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The Na^+/H^+ Exchanger NHE5 Is Sorted to Discrete Intracellular Vesicles in the Central and Peripheral Nervous Systems

Viktoria Lukashova, Tushare Jinadasa, Alina Ilie, David Verbich, Ellis Cooper, and John Orlowski

Abstract

The pH milieu of the central and peripheral nervous systems is an important determinant of neuronal excitability, function, and survival. In mammals, neural acid–base homeostasis is coordinately regulated by ion transporters belonging to the Na^+/H^+ exchanger (NHE) and bicarbonate transporter gene families. However, the relative contributions of individual isoforms within the respective families are not fully understood. This report focuses on the NHE family, specifically the plasma membrane-type NHE5 which is preferentially transcribed in brain, but the distribution of the native protein has not been extensively characterized. To this end, we generated a rabbit polyclonal antibody that specifically recognizes NHE5. In both central (cortex, hippocampus) and peripheral (superior cervical ganglia, SCG) nervous tissue of mice, NHE5 immunostaining was punctate and highly concentrated in the somas and to lesser amounts in the dendrites of neurons. Very little signal was detected in axons. Similarly, in primary cultures of differentiated SCG neurons, NHE5 localized predominantly to vesicles in the somatodendritic compartment, though some immunostaining was also evident in punctate vesicles along the axons. NHE5 was also detected predominantly in intracellular vesicles of cultured SCG glial cells. Dual immunolabeling of SCG neurons showed that NHE5 did not colocalize with markers for early endosomes (EEA1) or synaptic vesicles (synaptophysin), but did partially colocalize with the transferrin receptor, a marker of recycling endosomes. Collectively, these data suggest that NHE5 partitions into a unique vesicular pool in neurons that shares some

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characteristics of recycling endosomes where it may serve as an important regulated store of functional transporters required to maintain cytoplasmic pH homeostasis.

Keywords
Brain • pH homeostasis • Sodium/proton exchangers • Subcellular distribution • Imaging

34.1 Introduction

The central nervous system (CNS) is highly active metabolically relative to most other organ systems and is especially reliant on glucose and its metabolite lactate as fuel to generate the main form of biochemical energy, adenosine 5′-triphosphate (ATP), needed to sustain membrane excitability and synaptic transmission (Chih et al. 2001; Schurr et al. 1988; Tsacopoulos and Magistretti 1996; Wyss et al. 2011). Indeed, it is estimated that the brains of most vertebrate species consume between 2% and 8% of the total body energy production, but in humans this can increase to as much as 20% (Mink et al. 1981). Such elevated rates of metabolism would be anticipated to generate considerable CO_2 and H^+ (or acid equivalents) as waste products that are in dynamic equilibrium ($CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$) and must be efficiently buffered or removed to maintain a suitable pH milieu for stable protein activity and interactions, and ultimately neural function. In this regard, it is well documented that various depolarizing stimuli (e.g., repetitive spike activity, hormones, and neurotransmitters) increase metabolic acid production which elicits transient fluctuations in intra- as well as extracellular pH of many types of neurons and glial cells that, in turn, can further modulate membrane excitability (Chen and Chesler 1992a, b; Chesler 2003; Dulla et al. 2005; Hsu et al. 2000; Luckermann et al. 1997; Paalasmaa and Kaila 1996; Ransom 2000; Rose and Ransom 1996; Trapp et al. 1996). Mammals have also developed highly specialized chemosensitive cells located centrally (Mulkey et al. 2004; Putnam 2010; Severson et al. 2003; Williams et al. 2007) as well as peripherally (DeSimone and Lyall 2006; Gonzalez et al. 1992) that are exquisitely responsive to minute changes in arterial blood/interstitial CO_2/H^+ levels and are believed to modulate adaptive behaviors such as breathing, arousal, nociception, and sour taste. The underlying molecular mechanisms are not fully elucidated, but are thought to reflect alterations in the conductances of certain pH-sensitive neurotransmitter-gated receptors (Traynelis and Cull-Candy 1991; Zhai et al. 1998), voltage- and H^+-gated cation channels (Baron et al. 2002; Buckler et al. 2000; Church et al. 1998; Kiss and Korn 1999; Tombaugh and Somjen 1996; Tombaugh and Somjen 1997; Waldmann 2001), gap junctional coupling (Rorig et al. 1996), as well as activation of unique H^+-sensing G protein-coupled receptors (Huang et al. 2007; Seuwen et al. 2006). Abnormal disruptions of acid–base homeostasis have also been implicated in the progression of certain neuropathies, including ischemic and hypoxic injuries (Bondarenko and Chesler 2001; Li and Siesjo 1997; Pulsinelli 1992), cerebral edema (Kempski 2001), and seizures (Ali et al. 2006; Gu et al. 2001; Hentschke et al. 2006; Schuchmann et al. 2006; Vilas et al. 2009). Hence, precise regulation of neural pH is an important physiological process and may serve as a physiologically relevant signal to modulate nervous system function (Brookes 1997; Deitmer and Rose 1996; Putnam 2001; Putnam et al. 2004; Takahashi and Copenhagen 1996). It is therefore important to understand the mechanisms that generate and regulate neural pH homeostasis.

The molecular machinery responsible for acid–base regulation in the nervous system is similar to that of peripheral cell types and involves the coordinated activities of several distinct ion transporters resident at the plasma membrane,

principally Na^+/H^+ antiporters or exchangers (NHA/NHE/NHX) in combination with one or more HCO_3^--dependent transporters in the forms of Cl^-/HCO_3^- exchangers, Na^+-dependent Cl^-/HCO_3^- exchangers, and Na^+-HCO_3^- cotransporters (for comprehensive reviews, see Casey et al. 2010; Chesler 2003; Putnam et al. 2004; Romero et al. 2004). These pH-regulating transporters exist as multiple isoforms that are distributed throughout the nervous system in a ubiquitous or cell-specific manner, suggesting unique contributions to neural pH homeostasis (Bonnet et al. 2000; Douglas et al. 2001; Havenga et al. 1994; Kobayashi et al. 1994; Ma and Haddad 1997; Rickmann et al. 2007). However, their individual regulatory properties and functions have yet to be extensively characterized, often hindered in part by the lack of suitable isoform-specific molecular probes. For the purposes of this report, further discussions will focus on the mammalian NHEs, with a specific emphasis on the NHE5 isoform which is preferentially expressed in brain.

34.1.1 Diversity of Na^+/H^+ Exchangers

The mammalian Na^+/H^+ exchanger gene family (classified as the solute carrier SLC9 family by the HUGO Gene Nomenclature Committee) consists of at least 11 structural diverse isoforms (NHE1-9/SLC9A1-9 and NHA1-2/SLC9B1-2) that assemble as homodimers and mediate the countertransport of alkali cations (Li^+, Na^+, or K^+) for H^+ across biological membranes, though their substrate specificity can differ among isoforms (Brett et al. 2005; Casey et al. 2010). The NHEs are expressed in a ubiquitous or tissue-specific manner and are differentially sorted to the plasma membrane as well as various endomembrane compartments, reflecting their involvement in diverse physiological processes. The plasma membrane-type NHEs include NHE1 to NHE5 and are generally involved in cytoplasmic or systemic pH and fluid volume homeostasis. Kinetically, they share a common mode of operation, preferentially exchanging extracellular Na^+ (and in some cases Li^+) for cytoplasmic H^+ (i.e., Na^+-selective NHEs). By contrast, NHE6 to NHE9 are present in most tissues/cells where they accumulate predominantly in organelles along the secretory and endocytic-degradative pathways. Their roles are not well defined, but crude transport measurements indicate that may operate as nonselective monovalent cation (Li^+, Na^+, or K^+)/H^+ exchangers involved in organellar pH and/or cation homeostasis. Finally, NHA1 and NHA2 are the most divergent evolutionarily and have not been extensively characterized. Analyses of the subcellular localization of NHA2 have produced divergent results, with reports indicating that it resides at the apical surface of epithelial cells (Fuster et al. 2008; Xiang et al. 2007), the endolysosomal compartment and basolateral membrane of osteoclasts (Hofstetter et al. 2010), and the inner membrane of mitochondria (Battaglino et al. 2008; Fuster et al. 2008). The basis for these differences remains obscure.

34.1.2 NHE Expression and Function in Brain

The relative contributions of NHEs to CNS function have been a subject of considerable interest not only because of their fundamental importance to cellular pH homeostasis but also because they have been implicated in a number of neuropathies. For example, recent genetic studies in humans have linked mutations in the organellar NHE6 and NHE9 isoforms to distinct neurological diseases, including mental retardation (Garbern et al. 2010; Gilfillan et al. 2008), autism (Morrow et al. 2008), and attention deficit hyperactivity disorders (de Silva et al. 2003; Franke et al. 2009; Lasky-Su et al. 2008a; Lasky-Su et al. 2008b; Markunas et al. 2010). However, at present the mechanistic bases for the observed phenotypes are unknown.

Among the plasma membrane-type isoforms, NHE1 is distributed throughout the brain as well as peripheral tissues where it fulfills basic housekeeping functions, such as the regulation of cytoplasmic pH and maintenance of cell volume. Despite its ubiquitous expression, loss of NHE1 function produces a dominant neurological

phenotype. Mice containing a null NHE 1 mutation (*Nhe1*$^{-/-}$) exhibit ataxia, epileptic-like seizures, and significant postnatal mortality (Bell et al. 1999; Cox et al. 1997). These changes are associated with selective loss of neurons in the cerebellum and brainstem (Cox et al. 1997) as well as enhanced neuronal excitability in the hippocampal and cortical regions (Gu et al. 2001; Xia et al. 2003). By contrast, abnormal hyperactivation of NHE1 is thought to contribute to the progression of cerebrovascular injuries resulting from ischemic stroke. Moderate to severe reductions in cerebral blood flow significantly decrease the supply of glucose and oxygen required to maintain the high energy demands of the brain. As a consequence, ATP stores are rapidly depleted, and lactate, pyruvate, and protons accumulate due to anaerobic metabolism of the limited stores of glucose (Pulsinelli 1992). This results in rapid increases in both intracellular and extracellular acidity which disrupts the homeostasis of other ions; most notable is the excess accumulation of Na_i^+ (via the Na^+/H^+ exchanger) and Ca_i^{2+} (via the Na^+/Ca^{2+} exchanger acting in reverse mode) (Matsuda et al. 1996; Stys et al. 1991). This, in turn, precipitates a series of other cellular changes that lead to neuronal dysfunction and ultimately tissue damage, including free radical toxicity, cellular edema, apoptosis, and necrosis (Pulsinelli 1992; White et al. 2000). A role for NHE1 is most convincingly demonstrated by studies showing that specific NHE antagonists (Andreeva et al. 1992; Horikawa et al. 2001; Kuribayashi et al. 1999; Lee et al. 2009; Luo et al. 2006; Matsumoto et al. 2004; Park et al. 2005; Suzuki et al. 2002; Vornov et al. 1996), or NHE1 gene ablation (Luo et al. 2005; Wang et al. 2008), effectively mitigate neural injuries associated with ischemia both in vitro and in vivo. Taken together, these studies implicate the functional coupling of Na^+/H^+ and Na^+/Ca^{2+} exchange as critical upstream events in the pathogenesis of cerebral ischemic and reperfusion injuries.

Other plasma membrane isoforms such as NHE2 to NHE4, though mainly found in epithelia of peripheral tissues, are also present in certain areas of the brain where they likely fulfill more specialized roles (Ma and Haddad 1997). For example, NHE3 is present in cerebellar Purkinje and glial cells (Ma and Haddad 1997) as well as chemosensitive neurons of the ventrolateral medulla oblongata that modulate the rate of respiration (Wiemann et al. 1999). Its presence in the latter neurons is particularly intriguing in light of studies showing that relatively selective antagonists of NHE3 acidify and activate these neurons in vitro (Wiemann et al. 1999; Wiemann and Bingmann 2001) and elevate the central respiratory response to hypercapnia in vivo (Abu-Shaweesh et al. 2002; Kiwull-Schone et al. 2001, 2007; Wiemann et al. 2005), implicating a regulatory role for this isoform in the control of breathing rhythm.

NHE5 is unique among the plasma membrane-type isoforms by its preferential mRNA expression in the central nervous system (Attaphitaya et al. 1999; Baird et al. 1999). In transfected non-neuronal and neuronal cells, exogenous NHE5 is present and active at the plasma membrane, but interestingly the bulk of the protein (>85%) accumulates in a transferrin receptor (Tf-R)-associated recycling endosomal compartment (Szászi et al. 2002). Subsequent studies have identified a number of factors that regulate its trafficking to and from the cell surface, including the protein kinases PI3-K (Szászi et al. 2002) and CK2 (Lukashova et al. 2011) as well as ancillary interacting proteins such as secretory carrier membrane protein SCAMP2 (Diering et al. 2009), RACK1 (Onishi et al. 2007), and the clathrin endocytic adaptors β-arrestin1 and β-arrestin2 (Szabó et al. 2005). However, it remains unclear whether the subcellular distribution and regulation of the exogenously expressed NHE5 reflects the behavior of the endogenous protein in its native environment and, if so, what is the functional significance of this distribution.

In this report, we describe the subcellular location of the native NHE5 protein in selected regions of the central (cortex, hippocampus) and peripheral (superior cervical ganglia, SCG) nervous systems as well as in primary cultures of SCG neuronal and glial cells using a newly developed

NHE5 isoform-specific rabbit polyclonal antibody. Similar to transfected cell systems, the results show that NHE5 is sorted almost exclusively into a unique vesicular pool in neurons as well as glial cells that share some characteristics of recycling endosomes and which may serve as an important reservoir of functional transporters.

34.2 Experimental Procedures

34.2.1 Materials

C57BL/6 mice were from Jackson Laboratories. Restriction enzymes were from New England Biolabs. Polyvinylidene fluoride (PVDF) membrane, Millex-HV filter, and Amicon Ultra-4 centrifugation filter units were from Millipore. Lipofectamine™ was from Invitrogen (Life Technologies Corporation). α-MEM, fetal bovine serum, and penicillin–streptomycin were from GIBCO. Goat anti-rabbit or anti-mouse HRP-conjugated antibodies were from Jackson Immuno Research Laboratories, Inc. Monoclonal anti-synaptophysin was from Synaptic Systems. Monoclonal antibody against microtubule-associated protein 2 (MAP2) was purchased from Sigma-Aldrich. Monoclonal anti-EEA1, anti-transferrin antibodies were from BD Biosciences. Monoclonal anti-SMI 31 (anti-neurofilament) and anti-HA antibodies were from Covance. Goat anti-mouse or anti-rabbit Alexa Fluor secondary antibodies were from Molecular Probes (Life Technologies Corporation). Enhanced chemiluminescence system, Protein G-Sepharose 4B, glutathione sepharose 4B, and pGEX-2T bacterial expression vector were purchased from GE Healthcare Life Sciences. All other reagents were obtained from Fisher Scientific or Sigma.

34.2.2 Mouse Brain Cryosectioning and Primary Tissue Culture

All procedures for animal handling were carried out according to the guidelines of the Canadian Council on Animal Care (CCAC). Mice were anesthetized and sacrificed by cervical dislocation, followed by dissection of the brains which were immediately removed and placed on dry ice. Twenty micron thick sections were cut using a cryostat and mounted on gelatin subbed slides. Slides were dried at room temperature for 30 min. They were then kept frozen with desiccant until staining.

Mouse superior cervical ganglia (SCG) were dissected from neonatal C57BL/6 littermates and dissociated enzymatically and mechanically as previously described (Gingras et al. 2002). Briefly, ganglia were incubated for 45 min in Hank's Balanced Salt Solution/trypsin 3× (1 mg/ml) and gently triturated with a fire-polished Pasteur pipette. Dissociated cells were washed in plating media, isolated by centrifugation (800 rpm, 4 min), and resuspended in growth media. Neurons were then plated at a density of ~35 cells/mm^2 on laminin-coated ACLAR coverslips in a modified Petri dish. The neurons were incubated in 1.5 ml of Leibovitz's L-15 medium supplemented with 5% rat serum, vitamins, cofactors, penicillin, streptomycin, sodium bicarbonate, and nerve growth factor NGF 2.5 S (25 ng/ml). Neurons were maintained in 5% CO_2 at 37 °C and treated with 1-ß-D-arabinofuranoside (Ara-C, 5 mM; Sigma-Aldrich) for the first 2–3 days in culture to eliminate non-neuronal cells.

34.2.3 Polyclonal NHE5 Antibody Production

cDNA segments encoding amino acids 689–720, 689–789, and 789–896 of the cytoplasmic C-terminus of human NHE5 were amplified by polymerase chain reaction (PCR), excised with *Bam*HI and *Eco*RI, and inserted into the pGEX-2T bacterial expression vector in frame with the amino-terminal glutathione S-transferase (GST) coding sequence. After sequence confirmation, plasmids were transformed into BL21 *E. coli* cells for protein expression. Cells were lysed in 1×phosphate-buffered saline (PBS) supplemented

with 0.4 mM of lysozyme and purified by overnight incubation with glutathione-conjugated sepharose beads at 4 °C. Proteins were eluted with 10 mM glutathione in 1×PBS. Eluted proteins were concentrated using Amicon Ultra-4 centrifugal units (Millipore) and filtered via 0.45 μm Millex-HV filter. Rabbits were injected intradermally with 300 μg of antigen in complete Freund's adjuvant using standard Canadian Council on Animal Care (CCAC)-approved protocols. Subsequent booster injections were given in incomplete Freund's adjuvant every 2–3 weeks. Sera were obtained from the animals before each immunization and then every third week starting on day 30 after initial immunization.

Sodium azide was added to the sera to a final concentration of 0.05% followed by centrifugation at 15,000×*g* for 5 min at 4 °C to clear up the serum. Clarified serum was collected and added to a 2 ml Bio-Rad protein A chromatography column at an approximate drip rate of 2 ml per minute. The flow through was passed through the column twice and washed with TBS (50 mM Tris–HCl, pH 7.4; 150 mM NaCl, 0.05% sodium azide) at a volume 10 times greater than the loaded serum. The washes were repeated until the absorbance reached less than 0.2 units above background. The column was drained of TBS without allowing the column to stand dry. Fifteen milliliters of elution buffer pH 2.7 (50 mM Glycine-HCl, pH 2.7) was added to the column, and 1 ml fractions were collected in microcentrifuge tubes each containing 100 μl of neutralization buffer (1 M Tris–HCl, pH 8.0; 1.5 M NaCl; 1 mM EDTA; 0.5% sodium azide). Each fraction was mixed immediately and placed on ice prior to collecting the next fraction. Each fraction was measured for protein concentration, and fractions registering an absorbance greater than 0.2 units over background were combined. The combined fraction's pH was measured and adjusted to approximately pH 7.4.

34.2.4 Cell Culture

Chinese hamster ovary AP-1 cells were maintained in complete α-MEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 25 mM $NaHCO_3$, pH 7.4, and incubated in an humidified atmosphere of 95% air, 5% CO_2 at 37 °C. Cells were transiently transfected with HA epitope-tagged forms of NHE1-9 using Lipofectamine and incubated for a minimum of 48 h prior to Western blot analysis.

34.2.5 SDS-PAGE and Immunoblotting

AP-1 cells were solubilized in cell lysis solution (NaCl 150 mM, Tris 20 mM, EDTA 10 mM, 0.5% Triton X-100, pH 7.4) and added to an equal amount of sample buffer (1 M Tris-Cl, 1% SDS, 10% glycerol, 0.1% bromophenol blue) and then subjected to SDS-PAGE using 10% polyacrylamide gels. Proteins were then transferred to a PVDF membrane for immunoblotting. The PVDF membrane was incubated with skim milk–PBS (5% nonfat dry skim milk and 0.1% Tween 20 in PBS, pH 7.4) for 1 h at room temperature in order to block nonspecific binding. The PVDF membrane was subsequently incubated for 1 h with the primary antibody in skim milk–PBS–Tween 20 at the following concentrations: anti-NHE5 at 1:500 and monoclonal anti-HA at 1:5,000. The membrane was washed three times for 10 min with PBS–Tween 20, and an appropriate secondary antibody was added at a concentration of 1:5,000 for 1 h at room temperature. Goat polyclonal anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody from Upstate Biotechnology was used for blots probed with polyclonal anti-NHE5. Goat anti-mouse HRP-conjugated secondary antibody from Jackson ImmunoResearch Laboratories was used for blots probed with monoclonal anti-HA. Membranes were again washed with PBS–Tween 20 three times for 10 min each, and the immunocomplex was visualized using an enhanced chemiluminescence system (Amersham Biosciences).

34.2.6 Confocal Microscopy

Primary cultures and cryosections were fixed in 1% paraformaldehyde, permeabilized in 0.1%

Triton in 1× PBS, blocked in 10% goat serum in 1× PBS, and incubated with the primary antibody diluted in blocking solution at the following concentrations: anti-NHE5 at 1/500–1/1,000, anti-HA at 1/2,000, anti-synaptophysin 1/500, anti-neurofilament at 1/10,000, anti-MAP2 at 1/1,000, anti-transferrin at 1/300, and anti-EEA1 at 1/1,000. Images were analyzed by confocal laser scanning microscopy using a Zeiss IM 35 microscope with a 63× objective (Neofluar, N.A. 0.75). Images were analyzed using LSM AIM and Zen Software.

34.3 Results and Discussion

As mentioned above, NHE5 mRNA is widely distributed in the brain, but precise localization of the encoded protein awaits the development of isoform-specific antibodies. We demonstrated previously that an external triple HA epitope-tagged form of NHE5 ($NHE5_{HA}$) resides in both the plasma membrane and in transferrin receptor (Tf-R)-associated recycling endosomes when ectopically expressed in Chinese hamster ovary (CHO)-derived AP-1 cells (Szászi et al. 2002). Although these cells retain many exocytic and endocytic sorting mechanisms that are analogous to the more specialized membrane trafficking pathways found in polarized cells such as epithelia and neurons (Cameron et al. 1991; Chavez et al. 1996; Coorssen et al. 1996; D'Souza et al. 1998; Morimoto et al. 1995; Wilson and Colton 1997; Yoshimori et al. 1996), sorting of NHE5 in CHO/AP-1 cells may differ from that in neuronal cells. For example, the synaptic vesicle protein synaptophysin, which colocalizes with Tf-R-associated recycling endosomes when ectopically expressed in CHO cells (Cameron et al. 1991), only partially coincides with Tf-R in the somatodendritic region of hippocampal neurons and is selectively enriched in axonal synaptic vesicles that are devoid of Tf-R (Mundigl et al. 1993). Hence, it is conceivable that NHE5 may sort to discrete vesicular compartments in neuronal cells other than Tf-R-associated endosomes, and this merits further investigation.

To generate a specific antibody against NHE5, recombinant fusion proteins comprised of bacterial glutathione S-transferase (GST) linked to isoform-specific, evolutionarily conserved, C-terminal segments (amino acids 689–720, 689–789, and 789–896) of human NHE5 were constructed and injected into rabbits using standard protocols. Of the various constructs, the serum from rabbit #3568 injected with GST-NHE5(789–896) generated the strongest immunoreactive signal (*data not shown*). This serum was then subjected to affinity purification to enrich for anti-$NHE5_{3568}$ polyclonal antibodies (simply referred to as α-$NHE5_p$). To test for NHE isoform specificity, whole cell lysates of AP-1 cells stably expressing $NHE5_{HA}$ and its various HA epitope-tagged paralogs (NHE1–4, 6–9) were fractionated by SDS-PAGE and then subjected to immunoblotting using an anti-HA monoclonal antibody (α-HA_m) or α-$NHE5_p$ (Fig. 34.1a, *upper* and *lower* panels, respectively). As expected, the α-HA_m antibody detected all of the NHE isoforms which were well expressed in the AP-1 cells with the exception of $NHE4_{HA}$ which showed low levels of expression (Fig. 34.1a, *upper panel*). By contrast, when reprobing the blot with α-$NHE5_p$, a strong immunoreactive signal was detected only from cell lysates containing $NHE5_{HA}$, verifying the selectivity of the antibody for NHE5. Additional control experiments showed no detectable signal in NHE5-expressing cells labeled only with secondary antibodies or in untransfected cells (*data not shown*) as well as tissue incubated with competing NHE5 antigen (Fig. 34.1d).

To determine the distribution of NHE5 in native tissues, immunofluorescence studies were performed using murine tissue sections from the central (neonatal cortex and hippocampus CA3 region) and peripheral (superior cervical ganglia, SCG) nervous systems. Primary cultures of differentiated SCG neurons (>2 weeks) were also examined. Cryosections of each tissue were prepared

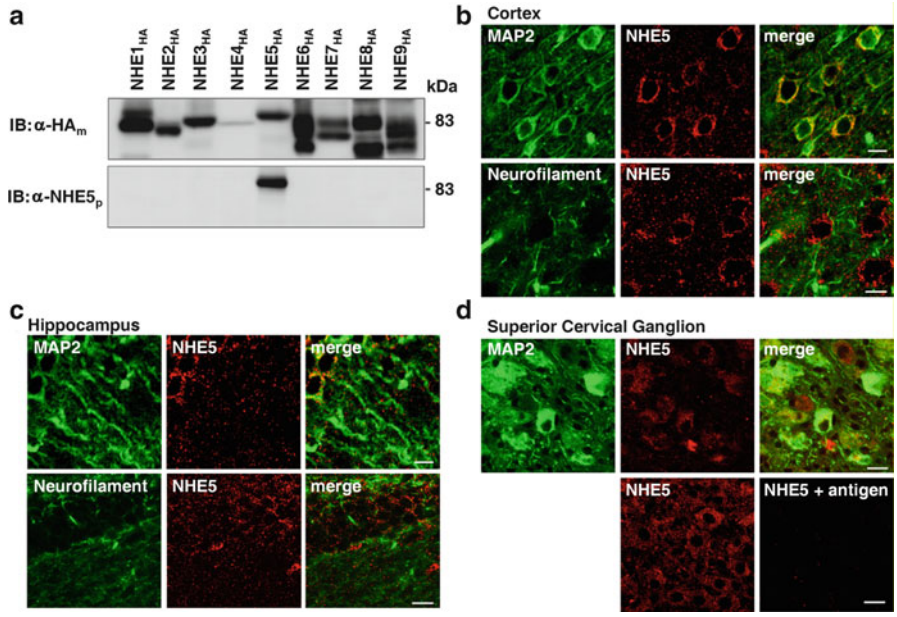

Fig. 34.1 *Immunolocalization of NHE5 in mouse nervous tissue*. (**a**) The specificity of an affinity-purified rabbit polyclonal antibody generated against amino acids 789–896 of NHE5 (α-NHE5p) was tested by probing immunoblots containing whole cell lysates of Chinese hamster ovary AP-1 cells transiently expressing HA-tagged constructs of the different NHE isoforms (NHE1-9_{HA}). The blots were probed with either a monoclonal anti-HA antibody (*upper panel*) or α-NHE5p (*lower panel*). (**b–d**) Cryosections of tissue from the cortex (**b**), hippocampus CA3 (**c**), and superior cervical ganglia (SCG) (**d**) were prepared from young mice (1 month old) and subjected to dual immunolabeling with a polyclonal antibody to native NHE5 (α-NHE5p) and either a mouse monoclonal antibody to microtubule-associated protein 2 (MAP2) to visualize cell bodies and dendritic processes or to neurofilament, a marker of axons, followed by incubation with appropriate Alexa-conjugated secondary antibodies. The SCG sections were also incubated with α-NHE5p alone, or in the presence of the NHE5-immunizing antigen for 1 h, which blocked the signal. The data reveal that NHE5 is preferentially localized in punctate vesicles in the cell bodies as well as dendrites. Scale bar: 10 μm.

from embryonic 18.5 day-old animals. For controls, some cryosections were processed with preimmune serum, without the primary antibody or with the primary antibody in the presence of the immunizing antigen. Cross-reactivity tests were also conducted with different combinations of the secondary antibodies. Sections were visualized using a Zeiss laser scanning confocal microscope.

NHE5 expression was readily detected in cortex (Fig. 34.1b), hippocampus CA3 (Fig. 34.1c), and SCG (Fig. 34.1d) and showed similar subcellular distributions, notably punctate immunostaining that was highly concentrated in the somas and dendrites, as revealed by co-staining with an antibody against microtubule-associated protein 2 (MAP2), a somatodendritic marker. Very little signal was detected in axons co-stained with an anti-neurofilament H, a recognized axonal marker. Importantly, the signal for NHE5 was blocked in the presence of excess immunizing antigen GST-NHE5(789–896), confirming the specificity of the antibody (Fig. 34.1d). Likewise, in primary cultures of differentiated SCG neurons, NHE5 localized predominantly to somatodendritic vesicles (Fig. 34.2a–d), though some signal was also detected in punctate vesicles present along the axons (Fig. 34.2e–h). Intriguingly, unlike in AP-1 cells, NHE5 showed colocalization with only a subset of vesicles containing the transferrin receptor Tf-R in the cell body and

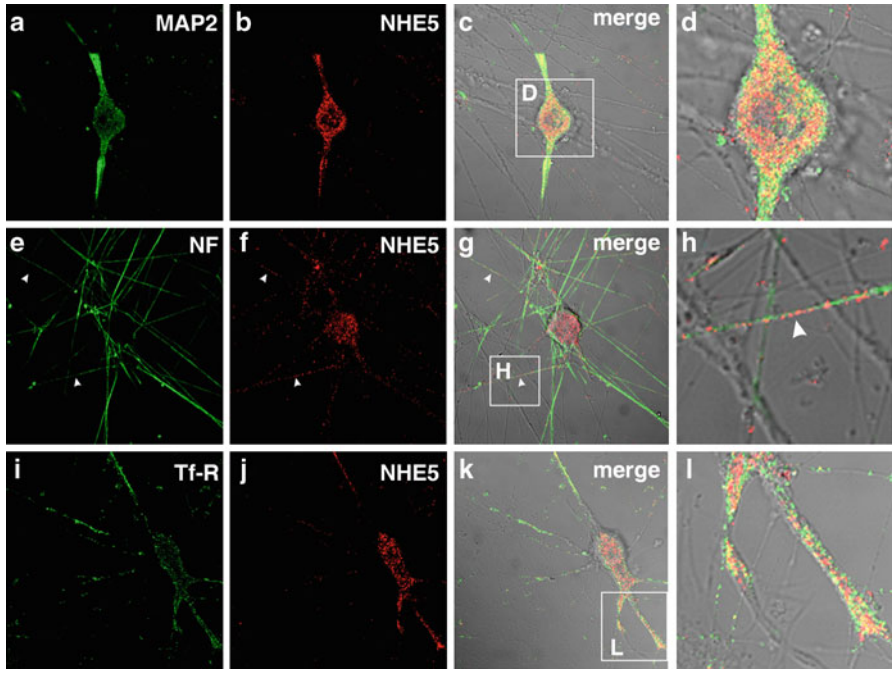

Fig. 34.2 *Localization of NHE5 in primary cultures of murine superior cervical ganglion (SCG) neurons*. Fixed and permeabilized primary cultures of SCG neurons were dual immunolabeled with α-NHE5p (**b**, **f**, **j**) and monoclonal antibodies to MAP2 (**a**) to visualize cell bodies and dendrites, neurofilament (NF) (**e**) to visualize axons, and transferrin receptor (Tf-R) (**i**) to label recycling endosomes. The merged images (**c**, **g**, **k**) were overlaid on the differential interference contrast (DIC) images of the neurons. The *boxed sections* within panels **c**, **g**, and **k** are enlarged as panels **d**, **h,** and **l**, respectively. Immunolabeling of NHE5 was detected predominantly in the soma and dendrites, and to some extent in axons (indicated by *arrowheads*)

dendrites (Fig. 34.2i–l). Moreover, NHE5 did not colocalize with the early endosome antigen 1 (EEA1) (Fig. 34.3a) nor with synaptophysin (Fig. 34.3b), a marker of synaptic vesicles. NHE5 was also detected predominantly in intracellular vesicles of cultured SCG glial cells. The presence of NHE5 at the cell surface was not readily evident in these confocal images.

During the course of the studies, Diering and colleagues (Diering et al. 2011) also reported that NHE5 is present almost exclusively in intracellular vesicles in dendrites and to a lesser extent in axons of cultured hippocampal neurons, similar to our observations. Intriguing, NHE5-containing vesicles were recruited to the cell surface of dendritic spines in response to glycine-induced activation of NMDA receptors where it modulated the pH homeostasis and activity-dependent growth of the spines, suggesting that NHE5 may play an important role in synaptic maturation and plasticity. However, the mechanistic basis for this translocation is unknown.

Collectively, these data suggest that NHE5 partitions into a discrete pool of intracellular vesicles in central and peripheral neurons as well as glial cells. With respect to neurons, these vesicles are distinct from early endosomes and synaptic vesicles and partially overlap with transferrin-containing recycling endosomes. More detailed analysis of the nature of the intracellular compartments containing NHE5 using other specific organelle markers is needed to better understand the physiological role of NHE5 in neuronal function.

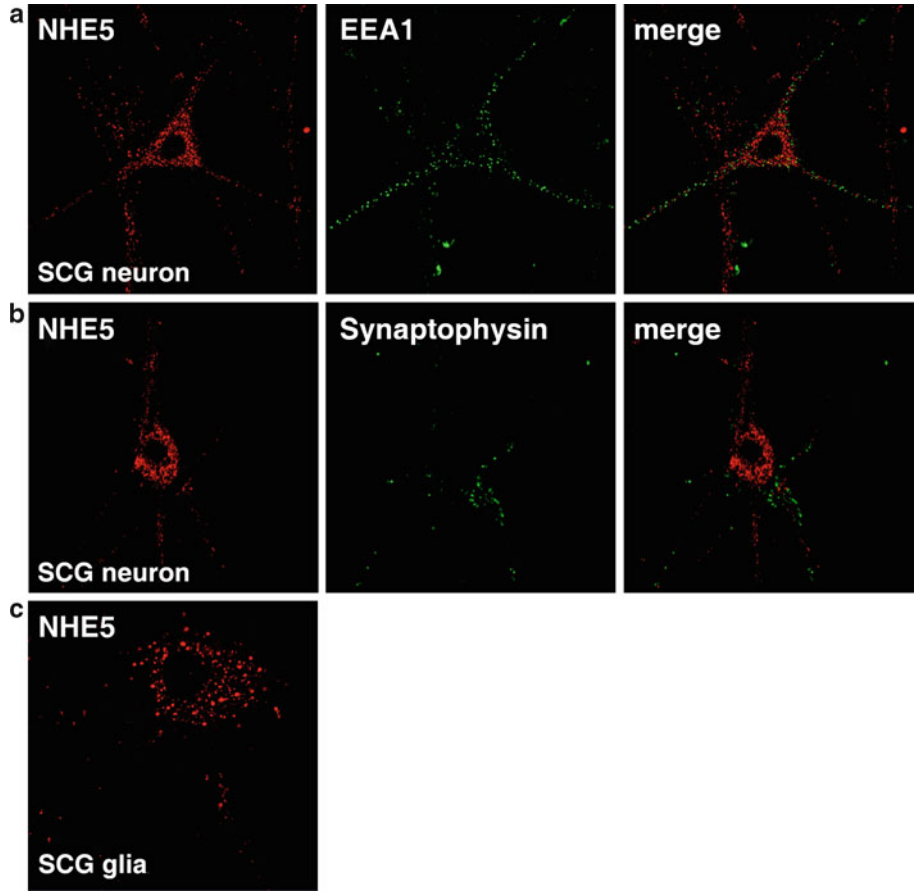

Fig. 34.3 *NHE5 is not localized to early endosomes or synaptic vesicles in primary cultures of superior cervical ganglion (SCG) neurons*. (**a**, **b**) Primary cultures SCG neurons were incubated simultaneously with α-NHE5p and monoclonal antibodies to synaptophysin and early endosomal antigen 1 (EEA1) followed by labeling with the corresponding Alexa-conjugated secondary antibodies. (**c**) Immunolabeling of NHE5 was also detected predominantly in intracellular vesicles of primary cultures of SCG glial cells

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