with glutamate, the role of CIC-3 in GABA uptake and its significance to synaptic transmission remains controversial (Stauber and Jentsch 2013).

The cation/H^+ exchange has been proposed to decrease ΔpH and thus increase $\Delta\Psi$. Such a process clearly diverges from anion conductance that would facilitate ΔpH. In an in vitro acidification assay, addition of Ca^{2+} reduced ΔpH once a stable baseline was established (Goncalves et al. 1999a, b). More recently, a cation/H^+ exchange mechanism was demonstrated in isolated SVs, and a Na^+/H^+ exchanger (NHE) detected in the SV proteome (Goh et al. 2011; Gronborg et al. 2010). NHE activity pushes out protons at the expense of Na^+ (or K^+) influx. As a result, ΔpH was decreased and $\Delta\Psi$ was facilitated, providing favorable conditions for glutamate uptake. Moreover, replacing cytoplasmic K^+ with a non-permeable cation ($NMDG^+$) attenuated quantal release of glutamate at the calyx of Held synapse, indicating the importance of cation/H^+ exchange for normal glutamate loading into SVs (Goh et al. 2011).

In addition to biochemical demonstrations of vesicular channels/transporters, it is possible to take electrophysiological recordings on SV membranes once they have been incorporated into liposomes or planner bilayers. From these preparations three cation conductances and one anion conductance were detected (Sato et al. 1992). Intriguingly, although not demonstrated in mammalian SVs, the ion conductances in the Torpedo electric organ SVs were sensitive to pH (Ahdut-Hacohen et al. 2004). Specifically, these channels were active at neutral pH and inactivated in acidic conditions. To date, the relationship between the ion conductances and indications from biochemical experiments remains enigmatic; furthermore, how these ion permeations translate into neurotransmitter loading is unknown.

12.6 Reconstitution: Simpler Is Better?

The cloning of VGAT and the three VGLUT isoforms has allowed for detailed investigation into their mechanism of action. By expressing the proteins in isolation, this system avoids complexities resulting from other SV proteins. For instance, their effect on $\Delta\mu H^+$ could be studied in the absence of potentially confounding influences from putative Cl^- channels and cation/H^+ exchanger proteins. The reconstitution of recombinant VGLUT1 protein with a F_0F_1-ATP synthase (which can be used as a proton pump) facilitated Cl^--induced acridine orange quenching in a dose-dependent manner (Schenck et al. 2009). This is consistent with the fact that overexpression and gene knockout of VGLUT1 resulted in facilitated and reduced Cl^--induced acidification, respectively (Bellocchio et al. 2000; Schenck et al. 2009), supporting that VGLUT1 has a Cl^- conductance. Having demonstrated that glutamate transport and the Cl^- conductance coexist in VGLUT1 proteins, it was subsequently shown that $\Delta\Psi$-driven (nigericin-resistant) glutamate uptake was markedly facilitated when liposomes were filled with high concentration of Cl^-. Thus, the Cl^- efflux pathway appears to be required for efficient glutamate influx by maintaining charge neutrality during transport cycle. In addition, VGLUT1 liposomes also exhibited a significant nigericin-sensitive uptake that depended on external Cl^- concentrations, indicating that VGLUT can also be driven by ΔpH. Remarkably, Cl^--filled VGLUT1 liposomes took up glutamate very efficiently even in the absence of external Cl^- and thus strongly opposed the previous hypothesis that Cl^- directly binds to the carrier and allosterically activates it (Hartinger and Jahn 1993). One intrinsic caveat of the experimental strategies of the study was that the composition of $\Delta\mu H^+$ could be affected when Cl^- was replaced with other anions such as gluconate. More recent reconstitution studies, however, have argued against several important conclusions summarized above. Juge and colleagues used a reconstitution system in which the proton pump was omitted (Juge et al. 2010). Instead, the authors created $\Delta\Psi$ by manipulating internal and external K^+ concentrations in the presence of the K^+ ionophore valinomycin. They demonstrated glutamate uptake occurred transiently and peaked within 2 min. Interestingly, glutamate transport elicited by the Val-induced K^+-diffusion potential was greatly reduced in the absence of external Cl^- and drastically increased by several mM of Cl^-, with a Hill coefficient of 3.3. Furthermore, when ΔpH was imposed by simply loading liposomes with a solution of pH 5.5 in the assay buffer (pH 7.0), there was no significant glutamate uptake. This led the authors to propose that VGLUT functions as a Cl^--activated glutamate uniporter whose driving force was solely provided by $\Delta\Psi$ (Fig. 12.2).

Juge and colleagues also investigated GABA transport by reconstituting VGAT into liposomes (Juge et al. 2009). Similar to glutamate transport by VGLUTs, GABA transport by VGAT also showed a biphasic dependency on external Cl^- when VGAT was co-reconstituted with a proton pump. However, they demonstrated that GABA uptake could be driven by $\Delta\Psi$ generated by the combination of K^+ gradient and valinomycin and, unexpectedly, stimulated by the presence of

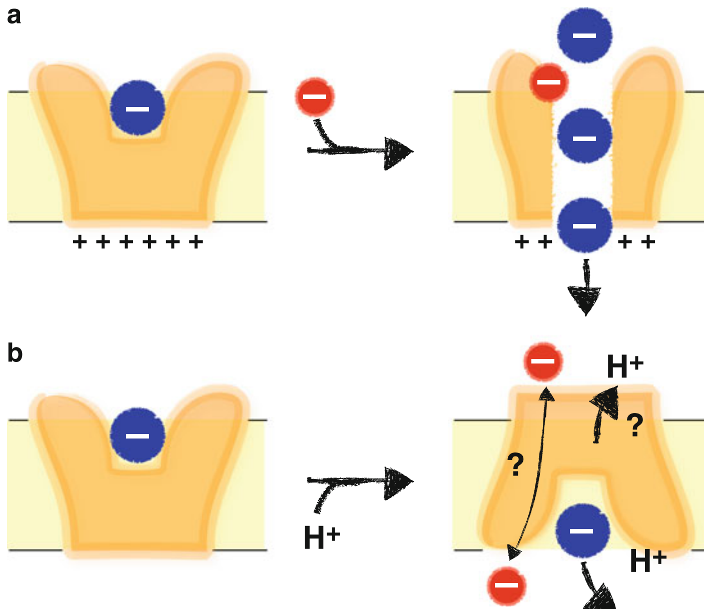

Fig. 12.2 Models for glutamate transport by VGLUT. (**a**) $\Delta\Psi$-driven transport. In this model, $\Delta\Psi$ solely drives glutamate transport. Negatively charged glutamate binds to VGLUT, but is not transported efficiently even when there is $\Delta\Psi$. Cl^- directly binds to VGLUT and promotes conformational change of VGLUT protein. Glutamate is transported electrophoretically as if VGLUT is a Cl^--activated glutamate uniporter. (**b**) ΔpH-driven transport. In outward-facing conformation, glutamate binds to the substrate-binding site that can shift VGLUT to inward-facing conformation. Protonation of the luminal side of VGLUT causes a release of bound glutamate released from VGLUT. Protons that are once bound to VGLUT are then released either the same side (glutamate symport) or the other side (glutamate/H^+ antiport) of VGLUT protein. VGLUT protein has a putative Cl^- permeation pathway which may help to facilitate ΔpH or to compensate charge movement associated with glutamate influx. *Blue balls* and *red balls* indicate glutamate and Cl^-, respectively

external Cl^-. Whereas Cl^- was proposed to directly activate VGLUTs, Cl^- was shown to be cotransported with GABA or glycine via VGAT with a GABA-Cl^- stoichiometry of 1:2. This led the authors to a compelling hypothesis that VGAT is a Cl^-/GABA cotransporter. Although the liposomes contained only VGAT protein and therefore represented the simplest assay system to monitor VGAT activity, it is not easy to reconcile all the biochemical data that clearly indicated ΔpH-driven GABA uptake into isolated SVs (Fykse et al. 1989; Hell et al. 1991; Kish et al. 1989) (Fig. 12.3). One possible drawback of the aforementioned reconstitution experiments is their use of high acetate concentrations as a substitute anion for Cl^-. The protonated form of acetate (CH_3COOH) is to some extent membrane permeable and can release H^+ when deprotonated. Careful consideration of the effects of acetate on pH is required to exclude the involvement of ΔpH on GABA transport. Furthermore, although the authors attempted to establish ΔpH by preloading a low acidic buffer into liposomes, number of free protons in a limited space, especially if

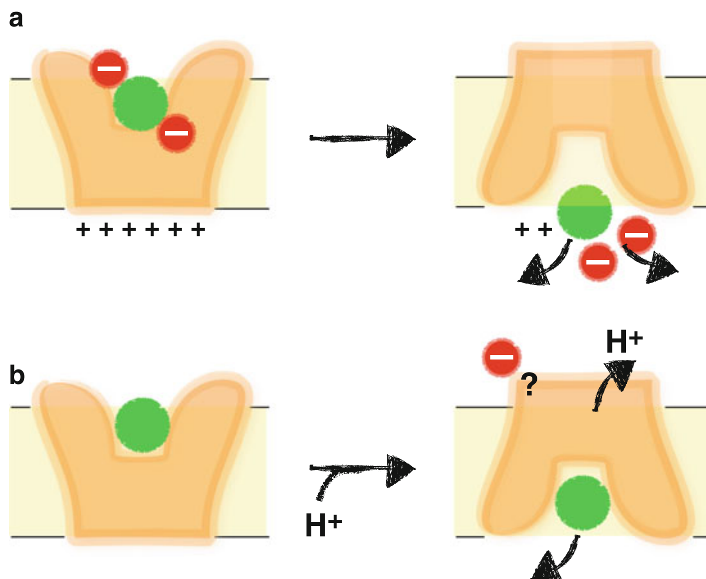

Fig. 12.3 Models for GABA transport by VGAT. (**a**) ΔΨ-driven transport. In this model, ΔΨ solely drives GABA transport. Unlike the ΔΨ-driven glutamate transport, GABA transport is coupled to Cl^- transport with a stoichiometry of GABA-Cl^- = 1:2. As such, GABA transport involves two negative charge movements, which would consume ΔΨ and facilitate ΔpH formation. (**b**) ΔpH-driven transport. A line of evidence suggested that ΔpH can drive GABA transport via GABA/H^+ antiport mechanism. Although Cl^- affects the efficacy of GABA transport, the role of Cl^- is largely unknown. *Green balls* and *red balls* indicate GABA and Cl^-, respectively

protons were coupled to substrate transport by an antiport mechanism, the reaction would have stopped immediately as the efflux of a single proton would evoke massive alkalization of the liposome lumen.

In summary, despite recent advances provided by the reconstitution of vesicular transporters, the exact mechanisms and bioenergetics of the transport processes are largely unknown. To improve understanding, experimental conditions require further refinement, including methods to produce proteoliposomes, buffer compositions, and a quantitative assay for the proton electrochemical gradient. Additionally, if pH plays a role in transport process, structural information of the transporters at various pH will open a new avenue for experimental design.

12.7 Regulations of Synaptic Vesicle Refilling and Quantal Size

Since postsynaptic receptors at the CNS synapses are not always saturated by neurotransmitters released from a single SV, alterations in the amount of neurotransmitter in a SV may potentially influence the efficacy of synaptic transmission.

Heterologous expression of VGLUT1 in hippocampal neurons increased both the amplitude and frequency of miniature EPSCs, suggesting that SVs with higher VGLUT1 expression can accumulate more glutamate (Wilson et al. 2005; Wojcik et al. 2004). However, increased expression of the transporter would in theory affect the kinetics of vesicle refilling rather than steady-state level of neurotransmitters (Edwards 2007). One explanation is that increased expression of VGLUT1 resulted in an enlargement of vesicles, leading to greater glutamate accumulation without changing its concentration. In fact, overexpression of *Drosophila* VGLUT (DVGLUT) in the neuromuscular junction of larvae increased the postsynaptic response in parallel with vesicle volume (Daniels et al. 2004). Yet, gradual reductions in DVGLUT expression did not alter mEPSP amplitude, but did produce a decrease in their frequency, indicating that only a single copy of DVGLUT was sufficient to fill SVs with glutamate to the normal level (Daniels et al. 2006). In this context, it should be noted that there were no detectable differences in mEPSCs in VGLUT1 heterozygous mice (Fremeau et al. 2004; Wojcik et al. 2004), supporting the idea that the expression of VGLUTs is not related to steady-state glutamate content. However, as VGLUT heterozygous mice exhibited a number of behavioral phenotypes, including clonic seizures, increased fear, and depression (Leo et al. 2009; Schallier et al. 2009; Tordera et al. 2007), the possibility remains that a reduction in VGLUT expression influences synaptic transmission.

Another parameter that might affect quantal size is concentration of neurotransmitter in the cytoplasm. Evidence from a radiotracer assay of isolated SVs revealed that increasing substrate concentration resulted in greater substrate accumulation such that transport kinetics could be adequately described by a Michaelis-Menten equation (Wilson et al. 2005; Wolosker et al. 1996). Furthermore, when the concentration of cytosolic glutamate was increased at the calyx of Held, glutamate content was also increased, demonstrated by an enhancement in EPSC amplitude (Ishikawa et al. 2002). Also at the calyx of Held, vesicle refilling rates were measured by flash photolysis of caged glutamate following washout of cytoplasmic glutamate (Hori and Takahashi 2012). The time constant of glutamate refilling was approximately 15 s, much faster than previous estimated from biochemical assays. As observed for glutamate uptake in isolated SVs, neurotransmitter-refilling rate at the synapse was also affected by cytoplasmic Cl^- concentrations, albeit to a lesser extent.

12.8 Trafficking of VGLUTs and VGAT to and Within Presynaptic Terminals

The expression of vesicular transporters on SVs can be regulated either at the point of biosynthesis in the soma or by factors that modulate their sorting and/or targeting to SVs. Given their crucial role in neurotransmitter release, the efficacy of their trafficking to required destinations may have profound physiological implications.

Although little is known concerning how VGLUTs and VGAT are conveyed from the soma to locations throughout the endoplasmic reticulum (ER)-Golgi network, VGLUT3 appears to utilize a unique sorting mechanism. Unlike VGLUT1, VGLUT2, and VGAT, which all preferentially localize on SVs at presynaptic sites, VGLUT3 also localizes at dendrites in multiple brain regions including the striatum, where it may promote retrograde glutamate signaling from the postsynaptic cells (Fremeau et al. 2002). Molecular mechanisms underlying this difference in subcellular localization are totally unexplored to date. At presynaptic sites, the ratio of the transporters on SVs and at the plasma membrane can be differentially regulated among VGLUT isoforms. When heterologously expressed in PC12 cells, VGLUT1 is targeted to secretory vesicles more efficiently than VGLUT2 (Fremeau et al. 2001). These vesicular transporters transiently remain within the plasma membrane upon exocytosis. Thus, the mechanism by which the endocytic machinery recognizes and initiates the incorporation of the transporters into newly endocytosed vesicles would determine their distributions at the presynaptic terminals. Interestingly, the kinetics of VGLUT1 recycling during and after stimulation is faster than that of VGLUT2 (Foss et al. 2013). How the recycling kinetics are controlled molecularly has been investigated through mutagenesis experiments. Essentially, both typical and atypical dileucine-like endocytic motifs in VGLUTs and VGAT moieties control transporter trafficking, albeit to different degrees (Foss et al. 2013; Santos et al. 2013). All VGLUT isoforms contain a dileucine motif at their C-terminus and disruption of this motif differentially affects distribution of VGLUT1 and VGLUT2 (Foss et al. 2013; Voglmaier et al. 2006). In case of VGLUT1, disruption of the motif slows down its activity-dependent recycling, although it does not entirely eliminate its endocytosis. In contrast, the disruption of the motif in VGLUT2 resulted in a dramatic redistribution of VGLUT2 proteins on the cell surface, suggesting that the reliance on the motif for effective recycling differs between VGLUT isoforms. Indeed, VGLUT1 contains two additional dileucine-like motifs at its N-terminus, which may also contribute to its endocytosis. Disruption of all three motifs resulted in limited accumulation of VGLUT1 at synaptic sites and impairment of its activity-dependent recycling, indicating that N-terminus dileucine motifs cooperatively function to sort VGLUT1 to presynaptic sites and, along with the C-terminal motif, promote its recycling. In contrast, when the N-terminus of VGLUT1 was mutated, no abnormalities in SV recycling were reported, suggesting these three motifs are functionally redundant. Yet, differences in surface expression of N-term and C-term mutants indicate that the motifs do have distinct and probably complex mechanisms that underlie transporter targeting and recycling. It has just emerged that VGAT also contains an atypical dileucine-like motif in its N-terminus, which is similarly required for its distribution at presynaptic sites, localization on SVs, and activity-dependent recycling (Santos et al. 2013). The question remains as to how these dileucine motifs dictate trafficking and recycling of vesicular transporter proteins, while other SV proteins can be correctly targeted in the absence of these structures.

While the general principle of vesicle targeting remains enigmatic, features of the specific trafficking mechanism for VGLUT1 have been determined. VGLUT1

has unique proline-rich domains (PRDs) at the C-terminus that interact with endophilin A family proteins at their SH3-domain. Disruption of this interaction had a subtle effect on the steady-state distribution of VGLUT1, but slowed its retrieval from the plasma membrane during high-frequency stimulations (Voglmaier et al. 2006). Furthermore, this interaction may confer lower release probability of VGLUT1-laden SVs compared to that of VGLUT2-laden SVs (Weston et al. 2011).

12.9 Concluding Remarks

Over the past several decades, we have learned much about the molecular basis of neurotransmitter refilling into SVs. Identification of vesicular transporters for glutamate and GABA has provided molecular probes suitable for studying the anatomy of neurotransmitter-specific neural circuits in the mammalian CNS. In addition, the ability to inactivate individual transporters in vivo has allowed researchers to impair synaptic transmission from specific neural populations, thereby greatly enhancing knowledge of how certain glutamatergic and GABAergic circuits contribute to brain function. However, much remains to be solved regarding precise mechanisms of how the transporters work and whether this process is limiting for synaptic physiology and pathology.

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