

Regular Article

Functional Significance of the C-terminal -ETTL Motif of NBCe1-C and Its Interaction with Sorting Nexin 27

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Received September 13, 2026; Accepted September 14, 2026

Electrogenic $\text{Na}^+/\text{HCO}_3^-$ cotransporter 1 (NBCe1), encoded by *SLC4A4*, regulates intra- and extracellular pH, and its dysfunction is associated with neurological manifestations. Among the major NBCe1 isoforms, NBCe1-C is predominantly expressed in the brain and possesses a unique C-terminal -ETTL sequence that constitutes a class I PDZ-binding motif. However, the functional significance of this motif remains unclear. Using an NBCe1-C-specific antibody, we found that NBCe1-C was predominantly expressed in astrocytes in primary rat hippocampal cultures and enriched at the distal tips of astrocytic processes. We therefore examined the role of the -ETTL motif in NBCe1-C stability, cell-surface expression, and endocytosis using heterologous expression systems. Disruption of the -ETTL motif did not affect protein stability but significantly reduced cell-surface expression. Unexpectedly, the motif also promoted NBCe1-C internalization, suggesting that it facilitates dynamic trafficking between the plasma membrane and intracellular compartments rather than simply stabilizing NBCe1-C at the cell surface. Under amino acid-starved conditions accompanied by LC3-II accumulation, cell-surface expression of wild-type NBCe1-C was markedly reduced, whereas that of the -ETTL mutant was largely unchanged. Furthermore, sorting nexin 27 (SNX27) interacted with NBCe1-C in a manner dependent on both the -ETTL motif and the SNX27 PDZ domain. These findings identify the -ETTL motif as a key determinant of NBCe1-C membrane trafficking and SNX27 as its PDZ-dependent binding partner, potentially contributing to dynamic regulation of NBCe1-C in astrocytic processes.

Key words electrogenic $\text{Na}^+/\text{HCO}_3^-$ cotransporter 1, PDZ binding motif, sorting nexin 27, astrocyte

INTRODUCTION

Electrogenic $\text{Na}^+/\text{HCO}_3^-$ cotransporter 1 (NBCe1) is a membrane transport protein encoded by the *SLC4A4* gene. At least five splice variants, designated NBCe1-A through -E, have been identified in mammals, among which NBCe1-A, -B, and -C are the major and best-characterized isoforms.¹⁻³ These isoforms exhibit distinct tissue distributions. NBCe1-A is predominantly expressed in renal proximal tubules, where it mediates HCO_3^- reabsorption and plays a critical role in maintaining systemic acid-base homeostasis.^{4,5} NBCe1-B is broadly distributed in tissues including the pancreas, cornea, ileum, heart, and brain,⁶ whereas NBCe1-C is predominantly expressed in the brain.^{7,8} We previously demonstrated that IRBIT, a negative regulator of inositol 1,4,5-trisphosphate receptors, interacts with both NBCe1-B and NBCe1-C through their shared N-terminal region encoded by exon 2 and enhances their transport activity.⁹⁻¹¹

Inactivating mutations in *SLC4A4* invariably cause prox-

imal renal tubular acidosis (pRTA)^{3,4,12} and are frequently accompanied by neurological and ocular manifestations, including intellectual disability, hemiplegic migraine, glaucoma, and cataract.^{5,13-16} These observations suggest that dysfunction of NBCe1 isoforms expressed in the brain, particularly NBCe1-C, may contribute to the neurological manifestations associated with *SLC4A4* deficiency. Immunohistochemical studies using NBCe1-C-specific antibodies have demonstrated that NBCe1-C is predominantly expressed at the plasma membrane of astrocytes in the brain.¹⁷ Consistent with this finding, single-cell RNA sequencing analyses have shown that *Slc4a4* expression in the brain is strongly enriched in astrocytes.¹⁸⁻²⁰ Astrocytic processes closely ensheath synapses and constitute the glial component of the tripartite synapse, where they are exposed to rapid changes in extracellular ion concentrations and pH associated with neuronal activity. NBCe1 expressed in astrocytes has been implicated in neuronal activity-dependent regulation of extracellular pH,²¹ suggesting that the localization and abundance of NBCe1-C at the

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astrocytic plasma membrane may need to be dynamically regulated in response to changes in the synaptic microenvironment.

NBCe1-C is generated by alternative splicing that excludes exon 24, giving rise to a unique C-terminal sequence.^{7,8,22} We previously reported that calcineurin binds to NBCe1-C through a PxIxIT motif within this unique C-terminal region and thereby enhances its cell-surface expression.²³ In the same study, several PDZ domain-containing proteins were identified as potential interaction partners of the NBCe1-C C-terminus.²³ Notably, the four C-terminal residues of NBCe1-C (-ETTL) conform to a consensus class I PDZ domain-binding motif.^{24–26} Given the well-established roles of PDZ domain-containing proteins in regulating the localization, stability, and trafficking of their binding partners,^{27,28} the -ETTL motif may be an important determinant of the subcellular localization and dynamic trafficking of NBCe1-C. However, the functional significance of this motif remains largely unknown.

To elucidate the functional role of the C-terminal -ETTL motif of NBCe1-C, we examined its contribution to protein stability, cell-surface expression, transport activity, and endocytosis by comparing wild-type NBCe1-C with variants in which the -ETTL motif was deleted or mutated in a heterologous expression system. We also searched for proteins that interact with the -ETTL motif and identified Sorting Nexin 27 (SNX27) as a candidate interacting protein.

MATERIALS AND METHODS

Reagents and Antibodies The cell-permeable pH-sensitive fluorescent indicator 5-(and-6)-carboxy SNARF-1 AM and the Pierce™ Cell Surface Protein Biotinylation and Isolation Kit were purchased from Thermo Fisher Scientific. Cycloheximide (CHX) and N-methyl-D-glucamine (NMDG) were purchased from FUJIFILM Wako Pure Chemical Corporation. 5-(N-Ethyl-N-isopropyl)amiloride (EIPA), poly-L-lysine (PLL; molecular weight, 30,000–70,000), and a 50% polyethyleneimine (PEI) solution (average molecular weight, 750,000), used for coating coverslips, were purchased from Sigma-Aldrich (Merck).

An NBCe1-C-specific antibody was generated in rabbits using a GST-fusion protein containing the C-terminal region of NBCe1-C as the immunogen.²³ The antibody was affinity-purified from the antiserum using a column containing covalently immobilized MBP-fusion protein corresponding to the antigenic region and used for both immunofluorescence staining (1:2000 dilution) and western blotting (1:1000 dilution). Rabbit polyclonal anti-NBCe1 antibody (#11885-1-AP, 1:500) was purchased from Proteintech. Mouse monoclonal anti-GFAP (clone GA5, #3670S, 1:1000) and rabbit polyclonal anti-LC3B (#2775, 1:500) antibodies were purchased from Cell Signaling Technology. Rabbit polyclonal anti-GLAST antibody was purchased from Thermo Fisher Scientific (#PA5-72895, 1:1000). Mouse monoclonal anti-human SNX27 antibody (clone 1C6, #ab77799, 1:500) was purchased from Abcam. Mouse monoclonal anti-HA antibody (clone 16B12, #901513, 1:1000) was purchased from BioLegend, and rat monoclonal high-affinity anti-HA antibody (clone 3F10, #11867423001, 1:1000) was purchased from Roche. Rabbit polyclonal anti-GFP antibody (#598, 1:1000) was purchased from MBL Life Science. Mouse monoclonal anti-β-actin antibody (clone AC-15, #sc-69879, 1:1000) was purchased from Santa Cruz Biotechnology.

Protein G Mag Sepharose (#28944008), horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (#NA931-1ML), and HRP-conjugated donkey anti-rabbit IgG (#NA934-1ML) were purchased from Cytiva. Highly cross-adsorbed Alexa Fluor™ 488- or Alexa Fluor™ 594-conjugated goat anti-rabbit IgG (H+L) antibody (#A-11034 and #A-11037, respectively) and goat anti-mouse IgG (H+L) antibody (#A-11029 and #A-11032, respectively), and Highly cross-adsorbed Alexa Fluor™ 488- or Fluor™ 647-conjugated donkey anti-rat IgG (H+L) antibody (#A-21208 and #A-78947) were purchased from Thermo Fisher Scientific. All other reagents were of analytical grade and obtained from commercial sources.

Plasmids Full-length human NBCe1-C cDNA was amplified from first-strand cDNA prepared from the human neuroblastoma cell line IMR-32, obtained from the JCRB Cell Bank (#JCRB9050), using the following primers: forward, 5'-CAGGAGGATGGAGGATGAAGC-3'; and reverse, 5'-GCGCTGTATTATTTGGCCTGTGACC-3'. The nucleotide sequence was deposited in the database under accession number AB470072. A mammalian expression plasmid encoding full-length NBCe1-C was constructed by subcloning its coding sequence into the pcDNA3.1 vector (Thermo Fisher Scientific). pcDNA3.1 expression plasmid encoding full-length human NBCe1-B was kindly provided by Dr. George Seki (Yaizu City Hospital, Japan). N terminally GFP-tagged human NBCe1-A, NBCe1-B, and NBCe1-C expression plasmids were prepared as previously described.⁹ HA-tagged NBCe1-B and NBCe1-C constructs were generated by inserting an HA epitope into the extracellular loop between Glu648 and Leu650 using a PCR-based method with the following primers: forward, 5'-TAATCTGGAACATCGTATGGATACTCTGGGGC-CAGTGTGGTG-3'; and reverse, 5'-TACGATGTTCCAGAT-TACGCTTTGCCAACTATGTCTTCTACTG-3'. This region is located within an extracellular loop of NBCe1,²⁹ and we previously demonstrated that insertion of the HA tag at this position did not affect NBCe1 transport activity.²³ An NBCe1-C ΔETTL mutant lacking the four C-terminal amino acids (ΔETTL) and an EAAA mutant in which the three residues following the C-terminal Glu were replaced with Ala residues were generated using PCR-based mutagenesis.

Full-length mouse SNX27 cDNA (clone ID A730053C13) was purchased from DNAFORM. An expression plasmid encoding C-terminally EGFP-tagged SNX27 (SNX27-EGFP; pEGFP-N1-SNX27) was generated by PCR amplification of the SNX27 coding sequence using the following primers: forward, 5'-GGGGAAGCTTAAGGCCACCATGGCGGACG-3'; and reverse, 5'-AAAGGATCCGGTGGCCACATCCCTCTGC-3'. The amplified product was subcloned into the HindIII/BamHI sites of the pEGFP-N1 vector. The SNX27-H112A-EGFP expression construct was generated by PCR-based site-directed mutagenesis of pEGFP-N1-SNX27 using the following primers: forward, 5'-GGCGACAGCCAAGCAGGTG-GTGGACC-3'; and reverse, 5'-CCTGCTTGGCTGTCGCCCTCAAC-3'. All constructs were verified by DNA sequencing.

Cell Culture and Transfection Primary hippocampal cultures were prepared from neonatal Wistar rat hippocampi using a previously described method.³⁰ Cells were maintained in Neurobasal medium supplemented with 1× GlutaMAX, 2% (v/v) B-27 supplement (both from Thermo Fisher Scientific), 0.5% fetal bovine serum (FBS; Sigma-Aldrich), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Nacalai Tesque).

For immunofluorescence staining, primary cultured cells were fixed on DIV 14.

HeLa and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% FBS and penicillin–streptomycin (100 IU/mL penicillin and 100 µg/mL streptomycin) at 37°C in a humidified atmosphere containing 5% CO₂.

Expression plasmids were amplified in *Escherichia coli* and purified using the PureLink™ HiPure Plasmid Midiprep Kit (Thermo Fisher Scientific). For immunoprecipitation and CHX chase experiments, plasmids were transiently transfected into HeLa or HEK293T cells using transfection-grade PEI (Polysciences; #23966). For immunofluorescence staining and live-cell pH imaging, HeLa cells were cultured on 12-mm glass coverslips (Matsunami Glass) coated with 0.02 mg/mL PLL or 0.02% (w/v) PEI and transfected using TransIT-2020 (Mirus Bio; #MIR5404). Cells were analyzed 24–48 h after transfection.

Cycloheximide Chase Assay HeLa cells were seeded in 12-well plates and transiently transfected with the indicated plasmids using PEI on the following day. At 48 h after transfection, cells were treated with 50 µg/mL CHX in complete growth medium and incubated for 0, 3, or 6 h. Whole-cell lysates were prepared at each time point, and the abundance of NBCe1 was determined by western blotting to assess protein degradation over time.

Immunofluorescence Staining

Standard Immunofluorescence Staining

Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After blocking with 2% normal goat serum (NGS) in PBS, cells were incubated with primary antibodies diluted in 2% NGS/PBS overnight at 4°C. After washing, immunoreactivity was visualized using the appropriate Alexa Fluor-conjugated secondary antibodies. Coverslips were mounted using VECTASHIELD mounting medium (Vector Laboratories). Confocal fluorescence images were captured using confocal microscope A1R (Nikon Instruments).

Immunofluorescence-Based Cell-Surface Expression Assay

HeLa cells were seeded onto coated coverslips and transfected with constructs encoding NBCe1 containing an HA epitope within an extracellular loop. At 24 h after transfection, living cells were incubated with rat anti-HA antibody in ice-cold DMEM for 15 min. Cells were then washed with ice-cold PBS and fixed with 4% PFA in PBS for 20 min. After washing, cells were incubated with Alexa Fluor 488-conjugated anti-rat IgG for 2 h at room temperature to label cell-surface NBCe1.

Cells were subsequently refixed with 4% PFA in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature, and blocked with 2% NGS in PBS. Cells were then incubated with anti-NBCe1 antibody (1:500) overnight at 4°C, washed with PBS, and incubated with Alexa Fluor 594-conjugated anti-rabbit IgG for 2 h at room temperature to label total NBCe1.

Fluorescence images were acquired using a confocal microscope system A1R (Nikon Instruments). For quantitative analysis, a region of interest (ROI) encompassing the entire cell was defined by tracing the outermost boundary of each cell using ImageJ. The fluorescence intensities corresponding to cell-surface NBCe1 (Alexa Fluor 488) and total NBCe1 (Alexa Fluor 594) within each ROI were quantified. Relative cell-surface expression was calculated as the ratio of cell-sur-

face to total NBCe1 fluorescence intensity.

Colocalization Analysis

Colocalization of heterologously expressed NBCe1 and endogenous SNX27 in HeLa cells was examined by immunofluorescence co-staining with antibodies against the HA epitope and SNX27, followed by incubation with the appropriate Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies. Fluorescence images were acquired by confocal microscopy, and the degree of colocalization between NBCe1 and endogenous SNX27 was quantified by calculating Pearson's correlation coefficient using ImageJ.

Cell-Surface Protein Biotinylation

Cell-Surface Expression Assay

HeLa cells were transiently transfected with the indicated plasmids and cultured until they reached approximately 90% confluence. Cell-surface proteins were biotinylated using the Pierce™ Cell Surface Protein Biotinylation and Isolation Kit. Briefly, cells were washed twice with ice-cold PBS and incubated with 0.25 mg/mL sulfo-NHS-SS-biotin in PBS for 30 min at 4°C. After removal of the labeling solution, unreacted biotin was quenched by incubation with Tris-buffered saline (TBS) for 20 min at 4°C.

Cells were lysed in lysis buffer containing 40 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and 1× protease inhibitor cocktail (Nacalai Tesque; #04080). Lysates were centrifuged at 15,000 × g for 15 min at 4°C, and the resulting supernatants were collected as cleared whole-cell lysates. Biotinylated proteins were isolated from the cleared lysates using NeutrAvidin™ Agarose (Thermo Fisher Scientific).

NBCe1 abundance in the cleared whole-cell lysates (total NBCe1) and biotinylated fractions (cell-surface NBCe1) was quantified by western blotting using anti-NBCe1 antibody. Relative cell-surface expression was calculated as the ratio of cell-surface NBCe1 to total NBCe1.

Endocytosis Assay

HeLa cells transfected with the indicated plasmids were surface-biotinylated as described above. One set of cells was immediately processed to determine the total amount of surface-biotinylated NBCe1 and was designated the total biotinylated fraction. In parallel, surface-biotinylated cells were incubated in complete DMEM at 37°C under 5% CO₂ for 30 min to allow endocytosis.

Cells were immediately placed on ice to terminate endocytosis and then washed with ice-cold PBS. Residual biotin attached to proteins remaining at the cell surface was removed by incubation with glutathione stripping buffer [50 mM reduced glutathione, 75 mM NaCl, 75 mM NaOH, 5 mM EDTA, 10% FBS, pH 8.6] on ice for 30 min.^{31–33} Cells were subsequently washed with ice-cold PBS and lysed in the aforementioned lysis buffer. Biotinylated proteins protected from glutathione stripping, representing internalized proteins, were isolated using NeutrAvidin Agarose and designated the internalized biotinylated fraction.

NBCe1 abundance in the total and internalized biotinylated fractions was quantified by western blotting using anti-NBCe1 antibody. The extent of NBCe1 endocytosis was expressed as the amount of internalized NBCe1 relative to the total amount of surface-biotinylated NBCe1.

Intracellular pH Measurement Intracellular pH (pH_i) measurements using the pH-sensitive fluorescent indicator SNARF-1 were performed essentially as described

previously.²³⁾ Briefly, at 48 h after transfection, cells were loaded with 20 μ M SNARF-1 AM in SNARF loading buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES-NaOH (pH 7.4) for 30 min at 37°C. After loading, cells were perfused with SNARF loading buffer for 5 min to remove extracellular dye and allow equilibration.

Time-lapse fluorescence imaging was then initiated. Cells were first perfused with SNARF-loading buffer for 2 min, followed by Na^+ -free buffer [Na^+ (–) buffer: 115 mM NMDG-Cl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 2.5 mM HEPES, and 25 mM tetraethylammonium bicarbonate] for 2 min. The perfusate was then switched to Na^+ -containing buffer [Na^+ (+) buffer: 115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 2.5 mM HEPES, and 25 mM $NaHCO_3$] for 2 min and subsequently returned to Na^+ (–) buffer for an additional 2 min. All perfusion solutions were maintained at 37°C. HCO_3^- -containing solutions were added with EIPA (10 μ M) to inhibit Na^+/H^+ exchanger activity and equilibrated with 5% CO_2 . SNARF-1 fluorescence intensities at emission wavelengths 580 and 640 nm with excitation at 488 nm was recorded using confocal microscope system A1R at 5s intervals. NBCe1 transport activity was defined as the change in the 640/580 ratio of SNARF-1 during the first 40 seconds (initial rate).

Immunoprecipitation HEK293T cells were seeded in 6-well plates and transiently transfected with the indicated plasmids using PEI (Polysciences) on the following day. At 48 h after transfection, cells were washed with PBS and lysed in 1 mL of lysis buffer [20 mM HEPES-NaOH (pH 7.4), 100 mM NaCl, 2 mM EDTA, 0.2% Triton X-100, Protease Inhibitor Cocktail for General Use (Nacalai Tesque)] on ice for 20 min. Lysates were centrifuged at $15,000 \times g$ for 20 min at 4°C, and the resulting supernatants were collected. An aliquot of each supernatant was retained as the input sample, and the remainder was incubated with 2 μ g of rabbit anti-GFP polyclonal antibody for 4 h at 4°C. 10 μ L of 50% slurry of Protein G Mag Sepharose (Cytiva) was then added, and samples were further incubated for 2 h at 4°C. After extensive washing with wash buffer [20 mM HEPES-NaOH pH 7.4, NaCl 100 mM, 2mM EDTA, and 0.1% Triton X-100], bound immunocomplexes were collected as immunoprecipitated (IP) samples. Input and IP samples were subjected to SDS-PAGE followed by western blot analysis.

Western Blotting Analysis An equal number of cells were washed once with ice-cold PBS and were lysed directly in $1 \times$ SDS sample buffer containing 50 mM Tris-HCl pH 6.8, 10% glycerol, 5% β -mercaptoethanol, 2% sodium dodecyl sulfate and 0.005% bromophenol blue. After boiling at 95°C for 7 min and centrifugation at $12,500 \times g$ for 10 min, total lysates were separated by 7.5–12.5% SDS/PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon®-P PVDF membrane, Merck Millipore). The membrane was blocked with 2.0% non-fat dry milk in PBS containing 0.05% Tween-20 (PBST) for 1 h and probed with the primary antibody for 1 h at room temperature or 16 hours at 4°C. After washing with PBST, the membranes were incubated with an appropriate HRP-conjugated secondary antibody, and signals were detected with Immobilon Forte Western HRP substrate (Sigma Aldrich). The ECL signals were captured by WSE-6100 LuminoGraph I (ATTO, Japan), and immunosignals were quantified by ImageJ using gel analysis tool.

Statistical Analysis Statistical analyses were performed using EZR version 1.65,³⁴⁾ a graphical user interface for R (version 4.5.2). Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test. A p value < 0.05 was considered statistically significant. Values in the graph are expressed as mean $\pm$ SD.

RESULTS

Astrocytic Expression and Subcellular Localization of NBCe1-C in Primary Hippocampal Cultures We first examined the expression and subcellular localization of NBCe1-C in primary cultures of rat hippocampal cells using an affinity-purified NBCe1-C-specific antibody. The specificity of the antibody was confirmed by western blotting using lysates of HEK293T cells expressing individual EGFP-tagged NBCe1 isoforms. The antibody selectively recognized NBCe1-C but not the other NBCe1 isoforms (Fig. S1A). In a 1% Triton X-100-solubilized membrane fraction prepared from mouse cerebellum, the antibody detected bands of approximately 130 and 160 kDa (Fig. S1B). Immunoreactive bands detected in both heterologous cells and mouse cerebellum disappeared when the antibody was preincubated with the antigen, further confirming the specificity of the antibody for NBCe1-C (Fig. S1A, B).

Primary hippocampal cultures at 14 days *in vitro* (DIV14) were immunostained for NBCe1-C and glial fibrillary acidic protein (GFAP), an astrocytic marker. Under our culture conditions, in which cells were maintained in Neurobasal medium supplemented with B-27 and 0.5% FBS, astrocytes developed extensively branched processes that were readily visualized by GFAP immunostaining (Fig. 1). NBCe1-C immunoreactivity was observed almost exclusively in GFAP-positive cells (Fig. 1A, upper panels). Within astrocytes, NBCe1-C was prominently enriched at the distal tips of astrocytic processes, where GFAP immunoreactivity was weak or undetectable (Fig. 1A, lower panels). The distribution of NBCe1-C closely resembled that of the glutamate transporter GLAST, which was also enriched in peripheral astrocytic processes (Fig. 1B).

These observations prompted us to investigate the molecular mechanisms regulating the subcellular localization of NBCe1-C, with particular attention to its unique C-terminal -ETTL motif.

The C-terminal -ETTL Motif Promotes Cell-Surface Expression and Internalization of NBCe1-C Wild-type NBCe1-C (WT) and two C-terminal mutants were expressed in HeLa cells: a deletion mutant lacking the terminal ETTL sequence (Δ ETTL) and an EAAA mutant in which the terminal TTL residues were replaced with AAA (Fig. 2A).

We first examined whether the -ETTL motif affects NBCe1-C protein stability using a cycloheximide (CHX) chase assay. HA-tagged long-IRBIT variant 3, included as a positive control, was rapidly degraded and decreased to 11.9% of its initial level by 3 h after CHX treatment (Fig. 2B, C), consistent with our previous findings.³⁵⁾ The abundance of all three NBCe1-C constructs decreased after CHX treatment, but their degradation profiles were similar. At 3 h, the remaining protein levels were 53.1% for WT, 49.7% for Δ ETTL, and 61.0% for EAAA (Fig. 2B, C). Thus, disruption of the -ETTL motif did not substantially affect the stability of NBCe1-C under basal conditions.

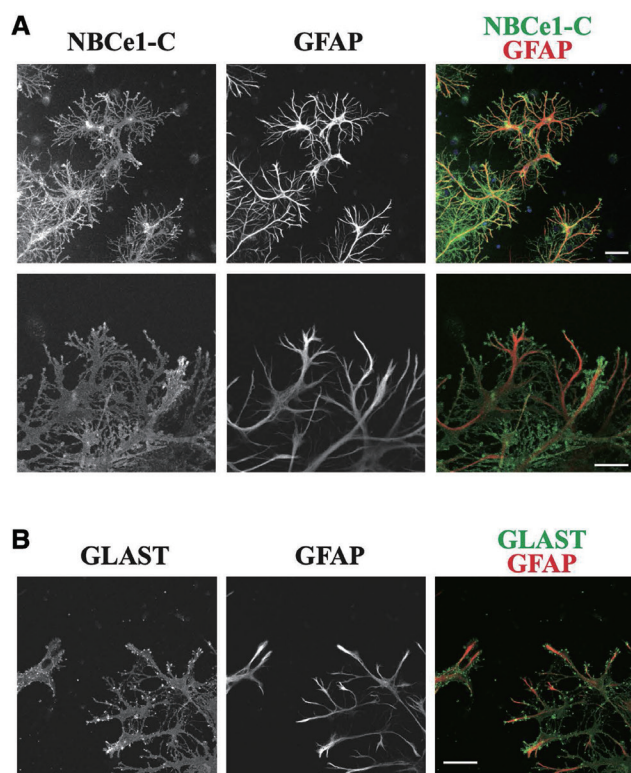


Fig. 1. Expression and Localization of NBCe1-C in Primary Cultures of Rat Hippocampal Cells

Primary rat hippocampal cultures at DIV14 were subjected to immunofluorescence staining.

A, Double immunostaining for NBCe1-C and GFAP. Upper panels show low-magnification images demonstrating the predominant expression of NBCe1-C in GFAP-positive astrocytes. Lower panels show higher-magnification images demonstrating enrichment of NBCe1-C at the distal tips of astrocytic processes, where GFAP immunoreactivity was weak or undetectable. Scale bars, 25 μ m (upper panels) and 10 μ m (lower panels).

B, Double immunostaining for GLAST and GFAP. GLAST immunoreactivity was also enriched at the distal tips of astrocytic processes. Scale bar, 10 μ m.

We next directly quantified cell-surface NBCe1 using an extracellular HA epitope introduced into an extracellular loop of NBCe1.^{23,29} Cell-surface NBCe1 was detected by anti-HA staining of non-permeabilized cells, whereas total NBCe1 was subsequently detected after permeabilization. Relative cell-surface expression was calculated as the ratio of cell-surface HA fluorescence to total NBCe1 fluorescence.

Strong cell-surface HA signals were detected in most cells expressing NBCe1-B, and its mean cell-surface expression was normalized to 1.0 (Fig. 3A, B). The mean surface expression of NBCe1-C WT was comparable to that of NBCe1-B, although individual NBCe1-C WT-expressing cells showed greater variability (1.29 ± 0.57 ; Fig. 3B). In contrast, surface HA signals were reduced in cells expressing either Δ ETTL or EAAA (Fig. 3A). Quantitative analysis showed that the relative cell-surface expression levels of Δ ETTL and EAAA were significantly lower than that of WT (0.65 ± 0.32 , $p < 0.0001$; and 0.52 ± 0.23 , $p < 0.0001$, respectively; Fig. 3B).

The contribution of the -ETTL motif to cell-surface expression was further examined using cell-surface biotinylation. The amount of surface-biotinylated NBCe1-C WT was significantly greater than that of the EAAA mutant (Fig. S2A, B), consistent with the immunofluorescence-based measurements.

We next used a cleavable biotin reagent to assess NBCe1-

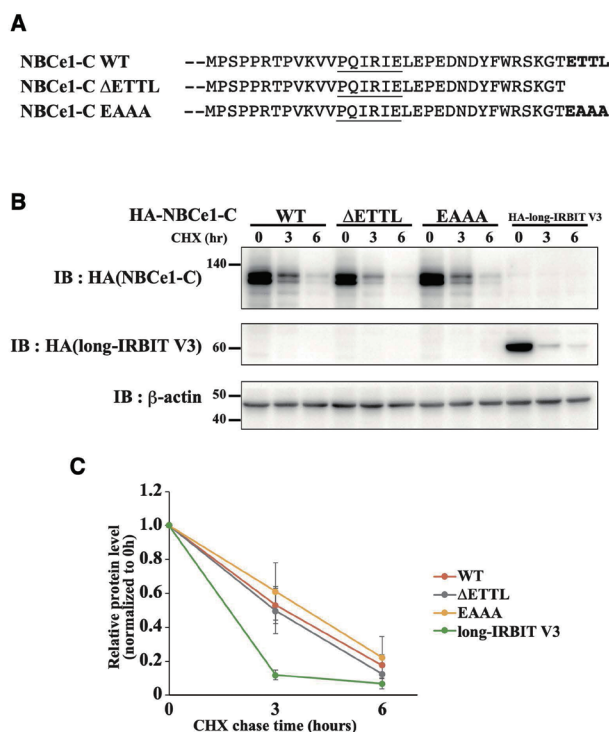


Fig. 2. Stability of NBCe1-C and its C-Terminal Mutants

A, Amino acid sequences of the C-terminal region of human NBCe1-C and the mutant constructs used in this study.

B, NBCe1-C WT, Δ ETTL, and EAAA were transiently expressed in HeLa cells and treated with 50 μ g/mL cycloheximide (CHX) for the indicated periods. Whole-cell lysates were prepared at 0, 3, and 6 h after CHX treatment, and NBCe1-C proteins were detected by western blotting using an anti-HA antibody. HA-tagged long-IRBIT variant 3 was included as a positive control for rapid protein degradation.

C, Protein stability was quantified by densitometric analysis of the immunoreactive bands. The signal intensity for each construct at 0 h was normalized to 1.0. Data were obtained from four independent experiments. Statistical significance was evaluated by one-way ANOVA followed by Tukey's HSD post hoc test.

C internalization. After allowing endocytosis at 37°C, biotin remaining on proteins exposed at the cell surface was removed using reduced glutathione, whereas internalized biotinylated proteins were protected from stripping. The fraction of internalized NBCe1-C was significantly greater for WT than for the EAAA mutant (Fig. S2A, C). Thus, the -ETTL motif contributed to both cell-surface expression and internalization of NBCe1-C.

Cell-Surface Expression of NBCe1-C Is Altered under Amino Acid-Starved Conditions We next examined whether the differences in cell-surface expression were reflected in NBCe1 transport activity. Transport activity was evaluated by monitoring intracellular pH (pH_i) using the fluorescent pH indicator SNARF-1. Na^+ gradient-dependent HCO_3^- transport through NBCe1 was initiated by switching the extracellular solution from Na^+ -free to Na^+ -containing buffer. This resulted in intracellular alkalinization, detected as an increase in the SNARF-1 emission ratio at 640 nm/580 nm.³⁶ Mock-transfected HeLa cells showed little or no increase in the fluorescence ratio after Na^+ addition, whereas cells expressing each NBCe1-C construct exhibited a clear increase (Fig. 4A). Unexpectedly, no significant difference in apparent transport activity was detected among NBCe1-C WT, Δ ETTL, and EAAA (Fig. 4A, B).

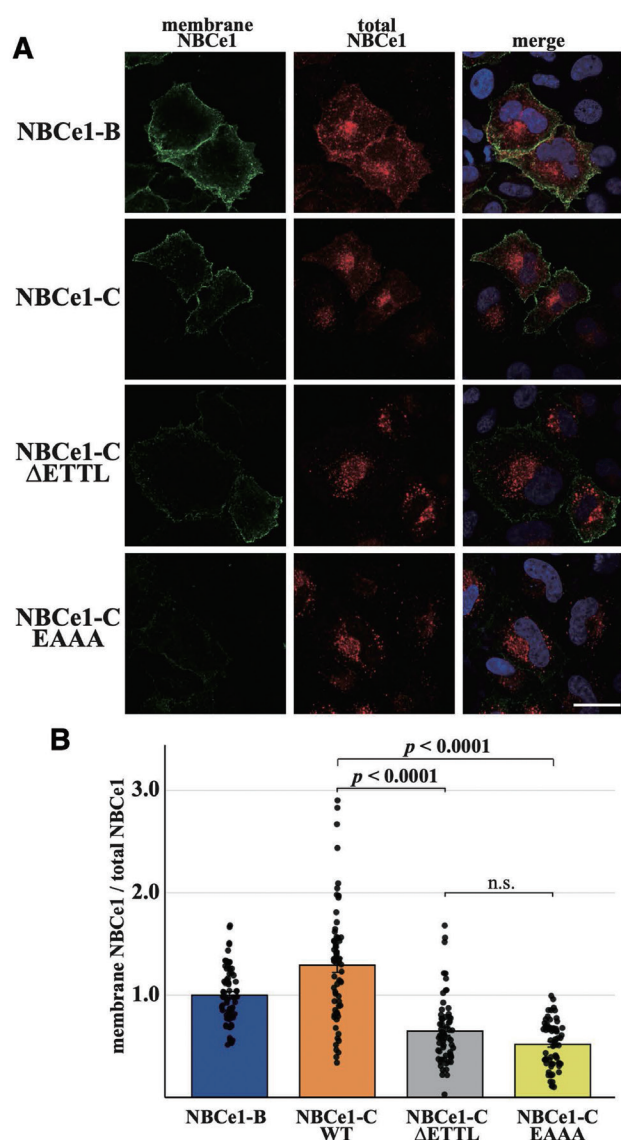


Fig. 3. Cell-Surface Expression of NBCe1-C and Its C-Terminal Mutants

A, NBCe1 constructs containing an HA epitope in an extracellular loop were transiently expressed in HeLa cells, and cell-surface expression was analyzed by sequential immunofluorescence staining. Living, non-permeabilized cells were first incubated with an anti-HA antibody at 4°C to label cell-surface NBCe1. After fixation with 4% PFA, bound anti-HA antibody was detected with a fluorescent secondary antibody. Cells were then refixed, permeabilized, and immunostained with an anti-NBCe1 antibody to detect total NBCe1. Scale bar, 25 μ m.

B, Cell-surface expression was quantified as the ratio of cell-surface HA fluorescence to total NBCe1 fluorescence. Values were normalized to the mean value for NBCe1-B, which was set to 1.0. Data were obtained from four independent experiments, with 16 cells analyzed per experiment for each construct (64 cells in total per construct). Statistical significance was evaluated by one-way ANOVA followed by Tukey's HSD post hoc test.

The experimental conditions used for transport measurements differed from those used for cell-surface expression assays. During SNARF-1 loading and pH_i measurements, cells were incubated for at least 40 min at 37°C in amino acid-free salt solutions containing glucose. Because amino acid starvation can rapidly alter autophagy-related pathways,³⁷⁻³⁹ we examined LC3 processing under these conditions. Incubation of HeLa cells in SNARF-loading buffer for 30 min at 37°C significantly increased the LC3-II/LC3-I ratio to 1.96-fold relative to cells maintained in complete medium ($p = 0.03$;

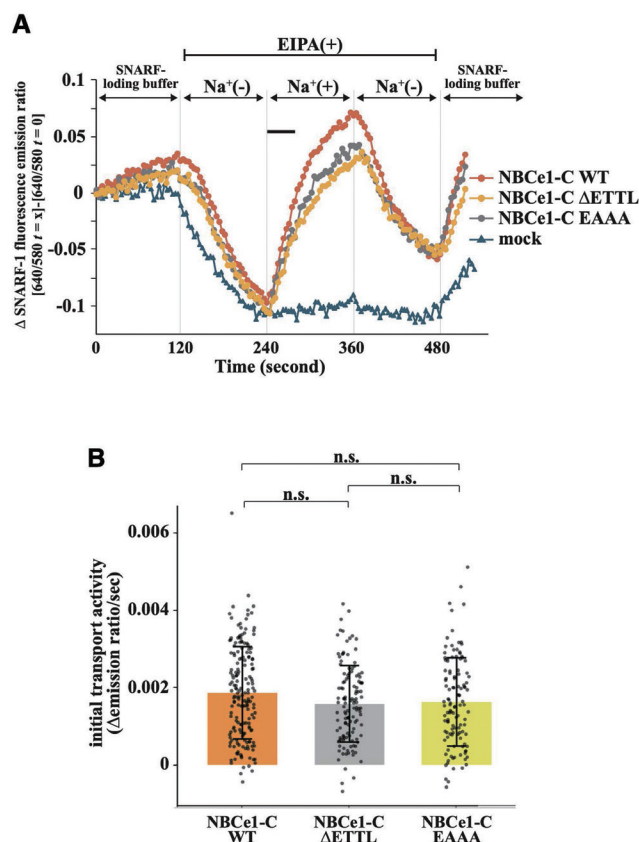


Fig. 4. Transport Activity of NBCe1-C and Its C-Terminal Mutants

HeLa cells were transiently transfected with the indicated NBCe1-C constructs.

A, Cells were loaded with 20 μ M SNARF-1 AM, and NBCe1-mediated Na⁺/HCO₃⁻ cotransport was initiated by switching the extracellular solution from Na⁺-free [Na⁺(-)] to Na⁺-containing [Na⁺(+)] buffer. Transport activity was monitored as the increase in intracellular pH, reflected by an increase in the SNARF-1 fluorescence emission ratio (640/580 nm). NBCe1 transport activity was defined as the change in the 640/580 ratio during the first 40 s after switching to Na⁺(+) buffer, as indicated by the bold bar. EIPA, 5-(N-ethyl-N-isopropyl)amiloride. Data were obtained from four independent experiments. The total numbers of cells analyzed were 179 for NBCe1-C WT, 127 for NBCe1-C ΔETTL, and 119 for NBCe1-C EAAA.

B, Comparison of NBCe1 transport activity among the indicated constructs. No significant differences were detected by one-way ANOVA.

Fig. 5A, B), consistent with accumulation of autophagosomal LC3-II.⁴⁰ In contrast, the total expression levels of the NBCe1 constructs remained largely unchanged after incubation in SNARF-loading buffer (Fig. 5C).

We therefore examined whether exposure to SNARF-loading buffer altered the cell-surface abundance of NBCe1-C. Cells expressing NBCe1-C WT or EAAA were either fixed immediately after removal from complete medium or incubated for 30 min in SNARF-loading buffer before fixation, and cell-surface NBCe1 was quantified by extracellular HA staining.

As observed above, NBCe1-C WT exhibited significantly greater cell-surface expression than EAAA in complete medium (Fig. 6A, B). After 30 min incubation in SNARF-loading buffer, however, the surface expression of NBCe1-C WT was markedly reduced (Fig. 6A, B). In contrast, the surface expression of EAAA was largely unaffected by incubation in SNARF-loading buffer and was comparable to that of WT after SNARF-loading buffer treatment. These findings indicate that the C-terminal -ETTL motif is required for the reduction

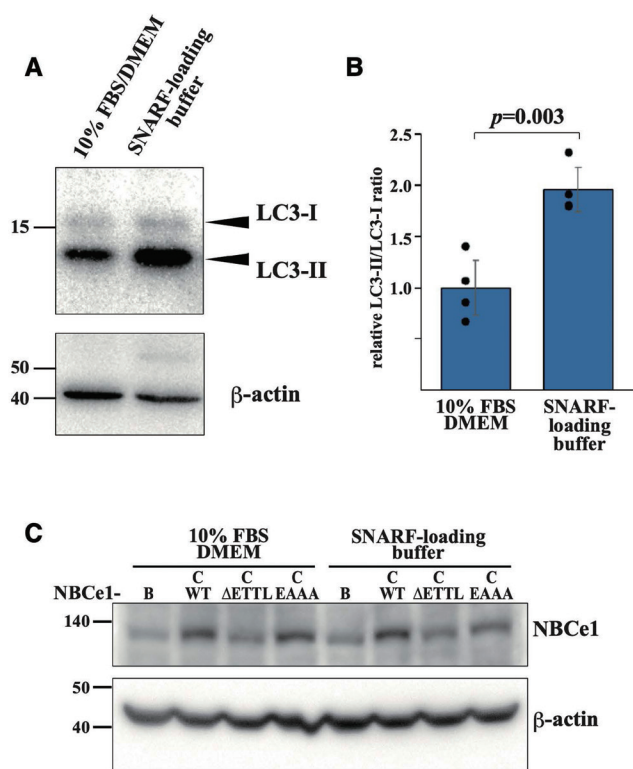


Fig. 5. Effects of pH_i Imaging Conditions on LC3-II Accumulation and NBCe1-C Protein Expression

A, HeLa cells were incubated for 30 min at 37 °C in either complete DMEM containing 10% FBS or amino acid-free SNARF-loading buffer containing 10 mM glucose. Whole-cell lysates were analyzed by western blotting for LC3-I and LC3-II.

B, Quantification of the LC3-II/LC3-I ratio under the two conditions. Data were obtained from four independent experiments. Statistical significance was evaluated using an unpaired two-tailed Student's *t*-test.

C, Effects of SNARF-loading buffer on the total expression levels of the indicated NBCe1-C constructs. Whole-cell lysates were analyzed by western blotting using an anti-NBCe1 antibody.

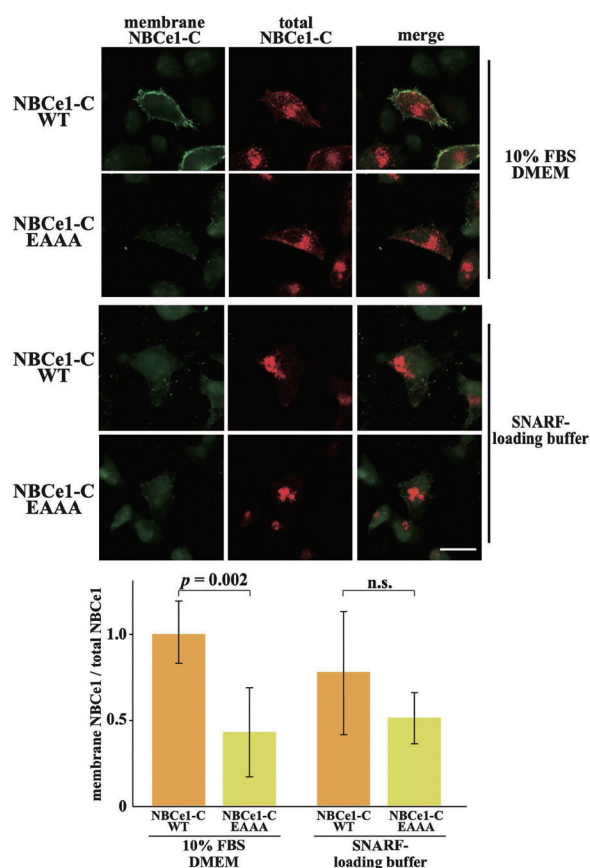


Fig. 6. Effects of pH_i Imaging Conditions on the Cell-Surface Expression of NBCe1-C

A, HeLa cells transiently expressing NBCe1-C WT or EAAA were incubated for 30 min at 37°C in either complete DMEM containing 10% FBS or SNARF-loading buffer. Cell-surface expression of NBCe1-C was analyzed by sequential immunofluorescence staining as described for Fig. 3. Scale bar, 25 μm.

B, Cell-surface expression was quantified as the ratio of cell-surface NBCe1-C fluorescence to total NBCe1-C fluorescence. Data were obtained from four sets of experiments. The total numbers of cells analyzed were 75 for NBCe1-C WT in complete medium, 58 for NBCe1-C WT in SNARF-loading buffer, 51 for NBCe1-C EAAA in complete medium, and 43 for NBCe1-C EAAA in SNARF-loading buffer. Statistical significance was evaluated using an unpaired two-tailed Student's *t*-test.

in NBCe1-C surface abundance observed under amino acid-starved conditions.

NBCe1-C Interacts with SNX27 through Its C-Terminal -ETTL Motif We next examined whether sorting nexin 27 (SNX27), previously identified as a candidate NBCe1-C-interacting protein,²³ associates with NBCe1-C through the -ETTL motif.

Co-immunoprecipitation experiments demonstrated that NBCe1-C WT, but not NBCe1-B, co-immunoprecipitated with SNX27-EGFP (Fig. 7). Neither NBCe1-C ΔETTL nor EAAA efficiently co-immunoprecipitated with SNX27-EGFP, indicating that the C-terminal -ETTL motif is required for the interaction.

A conserved histidine residue within the SNX27 PDZ domain, His112 in mouse SNX27, is critical for recognition of PDZ-binding motifs in cargo proteins.⁴¹⁻⁴³ Substitution of His112 with Ala (SNX27-H112A) abolished co-immunoprecipitation of NBCe1-C WT with SNX27 (Fig. 7), indicating that an intact SNX27 PDZ domain is required for the interaction.

NBCe1-C WT also showed substantial colocalization

with endogenous SNX27 in intracellular vesicular structures (Fig. 8A). Quantitative analysis using Pearson's correlation coefficient revealed significantly greater colocalization between NBCe1-C WT and SNX27 than between SNX27 and either NBCe1-C ΔETTL, EAAA, or NBCe1-B (Fig. 8B). Together, these findings demonstrate that NBCe1-C associates with SNX27 in a manner dependent on both the C-terminal -ETTL motif of NBCe1-C and an intact PDZ domain of SNX27.

DISCUSSION

In the present study, we investigated the functional significance of the unique C-terminal -ETTL motif of NBCe1-C. Our findings provide three principal observations. First, NBCe1-C was predominantly expressed in astrocytes in primary rat hippocampal cultures and was enriched at the distal tips of astrocytic processes. Second, disruption of the -ETTL motif reduced both cell-surface expression and internalization of NBCe1-C without substantially altering its protein stability. Third, SNX27 associated with NBCe1-C through a canonical interaction involving the -ETTL motif of NBCe1-C and the

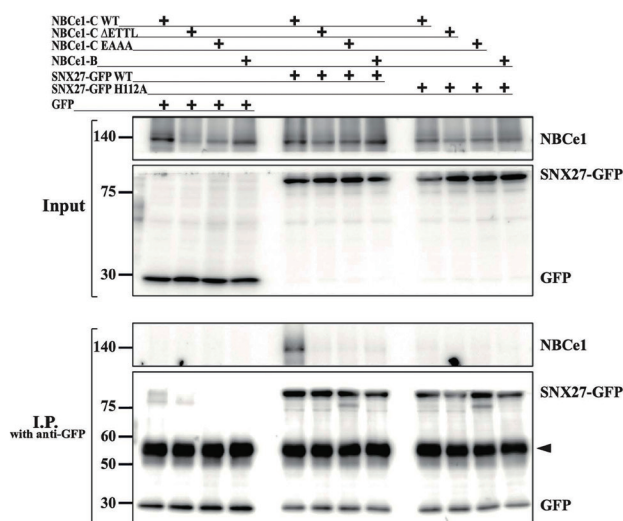


Fig. 7. Interaction of NBCe1-C with SNX27

HEK293T cells were transiently cotransfected with the indicated combinations of HA-tagged NBCe1 constructs and C-terminally EGFP-tagged SNX27 constructs. At 48 h after transfection, cells were lysed in buffer containing 0.2% Triton X-100, and SNX27-EGFP was immunoprecipitated using an anti-GFP antibody. Immunoprecipitates were analyzed by western blotting using an anti-GFP antibody to detect SNX27 and an anti-HA antibody to detect co-immunoprecipitated NBCe1.

PDZ domain of SNX27. Together, these observations identify the -ETTL motif as an important determinant of the dynamic membrane trafficking of NBCe1-C.

The prominent localization of NBCe1-C in astrocytes is consistent with accumulating evidence that Slc4a4 expression in the brain is strongly enriched in astrocytic populations. In our primary hippocampal cultures, NBCe1-C immunoreactivity was particularly prominent at the distal tips of astrocytic processes and closely resembled the distribution of GLAST, a transporter enriched in fine peripheral astrocytic processes.^{44,45} Earlier studies reported prominent NBCe1-C expression in primary cultured cortical neurons rather than astrocytes,^{7,17} suggesting that cell-type distribution may depend on brain region and/or culture conditions. Nevertheless, the localization observed here is compatible with a physiological role for NBCe1-C in fine astrocytic processes surrounding synapses. Because neuronal activity causes rapid local changes in extracellular ion concentrations and pH, positioning NBCe1-C in these processes may facilitate local regulation of acid-base homeostasis at or near tripartite synapses.

The C-terminal -ETTL motif exerted an unexpected dual effect on NBCe1-C trafficking. Disruption of the motif decreased cell-surface expression, indicating that -ETTL-dependent interactions facilitate delivery or retention of NBCe1-C at the plasma membrane. However, the same mutations also reduced NBCe1-C internalization. Thus, the -ETTL motif does not appear to function simply as a static plasma membrane retention signal. Instead, it may participate in continuous cycling of NBCe1-C between the plasma membrane and intracellular compartments. Such dynamic trafficking could account, at least in part, for the relatively large cell-to-cell variation in the surface abundance of WT NBCe1-C observed in the immunofluorescence-based assay.

The apparent transport activities of WT and mutant

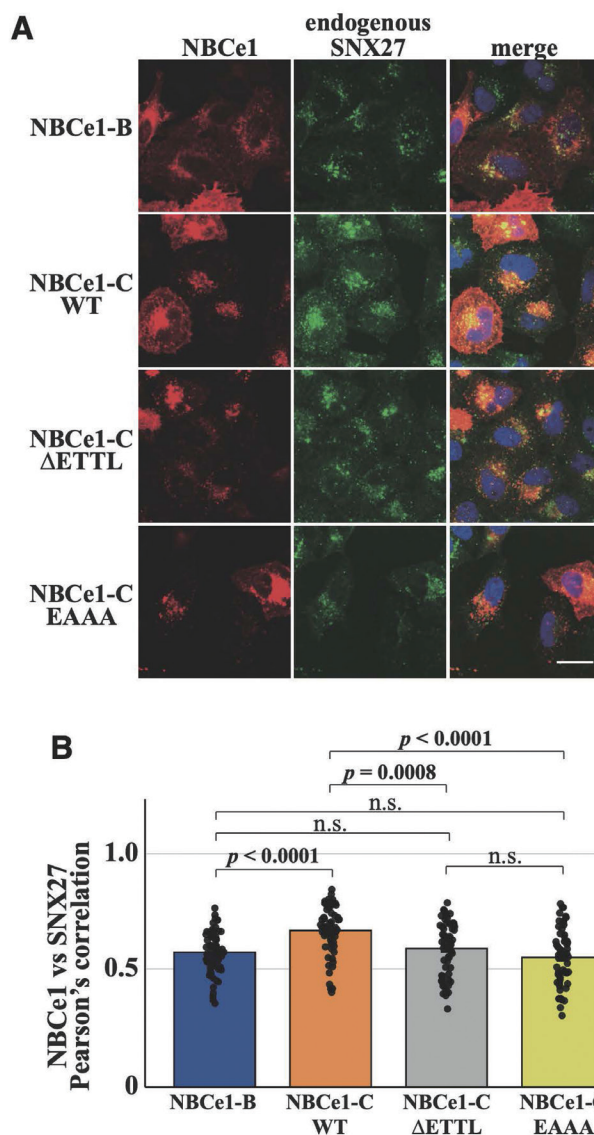


Fig. 8. Colocalization of NBCe1-C with Endogenous SNX27

A, HeLa cells were transiently transfected with the indicated HA-tagged NBCe1 constructs. Twelve hours after transfection, cells were replated onto glass coverslips and cultured for an additional 36 h. Cells were then fixed and double-immunostained for HA-tagged NBCe1 and endogenous SNX27. Confocal fluorescence images were acquired using an A1R confocal microscope. Scale bar, 25 μ m.

B, Colocalization of NBCe1 with endogenous SNX27 was quantified by calculating Pearson's correlation coefficient using ImageJ/Fiji. Sixty cells per construct without saturated fluorescence signals were analyzed.

NBCe1-C constructs were nevertheless similar under the conditions used for pH_i measurements. This initially appeared inconsistent with the lower surface expression of the C-terminal mutants. However, the transport assay required prolonged incubation of cells in amino acid-free solutions, conditions that markedly reduced WT NBCe1-C surface abundance. Under these conditions, the difference in surface expression between WT and EAAA was largely eliminated. Thus, the absence of a difference in transport activity may reflect convergence of their cell-surface expression levels during the pH_i assay rather than an absence of functional consequences of the -ETTL motif.

Incubation in the amino acid-free SNARF-loading buffer increased the LC3-II/LC3-I ratio, indicating substantial

alteration of autophagy-related pathways under these conditions. Amino acid starvation is a well-established stimulus for autophagy,³⁷⁻³⁹ and increasing evidence indicates extensive functional crosstalk between autophagic and endocytic pathways.⁴⁶⁻⁵¹ Notably, starvation reduced the surface abundance of WT NBCe1-C but had little effect on the EAAA mutant, suggesting that the -ETTL motif is required for starvation-associated redistribution of NBCe1-C. However, LC3-II accumulation alone does not establish that autophagic flux directly drives NBCe1-C internalization. The present results therefore support an association between amino acid starvation and -ETTL-dependent redistribution of NBCe1-C but do not establish a causal role for the autophagy machinery. Pharmacological or genetic manipulation of autophagy-related proteins will be required to address this issue directly.

SNX27 represents a plausible molecular partner for regulating the trafficking of NBCe1-C. SNX27 is an endosomal sorting protein that contains a PDZ domain and regulates recycling of numerous transmembrane cargo proteins, including several members of the solute carrier family.^{42,43,52-55} The C-terminal -ETTL sequence of NBCe1-C conforms precisely to a class I PDZ-binding motif recognized by SNX27.^{52,56} In agreement with this structural relationship, NBCe1-C WT associated with SNX27, whereas deletion or mutation of the -ETTL motif abolished the interaction. Conversely, mutation of His112 within the SNX27 PDZ domain also abolished association with NBCe1-C. These complementary loss-of-interaction experiments strongly support a canonical PDZ-dependent interaction between NBCe1-C and SNX27.

NBCe1-C WT also showed greater colocalization with endogenous SNX27 than either NBCe1-C mutants or NBCe1-B. Together with the co-immunoprecipitation data, this observation supports the presence of an NBCe1-C-SNX27 complex in intracellular vesicular compartments. Nevertheless, the present experiments do not establish whether SNX27 is responsible for the -ETTL-dependent effects on NBCe1-C surface expression, internalization, or recycling. Direct functional testing, such as SNX27 depletion or rescue with SNX27 mutants, will be required to determine whether SNX27 directly controls NBCe1-C trafficking.

The NBCe1-C-specific C-terminal region may constitute a regulatory platform integrating multiple trafficking signals. We previously showed that calcineurin binds to a PxIxIT motif within this region and enhances NBCe1-C cell-surface expression.²³ The present study adds the terminal -ETTL motif and SNX27 as an additional regulatory interaction. Because SNX27-mediated cargo recognition can itself be modulated by phosphorylation,⁵⁶ it is tempting to speculate that phosphorylation-dependent signaling and calcineurin-dependent dephosphorylation may influence NBCe1-C interaction with SNX27 or other PDZ domain-containing proteins. However, such functional interplay has not yet been demonstrated and remains an important subject for future investigation.

In conclusion, the present study identifies the C-terminal -ETTL motif as a key regulatory element controlling the membrane trafficking of NBCe1-C. The motif promotes both cell-surface expression and internalization of NBCe1-C and is required for the reduction in its surface abundance observed under amino acid-starved conditions. We further demonstrate that SNX27 binds to NBCe1-C through a canonical PDZ-dependent mechanism. These findings provide a molecular framework for understanding the dynamic regulation of

NBCe1-C localization and suggest that rapid redistribution of NBCe1-C may be particularly relevant in fine astrocytic processes exposed to continuously changing ionic and pH conditions at tripartite synapses.

Acknowledgments We greatly appreciate Dr. George Seki at Yaizu City Hospital, Yaizu, Japan, for providing the human NBCe1-A and NBCe1-B cDNA clones. We also thank all the laboratory members for their encouragement.

Funding This work was supported by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (JSPS KAKENHI Grant Number 16K07068 to A.M., and 15K08318 to K.H.).

Conflict of Interest Statement The authors declare no competing interests.

Data Availability Statement The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution Statement

A.M.: Conceptualization, Methodology, Investigation, Formal analysis, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing

Y.G.: Investigation, Formal analysis, Visualization, Writing – original draft

N.H.: Investigation

S.O.: Investigation

S.M.: Formal analysis, Writing – review & editing

K.H.: Formal analysis, Writing – review & editing

Ethics Approval All animal experiments were approved by the Institutional Animal Care and Use Committee of Showa Pharmaceutical University (Approval No.P-2021-12) and were performed in accordance with the relevant guidelines and regulations.

Informed Consent Statement Not applicable.

Supplementary Materials This article contains Supplementary Materials.

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