The H⁺-Coupled Electrogenic Lysosomal Amino Acid Transporter LYAAT1 Localizes to the Axon and Plasma Membrane of Hippocampal Neurons

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Recent work has identified a lysosomal protein that transports neutral amino acids (LYAAT1). We now show that LYAAT1 mediates H⁺ cotransport with a stoichiometry of 1 H⁺/1 amino acid, consistent with a role in the active efflux of amino acids from lysosomes. In neurons, however, LYAAT1 localizes to axonal processes as well as lysosomes. In axons LYAAT1 fails to colocalize with synaptic markers. Rather, axonal LYAAT1 colocalizes with the exocyst, suggesting a role for membranes expressing LYAAT1 in specifying sites for exocytosis. A protease protection assay and measurements of intracellular pH further indicate abundant expression at the plasma membrane, raising the possibility of physiological roles for LYAAT1 on the cell surface as well as in lysosomes.

Key words: LYAAT1; lysosome; neutral amino acid transport; exocyst; axon; neuronal ceroid lipofuscinosis

Introduction

After endocytosis the membrane proteins destined for degradation sort to lysosomes. Lysosomal degradation contributes to normal protein turnover and the elimination of damaged or misfolded proteins. It also helps to terminate signals initiated at the plasma membrane by inactivating internalized receptors. Within the lysosome hydrolytic enzymes break down membrane proteins into their constituent amino acids and sugars. Defects in these enzymes result in lysosomal storage disorders such as Tay–Sachs disease that affect multiple organs, including the nervous system (Gravel et al., 1995).

Continuing protein degradation by the lysosome requires a mechanism to clear breakdown products. Lysosomes indeed exhibit a number of transport activities, many of which serve to remove amino acids or carbohydrates to the cytoplasm (Pisoni and Thoenes, 1991; Mancini et al., 2000). Similar to defects in protein breakdown, defects in export also can produce lysosomal storage disease. Cystinosis involves the accumulation of cystine by multiple tissues and results from mutations in a lysosomal cystine transporter (Gahl et al., 1982; Town et al., 1998; Kalatzis et al., 2001). Sialic acid storage disorders similarly result from mutations in a lysosomal transporter for sialic acid (Mancini et al., 1991; Verheijen et al., 1999). Both of these efflux systems rely on H⁺ cotransport driven by the outwardly directed lysosomal H⁺ electrochemical gradient (Smith et al., 1987; Mancini et al.,

1989). However, the proteins responsible for most lysosomal transport activities remain unknown.

Identified several years ago, the vesicular GABA transporter (VGAT) defined a large family of mammalian proteins (McIntire et al., 1997). VGAT itself mediates the transport of GABA into synaptic vesicles and, like many lysosomal transport activities, relies on a H⁺ electrochemical gradient. In particular, transport by VGAT involves the exchange of H⁺ for GABA (McIntire et al., 1997). Closely related to VGAT, the amino acid transport systems N and A contribute to nitrogen metabolism and the glutamine–glutamate cycle involved in recycling glutamate and GABA for release at synapses (Chaudhry et al., 2002b). Consistent with its function at the plasma membrane, the system N transporter SN1 depends on Na⁺ but also mediates H⁺ exchange similar to VGAT (Chaudhry et al., 1999). System A transporters also reside on the plasma membrane, depend on Na⁺, and have retained the sensitivity to H⁺; however, they do not have the capacity for H⁺ translocation (Albers et al., 2001; Chaudhry et al., 2002a). The members of this family thus vary considerably in their ionic coupling and subcellular location. This family also includes the lysosomal amino acid transporter LYAAT1.

Although predominantly lysosomal in many cells, heterologous expression of LYAAT1 confers the uptake of radiolabeled GABA and many neutral amino acids from the extracellular medium, indicating localization to the plasma membrane as well (Sagne et al., 2001). Indeed, uptake by the cell is topologically equivalent to efflux from lysosomes. Consistent with H⁺ cotransport out of the lysosome, low pH stimulates uptake by LYAAT1 at the cell surface. We now have extended the analysis of LYAAT1 to direct measurements of H⁺ flux and charge movement. The addition of amino acid substrates reduces the pH of cells overexpressing LYAAT1, indicating that the protein mediates H⁺ flux. LYAAT1 expressed in *Xenopus* oocytes mediates inward charge movement in response to substrates, and this

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charge movement is coupled with 1:1 stoichiometry (charge/ amino acid). We also have found that LYAAT1, which is enriched in the brain, localizes to axons as well as the perikaryal lysosomes of neurons. Axonal LYAAT1 does not colocalize with lysosomal or synaptic vesicle markers. Rather, it colocalizes with subunits of the exocyst complex implicated in the specification of sites for exocytosis. In addition, a substantial proportion of endogenous LYAAT1 in neurons resides at the cell surface, as determined by both protease protection and the response of intracellular pH to extracellular substrates. The transporter thus may function at the plasma membrane as well as lysosomes in neurons and in other cells.

Materials and Methods

Molecular cloning and in situ hybridization. A fragment of the LYAAT1 cDNA amplified by PCR from brain cDNA was used to screen a rat brain

library. Several clones containing the entire open reading frame were isolated, sequenced, and compared with related sequences by using the PileUp⁺ program (GCG Wisconsin Package, Accelrys, San Diego, CA). For *in situ* hybridization a 595 bp LYAAT1 cDNA containing the last 26 codons of the protein-coding sequence plus 3'-UTR was amplified by PCR and subcloned into pBluescript II KS (pBS) at the *SalI*–*NotI* sites. *In situ* hybridization was performed as described previously (Freneau et al., 1992). Briefly, fresh-frozen brains from 21-d-old rats were sectioned at 20 μ m, postfixed in 4% paraformaldehyde/PBS, UV cross-linked, and hybridized to ³⁵S-labeled single-stranded RNA probes in 50% formamide for 16–18 hr at 53°C.

Transport assay. LYAAT1 was expressed in HeLa cells by using the vaccinia virus-T7 polymerase system (Povlock and Amara, 1998). Briefly, HeLa cells were plated on 24-well plates at a density of 100,000 per well and infected with vaccinia virus (1×10^6 pfu in 750 μ l of OptiMem media). Thirty minutes later the cells were transfected with 1 μ g of DNA/1.5 μ g of lipofectin (Invitrogen, San Diego, CA). Cells trans-

Figure 1. Expression of LYAAT1 in the brain. *A*, Predicted protein sequence of rat LYAAT1 aligned with the vesicular GABA transporter (VGAT), the system N transporter SN1, and the closely related LYAAT2. Horizontal lines over the sequence indicate nine putative transmembrane domains (TMD 1–9), and asterisks indicate potential N-linked glycosylation sites. *B, C*, *In situ* hybridization for LYAAT1 in rat brain slices with the use of antisense (*B*) and sense (*C*) probes. Ctx, Cortex; Cpu, caudate putamen; Hc, hippocampus; Th, thalamus. Scale bar, 2 mm. *D, E*, Dark-field microscopy of autoradiographic images from *D* and *E*, respectively, shows strong hybridization in the pyramidal cell layers of hippocampal fields CA1 and CA3. The granule cell layer of the dentate gyrus (DG) expresses lower levels. *F, G*, High-magnification dark-field image of the cerebellum hybridized with antisense (*F*) and sense (*G*) probes. Arrows indicate the strongly hybridizing Purkinje cell layer (Pc). Molecular (m) and granule (g) cell layers express lower levels. The white matter (w) shows essentially no labeling. Scale bar: G (for E–G), 500 μ m.

Figure 2. Proline transport by LYAAT1. *A*, A representative time course of specific ^3H -proline uptake by cells expressing LYAAT1 (minus untransfected). *B*, Representative saturable uptake of proline by LYAAT1. Multiple independent experiments indicate a K_m $870 \pm 419 \mu\text{M}$; $n = 9$. *C*, Inhibition of ^3H -proline uptake at 5 min by 10 mM amino acid. GB, GABA; sarc, sarcosine; single letters reflect standard amino acid nomenclature. *D*, Ionic and energetic dependence of proline uptake. Dissipation of ΔpH by nigericin (nig) reduces transport by LYAAT1 to a greater extent than dissipation of $\Delta\psi$ with valinomycin (val). *E*, Proline transport by LYAAT1 depends on low external pH (pH_o). Results are normalized to maximal uptake at pH_o 5.5. *F*, Replacement of choline chloride with 60 mM KCl/60 mM NaCl (+KCl) or NaCl does not reduce ^3H -proline uptake significantly. On the other hand, replacement with Na-glucuronate (Na gluc) reduces, but does not eliminate, transport. The results are normalized to uptake in choline chloride. * $p < 0.05$ relative to control (pH_o 5.5 in *E* or choline in *F*); ** $p < 0.001$; *** $p < 0.0001$. For all experiments the error bars indicate SEM.

fects with empty pBS plasmid were used as controls. After 16–20 hr the cells were washed three times with Krebs–Ringer solution, pH 7.4 [containing (in mM) 120 NaCl, 4.7 KCl, 2.2 CaCl_2 , 1.2 MgCl_2 , 1.2 KH_2PO_4 , and 10 HEPES plus 0.18% glucose], and incubated for 5 min (or the time indicated in the text) in 250 μl of Krebs–Ringer solution, pH 5.5, containing MES buffer, 120 mM choline chloride substituted for 120 mM sodium chloride (assay buffer), and 100 μM [2,3- ^3H]-proline (PerkinElmer Life Sciences, Emeryville, CA). Then the cells were rinsed three times with cold assay buffer, solubilized in 1% SDS for 30 min at room temperature, and mixed with 3 ml of Ecolume (ICN Biochemicals, Costa Mesa, CA). All measurements were made in triplicate. To assess inhibition, we added 2 mM competing amino acid to the standard assay mixture. To assess ionic dependence, we used choline chloride (120 mM), choline chloride/potassium chloride (60 mM each), sodium chloride (120 mM), or sodium gluconate (120 mM) in both the assay and wash buffers. For experiments that used nigericin (5 μM) and valinomycin (20 μM), choline chloride/potassium chloride (60 mM each) was used. Quantitation and statistics were performed by using the Prism program (GraphPad Software, San Diego, CA).

Measurement of intracellular pH in HeLa cells. The vaccinia virus-T7 polymerase system was used to express LYAAT1 in HeLa cells at a density of 40,000 cells per glass coverslip coated with Matrigel (Becton Dickinson, Mountain View, CA). The cells were loaded for 10 min with 5 μM BCECF-AM (Molecular Probes, Eugene, OR) in Krebs–Ringer solution, pH 7.4, and washed for 10 min in the same buffer before the pH of individual cells was measured by ratiometric methods at 440 and 490 nm excitation (Chaudhry et al., 1999). Approximately 20% of the transfected

Figure 3. Transport by LYAAT1 involves H^+ translocation. *A*, The pH_i of HeLa cells expressing LYAAT1 with the vaccinia virus-T7 polymerase system was determined by ratiometric analysis via the pH-sensitive dye BCECF. Amino acids (10 mM) were added to the cells for 5 min intervals at the times indicated by the horizontal bars above the trace. L-Proline, alanine, GABA, and D-proline, but not serine, produce an acidification that was not observed in cells transfected with empty vector. The pH_i was calibrated in the proton ionophore nigericin at different pH_o . *B*, The change in pH_i produced by different amino acids was quantitated as a proportion of the response to proline. ** $p < 0.001$ and *** $p < 0.0001$ relative to proline; ++ $p < 0.001$ relative to alanine. Error bars indicate SEM.

cells in a field of ~30 (and no control cells) showed pH changes with the addition of 10 mM amino acid substrate. pH calibration was performed by using buffers at various pH containing 60 mM KCl and 5 μM nigericin. Quantitation and statistics were performed with Prism (GraphPad Software).

Electrophysiology. *Xenopus* oocytes were prepared as described previously (Quick and Lester, 1994); they were washed extensively in (in mM) 96 NaCl, 2 KCl, 1 MgCl_2 , 5 HEPES, pH 7.4, and 1.8 CaCl_2 (ND96, pH 7.4) and injected with capped LYAAT1 cRNA transcribed from the pGEMHE plasmid (Liman et al., 1992) (Ambion, Austin, TX). After injection the oocytes were stored at 14°C in ND96 containing 2.5 mM sodium pyruvate, 0.5 mM theophylline, 5% heat-inactivated horse serum, and 50 $\mu\text{g}/\text{ml}$ gentamycin for 4–6 d before use. Two-electrode voltage-clamp recordings were performed with 0.1–1.0 M Ω electrodes via Geneclamp 500B (Axon Instruments, Union City, CA) at room temperature in ND96, pH 6.5 (except where noted in the text). Oocytes were held at –30 mV and stepped for 250 msec (with 100 msec between) from +40 to –140 mV. For charge/flux measurements the oocytes were incubated in ND96, pH 6.5, containing 0.625 mM L-[5- ^3H]-proline for 5 or 10 min, washed for 1 min, and solubilized in 100 μl of 1% SDS; the radioactivity was measured by scintillation counting in Ecolume. Background uptake by uninjected oocytes was subtracted from the uptake by oocytes expressing LYAAT1 to yield specific transport. The uptake of ^3H -proline by individual oocytes was compared with currents generated over the same time interval in the same oocyte. Quantitation and statistics were performed with Prism (GraphPad Software).

Antibody production. A glutathione S-transferase (GST) fusion containing the N terminus of rat LYAAT1 from residues +1 to +45 was

Figure 4. Transport by LYAAT1 is electrogenic. *A*, Proline produces inward currents in *Xenopus* oocytes injected with LYAAT1 cRNA. Increasing concentrations of proline (in mM) added at the times indicated by the horizontal bars above the trace cause progressively larger currents. *B*, At a holding potential of -70 mV the currents produced by proline are saturable. Normalizing to the maximal response (I_{max}) extrapolated from the data, the data reveal an EC_{50} 1.65 ± 0.25 mM; $n = 7$. *C*, Current–voltage relationship (I – V curve) of currents produced by 10 mM proline in a range of pH_o . *D*, Dependence of currents associated with LYAAT1 on low pH_o at a holding potential of -70 mV. The currents were normalized to those observed at pH_o 6.5; $n = 9$. *E*, Currents produced by different amino acids (10 mM) at -70 mV normalized to those produced by proline. $n = 6$ for all except *glu*, in which $n = 3$. *F*, Charge/flux ratio for proline transport. Oocyte membrane potential was held at -70 mV (triangles) and -30 mV (squares); the current produced by 0.625 mM 3H -proline was integrated over a 5 or 10 min interval. The 3H -proline accumulated during this time was used to calculate the amino acid uptake, and the results were fit by linear regression to yield a slope of 0.91 ± 0.07 ($r^2 = 0.493$). For all experiments the error bars indicate SEM. * $p < 0.05$, ** $p < 0.001$, and *** $p < 0.0001$ relative to pH_o 6.5 (*D*) and proline (*E*).

produced in the BL21 strain of *Escherichia coli* and purified by chromatography over glutathione-agarose; ~ 250 μ g of the protein was inoculated into rabbits at monthly intervals, and bleeds were performed 10–12 d after the boosts (QCB, Hopkinton, MA).

Western blot analysis. LYAAT1 was expressed in HeLa cells via the vaccinia virus-T7 polymerase system. Cells were harvested 20 hr after transfection and homogenized in 0.32 M sucrose buffer and 10 mM HEPES, pH 7.4, containing 1% Triton X-100, 20 mM NH_4Cl , and protease inhibitors. To prepare brain extracts, we froze an adult male rat brain in liquid nitrogen and homogenized it in the same buffer; the nuclei were sedimented at $1000 \times g$ for 10 min. The resulting HeLa cell (10 μ g) or brain extracts (30 μ g) were separated by electrophoresis through 10% acrylamide, transferred to polyvinylidene difluoride, and immunostained as described previously (Krantz et al., 2000), using the rabbit LYAAT1 antibody at 1:10,000, a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase, and the SuperSignal West Pico chemiluminescence detection system (Pierce, Rockford, IL).

Immunofluorescence. For immunofluorescence the HeLa cells were

Figure 5. LYAAT1 localizes to lysosomes. *A*, Western blot analysis of extracts from HeLa cells transfected with the LYAAT1 cDNA or empty plasmid (*con*) shows that the LYAAT1 antibody recognizes an ~ 55 kDa protein only in cells expressing LYAAT1. *B*, Immunoblots of extracts from HeLa cells transfected with the LYAAT1 and 2 cDNAs (*top*) indicate that the LYAAT1 antibody (α -LYAAT1) specifically recognizes LYAAT1 and not LYAAT2. LYAAT2 protein nonetheless is detectable by using an antibody to LYAAT2 (α -LYAAT2). *C*, In brain extracts α -LYAAT1 recognizes proteins of ~ 35 , 40, and 90 kDa, but adsorption with the GST fusion protein used to produce α -LYAAT1 (*GST-LYAAT1*) eliminates only the two smaller species, indicating that recognition of the ~ 90 kDa protein is nonspecific. Markers on the left of *A* and *B* indicate kilodaltons. *D*, Immunofluorescence in transfected HeLa cells shows that LYAAT1 (red) colocalizes almost completely with the lysosomal protein LAMP-1 (green).

transfected with the LYAAT1 cDNA in pcDNA3.1 (Invitrogen). Primary hippocampal cultures prepared from 1-d-old rats were grown for 10 d on glass coverslips coated with poly-D-lysine. All cells were fixed in 4% paraformaldehyde for 5 min at room temperature; 100% methanol at $-20^\circ C$ for 5 min; rinsed in PBS; blocked in PBS containing 2% normal goat serum, 2% bovine serum albumin, 1% fish skin gelatin, and 0.02% saponin; and incubated in the same buffer with 1% normal goat serum and the LYAAT1 antibody at 1:2000 for 1 hr at room temperature or overnight at $4^\circ C$. For double labeling in HeLa cells a mouse monoclonal CD107a antibody (LAMP-1; PharMingen, San Diego, CA) was used at a dilution of 1:2000. In hippocampal cultures a mouse monoclonal antibody to LAMP1 (Stressgen Biotechnologies, San Diego, CA) was used at 1:7500 and a mouse monoclonal antibody to synaptophysin (p38) at 1:2000. Subsequent washes and incubations were performed at room temperature in the same buffer lacking horse serum. Secondary antibodies (goat anti-mouse or goat anti-rabbit) were conjugated to either Alexa 488 or Alexa 594 fluorophores (Molecular Probes). In the hippocampal cultures the final washes contained 1% glycine to reduce background fluorescence. The fluorescence was examined by confocal laser microscopy.

Trypsin digestion of cultured hippocampal neurons. Dissociated hippocampal neurons were plated onto glass coverslips at a density of 50–100,000 per well in a 24-well plate. After 10 d *in vitro* the cells were rinsed twice for 10 min each in PBS (with Ca^{2+} and Mg^{2+}) at $37^\circ C$ and 5% CO_2 and were incubated for 10 min at $37^\circ C$ in 100 μ l of Ca^{2+}/Mg^{2+} -free PBS $\pm$ 100 μ g/ml trypsin $\pm$ 1% Triton X-100. The reaction was terminated by the addition of EDTA, multiple protease inhibitors (PMSF, trypsin inhibitor, leupeptin, antipain), and SDS sample buffer (Pierce) and by boiling for 5 min (Renick et al., 1999). Approximately one-third of the extract from each coverslip was subjected to electrophoresis through 4–20% acrylamide, transferred to a nylon filter, and stained with LYAAT1 antibody at 1:10,000 or a rabbit synaptophysin antibody at 1:5000; the deposits were visualized with HRP-conjugated secondary antibodies and SuperSignal West Pico chemiluminescent detection system (Pierce).

Measurement of intracellular pH in neurons. Previous studies have shown that the green fluorescent protein (GFP) is an efficient indicator of

Figure 6. LYAAT1 localizes to axonal processes as well as somatodendritic lysosomes. Shown is double labeling of hippocampal neurons for LYAAT1 (*A, D, G, J*) and for LAMP1 (*B, E*), synaptophysin (*syp; H*), and sec6 (*K*). Merged images are shown in *C, F, I*, and *L*; all images were visualized by confocal laser microscopy. *A–C*, LYAAT1 colocalizes with LAMP1 in cell bodies (*arrows, C*). However, several LAMP1⁺ structures do not label for LYAAT1 (*arrowhead*), and in processes that generally contain little if any LAMP1 the LYAAT1⁺ structures do not label for LAMP1 (*asterisk*). *D–F*, Adsorption of the antibody with the GST-LYAAT1 fusion protein eliminates the staining for LYAAT1. *G–I*, In processes the LYAAT1 shows only partial colocalization with synaptophysin (*arrowheads*). Most structures label only for LYAAT1 or synaptophysin. *J–L*, The LYAAT1 in processes colocalizes extensively with exocyst subunit sec6. However, the colocalization does not extend to cell bodies (*asterisk*), which label only for LYAAT1 and not for sec6. Scale bar in *L* applies to all images.

intracellular pH (Kneen et al., 1998). Dissociated hippocampal cultures grown for 7 d *in vitro* were transfected with the GFP cDNA under the control of the chicken actin promoter with the use of Effectene (Qiagen, Chatsworth, CA) and were examined 3–7 d later with a Zeiss (Oberkochen, Germany) Pascal LSM 5 laser-scanning microscope. Coverslips containing transfected cells were mounted in a laminar-flow chamber and perfused with a Ringer's solution [containing (in mM) 115 NaCl, 5.0 KCl, 1.0 MgCl₂, 1.8 CaCl₂, 5.0 HEPES, 5.0 glucose, pH 7.4]. In Na⁺-free solutions Na⁺ was replaced by equimolar amounts of Li⁺. With the use of a 488 nm argon laser under low power to avoid photobleaching and a 63× water immersion objective (0.95 numerical aperture), GFP-expressing cells with the characteristic morphology of pyramidal neurons were imaged (512 × 512 pixels) every 10–15 sec. After the

experiment the regions of interest corresponding to the cell body or processes were selected; the average intensity was computed for each. The addition of NH₄Cl at the end of experiments produced the expected rapid alkalization, and its removal produced the expected acidification.

Results

To determine the distribution of LYAAT1 in the brain, we performed *in situ* hybridization. The cortex, thalamus, pyramidal cell layer of the hippocampus, and Purkinje cells of the cerebellum strongly and specifically label for LYAAT1 mRNA (Fig. 1*B–G*). The absence of hybridization to white matter, together with the labeling of specific cell populations in hippocampus and cerebellum, suggests that expression is restricted to neurons. However, the hybridization to hippocampal pyramidal cells and cerebellar Purkinje cells indicates that both glutamatergic and GABAergic neurons express LYAAT1.

Neutral amino acid transport

Previous studies have shown that the overexpression of LYAAT1 can result in cell surface expression, enabling the measurement of uptake into intact cells (Sagne et al., 2001). We used the vaccinia virus-T7 polymerase system to overexpress LYAAT1 in HeLa cells and confirmed the plasma membrane localization by immunofluorescence (data not shown). By measuring uptake at an external pH (pH_o) of 5.5, we created conditions analogous to efflux from acidic lysosomes. Under these conditions we observed time-dependent, saturable uptake of ³H-proline with a *K_m* 870 ± 419 μM (Fig. 2*A,B*), similar to the previously reported *K_m* for GABA ~500 μM (Sagne et al., 2001). With the use of 2 mM amino acid to compete with the uptake of ³H-proline, GABA was the most potent inhibitor, followed by proline, sarcosine, glycine, and alanine (Fig. 2*C*). Among the small neutral amino acids only serine is not recognized by LYAAT1.

To characterize the ionic coupling of amino acid transport by LYAAT1, we focused on H⁺ because lysosomes maintain an outwardly directed H⁺ electrochemical gradient.

The proton ionophore nigericin, which dissipates the chemical component of this gradient (ΔpH) but leaves the electrical component (ΔΨ) intact, reduces ³H-proline uptake by ~65% (Fig. 2*D*), indicating the dependence of transport on ΔpH. Addition of the ionophore valinomycin, which specifically abolishes ΔΨ, reduces transport activity, suggesting dependence on ΔΨ but to a much lower extent (~14%). Consistent with a role for H⁺ cotransport in LYAAT1 function, increases in pH_o over 6.0 reduce uptake (Fig. 2*E*), and the elimination of Na⁺ and K⁺ have no significant effect (Fig. 2*F*). However, substitution of Cl[−] with gluconate reduces transport by ~50%. Chloride thus is

not required for transport but appears to modulate LYAAT1 activity.

Electrogenic H^+ cotransport

We previously have used the pH-sensitive dye BCECF to show that the system N transporter SN1 mediates the exchange of H^+ for amino acid substrate (Chaudhry et al., 1999). To assess more directly the coupling of LYAAT1 to H^+ , again we have used BCECF. In these experiments large concentrations of potential substrates are used to drive the movement of coupled H^+ . The low external pH important for the uptake of small amounts of radiolabeled compounds therefore is not required. Ratiometric measurements in HeLa cells expressing LYAAT1 show that 10 mM L- and D-proline, alanine, and GABA reduce intracellular pH (pH_i) (Fig. 3A), indicating that they are all substrates. None of these amino acids produces a significant change in the pH_i of mock-transfected HeLa cells (data not shown), indicating the specificity of changes for LYAAT1. In addition, serine, threonine, and other amino acids that failed to inhibit proline uptake do not change pH_i (Fig. 3B). Except for alanine, the various amino acid substrates showed no significant difference in the magnitude of change that was produced (Fig. 3B), consistent with the equilibrium nature of the pH_i measurement and similar flux coupling for all amino acid substrates.

The cotransport of neutral amino acids and H^+ suggests that LYAAT1 moves net charge and may generate currents coupled to amino acid flux. We therefore used two-electrode voltage clamp to measure the currents produced by amino acids in *Xenopus* oocytes expressing LYAAT1. The addition of known substrates for LYAAT1 to cells injected with LYAAT1 cRNA, but not uninjected oocytes, produces concentration-dependent inward currents (Fig. 4A,B). The EC_{50} for proline-induced currents is 1.65 ± 0.25 mM, which is not significantly different from the K_m for the uptake of radiolabeled proline (870 ± 419 μ M). High concentrations of sarcosine, alanine, and GABA also generate currents in oocytes expressing LYAAT1 (Fig. 4E), consistent with their recognition as substrates. Although GABA appears to be recognized with highest affinity, it produces only $\sim 60\%$ of the current caused by proline, suggesting that GABA is transported more slowly than proline.

The currents associated with LYAAT1 exhibit a dependence on pH_o similar to the measurements of flux (Fig. 4C,D). The relationship of substrate-induced currents to voltage also shows strong rectification: if the currents are coupled stoichiometrically to transport, the addition of external amino acid can generate only inward currents. To characterize the flux coupling further,

Figure 7. Double staining for LYAAT1 and synaptophysin in hippocampal cultures at different times *in vitro*. Hippocampal neurons grown for 4 (A–C), 6 (D–F), 9 (G–I), and 12 (J–L) d *in vitro* were immunostained for LYAAT1 (A, D, G, J) and synaptophysin (syp; B, E, H, K). The immunofluorescence was detected by confocal laser microscopy; merged images are shown in C, F, I, and L. In general, LYAAT1 expression increases with time in culture. At 4 d *in vitro* LYAAT1 and synaptophysin appear to be segregated to different processes. Although segregation persists at later times as well, LYAAT1 and synaptophysin progressively localize to the same processes. Despite partial colocalization the distribution of the two proteins within common processes generally remains distinct. The strip beneath each panel is magnified $4\times$ to illustrate more clearly the segregation within processes. The scale bar in L applies to all main panels.

we measured the ratio of charge moved per amino acid. Fitting the combined data at both -30 and -70 mV (Fig. 4F) by linear regression shows a charge/flux ratio 0.91 ± 0.07 ($r^2 = 0.493$), demonstrating that all of the observed currents are coupled stoichiometrically to transport and indicating a stoichiometry of 1 proton/1 amino acid.

Localization in somatic lysosomes and axonal processes

To characterize the subcellular location of LYAAT1, we raised an antibody to the cytoplasmic N terminus expressed as a fusion

Figure 8. Quantitation of LYAAT1 colocalization with synaptophysin. *A*, In hippocampal cultures that were double labeled for LYAAT1 and synaptophysin (syp), the number of independent processes (originating at distinct sites from the cell body) labeled for LYAAT1 alone, LYAAT1 and synaptophysin (syp), or synaptophysin alone was counted at 4, 6, 9, and 12 d *in vitro* and plotted as a proportion of total processes at that time point. The percentage of double-labeled processes increases with time in culture, and the number of LYAAT1⁺-only processes declines. The total number of processes counted at each time point was 200–300. **p* < 0.05 and ****p* < 0.0001 relative to LYAAT1⁺/syp⁺ processes. *B*, At 12 d *in vitro* the colocalization of LYAAT1 and synaptophysin within double-labeled processes was quantitated by counting the number of puncta labeled for LYAAT1 alone, LYAAT1 and synaptophysin, and synaptophysin alone. The two proteins remain mainly segregated at this late time in culture; 300–1300 puncta were counted. Error bars indicate SEM; *n* = 4.

protein in bacteria. The antiserum recognizes an ~55 kDa protein in transfected HeLa cell extracts, but not in the extracts from cells transfected with vector alone (Fig. 5*A*). The additional bands observed in untransfected membranes may represent endogenous LYAAT1 or cross-reactivity with an unrelated protein. However, the antibody does not recognize closely related LYAAT2 expressed in HeLa cells by transfection (Fig. 5*B*). In brain extracts the antibody recognizes proteins of ~35, 40, and 90 kDa, but adsorption with the GST fusion protein eliminates only the ~35 and 40 kDa species, indicating that recognition of the ~90 kDa form is not specific (Fig. 5*C*). The antibody also detects LYAAT1 by immunofluorescence (Fig. 5*D*), and adsorption again eliminates the signal (Fig. 5*D*). In HeLa cells the LYAAT1 colocalizes entirely with the lysosomal protein LAMP1 (Fig. 5*D*), and this was observed in cells expressing low as well as high levels of LYAAT1. Further, LYAAT1 localizes to the same LAMP1⁺

compartment when expressed in PC12 and COS7 cells (data not shown). Lysosomal degradation indeed may account for the lower molecular weight species of LYAAT1 observed from HeLa cells and brain extracts in Western blots.

We immunostained primary hippocampal cultures to assess the localization of LYAAT1 in neurons. Double staining with the neuronal marker MAP2 (data not shown) confirmed the localization to neurons suggested by *in situ* hybridization. However, LYAAT1 colocalizes with MAP2 only in cell bodies, not in dendritic processes (data not shown). Within neuronal cell bodies, very similar to HeLa cells, LYAAT1 colocalizes extensively with LAMP1 (Fig. 6*A–C*, arrows), and adsorption with GST fusion protein eliminates the signal (Fig. 6*D–F*). However, LYAAT1 does not colocalize with LAMP1 at all sites. Near the cell body some membranes label only for LAMP1 (Fig. 6*C*, arrowhead), and others label only for LYAAT1 (Fig. 6*C*, asterisk). Further, axonal processes contain substantial LYAAT1 but very little if any LAMP1 (data not shown). Within axonal processes the LYAAT1 has a punctate distribution, suggesting possible localization at synapses. Several puncta indeed label for both LYAAT1 and the synaptic vesicle protein synaptophysin (Fig. 6*G–I*, arrowheads). However, many puncta stain only for LYAAT1 or only for synaptophysin, indicating that much of the LYAAT1 in axons does not appear to reside at synapses or even at clusters of synaptic vesicles.

Previous work has shown that a complex of proteins termed the exocyst identifies the sites at which secretory vesicles fuse with the plasma membrane (Finger and Novick, 1998; Hsu et al., 1999). In yeast the exocyst acts upstream of other proteins involved in secretion, and it marks the site of membrane addition to the growing bud early in the cell cycle and the neck between mother and daughter cells later in the cycle (TerBush and Novick, 1995; Finger et al., 1998). Interestingly, the components of the exocyst do not always colocalize with synaptic markers in neurons. In particular, the rat sec6/sec8 exocyst complex appears at the growth cone and at periodic sites along the axon early in development but downregulates during subsequent synapse formation (Hazuka et al., 1999). Although axonal, the complex thus does not colocalize extensively with synaptic markers, raising the possibility that LYAAT1 might colocalize with the exocyst. To test this possibility, we double stained 12-d-old hippocampal cultures for sec6 as well as LYAAT1 and observed extensive colocalization (Fig. 6*J–L*). Only the perikaryal labeling for LYAAT1 in lysosomes does not double stain for sec6 (Fig. 6*L*).

Because the subcellular location of sec6 and sec8 in mammalian neurons changes during development, we examined the colocalization of LYAAT1 with synaptophysin and sec6 in cultures grown for different times *in vitro*. Figure 7 shows that the intensity of staining for both LYAAT1 and synaptophysin increases with days in culture. However, even at the earliest times (Fig. 7*A–C*) distinct structures appear to label for the two proteins. The segregation occurs at the level of entire processes, with some labeling predominantly if not exclusively for either LYAAT1 or synaptophysin. To quantitate the segregation to different processes as a function of time, we counted processes expressing only LYAAT1, both LYAAT1 and synaptophysin, and synaptophysin alone at 4, 6, 9, and 12 d *in vitro*. Although the total expression of LYAAT1 increases over time, Figure 8*A* shows that the number of LYAAT1-only processes declines. LYAAT1 thus may have roles in early as well as later stages of process outgrowth and differentiation. Nonetheless, most processes contain both proteins. Within processes expressing both LYAAT1 and synaptophysin, however, the proteins localize to distinct structures. Quantifying the extent

of colocalization of puncta within processes of day 12 cultures, we observe almost equal numbers labeling for LYAAT1 alone, synaptophysin alone, and both proteins (Fig. 8B).

We also examined the colocalization of LYAAT1 with sec6 as a function of time in culture. Unlike LYAAT1 and synaptophysin, sec6 is expressed strongly at early times, consistent with previous observations (Hazuka et al., 1999). From day 6 on, however, LYAAT1 colocalizes extensively with sec6 (Fig. 9).

Expression at the cell surface

Heterologous expression of LYAAT1 results in the H^+ -dependent uptake of neutral amino acids from the extracellular medium and hence localization to the cell surface, but the extent to which endogenous LYAAT1 localizes to the plasma membrane remains unclear, particularly in neurons. We therefore used a protease protection assay to assess the amount of LYAAT1 expressed on the surface of neurons. The addition of trypsin to dissociated hippocampal cultures results in degradation of most, but not all, LYAAT1 in the culture (Fig. 10, top panel). As expected, the addition of Triton X-100 results in the complete loss of LYAAT1 by making intracellular pools available to the exogenous protease. The amount of trypsin used was thus fully capable of degrading all of the accessible LYAAT1. To assess the specificity of degradation by exogenous protease, we used the synaptic vesicle protein synaptophysin, which resides at low levels on the cell surface. Indeed, the amount of trypsin that was used does not appear excessive because it failed to degrade all but a small fraction of the synaptic vesicle protein synaptophysin in the absence of detergent although it could degrade all of the synaptophysin in the presence of detergent (Fig. 10, bottom panel).

The cotransport of H^+ by LYAAT1 provides an additional way to assess cell surface expression in neurons. If LYAAT1 resides at the plasma membrane, substrates should produce the same changes in pH_i observed in transfected HeLa cells. To monitor pH_i , we used the pH-sensitive GFP expressed in dissociated hippocampal neurons by transfection (Kneen et al., 1998). Because of the small volume of neuronal processes, the fluorescence of GFP provides a much stronger fluorescent signal than BCECF. Similar to HeLa cells monitored with the pH-sensitive dye BCECF, external sarcosine reduces the pH_i of hippocampal neurons in both the cell body and processes (Fig. 11A,B). Proline also acidifies the neurons, and, consistent with a role for LYAAT1, the pH_i shifts occur in LiCl as well as NaCl and hence do not depend on Na^+ . Physiological studies of pH_i thus further support the cell surface expression of LYAAT1 by neurons.

Figure 9. Colocalization of LYAAT1 with sec6 from 4 through 12 d *in vitro*. Hippocampal neurons grown for 4 (A–C), 6 (D–F), 9 (G–I), and 12 (J–L) d *in vitro* were immunostained for LYAAT1 (A, D, G, J) and sec6 (B, E, H, K). The immunofluorescence was detected by confocal laser microscopy; merged images are shown in C, F, I, and L. In contrast to the low levels of LYAAT1 expression in day 4 cultures, sec6 already is expressed at high levels. Nonetheless, essentially all of the LYAAT1 detected at day 4 colocalizes with sec6, and this pattern persists through day 12. The strip beneath each main panel is magnified 4 $\times$ to illustrate the colocalization. The scale bar in L applies to all main panels.

Discussion

The functional characterization of LYAAT1 is consistent with a role in amino acid efflux from lysosomes. Lysosomes exhibit an outwardly directed H^+ electrochemical gradient capable of driving efflux via a mechanism that couples substrate efflux to H^+ efflux. The dependence of amino acid uptake by LYAAT1 on low external pH indeed suggests that H^+ is cotransported with amino acid. Other lysosomal transport activities exhibit a similar dependence on pH (Pisoni and Thoenes, 1991; Mancini et al., 2000). However, many transporters show a sensitivity to pH without actually translocating H^+ . For example, external pH dramatically

Figure 10. Accessibility of neuronal LYAAT1 to exogenous protease. Neonatal rat hippocampal cultures maintained for 10 d *in vitro* were treated with trypsin (100 μ g/ml) in the presence or absence of 1% Triton X-100. Treatment with trypsin resulted in degradation of most LYAAT1 even in the absence of detergent (*top panel*). In contrast, the synaptic vesicle protein synaptophysin (*syp*) undergoes minimal degradation in response to exogenous protease (*bottom panel*). However, both proteins show full sensitivity to trypsin in the presence of detergent. The arrows indicate apparent full-length LYAAT1 and synaptophysin, and the arrowheads indicate degradation products of LYAAT1. Markers on the right indicate kilodaltons.

affects the system A transporters SAT1 and 2, but these carriers do not move H^+ (Albers et al., 2001; Chaudhry et al., 2002a). We now show that the addition of substrate to cells expressing LYAAT1 decreases pH_i , demonstrating that LYAAT1 mediates H^+ as well as amino acid flux. Coupling to H^+ also predicts that LYAAT1 will transport substrates against a concentration gradient. However, the extent of concentration depends on the stoichiometry of coupling. Assuming that LYAAT1 transports only amino acids and H^+ and not other ions, the observed charge/flux ratio of 1 predicts a stoichiometry of 1 H^+ /1 amino acid. Given this coupling mechanism, a lysosomal pH ~ 5.5 will produce an effective concentration gradient of amino acid ~ 100 -fold higher in the cytoplasm than in the lumen. On the other hand, transport by LYAAT1 is also electrogenic and involves the movement of +1 net charge into the cell for each amino acid taken up. Assuming a potential across lysosomal membranes approximately +50 mV, similar to that across endosomes and neurosecretory vesicles (Holz, 1979; Anderson and Orci, 1988), LYAAT1 should concentrate substrates almost 10-fold more than predicted solely by ΔpH , i.e., almost 1000-fold. Similar ionic coupling has been reported recently for the mouse orthologue of LYAAT1 (Boll et al., 2002). However, glycine produced outward currents at depolarizing potentials with the mouse cDNA, raising the possibility of an uncoupled ionic conductance in addition to currents coupled stoichiometrically to amino acid flux. In contrast, the currents produced by proline in oocytes expressing rat LYAAT1 rectify strongly and do not reverse. Different substrates thus may interact differently with LYAAT1. Nonetheless, the ionic coupling observed for both rat and mouse orthologues predicts that LYAAT1 may serve to maintain extremely low concentrations of amino acid substrates within the lumen of lysosomes.

The cotransport of H^+ and amino acids by LYAAT1 contrasts with the activity of many related amino acid transporters. The VGAT, which defined the family of mammalian proteins including LYAAT1, functions as a H^+ exchanger rather than cotrans-

Figure 11. LYAAT1-dependent pH_i changes in neuronal cell bodies and processes. Dissociated hippocampal neurons maintained for 7 d *in vitro* were transfected with a GFP cDNA and imaged by confocal laser microscopy 3–7 d later. In both the cell body (*A*) and processes (*B*) 10 mM sarcosine causes acidification. The addition of NH_4Cl (15 mM) produces the anticipated alkalinization, and its removal produces the expected acidification. *C, D*, Proline (10 mM) also causes acidification in both cell body (*C*) and processes (*D*), and the acidification occurs in Ringer's solution, with LiCl replacing NaCl (*horizontal bars* designate Li^+) as well as in NaCl.

porter (McIntire et al., 1997). However, this coupling mechanism is consistent with the role of VGAT in packaging neurotransmitter into secretory vesicles, a process that, given the outwardly directed H^+ electrochemical gradient produced by the vacuolar H^+ -ATPase (Forgacs, 2000), can be driven by H^+ exchange, but not cotransport. In contrast, the efflux from lysosomes mediated by LYAAT1 can be driven by H^+ cotransport, but not exchange. A family of yeast proteins related to VGAT also functions in transport across the membrane of the vacuole (a yeast organelle equivalent to the vertebrate lysosome) (Russnak et al., 2001). Interestingly, this family includes transporters that mediate both uptake into (AVT1) and efflux from the vacuole (AVT3, 4, and 6), and the dependence on a H^+ electrochemical gradient suggests that closely related proteins can couple both to H^+ exchange (AVT1) or H^+ cotransport (AVT6) (Russnak et al., 2001).

LYAAT1 appears to correspond most closely to lysosomal transport system f described previously by Pisoni et al. (1987).

System f recognizes small neutral amino acids, whereas system p, another lysosomal transport activity, is specific for proline. However, systems f and p are stereoselective for L-proline, whereas LYAAT1 also recognizes D-proline. In addition, LYAAT1 has a lower apparent affinity ($K_m \sim 900 \mu\text{M}$) than systems f and p ($K_m \sim 10\text{--}70 \mu\text{M}$) (Pisoni et al., 1987). LYAAT1 in fact may correspond better to previously characterized plasma membrane transport activities than to lysosomal system f. Membrane vesicles from the proximal tubule of the kidney exhibit Na^+ -independent, but H^+ -dependent, electrogenic uptake of D-proline with an apparent affinity $\sim 1 \text{ mM}$ (Roigaard-Petersen et al., 1989). Human intestinal Caco-2 cells also show Na^+ -independent, H^+ -dependent, electrogenic uptake of small neutral L- and D-amino acids (Thwaites et al., 1993, 1995; Thwaites and Stevens, 1999). It remains to be determined whether LYAAT1 or a related gene such as LYAAT2 accounts for these plasma membrane transport activities. However, the system N transporters belong to the same family of proteins defined by VGAT and also reside at the plasma membrane where they mediate H^+ exchange (Chaudhry et al., 1999; Nakanishi et al., 2001). The detection of activity at the plasma membrane thus raises the possibility that LYAAT1 also has a physiological role at the cell surface. Interestingly, we have observed that LYAAT1 localizes to more than conventional lysosomal membranes.

By immunofluorescence LYAAT1 localizes to axonal processes as well as to the cell bodies and dendrites of hippocampal neurons in culture. However, we did not detect the lysosomal protein LAMP-1 in axons. To identify the axonal structures immunoreactive for LYAAT1, we first double labeled for the synaptic vesicle protein synaptophysin and observed only partial colocalization. In contrast, LYAAT1 colocalizes extensively with sec6, a subunit of the exocyst. The exocyst appears to define plasma membrane sites for the fusion of secretory vesicles (Finger and Novick, 1998), but components of the exocyst downregulate during synapse maturation (Hazuka et al., 1999). The exocyst apparently marks sites that eventually develop into synapses (Hsu et al., 1999). The relative lack of LYAAT1 colocalization with synaptophysin is thus consistent with known properties of the exocyst. On the other hand, somatodendritic LYAAT1 does not colocalize with sec6, as anticipated from the localization to lysosomes in this region of the cell. In addition, the level of LYAAT1 expression increases dramatically with days *in vitro*, unlike sec6, which is highly expressed at early times. The more prominent early expression of sec6 than LYAAT1 further suggests that the exocyst may recruit membranes containing LYAAT1 to specific sites at the cell surface, which eventually release transmitter. The partial colocalization of LYAAT1 with synaptophysin indeed may reflect the role of LYAAT1-containing membranes during intermediate stages of synapse development. Consistent with this possibility, the proportion of processes containing LYAAT1, but not synaptophysin, declines with time in culture. The presence of rare LYAAT1⁺ but synaptophysin[−] processes even at late times simply may reflect the formation of new processes or branching. Importantly, lysosomes have been implicated in synapse development.

Ultrastructural studies have revealed substantial accumulation of lysosomal membranes at the nerve terminal during early development (Rees et al., 1976). The lysosomal structures observed include multivesicular bodies, residual bodies, autophagosomes, and multilamellated bodies. Synapse maturation involves clearance of these membranes. More recent studies have confirmed that lysosome accumulation precedes synapse maturation and have suggested a role in synapse remodeling and elim-

ination (Missler et al., 1993). However, the properties of axonal lysosomes have remained unclear. The localization of LYAAT1 to axonal membranes that do not double label for LAMP1 as well as to somatodendritic lysosomes now suggests that axonal processes may contain a variant of lysosomes. Interestingly, a distinct subset of lysosomes termed "secretory lysosomes" has been shown to mediate the release of many biologically active molecules, including histamine from mast cells, serotonin from basophils, clotting factors from platelets, and melanin from melanocytes (Blott and Griffiths, 2002). In addition, osteoclasts digest bone by directly exposing their lysosomes at the cell surface (Kornak et al., 2001). Consistent with their more specialized function, secretory lysosomes have been reported to contain several proteins not found in conventional lysosomes (Peters et al., 1991; Medley et al., 1996; Iida et al., 2000). Nonetheless, secretory lysosomes also contain many proteins present in conventional lysosomes (Blott and Griffiths, 2002). The location of LYAAT1 in lysosomes as well as axons thus suggests that LYAAT1 may define a novel class of secretory lysosome in neurons. Taken together with the similarity to H^+ -driven epithelial transporters noted above, colocalization with the exocyst, a protein complex involved in exocytosis, and detection of transport activity at the plasma membrane further suggest that LYAAT1 may have a physiological role at the cell surface. Interestingly, a mouse protein closely related to LYAAT1 appears to localize at the cell surface, at least when tagged with GFP (Boll et al., 2002). In addition, a number of the genes implicated in neuronal ceroid lipofuscinoses, autosomal recessive disorders of childhood referred to collectively as Batten disease (Dawson and Cho, 2000; Mitchison and Mole, 2001), encode polytopic membrane proteins with a subcellular distribution reminiscent of LYAAT1. They localize specifically to lysosomes in many cells, but in neurons they also appear in axonal processes and colocalize only partially with synaptic vesicle markers (Jarvela et al., 1998; Luiro et al., 2001).

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