JCB Mini-Review

The glutamine commute: take the N line and transfer to the A

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The transfer of glutamine between cells contributes to signaling as well as to metabolism. The recent identification and characterization of the system N and A family of transporters has begun to suggest mechanisms for the directional transfer of glutamine, and should provide ways to test its physiological significance in diverse processes from nitrogen to neurotransmitter release.

In multicellular organisms, the diverse function of specialized tissues requires mechanisms to coordinate their activity. This generally involves signaling between cells. In many cases, a soluble signal released by one cell activates the receptor in a second cell. In other cases, the signal involves contact with extracellular matrix rather than a soluble mediator or direct contact between cells in the case of adhesion molecules. In addition, many tissues interact through an entirely distinct mechanism—the transfer of metabolic intermediates. In this case, release often involves transport across the plasma membrane rather than exocytosis and the response uptake rather than activation of a cell surface receptor. The transferred substance has direct effects on cell function and may not require second messengers. The molecular basis and physiological role of many such transfer reactions remain poorly understood relative to other forms of signaling. In this review, we focus on the transfer between cells of the amino acid glutamine. Although involved in metabolism, glutamine transfer also participates in signal transduction.

Glutamine transfer between cells

Glutamine contributes to many metabolic pathways. The biosynthesis of other amino acids, proteins, nucleotides, and amino sugars all requires glutamine. Rapidly dividing cells such as in the gut and hair prefer glutamine as a carbon source for growth. The standard growth of cultured cells in high (5 mM) concentrations of glutamine attests to its importance. In vivo, skeletal muscle produces glutamine (through the action of glutamine synthetase) for consumption as a source of energy by several other tissues including the gut (Kvamme, 1988). Indeed, glutamine is the most abundant amino acid in blood, contributing one fifth of the total amino acid. These high levels presumably reflect the importance of glutamine transfer from producing to consuming tissues.

Glutamine also participates in more specialized metabolic pathways. In the kidney, the ammonia produced from conversion of glutamine to glutamate (by the enzyme glutaminase) promotes acid excretion (Bender, 1975). In the liver, glutamine has a central role in nitrogen metabolism. Together with the ammonia derived from portal blood, the ammonia produced by glutaminase helps to drive the urea cycle. Excess ammonia not converted into urea is then incorporated into glutamine through the action of glutamine synthetase. However, glutaminase and the urea cycle enzyme activities reside in a distinct set of hepatocytes from glutamine synthetase (Haussinger, 1990). Periportal hepatocytes, the vast majority of cells in the liver, express glutaminase. In contrast, the less abundant perivenous hepatocytes expressing glutamine synthetase occur downstream in the vascular system in good position to scavenge the ammonia not consumed by periportal cells. The liver thus exhibits a glutamine–glutamate cycle in which periportal cells convert glutamine to glutamate (and ammonia) and perivenous cells transform glutamate into glutamine (Fig. 1 A). The brain exhibits a similar glutamine–glutamate cycle but for an entirely different purpose than nitrogen metabolism.

Synaptic transmission involves the regulated release of neurotransmitter by exocytosis. Since neurosecretory vesicles contain large amounts of transmitter (up to molar concentrations in certain cases), the high rates of firing observed at many synapses require mechanisms to replenish the depleted stores. Most classical transmitters are taken back up directly into the nerve terminal. Indeed, disruption of the gene responsible for dopamine reuptake results in depletion of dopamine stores despite increased extracellular levels (Giros et al., 1996). However, transporters for the principal excitatory transmitter

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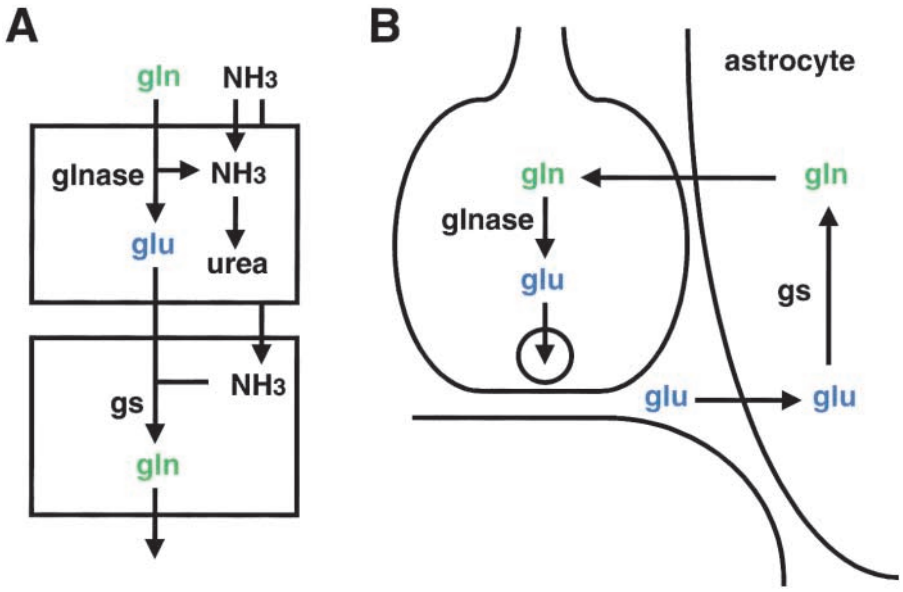

Figure 1. **The glutamine–glutamate cycles in the liver and nervous system.** (A) In periportal cells (top) of the liver, glutaminase (glnase) converts glutamine to glutamate and ammonia. Along with the ammonia directly supplied from the portal circulation, the ammonia derived from glutamine feeds into the urea cycle. The glutamine synthetase (gs) in perivenous cells (bottom) converts the ammonia that escapes the urea cycle into glutamine. (B) In the nervous system, the glutamate released by exocytosis from nerve terminals is taken up by astrocytes through known excitatory amino acid transporters and converted to glutamine by glutamine synthetase. Glutamine is then transferred from astrocytes to neurons and converted back to glutamate by glutaminase before packaging into vesicles.

glutamate reside primarily on astrocytes, and those expressed by neurons are, with certain exceptions (Gundersen et al., 1996; Arriza et al., 1997), generally postsynaptic (Rothstein et al., 1994; Dehnes et al., 1998). Glutamate thus requires an alternative mechanism for recycling. After release from the nerve terminal, glutamate accumulates in astrocytes through the action of known glutamate transporters (Fig. 1 B). Glutamine synthetase in astrocytes then converts glutamate to glutamine (Rothstein and Tabakoff, 1984). After transfer back to neurons, the glutaminase expressed by excitatory neurons converts glutamine to glutamate before repackaging into vesicles. Supporting this mechanism, the bulk of glutamate released as transmitter derives from glutamine (Hamberger et al., 1979; Thanki et al., 1983), and the inhibition of glutaminase depletes glutamate stores (Conti and Minelli, 1994). Exogenous glutamine can also maintain synaptic activity in the absence of glutamine synthetase activity (Barnett et al., 2000). Since γ–aminoisobutyric acid (GABA) derives from glutamate, the glutamine–glutamate cycle may contribute to inhibitory and excitatory neurotransmission (Pow and Robinson, 1994; Laake et al., 1995). Further, the cerebrospinal fluid contains ~0.5 mM glutamine, whereas no other amino acid exceeds 50 μM (McGale et al., 1977), indicating the importance of glutamine transfer in the brain. However, despite investigation over several decades the mechanisms involved in glutamine transfer and the actual role of the glutamine–glutamate cycle in the regulation of amino acid release have remained uncertain.

Classical studies have identified multiple distinct activities that transport glutamine across the plasma membrane of mammalian cells. Christensen and colleagues originally demonstrated the existence of two independent neutral amino acid uptake activities that they referred to as systems L and A (Oxender and Christensen, 1963). System L has a preference for leucine but also recognizes neutral amino acids including glutamine. Recent work has shown that system L is a heterodimer composed of the 4F2 heavy chain and a light chain, either the ubiquitously expressed broad specificity LAT1 or LAT2, which is expressed at the basolateral surface of epithelia (Verrey et al., 2000; Chillaron et al., 2001; Wagner et al., 2001). Although widespread, system L activity contributes relatively little to glutamine transport by most cells. In addition, system L is Na^+ independent and does not appear to mediate net amino acid flux. Rather, it acts principally to exchange extracellular for cytoplasmic amino acid and hence relies on other active transporters for the cytoplasmic store of amino acid that can be exchanged.

In contrast to system L, system A depends on Na^+ and can actively transport amino acids against a concentration gradient. Therefore, it contributes to the cytoplasmic amino acid exchanged by system L. System A has a preference for alanine but also recognizes neutral amino acids with short, polar, or linear side chains, including glutamine, and shows particular sensitivity to inhibition by low pH. Unlike many other amino acid transport systems, system A recognizes *N*-methylated amino acids. In addition, system A is highly regulated (McGivan and Pastor-Anglada, 1994). Extracellular amino acid concentrations, hormones, the cell cycle, and osmolarity control system A activity through a combination of short and long term mechanisms. Association with the cytoskeleton may account for several of these properties (Handlogten et al., 1996). Despite its general importance and interesting regulation, system A has eluded molecular characterization (Palacin et al., 1998) until recently.

In the liver, glutamine uptake depends on Na^+ but exhibits a component resistant to inhibition by *N*-methylated amino acids, distinct from system A. This activity has the highest affinity for glutamine, followed by histidine and asparagine, and was designated system N (Kilberg et al., 1980). Although not initially detected in other tissues, related activities were subsequently described in muscle (Nm) and brain (Nb) (Hundal et al., 1987; Tamarappoo et al., 1997). Interestingly, system N resembles system A in its sensitivity to low pH and regulation by osmolarity (Bode and Kilberg, 1991). It has also eluded molecular analysis. Several other proteins transport glutamine as well, including the system ASC exchanger ASCT2, which belongs to the family of EAATs (Ut-

sunomiya-Tate et al., 1996) and the system $B^{0,+}$ transporter $ATB^{0,+}$ (Sloan and Mager, 1999). Despite many attempts at biochemical purification and expression cloning, the first protein responsible for system N activity was identified through the analysis of an orphan transporter.

System N mediates efflux

Classical neurotransmitters are synthesized in the cytoplasm and require uptake by secretory vesicles for exocytotic release. In general, vesicular transport depends on an H^+ electrochemical gradient across the vesicle membrane generated by the vacuolar H^+-ATPase (Schuldiner et al., 1995; Liu and Edwards, 1997). Specifically, the uptake of transmitter involves exchange for lumenal protons, and molecular cloning has now identified three families of proteins responsible (Reimer et al., 2001). The vesicular GABA transporter (VGAT)* defined the second of these families with many members in organisms from yeast to mammals (McIntire et al., 1997; Sagne et al., 1997).

To identify proteins that might be involved in synaptic transmission, we focused on one VGAT-related sequence expressed by astrocytes (Chaudhry et al., 1999). In contrast to VGAT, this protein localizes to the plasma membrane. However, heterologous expression of the cDNA did not appear to confer uptake of potential substrates such as amino acids even though the only proteins of known function related to VGAT were a series of amino acid permeases in plants that included the auxin transporter (Bennett et al., 1996). On the other hand, VGAT and these plant proteins all use a H^+ electrochemical gradient to drive transport. This coupling mechanism suggested the possibility of using proton flux to identify the substrate: although ionic gradients normally drive the accumulation of substrate, gradients of substrate can drive the movement of ions, and this has been used on several occasions to identify the substrate for an orphan transporter (Pajor and Wright, 1992; Sloan and Mager, 1999). Therefore, we imaged intracellular pH (pH_i) and found that glutamine, histidine, and to a lesser extent asparagine increase the pH_i of cells expressing the transporter but not of untransfected cells (Chaudhry et al., 1999). The protein thus exchanges H^+ for amino acid. In addition, uptake requires extracellular Na^+. Li^+ also supports uptake, and since Li^+ suppresses the background uptake of glutamine by other systems that do not tolerate Li^+ substitution for Na^+, Li^+ could also be used to demonstrate glutamine uptake by the protein. Indeed, the orphan transporter confers Na^+-dependent transport of glutamine with all the properties described previously for transport system N (Chaudhry et al., 1999; Gu et al., 2000), and the proton exchange mechanism accounts for the pH sensitivity of system N. It is also expressed in the liver, kidney, and brain, consistent with the original description of system N in the liver (Kilberg et al., 1980). However, characterization of this system N transporter (SN1) also showed one feature that was not anticipated.

In the absence of external amino acid substrates, the pH_i of cells expressing SN1 (but not untransfected cells) declines to extremely low levels ($\sim$6.5). This suggests flux reversal with H^+ uptake coupled to amino acid efflux. Indeed, transfected cells require an external glutamine concentration $\sim$0.4 mM to maintain a pH_i of $\sim$7.4 (Chaudhry et al., 1999), indicating that efflux can occur under physiological conditions. Since most Na^+ cotransporters mediate essentially unidirectional uptake, the flux reversal observed for SN1 is quite surprising. It may reflect high intracellular glutamine levels, but a typical Na^+-dependent electrogenic transporter should produce a concentration gradient >100-fold due to the combined driving forces of the Na^+ concentration gradient and resting membrane potential. Regardless of the mechanism, the efflux of glutamine mediated by SN1 together with its localization to the liver strongly suggests that it could mediate aspects of the glutamine flux required for the urea cycle. SN1 may even localize preferentially to perivenous rather than periportal cells (Gu et al., 2000), suggesting more of a role in glutamine uptake than efflux, but all hepatocytes appear to express SN1. However, the expression by astrocytes and not by neurons strongly suggests a role in the glutamine efflux required for the glutamine–glutamate cycle involved in replenishing released amino acid transmitter. In astrocytes, SN1 localizes to the processes that surround synapses (Chaudhry et al., 1999), and previous work has shown robust system N activity in cultured astrocytes (Nagaraja and Brookes, 1996). In addition, glutamine and asparagine increase the pH_i of astrocytes with the characteristics expected for SN1 (Chaudhry et al., 2001). Another system N transporter (SN2) has been reported recently to occur in the brain as well, but the cellular pattern of expression is not known, and the affinity for glutamine is considerably lower than that of SN1 (Nakanishi et al., 2001). Nonetheless, like SN1, SN2 appears to mediate H^+ exchange. In contrast, systems Nm and Nb may not belong to this family of proteins because Nm and Nb do not show sensitivity to pH and do not tolerate Li^+ substitution for Na^+ (Hundal et al., 1987; Tamarappoo et al., 1997).

System A is related to system N

The known sensitivity of system A to inhibition by low pH suggested that proteins closely related in sequence to SN1 might encode system A. Indeed, SN1 defines a subfamily of proteins related to VGAT and several confer neutral amino acid transport with the properties expected for system A. One isoform (SAT2, ATA2, or SA1) exhibits almost ubiquitous expression and recognizes other neutral amino acids as well as or better than glutamine (Reimer et al., 2000; Sugawara et al., 2000; Yao et al., 2000). Another (SAT3 or ATA3) occurs primarily in the liver but recognizes glutamine poorly (Hatanaka et al., 2001). Like classical system A, all of the SAT isoforms recognize methylated amino acids, depend on Na^+, tolerate Li^+ substitution, and show sensitivity to low pH, very similar to SN1. However, a third isoform (SAT1, GlnT, ATA1, or SA2) has a more restricted distribution in the brain (and heart) and preferentially recognizes glutamine and asparagine as substrates (Varoqui et al., 2000; Albers et al., 2001; Chaudhry et al., 2002), suggesting a more specific role in glutamine uptake by neurons required for the glutamine–glutamate cycle. Indeed, SAT1 is expressed almost exclusively by neurons rather than glia. It exhibits particularly high levels of expression by inhibitory neurons, and glutamine affects inhibitory transmission in

*Abbreviation used in this paper: VGAT, vesicular GABA transporter.

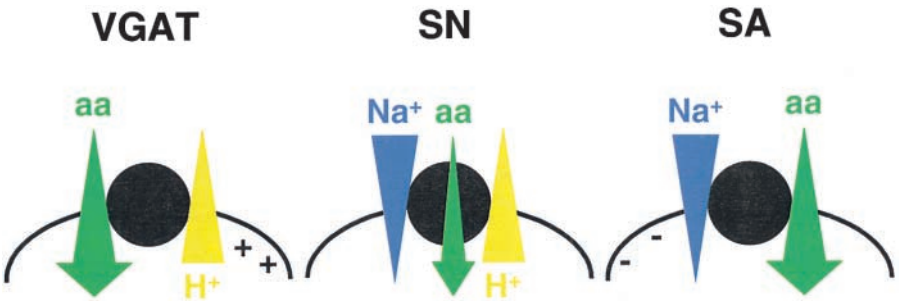

Figure 2. **Ionic coupling of VGAT, system N, and system A transporters.** VGAT (left) couples the uptake of GABA by synaptic vesicles to the movement of H^+ down their electrochemical gradient (out of vesicles). The positive charge inside synaptic vesicles contributes to the driving force on H^+. System N transporters (middle) mediate the Na^+-dependent uptake of glutamine in exchange for H^+. Electroneutrality appears to contribute to the shallow amino acid gradients achieved. In contrast, system A transporters (right) mediate only Na^+ cotransport with amino acid. Since amino acid substrates are generally neutral, transport is electrogenic, and the resting membrane potential contributes to the driving force on Na^+, predicting the accumulation of amino acid to high concentrations.

the hippocampus at a presynaptic locus (Chaudhry et al., 2002), consistent with the location of SAT1 at the nerve terminal.

If SN1 and SAT1 exhibit similar functional characteristics, how can they mediate the transfer of glutamine both out of cells (astrocytes) and into other cells (neurons)? They differ in one important respect: in contrast to SN1 and 2, system A transporters SAT1 and -2 do not mediate H^+ flux (Albers et al., 2001; Chaudhry et al., 2002) (Fig. 2). Their sensitivity to low pH may thus represent a vestige of the H^+ exchange mechanism. In the case of system N, H^+ binding can result in H^+ translocation but it cannot for system A. Indeed, low external pH increases the Km of SAT2 and particularly SAT1 for Na^+, suggesting that H^+ may compete with Na^+ for binding (Albers et al., 2001; Chaudhry et al., 2002). Since binding to SAT1 and -2 appears ordered with Na^+ preceding amino acid, H^+ appear to interfere with the first binding event (Albers et al., 2001; Chaudhry et al., 2002). In addition, these observations suggest that the glutamine-gated Na^+/H^+ exchanger SN1 is an evolutionary intermediate between VGAT, which mediates only H^+ exchange, and the SATs, which mediate only Na^+ cotransport (Fig. 2). But why would differences in H^+ translocation confer differences in the direction of glutamine transport by system N and A transporters?

Coupled and uncoupled charge movement

Differences in charge movement appear to account for the different directions of flux mediated by system N and A transporters. System A proteins all mediate electrogenic transport due to the uptake of Na^+ and neutral amino acid unopposed by H^+ (Reimer et al., 2000; Sugawara et al., 2000; Yao et al., 2000; Albers et al., 2001; Chaudhry et al., 2002). Although the stoichiometry for Na^+ appears not to exceed 1, the electrogenic nature of transport by system A enables membrane potential and Na^+ gradient to provide the driving force for amino acid uptake (Fig. 2). This should generate a glutamine concentration gradient >100-fold at resting membrane potential and predicts that SAT1 and -2 will not reverse under physiological conditions.

In contrast to system A transporters, the H^+ exchange mediated by SN1 appears to confer electroneutrality by balancing the Na^+ cotransported with amino acid (Chaudhry et al., 1999). In this case, membrane potential does not contribute to the driving force, predicting a much shallower concentration gradient of glutamine (~12-fold) and flux reversal under physiological conditions (Fig. 1). The high glutamine concentrations in glia (5–8 mM) may also contribute to efflux (Schousboe et al., 1979; Patel and Hunt, 1985; Storm-Mathisen et al., 1992). However, we and others have observed currents associated with SN1 expressed in *Xenopus* oocytes (Fei et al., 2000; Chaudhry et al., 2001), suggesting that transport is electrogenic rather than electroneutral. Indeed, glutamine induces currents with the same properties as transport by SN1: dependence on Na^+ and sensitivity to low pH (Fei et al., 2000; Chaudhry et al., 2001). On the other hand, whereas SAT1 and -2 show fixed charge to amino acid flux ratios at ~1 (Chaudhry et al., 2001, 2002), the charge movement by SN1 can be dissociated from amino acid flux. In addition, substrates can induce outward charge movement at positive potentials in oocytes expressing SN1. Further, the outward charge movement has been observed by multiple groups and for SN2 and SN1 (Nakanishi et al., 2001). It has been claimed that only certain substrates can produce these outward currents, but we have observed them with both glutamine and particularly asparagine in the case of SN1 (Chaudhry et al., 2001). If charge movement were strictly coupled to transport, it would not be possible for external amino acid to induce outward currents, strongly suggesting the existence of an ionic current not stoichiometrically coupled to transport. Ion substitution experiments show that the conductance is almost entirely selective for H^+ (Chaudhry et al., 2001). Importantly, the concentration of Na^+ and amino acid affect the magnitude of these currents, consistent with gating by the transport cycle, but do not affect their reversal potential, consistent with the selectivity of the conductance for H^+. In contrast, external pH affects the magnitude of the currents and their reversal potential, as anticipated from the coupling of amino acid transport to H^+ exchange and gating of the conductance by the transport cycle. Protons thus permeate the carrier both coupled to and uncoupled from transport. At positive membrane potentials, glutamine produces strong alkalinization due to the combined effects of H^+ exchange and outward H^+ currents. However, at negative potentials the oocytes alkalinize to a lesser extent because the uncoupled inward currents carried by H^+ offset the outward coupled H^+ movement (Chaudhry et al., 2001). In other words, SN1 can mediate H^+ movement in opposite directions through coupled and uncoupled mechanisms. This distinguishes SN1 from many other transporters, which exhibit

an uncoupled conductance selective for an ion not coupled to the transport mechanism. For example, plasma membrane glutamate transporters exhibit a chloride conductance but do not require chloride for transport (Sonders and Amara, 1996).

Although SN1 exhibits currents uncoupled from transport, this does not indicate that transport is not electrogenic. However, we have repeatedly found using a variety of different substrates and conditions that depolarization does not inhibit uptake by SN1 (Chaudhry et al., 2001). This may reflect the electroneutrality of a rate-limiting step in the transport cycle despite net charge movement, and other groups have suggested inhibition by depolarization (Fei et al., 2000), but depolarization does inhibit uptake by the more clearly electrogenic and closely related SAT1 and -2 (Chaudhry et al., 2002). SN1 thus appears to be electroneutral. On the other hand, substantial evidence indicates cooperative activation of SN1 flux by Na^+ (Fei et al., 2000), suggesting a stoichiometry for $Na^+ > 1$. More recently, measurement of flux with $^{22}Na^+$ confirmed the uptake of 2–3 Na^+ per amino acid, but transport again did not appear to involve net charge movement, particularly at low concentrations of substrate (Broer et al., 2002). Since electroneutrality presumably requires the movement of an equal number of H^+ in the opposite direction to Na^+, the observations predict a stoichiometry for H^+ that is also 2–3, and this has not been assessed. However, even with electroneutrality a stoichiometry for Na^+ of 2–3 predicts very large concentration gradients of amino acid, inconsistent with the ready flux reversal that we and others have observed (Chaudhry et al., 1999; Broer et al., 2002). Indeed, glutamine concentrates in oocytes expressing SN1 to an extent consistent with a stoichiometry of 1 Na^+ rather than 2 (Broer et al., 2002). Questions thus remain about the basic function of SN1. Since it is difficult to control the concentration of substrates and coupled ions in intact cells, reconstitution of the purified protein in artificial membranes may help to clarify the ionic coupling. The study of astrocytes will also help to assess the physiological significance of the uncoupled currents. Nonetheless, H^+ exchange appears to confer the electroneutrality of transport required for shallow gradients and the efflux of glutamine from glia. In the liver, SN1 may mediate both glutamine uptake and release. In contrast, the electrogenic nature of the SATs presumably confers the unidirectional uptake of glutamine by hepatocytes and neurons. Interestingly, SAT2 also exhibits an uncoupled Na^+ conductance (Albers et al., 2001; Chaudhry et al., 2002), but this is detectable only in the absence of substrate, and the transport-associated currents are entirely coupled.

What is the purpose of the uncoupled conductance associated with SN1? In addition to conferring electroneutrality, the coupling to H^+ exchange appears to provide a mechanism for regulation. During synaptic transmission, the pH_i of astrocytes increases due to the activation of a $Na^+/HCO3^-$ cotransporter (Chesler and Kaila, 1992; Deitmer and Rose, 1996). This would increase the inward driving force on H^+ and promote glutamine efflux by SN1, linking the glutamine–glutamate cycle to the exocytosis of transmitter. Proton coupling may thus confer an important form of regulation. However, we have also observed that it can cause dramatic swings in pH_i. Indeed, H^+ occur at much lower concentrations (100 nM at pH 7) than either the Na^+ (140 mM) or glutamine (0.5 mM) also translocated by SN1, and high rates of transport may disturb the pH homeostasis of cells expressing SN1. The uncoupled H^+ conductance may therefore serve to short-circuit these pH_i changes and protect the cell. Interestingly, the conductance appears to be activated by amino acid uptake rather than efflux, and the negative resting membrane potential would specifically enable uncoupled inward H^+ movement to prevent excessive alkalinization due to coupled H^+ efflux.

Future directions

Molecular characterization of the system N and A transporters has suggested specific roles in glutamine efflux and uptake. However, many questions remain about the function and regulation of these proteins. First, the ionic mechanism of SN1 remains uncertain: the apparent stoichiometry for Na^+ still needs to be reconciled with the propensity for flux reversal. Second, does the uncoupled H^+ conductance occur in astrocytes, hepatocytes, and oocytes? In addition, several cells including astrocytes and hepatocytes express both system N and A transporters. If one promotes glutamine efflux and the other uptake, how is their activity coordinated? Perhaps they are expressed in distinct microdomains on the cell surface. We also do not understand the mechanisms involved in regulation of these transporters. System A substrates regulate the transcription of SAT genes (Ling et al., 2001), indicating their role in signaling and metabolism. However, the mechanism by which transport regulates gene expression remains unknown. In addition, classical studies have shown that cell swelling can regulate transport post-transcriptionally (Bode and Kilberg, 1991), but again the mechanisms remain to be elucidated. The analysis of system N and A transporters will also help us to understand the physiological role of the glutamine–glutamate cycle, particularly in synaptic transmission. How important is the cycle in transmitter release, and do activities other than system N and A also have a role? Further, what are the advantages of this indirect recycling mechanism over direct reuptake by the nerve terminal? Perhaps the cycle provides greater capacity, speed, and potential for regulation. Genetic and pharmacologic manipulation in vivo will allow us to test these possibilities.

Understanding the glutamine–glutamate cycle will in turn address several biomedical problems. Liver failure produces encephalopathy, but the mechanism has remained speculative for decades. Interestingly, elevated cerebrospinal fluid glutamine levels correlate better with the condition than any other marker (Plum, 1971), suggesting that a disturbance of the glutamine–glutamate cycle in the liver may result in a disturbance of the related cycle in the brain. The activation of system N by cell swelling may also contribute to the brain edema associated with fulminant hepatic failure. Further, the pH shifts associated with the glutamine–glutamate cycle may contribute to the pH changes that accompany spreading depression in migraine.

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