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Molecular Analysis of System N Suggests Novel Physiological Roles in Nitrogen Metabolism and Synaptic Transmission

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Summary

The amino acid glutamine has a central role in nitrogen metabolism. Although the molecular mechanisms responsible for its transport across cell membranes remain poorly understood, classical amino acid transport system N appears particularly important. Using intracellular pH measurements, we have now identified an orphan protein related to a vesicular neurotransmitter transporter as system N. Functional analysis shows that this protein (SN1) involves H^+ exchange as well as Na^+ cotransport and, under physiological conditions, mediates glutamine efflux as well as uptake. Together with the pattern of SN1 expression, these unusual properties suggest novel physiological roles for system N in nitrogen metabolism and synaptic transmission.

Introduction

The amino acid glutamine occupies a central place in nitrogen metabolism. It is the most abundant free amino acid in both plasma and cerebrospinal fluid (Fishman, 1992). In the liver, glutamine contributes to the detoxification of ammonia and the production of urea (Bender, 1975), requiring the sequential uptake and release of glutamine by distinct populations of hepatocytes (Haussinger, 1990). In the kidney, the metabolism of glutamine contributes to the secretion of acid (Bender, 1975). Glutamine also serves as a nitrogen donor for other amino acids, purines, pyrimidines, and amino sugars (Tate and Meister, 1973). In the central nervous system, glutamine serves as a precursor for the principal excitatory neurotransmitter glutamate (Hamberger et al., 1979a; Thanki et al., 1983; Conti and Minelli, 1994). Since glia synthesize the glutamine used by neurons, this requires transfer of the amino acid between the two cell types (Ottersen et al., 1992). The role of glutamine in nitrogen metabolism and synaptic transmission thus involves transport across the membrane of many cells.

Previous studies have identified several transport systems for glutamine. These include Na^+-independent transport by system L that mediates exchange rather than net uptake, and Na^+-dependent transport by systems A and ASC (Collarini and Oxender, 1987; Palacin et al., 1998). However, system N appears almost solely responsible for the Na^+-dependent uptake of glutamine by hepatocytes (Kilberg et al., 1980). It also contributes to glutamine transport across the blood–brain barrier (Ennis et al., 1998). Related but distinct activities mediate the uptake of glutamine in neuronal culture (Tamarappoo et al., 1997) and by muscle (Hundal et al., 1987). Although system N has been expressed in *Xenopus* oocytes using liver mRNA (Taylor et al., 1992), the protein responsible for its activity has not been identified. We now find that the protein mediating system N belongs to a family of neurotransmitter transporters.

In contrast to reuptake systems for neurotransmitters that terminate synaptic transmission and involve Na^+ cotransport (Amara and Arriza, 1993; Kanner, 1994), transport into synaptic vesicles is required for the exocytotic release of classical transmitters and involves the exchange of lumenal protons for cytoplasmic transmitter (Schuldiner et al., 1995; Liu and Edwards, 1997). The vesicular neurotransmitter transporters in turn belong to two distinct families. One includes the transporters for monoamines and acetylcholine (ACh). The protein that transports γ-aminobutyric acid (GABA) and glycine into synaptic vesicles defines a second, larger family with many members in organisms ranging from nematodes to mammals (McIntire et al., 1997). Aside from the vesicular GABA transporter (VGAT) and the related plant amino acid permeases (Young et al., 1999), however, the function of these putative transport proteins has remained unknown. We now report that one member of this family resides on the plasma membrane rather than on secretory vesicles. Using intracellular pH measurements, we show that this protein has the functional characteristics previously described for system N. In addition, it mediates the exchange of glutamine for protons as well as cotransport with Na^+, accounting for its relationship with vesicular neurotransmitter transporters and the extreme pH sensitivity previously described for system N (Kilberg et al., 1980). Its expression by brain astrocytes, the liver, and the kidney further supports a role in neurotransmission as well as nitrogen metabolism.

Results

Novel Transporter Related to VGAT

The role of VGAT in packaging GABA for exocytotic release suggested that additional family members might

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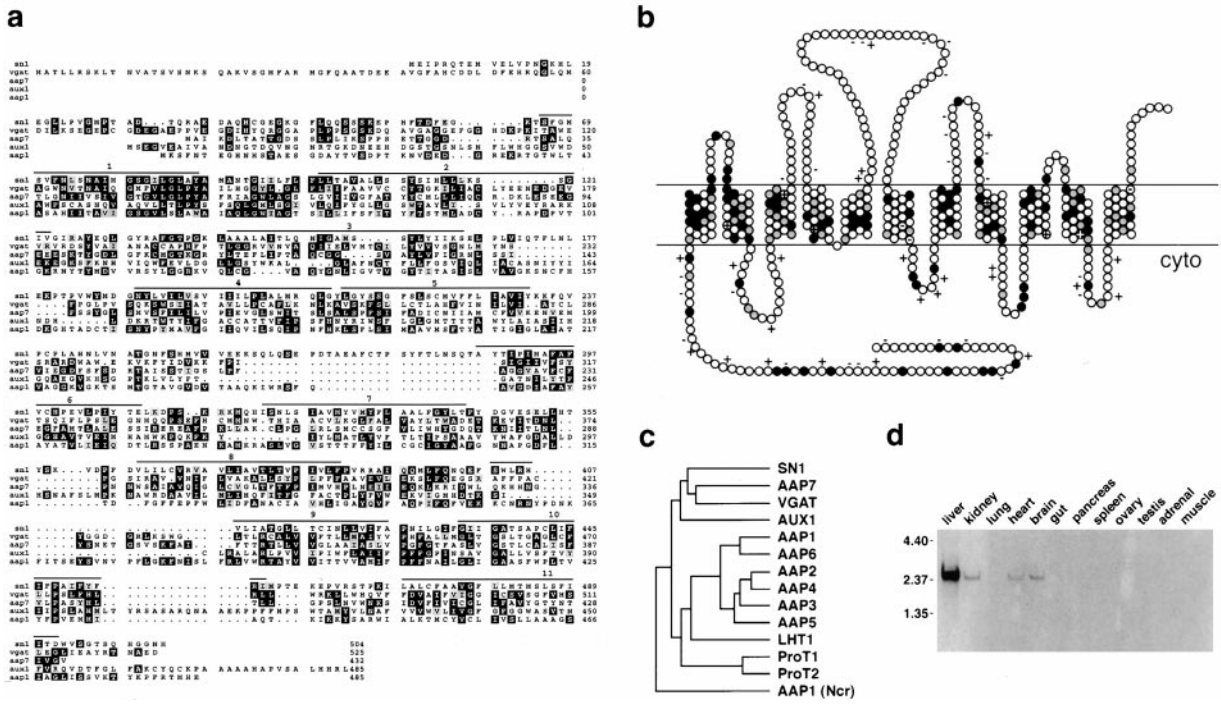


Figure 1. Tissue-Specific Expression of a Novel Protein Related to the Vesicular GABA Transporter (VGAT)

(a) The sequence of a novel rat cDNA (sn1) predicts a protein with similarity to rat VGAT, two amino acid permeases (aap7 and -1) and the auxin transporter (aux1) from *Arabidopsis thaliana*. The bars indicate predicted transmembrane domains and the numbers in the right margin the position of amino acid residues relative to the translation start site. Black boxes indicate identical residues and the gray conservative substitutions.

(b) Predicted secondary structure of rat SN1, with the membrane represented between the two horizontal lines, the extracellular space above and the cytoplasm (cyto) below. Minus signs (−) represent acidic residues and plus signs (+) basic residues. Filled circles indicate residues identical (black) or similar (gray) to those in VGAT.

(c) Dendrogram for proteins of the VGAT and amino acid/auxin permease (AAAP) family. AAP1–7 denote amino acid permeases 1–7, VGAT the vesicular GABA transporter, AUX1 the auxin transporter 1, LHT1 the lysine histidine transporter, ProT1–2 the proline transporters 1 and 2.

(d) Tissue-specific expression of SN1 mRNA. Northern analysis of 5 μg poly(A)$^+$ RNA shows a 2.4 kb transcript only in the liver, kidney, heart, and brain. The standards (in kb) are indicated on the left.

also contribute to synaptic transmission. We therefore searched the available databases with the rat VGAT sequence and identified a distinct but related human cDNA (SN1). Using a probe obtained by polymerase chain reaction amplification of the human sequence, we isolated a rat cDNA. The nucleotide sequence predicts a protein of 504 amino acids with 17% identity and 28% similarity to VGAT (Figure 1). The amino acid sequence also predicts 10–11 amphipathic α helices suggestive of transmembrane domains, with the N terminus in the cytoplasm (Figures 1a and 1b). In addition, the protein shows similarity to a series of amino acid permeases in plants that include an auxin transporter (Figure 1c) (Young et al., 1999). The function of several of these plant proteins has been determined and they all appear to use a H^+ electrochemical gradient to drive the active transport of amino acids and related compounds (Fischer et al., 1995; Bennett et al., 1996; Boorer et al., 1996; Rentsch et al., 1996; Boorer and Fischer, 1997; Chen and Bush, 1997). These functional characteristics support the significance of their sequence similarity to VGAT, which uses proton exchange to package GABA into synaptic vesicles (McIntire et al., 1997).

To assess the potential function of the novel VGAT-related sequence, we first determined its tissue distribution. Northern analysis shows strong expression of an ~2.5 kb SN1 mRNA by the liver, with lower levels of expression by kidney, heart, and brain (Figure 1d). The orphan transporter thus has a more diffuse distribution than VGAT, which is expressed essentially only in the brain (McIntire et al., 1997). However, the gut, lung, spleen and muscle contain no detectable transcripts, indicating that expression is not ubiquitous. In situ hybridization shows diffuse but specific labeling throughout the rostrocaudal extent of sagittal brain sections (Figures 2a and 2b). At higher magnification, the label is seen over small cells but not over large perikarya. The labeling in the cerebellum is particularly strong over the granule cell layer, but the labeled cells are too few to represent granule cells, and only scattered positively hybridizing cells appear in the molecular layer (Figures 2e and 2f). This pattern suggests expression by astrocytes rather than interneurons, which distribute more equally between granule and molecular layers (Chaudhry et al., 1995, 1998), and the diffuse labeling of cortex, hippocampus, striatum, and olfactory bulb supports this conclusion (Figures 2g–2j). The ependyma and choroid plexus also show strong hybridization (Figure 2k). In the liver, expression appears ubiquitous in all hepatocytes (Figure 2l) whereas the kidney shows hybridization restricted to the medulla (Figures 2c and 2m).

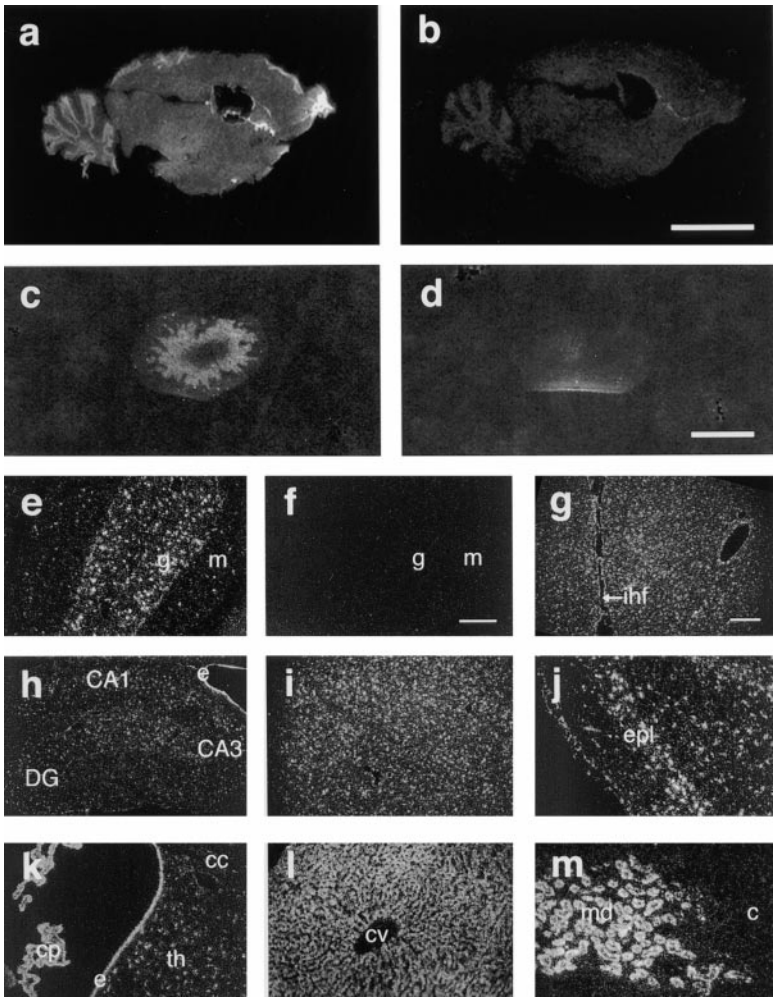


Figure 2. In Situ Hybridization Shows Cell-Specific Expression of SN1 in the Brain, Liver, and Renal Medulla

(a–d) In situ hybridization of sagittal sections from the brain (a and b) and kidney (c and d) with ^{35}S-labeled antisense (a and c) and sense (b and d) RNA probes for SN1. All brain regions show diffuse, specific hybridization, but the granular cell layer of the cerebellum and the renal medulla label particularly strongly.

(e–m) In situ hybridization of tissue sections with ^{35}S-labeled antisense (e and g–m) and sense (f) RNA for SN1, followed by counterstaining with cresyl violet and visualization by dark-field illumination. In the cerebellum (e and f), individual cells of the granular layer (g) and to a much lesser extent the molecular layer (m) label specifically with the antisense probe. (g) In the cerebral cortex, the antisense probe hybridizes specifically with small cells in all six layers. No labeling appears in the pyramidal cells. ihf, interhemispheric fissure. (h) The hippocampus contains small positively hybridizing cells scattered throughout the molecular layer of the dentate gyrus (DG) and in the neuropil layers of CA1 through CA3. The granular and pyramidal cells are unlabeled. (i) The caudate-putamen contains scattered labeled cells. (j) In the olfactory bulb, high levels of SN1 expression occur in the external plexiform layer (epl). (k) The choroid plexus (cp), ependyma (e), and cells in the gray matter of the thalamus (th) hybridize with the antisense probe. Less intense hybridization occurs in the white matter (cc, corpus callosum). (l) Hepatocytes strongly express SN1 mRNA. (m) In the kidney, SN1 mRNA appears restricted to tubules in the medulla (md), with little expression in the cortex (c).

The bars in (b) and (d) indicate 0.5 cm and also apply to (a) and (c), respectively. The bar in (f) indicates 0.5 mm and applies as well to (e), (j), (l), and (m). The bar in (g) indicates 1 mm and applies as well to (h), (i), and (k).

Expression by nonneuronal cells suggested that the putative transport protein might differ from VGAT in additional ways such as in subcellular location. We therefore raised antibodies to a bacterial fusion protein containing the N terminus of SN1. Immunofluorescence of transfected rat pheochromocytoma PC12 cells shows a diffuse cell surface distribution distinct from that of the synaptic vesicle marker synaptophysin (Figures 3a–3c). In primary hippocampal culture, the antibody labels glial rather than neuronal cells, and also in a plasma

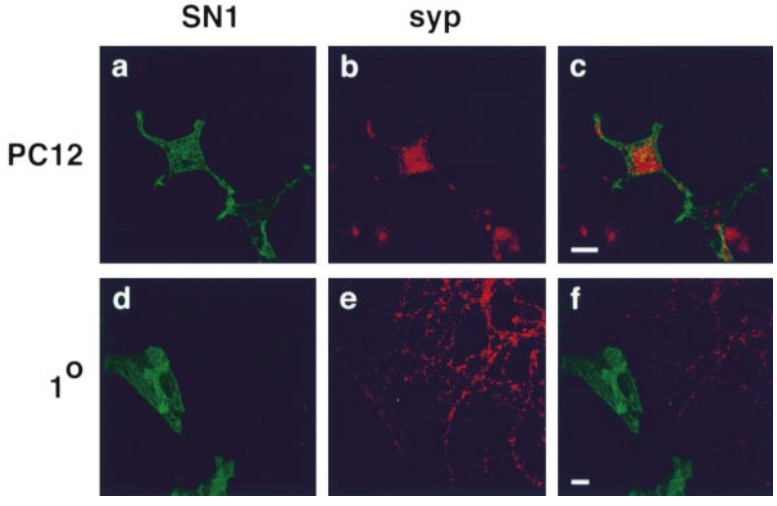
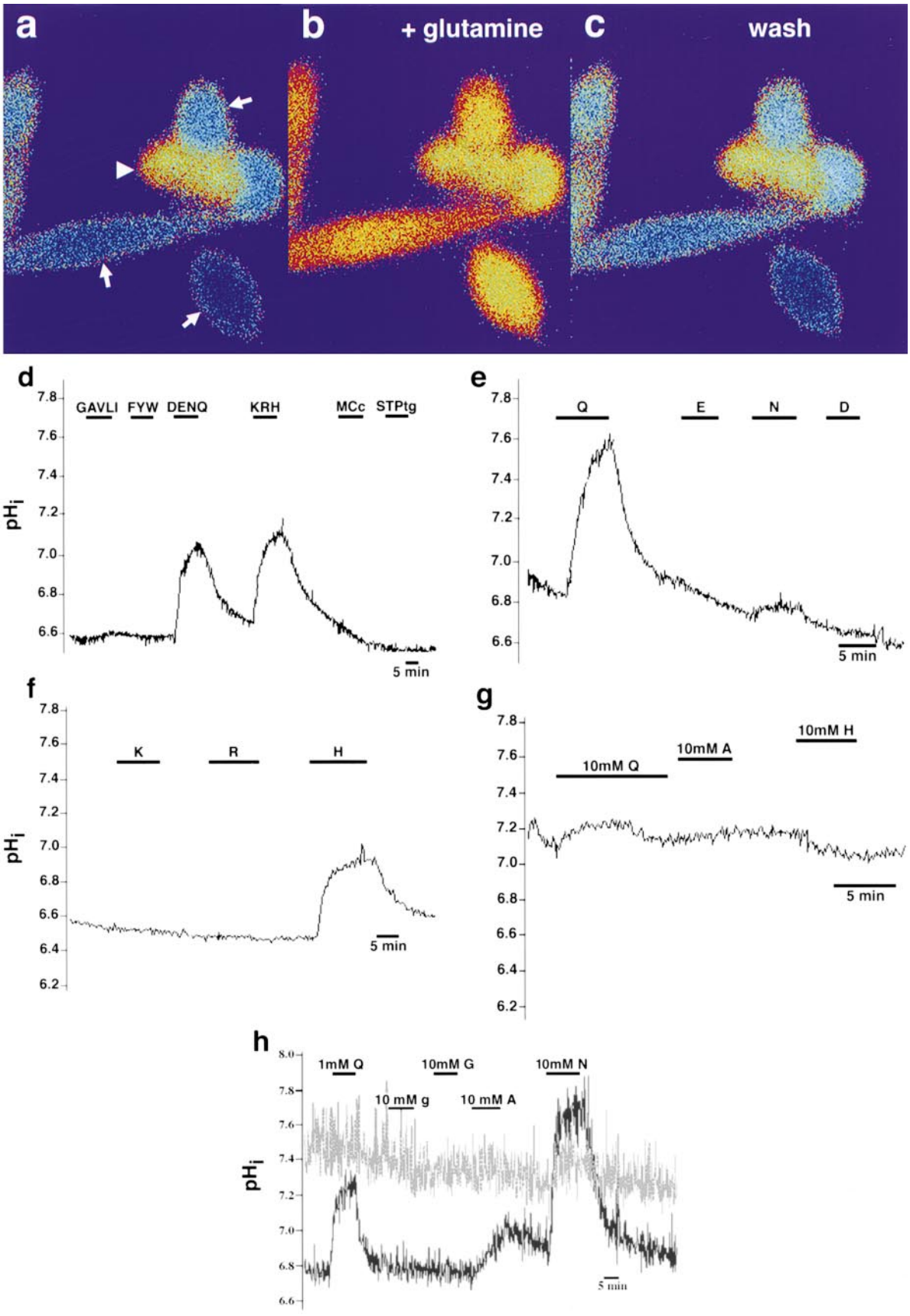


Figure 3. SN1 Localizes to the Plasma Membrane

(a–c) PC12 cells stably expressing SN1 and treated with NGF (a–c) were double stained with a polyclonal antibody to SN1 (a) and a monoclonal antibody to the synaptic vesicle protein synaptophysin (b). In contrast to the expression of synaptophysin on intracellular membranes, SN1 localizes to the cell surface as shown in the overlap (c).

(d–f) Double staining of the endogenous SN1 in primary hippocampal culture indicates expression on the plasma membrane of glial cells (d), with no expression by neurons or at synapses (e and f). Double staining for SN1 and the glial fibrillary acidic protein confirmed the expression of SN1 by astrocytes (data not shown).

The bars in (c) and (f) both indicate 0.5 μm.


a
b
+ glutamine
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g
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membrane pattern (Figures 3d–3f). The novel transporter thus differs from the synaptic vesicle protein VGAT in subcellular location as well as in tissue distribution.

Proton Exchange

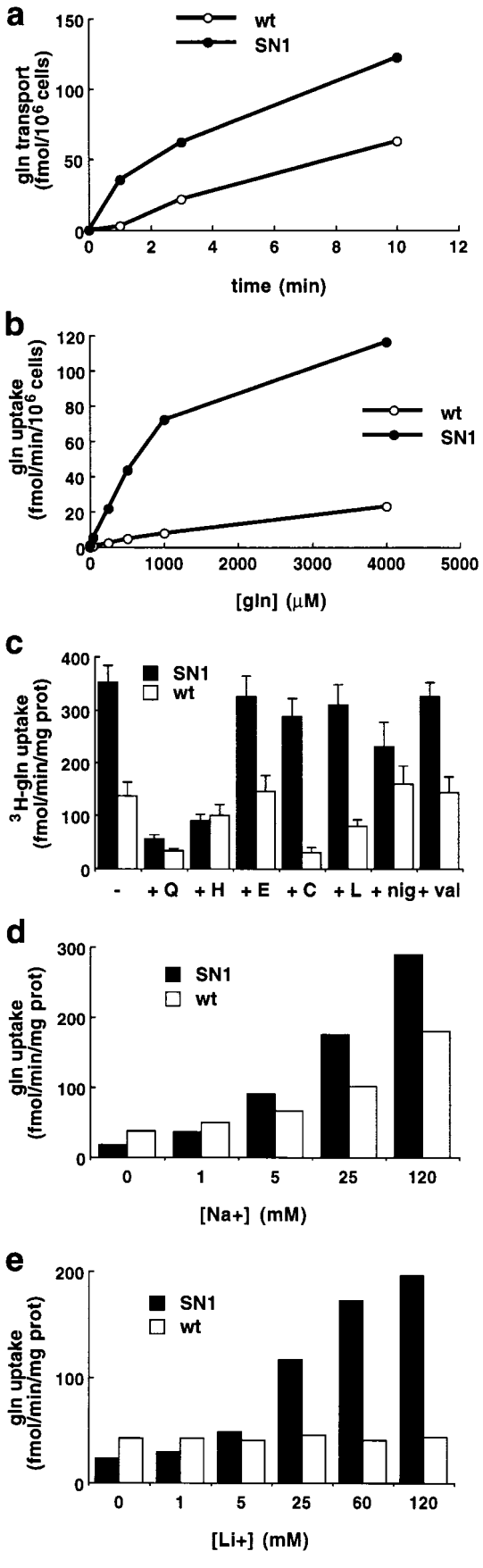
To determine the function of the novel protein, we took advantage of its sequence similarity to previously characterized transporters. All of the plant permeases listed in the dendrogram of Figure 1c recognize amino acids or related compounds as substrates. In addition, they use a proton electrochemical gradient to drive active transport (Fischer et al., 1995; Bennett et al., 1996; Boorer et al., 1996; Rentsch et al., 1996; Boorer and Fischer, 1997; Chen and Bush, 1997). Addition of the appropriate, amino acid–related substrate to cells expressing these proteins should therefore induce a change in intracellular pH. However, the presence of homeostatic mechanisms regulating intracellular pH may obscure these changes, so we stably expressed the SN1 cDNA in PS120 cells, which are deficient in Na^+/H^+ exchange activity (Pouysségur and Franchi, 1987). A surprisingly large percentage (~70%) of the stable transformants expressed the novel protein, suggesting that expression of the putatively proton-coupled transporter helped the relatively acidic PS120 cells to survive, perhaps by catalyzing proton efflux. The stable transformants also appeared to grow more rapidly than the slowly growing parental PS120 cells.

To identify substrates for the putative transporter, we loaded stably transfected cells with the pH-sensitive dye BCECF-AM and monitored the response to amino acids in Krebs-Ringer solution buffered with HEPES. Figure 4 shows the imaging of pH_i in stably transfected cells. Pools of small and aromatic amino acids (1 mM each amino acid) failed to produce a change of pH_i in these cells (Figure 4d). However, a pool containing aspartate, glutamate, asparagine, and glutamine increased pH_i to ~7.1, as did a pool of basic amino acids. Fractionation of these pools showed that glutamine produces a large increase in pH_i (Figures 4a–4c and 4e), followed by histidine and asparagine (Figures 4e and 4f). These amino acids cause a similar, large increase in the pH_i of most transfected cells but no substantial change in the pH_i of all untransfected cells examined ($n = 150$), even at 10-fold higher concentrations (Figure 4g). At the higher concentration, however, asparagine can increase the pH_i of transfected cells as much as glutamine, suggesting that the two substrates differ in apparent affinity, not in the maximal rate of transport (Figure 4h). Alanine also increases the pH_i in transfected cells but to a much lesser extent than the other substrates (Figure 4h). Other standard amino acids, GABA, taurine, ornithine, citrulline, betaine, sarcosine, formiminoglutamate, dihydroxyalanine, citrate, and α-ketoglutarate had no effect on pH_i (data not shown). Thus, the SN1 protein catalyzes the exchange of intracellular protons for extracellular glutamine, histidine, and asparagine.

Although the novel transporter appears to help PS120 cells grow, presumably by promoting proton efflux, the stably expressing cells show a baseline pH_i considerably below the parental cells. Ratiometric measurements of pH_i from individual, stably transfected cells further enabled us to distinguish cells with low pH_i that express SN1 from contaminating cells with a higher pH_i that do not. Figures 4a–4c show that most of the stably transfected cells (arrows) increase pH_i in response to extracellular glutamine. However, certain cells (arrowhead) exhibit a higher resting pH_i that does not detectably change with glutamine application, similar to all of the untransfected cells. Confirming these observations, Figure 4h shows pH_i measurements from two stably transfected cells, one that responds to glutamine and asparagine and another that does not. The responsive cell has a much lower baseline pH_i (~6.7) than the nonresponsive cell (~7.2), consistent with the qualitative observations of Figures 4a–4c. Since glutamine can increase pH_i by promoting proton efflux, the low pH_i observed at baseline in the absence of external glutamine presumably results from flux reversal, with the efflux of glutamine from large intracellular stores driving proton influx. Growth of the stably expressing cells in tissue culture medium that contains glutamine enables the transporter to maintain normal pH_i, but in the absence of glutamine, SN1 causes an even greater reduction in pH_i than observed in parental PS120 cells. The results suggest that the novel transporter may mediate amino acid flux in either direction under physiological conditions. Glutamine levels of ~400 μM produce roughly physiological pH_i (Figure 6a), further suggesting that at least in these

Figure 4. Identification of the Substrates for SN1 by Monitoring Intracellular pH

Extracellular glutamine regulates the pH of PS120 cells expressing SN1. Stable PS120 transformants expressing the SN1 protein were loaded with 5 μM BCECF in Krebs-Ringer solution buffered with 10 mM HEPES, pH 7.4, for 10 min, washed for 20 min in the same buffer and the intracellular pH (pH_i) measured by ratiometric methods in the absence (a) and presence of 1 mM glutamine (b), and after washing for 10 min (c). Most of the cells (arrows) show increased fluorescence in the presence of glutamine, indicating an elevation in pH_i. However, several cells (arrowhead) show a higher pH_i in the absence of glutamine and do not change significantly with the addition of glutamine or after the wash, consistent with contamination of the stable transformants by cells not expressing SN1. Blue indicates lower pH_i and yellow higher pH_i. (d–h) Quantitative analysis of intracellular pH (pH_i) shows that only two pools of amino acids (at 1 mM each amino acid) elicit an increase in cell pH_i (d). Amino acids are indicated by the standard letter code, with c representing cysteine, t taurine, and g GABA. (e and f) Identification of the substrates for SN1. Subdivision of one pool indicates that 1 mM glutamine and to a much lesser extent 1 mM asparagine increase intracellular pH_i (e). Among the basic amino acids, only 1 mM histidine (H) induces an increase in cell pH_i (f). (g) High concentrations (10 mM) of glutamine (Q), alanine (A) or histidine (H) do not affect the pH_i of untransfected cells. (h) Untransfected cells (top trace) have a substantially higher resting pH_i than those expressing SN1 (lower trace), presumably because in the absence of extracellular glutamine, SN1 catalyzes the efflux of cytosolic glutamine in exchange for the uptake of extracellular protons, acidifying the SN1-positive cells relative even to the Na^+-H^+-exchanger-deficient parental PS120 cells. Note the slight increase in pH_i observed with high concentrations of alanine observed only in those cells that also respond to glutamine and asparagine.



cells, increases in glutamine above this concentration will cause uptake and reductions below this concentration will induce efflux.

Na^+-Dependent Flux

The analysis of intracellular pH predicts that the SN1 protein transports glutamine, histidine, and asparagine. We therefore used the stably transfected PS120 cells to measure the uptake of ^{3}H-glutamine in standard flux assays. Figure 5a shows that transfected cells exhibit a time-dependent uptake of ^{3}H-glutamine exceeding that of untransfected cells. The outwardly directed pH gradient of these cells presumably helps to drive the uptake of glutamine and accounts for the difficulty detecting transport in wild-type CHO cells, which lack this driving force (data not shown). Further, transport of glutamine by the transfected cells is saturable with a K_m ~1.1 mM (Figure 5b). We have also used flux measurements to reassess the other substrates of this transporter. Figure 5c shows that 10 mM nonradioactive glutamine dramatically reduces the uptake of ^{3}H-glutamine by both transfected and untransfected cells, indicating endogenous mechanisms for glutamine transport by PS120 cells in addition to the introduced SN1. Histidine—but not glutamate, cysteine, or leucine—also reduces ^{3}H-glutamine uptake in the transfected cells to a greater extent than in the untransfected, consistent with the identification of substrates by measurement of pH_i. The H^+ ionophore nigericin reduces the transport of ^{3}H-glutamine (Figure 5c) by abolishing the pH gradient that drives uptake. Inhibition of glutamine flux by nigericin also militates against the possibility that the intracellular metabolism of glutamine increases pH_i by releasing ammonia to buffer free H^+, and supports instead a direct role for proton translocation in amino acid uptake. In contrast, dissipation of the membrane potential by increasing extracellular K^+ or by adding the K^+ ionophore valinomycin (Figure 5c) does not substantially affect glutamine uptake, suggesting that transport mediated by SN1 is electroneutral.

Figure 5. Flux Measurements of SN1 Transport Activity

(a) Time-dependent uptake of ^{3}H-glutamine uptake by stable PS120 transformants expressing SN1 (filled circles) relative to untransfected controls (open circles).

(b) Saturable transport of glutamine by cells expressing SN1. Lineweaver-Burke analysis indicates a K_m ~1.1 mM.

(c) Inhibition of ^{3}H-glutamine uptake. Unlabeled glutamine (10 mM) inhibits transport mediated by SN1 (closed bars) as well as background uptake (open bars). Similar concentrations of histidine block the transport of ^{3}H-glutamine by transfected and untransfected cells whereas glutamate has little effect on either. Cysteine and leucine affect ^{3}H-glutamine transport to the same small extent in untransfected as well as transfected cells. However, the proton ionophore nigericin (5 μM) selectively reduces transport by the stable transformants expressing SN1 rather than the untransfected cells. On the other hand, valinomycin (20 μM) dissipates the membrane potential and does not affect flux mediated by SN1, suggesting electroneutrality. Na^+ dependence of transport by SN1.

(d) Transport by SN1 requires extracellular Na^+ (closed bars) but even low concentrations of Na^+ (5 mM) suffice. Increasing concentrations of Na^+ also support a glutamine transport activity present in untransfected cells (open bars).

(e) Li^+ supports SN1-mediated glutamine transport almost as well as Na^+.

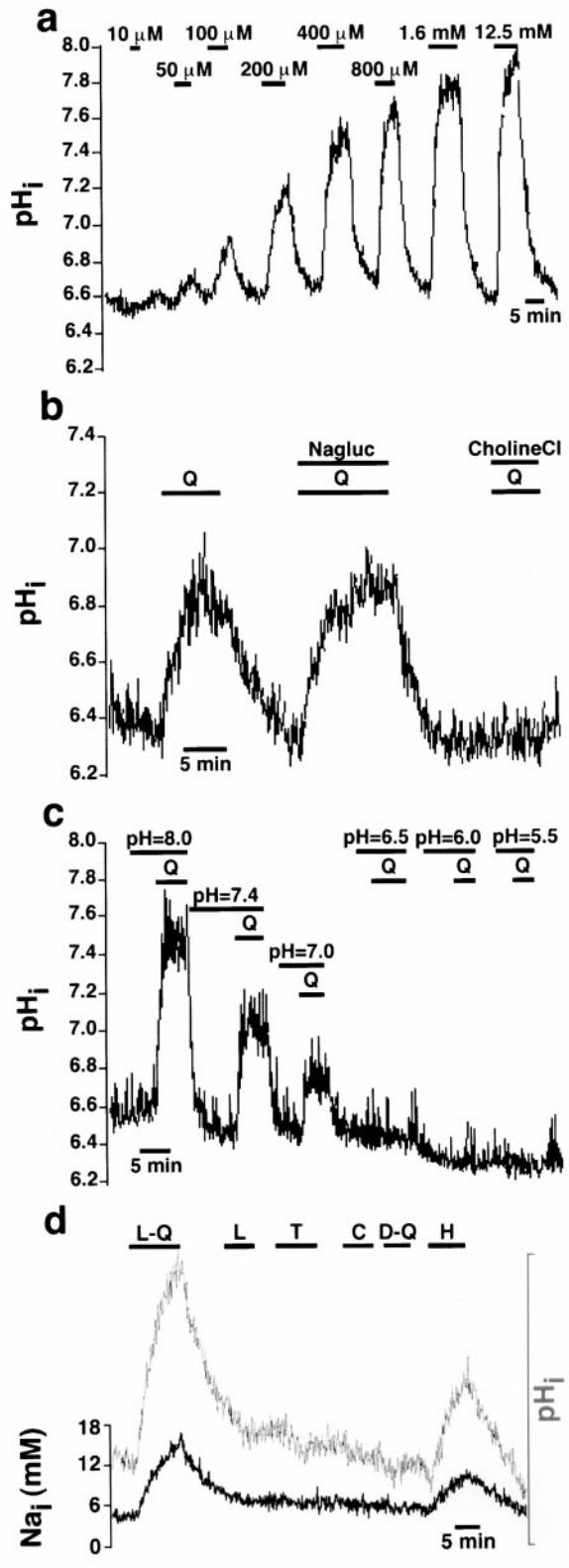


Figure 6. Roles of Glutamine, Na^+, and pH in SN1 Function

(a) Concentration dependence of intracellular pH on extracellular glutamine in cells expressing SN1. Stable PS120 transformants were loaded with BCECF-AM as described in Figure 4 and the cell pH determined in Krebs-Ringer solution buffered by HEPES, pH 8 in the presence of increasing concentrations of glutamine.

Since glutamine is a zwitterion at physiological pH, the electroneutrality of exchange for protons requires the movement of an additional charge. As shown in Figure 6b, replacement of chloride by gluconate does not abolish the increase in intracellular pH induced by extracellular glutamine. However, replacement of Na^+ by choline entirely prevents the increase in pH_i, strongly suggesting that Na^+ is cotransported with glutamine in exchange for H^+. This would account for the observed electroneutrality of transport by SN1. Interestingly, concentrations of Na^+ as low as 5 mM that do not provide a driving force for the cotransport of glutamine in the face of $[Na^+]_i \sim$5–10 mM still activate glutamine transport relative to untransfected cells (Figure 5d). Na^+ might simply activate glutamine transport by binding to a site on the external face of the transporter, but increasing concentrations of Na^+ produce higher levels of ^{3}H-glutamine uptake (Figure 5d), excluding a high-affinity binding site for Na^+. To examine the possibility of a low-affinity Na^+ binding site, we used the Na^+-sensitive dye SBFI to monitor intracellular Na^+ ($[Na]_i$). Figure 6d shows that the addition of 1 mM L-glutamine and histidine but not D-glutamine or several other amino acids increases the $[Na]_i$ of PS120 cells expressing SN1 by 2- to 4-fold. Thus, Na^+ translocation accompanies glutamine uptake by SN1. Consistent with a role for Na^+ cotransport, nigericin does not completely abolish the uptake of ^{3}H-glutamine by SN1 (Figure 5c). Nonetheless, the pH gradient appears to be a major driving force for transport, at least in these cells. Lowering pH_o also dramatically reduces the change in intracellular pH produced by external glutamine (Figure 6c).

Synaptic Localization

Glutamate serves as both the principal excitatory neurotransmitter in the mammalian brain and the precursor for the major inhibitory transmitter GABA. The glutamate used as neurotransmitter in turn derives largely from glutamine (Hamberger et al., 1979a; Thanki et al., 1983; Conti and Minelli, 1994). Since glia synthesize the glutamine used by neurons (Ottersen et al., 1992), the expression of a glutamine transporter by glia suggested by the

(b) Transport by SN1 requires extracellular Na^+ but not Cl^-. Glutamine (1 mM) causes an increase of pH_i in the presence of Krebs-Ringer solution (120 mM NaCl) and when the NaCl is replaced by 120 mM Na gluconate but not with replacement by 120 mM choline chloride. Glutamine uptake by SN1 thus requires extracellular Na^+.

(c) Uptake of glutamine by SN1 requires an outwardly directed pH gradient. Using Krebs-Ringer buffer, high extracellular pH (8) promotes the alkalinization by extracellular glutamine (1 mM) of cells stably expressing SN1. Neutral extracellular pH (7.4 and 7) also supports alkalinization but pH 6.5 and lower do not, indicating that the inwardly directed Na^+ gradient does not alone suffice for glutamine uptake by SN1.

(d) Stable PS120 transformants expressing the SN1 protein were loaded with 5 μM BCECF AM and 20 μM SBFI AM in Krebs-Ringer solution buffered with 10 mM HEPES, pH 7.4, for 30 min, washed for 20 min, and both the pH_i (light trace) and the intracellular Na^+ (Na_i) (dark trace) measured by ratiometric methods. L-glutamine (L-Q) and histidine but not leucine, threonine, cysteine, or D-glutamine (D-Q) (all 1 mM) increase $[Na]_i$ 2- to 4-fold. The changes in $[Na]_i$ parallel those in pH_i. Untransfected PS120 cells showed no change in $[Na]_i$ or pH_i in response to these amino acids.

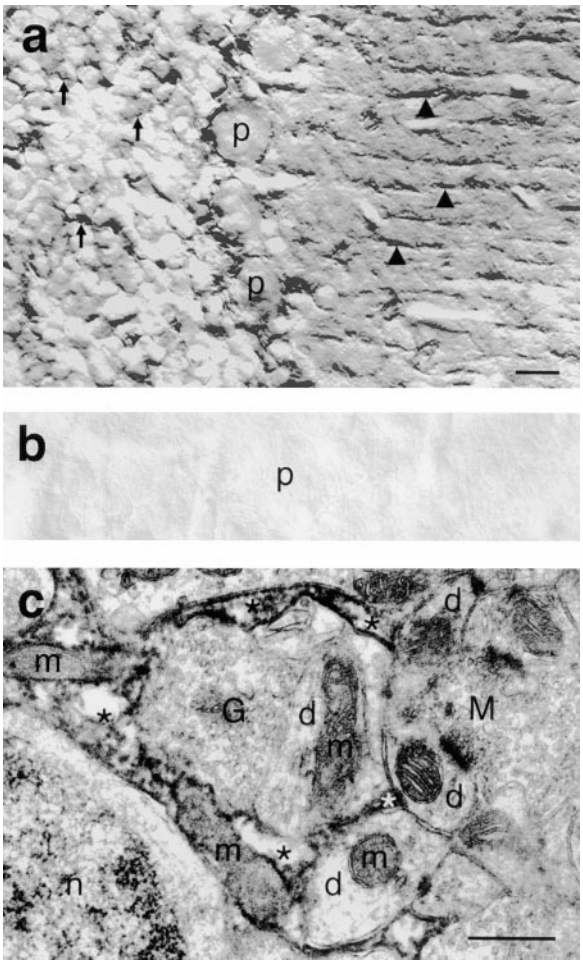


Figure 7. Localization of SN1 to Glial Cells Surrounding Central Synapses

(a) SN1 localizes to two types of astroglial cells in the rat cerebellum. Immunostaining for SN1 shows expression by Bergmann glial fibers (arrowheads) in the molecular layer of the cerebellum. SN1 also resides in astrocytic processes of the granular layer (arrows). Purkinje cells are indicated by p. Adsorption of the SN1 antibody with the bacterial fusion protein containing the N terminus of SN1 that was used to generate this antibody abolishes the immunoreactivity in sections from the cerebellum (b) and other brain regions (not shown). (c) Visualization of immunoperoxidase staining by electron microscopy shows expression of SN1 on astroglial processes (*) ensheathing a Golgi cell terminal (G) and close to a mossy fiber terminal (M), with both terminals synapsing on the dendritic digits of granule cells (d). m, mitochondria; n, the nucleus of a granule cell (labeled by uranyl acetate, not peroxidase). The bar in (a) indicates 50 μm and in (b) 0.5 μm.

in situ hybridization implicates the protein in transmitter synthesis and release. We have therefore localized SN1 by both light and electron microscopy. In the cerebellum, the protein appears on two classes of astroglial cells (Figure 7a). Bergmann glial fibers in the molecular layer and astrocytes in the granule cell layer both express the transporter. Bergmann glial fibers surround dense glutamatergic projections onto Purkinje cell dendrites, strongly supporting localization of the transporter to excitatory synapses. Astrocytic processes surrounding synaptic inputs onto granule cells also label for the transporter. In addition, dense staining around Purkinje cell bodies suggests a role in the GABAergic synapses made at these sites. Other brain regions such as the hippocampus show a similar glial localization in the vicinity of synapses (data not shown). Immuno–electron microscopy in the cerebellum confirms the localization to inhibitory as well as excitatory synapses, with intense immunoperoxidase staining of astrocyte processes ensheathing GABAergic Golgi cell terminals and approaching glutamatergic mossy fiber terminals in the granule cell layer (Figure 7b).

Discussion

System N

Classical studies have identified a series of Na^+-dependent transport activities for zwitterionic amino acids (Palacin et al., 1998). System A transports neutral amino acids including glutamine and, like the VGAT-related glutamine transporter, shows great sensitivity to pH. Although it has not been identified at the molecular level, system A recognizes as substrates serine and other amino acids that are not recognized by the transporter described here (Guidotti and Gazzola, 1992). In addition, system A activity expressed by hepatocytes does not recognize glutamine (Kilberg et al., 1980), further militating against the identity of the VGAT-related glutamine transporter as system A. System ASC has been cloned but recognizes multiple substrates not recognized by SN1 (Arriza et al., 1993; Shafqat et al., 1993; Utsunomiya-Tate et al., 1996). Additional Na^+-dependent amino acid transport activities also differ from the novel glutamine transporter in terms of substrate recognition.

In contrast to other classically described transport systems, the functional characteristics of system N correspond very well with the novel glutamine transporter. Like SN1, system N recognizes as substrates glutamine, histidine, and asparagine (Kilberg et al., 1980). In addition, both activities have a similar apparent affinity for glutamine ($K_m \sim 1.1$ mM). System N also shows a sensitivity to low pH very similar to the VGAT-related protein. Since system N has the relatively unusual ability to tolerate the substitution of Li^+ for Na^+ (Kilberg et al., 1980), we have further tested SN1 for this property. Figure 6e indeed shows that Li^+ supports glutamine transport almost as well as Na^+. The other Na^+-dependent glutamine transport activities present in this cell line apparently do not tolerate Li^+ substitution, accounting for the lower background observed in untransfected cells (Figure 5e).

The tissue distribution of system N also corresponds extremely well with SN1. System N was originally described in the liver (Kilberg et al., 1980), and Northern analysis for SN1 shows the highest level of expression in this tissue. In addition, the transport of glutamine across the blood–brain barrier has been attributed to system N (Ennis et al., 1998; Xiang et al., 1998), and we observe strong expression of SN1 by the choroid plexus. Importantly, previous reports have described the expression of activities related to but distinct from system N in muscle and neurons (Hundal et al., 1987; Tamarappoo et al., 1997), and we have not detected the VGAT-related mRNA in these cells. The glutamine transporter

described here thus accounts for essentially all of the functional characteristics and tissue distribution previously ascribed to amino acid transport system N.

The functional analysis of SN1 helps to account for some of the unusual features previously demonstrated for system N. First, the pH sensitivity of system N apparently derives from its function as a proton exchanger. Low external pH and nigericin eliminate the outwardly directed pH gradient that helps to drive glutamine uptake by SN1 in the acidic PS120 cells. The countertransport of a proton presumably also accounts for the difficulty detecting SN1-mediated transport in less acidic wild-type CHO cells. In addition, concentrations of external Na^+ similar to those found inside cells (and hence not capable of driving cotransport) still allow SN1-mediated glutamine uptake by the acidic PS120 cells. A pH gradient thus suffices to drive amino acid transport by SN1. On the other hand, nigericin does not completely abolish specific uptake by transfected cells, indicating that the inwardly directed Na^+ gradient can still drive transport in the absence of a pH gradient. The apparent electroneutrality further suggests the cotransport of 1 Na^+ with glutamine in exchange for 1 H^+.

The identification of system N as a proton exchanger presumably accounts for the sequence similarity between SN1 and VGAT, a documented proton exchanger (McIntire et al., 1997). The similar sensitivity of system A to low pH also suggests that the proteins responsible for this activity will show sequence similarity to SN1, whereas the pH-insensitive systems N_m and N_b (Hundal et al., 1987; Tamarappoo et al., 1997), although considered similar to system N, may in fact belong to a distinct family of transporters. The countertransport of H^+ by SN1 further indicates that system N contributes to pH regulation as well as glutamine transport. Indeed, the protein appears to behave as a glutamine-gated Na^+/H^+ exchanger.

Flux Reversal

The analysis of SN1 indicates the potential for flux reversal under physiological conditions. Stably expressing cells grow more rapidly than parental PS120 cells, presumably because SN1 promotes proton efflux and hence relieves their acidity. However, the cells expressing SN1 have a much lower intracellular pH ($\sim$6.5) than untransfected cells ($pH_i \sim 7.2$) when placed into medium without glutamine. In the absence of external glutamine, the relatively high intracellular levels apparently provide a concentration gradient for glutamine efflux that drives the uptake of protons, thereby acidifying the cell to a much greater extent than simple elimination of the Na^+/H^+ exchanger. In contrast, the presence of 5 mM glutamine in the tissue culture medium reduces or reverses the outwardly directed glutamine gradient, enabling proton efflux in exchange for external glutamine. Low extracellular glutamine thus induces flux reversal by SN1 despite the large inwardly directed driving force for Na^+ and outwardly directed force for H^+ present in PS120 cells that would both tend to prevent amino acid efflux. To assess the physiological relevance of these observations, the question then is at what concentration of extracellular glutamine does SN1 mediate flux reversal? Although we do not know the intracellular glutamine concentration of the stably expressing PS120 cells, Figure 7a shows that an extracellular concentration $\sim$400 μM restores steady-state pH_i close to 7.4. Thus, a $[gln]_o$ greater than $\sim$400 μM produces amino acid uptake and less than $\sim$400 μM produces efflux. The acidic nature of PS120 cells further suggests that cells with intact pH_i regulation and hence less driving force for uptake will reverse flux at even higher $[gln]_o$. Since extracellular glutamine concentrations range $\sim$500 μM in plasma and cerebrospinal fluid (Fishman, 1992), deviations above and below this level have the potential to influence the direction of flux mediated by SN1. Changes in intra- or extracellular pH will also affect the direction of flux. SN1 may therefore have a physiological role in amino acid efflux as well as uptake, maintaining higher glutamine concentrations inside the cell than outside but allowing changes in intra- or extracellular pH to adjust the steepness of this gradient. The analysis also indicates that changes in glutamine concentrations will influence cell pH.

Biological Roles

Detoxification of ammonia by the liver involves both glutamine uptake and efflux (Haussinger, 1990). Periportal hepatocytes take up glutamine from the portal circulation, and metabolism within these cells by the enzyme glutaminase provides ammonia for the urea cycle. In contrast, perivenous hepatocytes take up ammonia and glutamine synthetase expressed by these cells converts the ammonia to glutamine, which is then released into the circulation. Although glutaminase and glutamine synthetase show a complementary distribution in periportal and perivenous hepatocytes, respectively, SN1 occurs in all hepatocytes, suggesting that it mediates both glutamine uptake by the periportal cells and glutamine efflux by the perivenous cells. Indeed, glutamine metabolism by glutaminase in the periportal cells presumably promotes glutamine uptake by system N whereas glutamine production by glutamine synthetase in perivenous cells presumably promotes efflux. Since SN1 also mediates proton exchange, these fluxes will affect intracellular pH, and changes in pH will in turn influence amino acid flux. The nervous system exhibits a similar cycle involving glutamine uptake and release by distinct cell populations that helps to maintain synthesis and release of the excitatory transmitter glutamate.

The high levels of activity observed at many synapses require the efficient recycling of neurotransmitter as well as synaptic vesicles. For many classical transmitters, recycling depends on uptake by plasma membrane transporters expressed at the nerve terminal (Giros et al., 1996; Jones et al., 1998). However, most of the cloned neuronal glutamate transporters appear post- rather than presynaptically (Rothstein et al., 1994; Dehnes et al., 1998) and presynaptic transporters have not been cloned (Gundersen et al., 1996), raising questions about the mechanism of recycling for this excitatory transmitter. Indeed, considerable work has suggested that glutamate recycles indirectly, through glutamine (Hamberger et al., 1979a; Thanki et al., 1983; Conti and Minelli, 1994). Glutamine in turn derives from glia (Ottersen et al., 1992), where glutamine synthetase converts glutamate recovered from the synaptic space by well-characterized glutamate transporters (Rothstein and Tabakoff, 1984;

Chaudhry et al., 1995) into glutamine. Glutamate also serves as the precursor for GABA (Storm-Mathisen et al., 1986), implicating the glutamine–glutamate cycle in inhibitory as well as excitatory neurotransmission.

The mechanisms involved in glutamine transfer from glia to neurons have remained unknown. System N_b, an activity distinct from system N, has been proposed to mediate the accumulation of glutamine by neurons (Tamarappoo et al., 1997), but the mode of efflux from glia has not been characterized. Previous work in primary cell culture has documented the glial uptake of glutamine through systems L and ASC, but system N appears to predominate under physiological conditions (Nagaraja and Brookes, 1996). Glutamine transport by glia tolerates substitution of Na^+ by Li^+, shows greater sensitivity to inhibition by histidine than cysteine, and exhibits inhibition by low pH, implicating system N in particular (Nagaraja and Brookes, 1996). Indeed, the ASCT1 isoform of system ASC expressed in the brain does not take up glutamine or mediate glutamine heteroexchange (Zerangue and Kavanaugh, 1996), whereas the ASCT2 isoform that recognizes glutamine as a substrate has not been detected in the brain (Utsunomiya-Tate et al., 1996). Previous work has focused on system ASC for its ability to mediate glutamine efflux through heteroexchange, but the evidence of an exchange mechanism for glutamine release by glia has been controversial (Ramaharobandro et al., 1982; Albrecht, 1989; Nagaraja and Brookes, 1996). The ability of SN1 to mediate flux reversal under physiological conditions now indicates that transport system N, previously demonstrated to mediate the bulk of glutamine uptake by glia, may also mediate most of the efflux. Together with its expression by astrocytes and ultrastructural localization to the processes surrounding synapses, SN1 may thus contribute to the glutamine–glutamate cycle required for amino acid transmitter release. In addition, synaptic transmission involves an alkalinization of glia that increases the driving force for glutamine efflux by system N (Chesler and Kaila, 1992; Deitmer and Rose, 1996), providing a mechanism to link glutamine efflux with neural activity through changes in intracellular pH.

Experimental Procedures

Cloning of SN1

A database search for cDNAs encoding proteins with sequence similarity to the vesicular GABA transporter (VGAT) identified a human cDNA (accession number U49082) designated g17. A fragment corresponding to bases 286 to 1200 of g17 was amplified by the polymerase chain reaction (PCR) from a human brain cDNA library and used to screen a rat brain cDNA library (McIntire et al., 1997). The positively hybridizing clones were purified and two overlapping inserts were joined by ligation at a common SacI site to reconstruct the full open reading frame. The topology was predicted using TopPred2 (Stockholm), SOSUI-Classification, and Secondary Structure Prediction of Membrane Proteins (Tokyo) and fitted to the model proposed for amino acid permease 1 from *A. thaliana* (Chang and Bush, 1997).

RNA Analysis

Five micrograms poly(A)$^+$ RNA prepared from different rat tissues was separated by electrophoresis through formaldehyde-agarose, transferred to nylon, hybridized in 50% formamide to a ^{32}P-labeled probe derived from nucleotides 1–1265 of the rat homolog of g17 and submitted to autoradiography with enhancement for 12 hr at −80°C (Liu et al., 1992). In situ hybridization was performed as previously described (McIntire et al., 1997), with 15 μm sections hybridized to ^{35}S-labeled RNA probes at 52°C in 50% formamide before washing in 50% formamide, digestion with RNase A and autoradiography.

Immunofluorescence

PC12 cells treated with NGF (50 ng/ml) for 3 days and primary hippocampal cultures grown for 10 days on glass coverslips coated with poly-D-lysine were immunostained as previously described (McIntire et al., 1997) with polyclonal rabbit antibodies generated to a bacterial fusion protein containing the N terminus of SN1 and mouse monoclonal antibodies to synaptophysin. After washing, the cells were incubated with anti-rabbit antibodies conjugated to FITC and anti-mouse antibodies conjugated to rhodamine, and viewed under epifluorescence.

pH_i Measurement

Na^+/H^+ exchange-deficient PS120 cells derived from the hamster lung fibroblast line CCL-39 (Pouysségur and Franchi, 1987) were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, transfected with the SN1 cDNA in pcDNA3 (InVitrogen) and stable transformants selected for resistance to the neomycin analog G418. Screening by immunofluorescence with the antibody to SN1 identified a series of cell clones with more than 50% of the cells expressing the SN1 protein. For the analysis of pH_i, 15,000–100,000 cells were plated onto glass coverslips, incubated overnight in standard media, loaded for 10 min with 5 μM BCECF-AM (Molecular Probes) and washed for 10 min in Krebs-Ringer solution, pH 7.4. Ratiometric measurements were made in individual cells by excitation at 440 and 490 nm, imaging up to 40 cells per field of view. Calibration was performed using 50 μM nigericin in Krebs-Ringer containing 100 mM KCl as described previously (Krizaj and Copenhagen, 1998).

Transport Assay

Wild-type and stably transfected PS120 cells expressing SN1 were plated at 250,000 per well of a 24-well plate, grown to 70%–80% confluence, preincubated for 10 min in Krebs-Ringer solution buffered by 10 mM HEPES, pH 6.5, and then incubated in same buffer at pH 8.0 plus 1 mM ^{3}H-glutamine with or without additional amino acids or ionophores. The reaction was terminated by two cold washes in the same buffer, the cells were lysed in 1% SDS and the radioactivity was measured by scintillation counting in 2.5 ml Cytoscint (ICN).

Na_i Measurement

Cells were plated onto glass coverslips, incubated overnight in standard media, loaded for 30 min with 4 μM BCECF-AM and 20 μM SBFI-AM (Molecular Probes) and washed for 10 min in Krebs-Ringer solution, pH 7.4. Ratiometric measurements were made in individual cells as described above for imaging of pH_i with BCECF and by excitation at 340 and 380 nm for the ratiometric imaging of $[Na]_i$. Calibration was performed using 10 μM monensin in Krebs-Ringer containing 0, 5, 10, and 20 mM NaCl (in choline chloride) as previously described (Rose et al., 1998).

Immunocytochemistry

The tissue was prepared and the labeling performed as previously described (Chaudhry et al., 1998) using polyclonal antibodies to SN1 (at 1:200–1:500) and biotinylated secondary donkey anti-rabbit antibodies (1:100). For light microscopy, the sections were mounted using glycerol-gelatin, and visualized by differential interference contrast. Adsorption was performed by preincubation of the diluted SN1 antibody for 2 hr before immunostaining with 100 ng of bacterial fusion protein containing the N terminus of SN1 that was used to generate this antibody. For electron microscopy, ~1–3 mm^3 pieces of tissue were excised from the immunostained brain sections, postfixed in 2.5% glutaraldehyde, immersed in 10 mg/ml OsO_4 for 30 min, dehydrated in graded ethanol and propylene oxide, and embedded in Durcupan ACM. The contrast of ultrathin sections was enhanced by incubation with 10 mg/ml uranyl acetate for 10 min and

3 mg/ml lead citrate for 1 min before visualization using a Phillips CM10 electron microscope.

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