Exploring the Physiological Function of the Brain-enriched Na\(^+\)/H\(^+\) Exchanger

**NHE5**

by

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Abstract

In general, electrical activity in the brain is enhanced or suppressed by increases or decreases in pH respectively. Moreover, many components of the synaptic machinery are sensitive to changes in pH within the physiological range. Therefore, given this sensitivity to pH, it is likely that pH-regulatory proteins at the synapse may play an essential role in synaptic transmission and synaptic plasticity. However, the identity of the molecules responsible for regulating pH at the synapse has never been determined. NHE5 is a unique member of the Na⁺/H⁺ exchanger (NHE) gene family that is highly enriched in brain, yet a physiological role for NHE5 has never been described.

In this thesis work, I show for the first time that NHE5 protein is expressed in neurons. In these cells a portion of NHE5 is targeted to synapses; NHE5 abundance at synapses and on the neuronal cell-surface increases in response to synaptic activity. Further I found that dendritic spines, the location of the excitatory post-synapse, experience an activity and NHE5-dependent change in local pH. Blocking NHE5 activity by expression of a transport-deficient dominant-negative mutant or by knock-down resulted in spontaneous exuberant dendritic spine outgrowth, suggesting that NHE5 is a negative regulator of dendritic spine growth. Interestingly, this spine growth required an active NMDA receptor, a pH-sensitive protein, suggesting that the action of NHE5 is mediated through NMDA receptors. Thus, I propose that NHE5 is recruited to synapses during synaptic activity as part of a negative feed-back loop in order to regulate nearby pH-sensitive synapse components to control or stabilize dendritic spine growth.

I have also expanded on the current understanding of the molecular regulation of NHE5 by the identification and characterization of an NHE5-SCAMP2 interaction. SCAMP2 binds to NHE5 in recycling endosomes and promotes its delivery to the cell surface, in a pathway that requires the small GTPase Arf6. Therefore, I have uncovered part of the physiological function of NHE5 and also identified the very first molecule involved specifically in regulation of pH at the synapse. Future work on the action of NHE5 at synapses and the regulation of NHE5 by protein binding partners and signaling pathways is discussed.
Preface

A portion of the work presented in this thesis has been a collaborative effort between myself, my supervisor Dr. Masayuki Numata, and our collaborators Dr. John Church, Dr. Shernaz X. Bamji, and her doctoral student Fergil Mills. I performed all of the experiments shown in this thesis as well as prepared all of the data figures. However the preparation of many of the materials used as well as the design of all the experiments was very much a collaborative effort. My supervisor Dr. Numata, and Dr. Bamji and Fergil Mills assisted with the design of experiments shown in chapter 3 and chapter 4. The experiments shown in chapter 5 were designed by myself together with Dr. Numata and Dr. Church. The coronal mouse brain sections used for immunohistochemistry shown in figure 3-1 were prepared by Fergil Mills. In addition, figure 4-12, the summary figure to chapter 4 was prepared by Fergil Mills. All of the cultured neurons used in chapter 3 and chapter 4 were generously prepared on my behalf by members of the Bamji lab. Some of the cDNA constructs used in this work such as cDNA for SCAMP1, SCAMP2, SCAMP5, Rab11 and Arf6 were cloned by my supervisor Dr. Numata. I received training and technical assistance from many colleagues, but in particular from my supervisor Dr. Numata, Dr. Church, Dr. Bamji and my fellow graduate students Dr. Paulo Lin (Numata lab), and Fergil Mills and Stefano Brigidi (Bamji lab).

Some of this thesis work has been prepared as manuscripts for publication. I wrote the first drafts of these manuscripts which were then revised together with Dr. Numata and our collaborators Dr. Bamji and Fergil Mills (chapter 3 and chapter 4) and Dr. Church (chapter 5).


The research presented in this thesis was approved by the UBC Bio-Safety Committee (Certificate B09-0182) and the Radiation Safety Committee (Certificate BIOC-3155-12)
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List of abbreviations

AE, anion exchanger
AMPA, $\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AP2, clathrin adaptor protein 2
AP5, 2-amino-5-phosphonopentanoic acid
ASIC, acid-sensing ion channel
BCECF, 2',7'-Bis-(2-carboxyethyl)-5-(And-6)-carboxyfluorescein
Ca, carbonic anhydrase
CaMKII, Ca$^{++}$-calmodulin dependent protein kinase
CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO, Chinese hamster ovary
CPA, cation proton antiporter
CNS, central nervous system
CSF, cerebral spinal fluid
DIV, days in vitro;
EDTA, ethylenediaminetetraacetic acid
EAAT, excitatory amino acid transporter
EH, epsin homology domain
EIPA, 5-(N-Ethyl-N-isopropyl)amiloride
EM, electron microscopy
ERM, ezrin-radixin-moesin proteins
FBS, fetal bovine serum
GABA, $\gamma$-amino butyric acid
GAP, GTPase-activating protein
GEF, guanine nucleotide exchange factor
GFAP, glial fibrillary acidic protein
GFP, green fluorescent protein
GKAP, guanylate-kinase-associated protein
GPiCR, G protein coupled receptor
GST, glutathione-S-transferase
HA, hemagglutinin
HBSS, Hank’s buffered salt solution
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFS, high-frequency stimulation
IPTG, isopropyl-thio-\beta\text{-}galactopyranoside
LFS, low-frequency stimulation
LTD, long-term depression
LTP, long-term potentiation
MAPK, mitogen-activated protein kinase
MCT, mono carboxylate transporter
MDCK, Madin-Darby Canine Kidney
MES, 2-(N-morpholino)ethanesulfonic acid
MEM, minimum essential media
mEPSC, miniature excitatory post-synaptic currents
NBC, Na$^+$/bicarbonate cotransporter
NCBE, Na$^+$-coupled chloride bicarbonate exchanger
NDCBE, Na$^+$-driven chloride bicarbonate exchanger
NHE, Na$^+$/H$^+$ exchanger
NHERF, NHE-regulatory factor
NMDA, N-methyl-D-aspartate
NSF, N-ethylmaleimide sensitive factor
PAGE, polyacrylamide gel electrophoresis
PBS, phosphate buffered saline
PCR, polymerase chain reaction
PDZ, PSD95, Drosophila disc large tumor suppressor, Zonula occludens-1 domain
PI3K, phosphatidylinositol-3-kinase
PIP, phosphatidylinositol phosphate
pK_a, acid-dissociation constant
PKA, protein kinase A; cAMP-dependent protein kinase
PKC, protein kinase C
PMCA, plasma membrane Ca**-ATPase
PNS, peripheral nervous system
PPF, paired-pulse facilitation
PSD, post-synaptic density
PTH, parathyroid hormone
PVDF, polyvinylidene fluoride
RACK1, receptor for active C-kinase
ROCK, rho-activated protein kinase
RTK, receptor tyrosine kinase
SCAMP, secretory carrier membrane protein
SDS, sodium dodecyl sulphate
SERCA, sarcoplasmic/endoplasmic reticulum Ca**-ATPase
Syt1, synaptotagmin1
TGN, trans-Golgi network
TTX, tetrodotoxin
v-ATPase, vacuolar-type H+\( ^+\)-ATPase
VGCC, voltage-gated Ca**-channel
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interest in the process of fermentation is what led me to pursue a career in biochemistry in the first place.

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1. Introduction

1.1 Cellular pH regulation and homeostasis

1.1.1 Introduction to pH biology

All cells must maintain an ionic environment within their plasma membrane in a narrow range in order to perform their functions and grow. For eukaryotes, this requirement holds for the intracellular organelles as well as the cytoplasm, and for multicellular organisms, regulation of the ionic environment outside the cell is also crucial. H+ ion or pH homeostasis is of particular importance as pH can affect the charge, structure and function of most macromolecules. For instance, the structure and function of all proteins is sensitive to pH to a degree, with some having a particularly noted sensitivity to pH within the physiological range. In addition, the proton motive force, differences in pH across biological membranes, has evolved as a source of energy for multiple purposes such as ATP synthesis and the transport of solutes across membranes. Transient and localized changes in pH can also play an important signaling function for instance by acting on pH-sensitive ion channels.

1.1.2 Cellular buffering

It is important to consider the role of pH buffers in the cell. Cellular buffering capacity $\beta_{\text{total}}$, can be divided into two parts: intrinsic buffering capacity $\beta_i$, and bicarbonate buffering capacity $\beta_{\text{HCO}_3^-}$. $\beta_i$ is made up of all the intracellular weak acids and bases including phosphate, free amino acids, and ionizable side chains of amino acid residues (Boyarsky et al., 1988; Casey et al., 2010; Chesler, 2003; Roos and Boron, 1981). Phospho-groups with free hydroxyls such as those found on the lipids phosphatidic acid or phosphatidylinositol phosphates or on phosphorylated proteins can
also act as pH buffers. However, as the acid dissociation constant (pKₐ) of the majority of the ionizable groups in the cell are considerably above or below resting cellular pH, βᵢ is considerably low. Therefore the second component β₃, made up from HCO₃⁻/CO₂ is of considerable importance. Mammalian cells are constantly exposed to CO₂ which can readily cross biological membranes. When exposed to water, CO₂ undergoes the reversible reaction to form carbonic acid which almost instantly releases a proton to form bicarbonate (equation 1).

\[
(1) \quad \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^- 
\]

The hydration of CO₂ to form carbonic acid, although thermodynamically spontaneous, occurs slowly with forward and reverse time constants of approximately 20s and 1s respectively (Chesler, 2003). Thus the attainment of equilibrium following an acid or base challenge could require minutes. To overcome this, cells utilize various forms of the enzyme carbonic anhydrase (CA) which catalyzes both the hydration and dehydration steps. In humans, 11 isoforms of CA exist that can be found in the cytosol, mitochondria, anchored to the cytoplasmic or extracellular face of the plasma membrane or secreted (Casey et al., 2010; Chesler, 2003; Spitzer et al., 2002). CA thus regulates the availability of the buffer HCO₃⁻ protecting cells from dangerous shifts in pH.

Even in the presence of a large buffering capacity and CA enzymes, localized pH shifts and even pH gradients can occur in cells. Despite their extremely small size, protons have a surprisingly slow diffusion rate in cytoplasm (diffusion constant 0.4-2.2μm²/s) (Casey et al., 2010; Spitzer et al., 2002; Vaughan-Jones et al., 2002). This is thought to be due to the rapid association-dissociation of protons with ionizable groups on macromolecules that move slowly through the cytoplasm. Additionally, despite the activity of CA, rapid acid-base loads can transiently alter cellular pH before the cellular buffering can reach equilibrium (DeVries, 2001; Fedirko et al., 2007; Makani and
Chesler, 2007). Together, these findings suggest that transient and localized or even sustained pH gradients can exist in the cell given certain circumstances, such as the localized action of pH regulatory proteins (see below).

1.1.3 pH regulatory mechanisms

The majority of cellular pH regulatory mechanisms involve integral membrane proteins which transport $\text{H}^+$ or $\text{H}^+$-equivalents across biological membranes. For eukaryotes, these systems exist on the plasma membrane to regulate the pH of the cytoplasm and the extracellular space, as well as in the organelle membrane to regulate the pH of the organelle lumen and cytoplasm. pH regulatory proteins either import protons into the cell/organelle (acid loaders) or export protons from the cell/organelle (acid extruders). Alternatively, acid loaders or extruders can also function by the export or import of alkali respectively (Fig. 1-1). The major pH-regulatory transport proteins are the $\text{Na}^+$/H$^+$ exchanger (NHE), $\text{Cl}^-$/HCO$_3^-$ exchanger (anion exchanger, AE), $\text{Na}^+$/HCO$_3^-$ cotransporter (NBC), and $\text{Na}^+$-driven $\text{Cl}^-$/HCO$_3^-$ exchanger (NDCBE) (Orlowski and Grinstein, 2004; Pushkin and Kurtz, 2006; Romero et al., 2004). Note that NBCs can be electroneutral or electrogenic depending on the isoform (Pushkin and Kurtz, 2006; Romero et al., 2004). Also of great importance is the vacuolar-type proton ATPase (v-ATPase), also called the proton-pump, which uses the energy generated from ATP hydrolysis to transport protons across biological membranes in an electrogenic fashion.

In addition to these core pH-regulatory molecules, several other proteins are also important in cellular pH homeostasis. Carbonic anhydrase (CA), as mentioned above, is crucial for cellular buffering. The $\text{Na}^+$/K$^+$-ATPase (NKA) uses the energy of ATP hydrolysis to pump 3Na$^+$ ions out of the cytoplasm in exchange for 2K$^+$ ions, thereby
establishing the resting membrane potential and inward directed Na\(^+\) gradient, that powers secondary transporters such as NHE.

Several other membrane transport proteins are also known to transport protons in addition to their other substrates. Monocarboxylate transporters (MCT) cotransport H\(^+\) together with organic acids such as lactate (Halestrap and Meredith, 2004); excitatory amino acid transporters (EAATs) perform Na\(^+\)-dependent transport of glutamate or aspartate coupled to H\(^+\) influx (Broer, 2002); Ca\(^{++}\)-ATPase pumps such as the plasmalemmal Ca\(^{++}\)-ATPase (PMCA) or the sarcoplasmic/endoplasmic reticulum Ca\(^{++}\)-ATPase (SERCA) use the energy of ATP hydrolysis to transport a Ca\(^{++}\) ion in exchange for 2H\(^+\) ions (Makani and Chesler, 2010; Shull et al., 2003). Though these proteins do indeed transport protons and can affect local pH, these proteins do not function primarily as regulators of pH but rather for the transport of their other solutes (Casey et al., 2010).

### 1.1.4 Cytoplasmic pH

In mammalian cells, the resting cytoplasmic pH can vary depending on cell-type and various physiological parameters, but is typically close to 7.2, slightly more acidic than the surrounding extracellular pH, which is 7.3-7.4. Despite being surrounded by buffered solution of higher pH, mammalian cells must guard against excess acidification of the cytoplasm. At resting membrane potential, which is negative inside, there is a net electrochemical driving force for the uptake of protons into the cell. In addition, cells continuously produce acid through their normal metabolism. For these reasons cells must continuously and actively remove excess protons from the cytosol. This is typically accomplished by the acid extruders: NHE that exports protons, or NBC and NDCEBE, that import HCO\(_3^-\) base that can absorb cytoplasmic protons to form CO\(_2\) (Casey et al., 2010; Pushkin and Kurtz, 2006; Romero et al., 2004) (Fig. 1-1). These are secondary ion
transport proteins that use the inward Na\(^+\) electrochemical gradient as a source of energy. Protons can also be exported from the cell by H\(^+\)-ATPase pumps. Such ATPase pumps can be found in certain epithelia where they function to acidify lumen compartments such as the stomach, or in other specialized cells, such as osteoclasts, where H\(^+\)-ATPase pumps provide the necessary acidification for bone resorption (Forgac, 2007). While these ATPase pumps do remove protons from the cytosol their primary function is not in cytoplasmic pH regulation but rather to fulfill specific physiological requirements of specialized cell types. Accordingly, H\(^+\)-ATPase pumps are not found universally on the plasma membrane of all cells, but rather on the surface of specific cell types (Brown et al., 2009).

In addition to acid extruders, most cells also contain acid loaders to protect against cytosolic alkalization. AEs exchange extracellular Cl\(^-\) ions for intracellular HCO\(_3\)\(^-\) (Pushkin and Kurtz, 2006; Romero et al., 2004). In some cases electrogenic NBCs can also function as acid loaders depending on transport stoichiometry (Pushkin and Kurtz, 2006; Romero et al., 2004). The removal of an alkali ion is equivalent to the import of a proton and serves to lower cytosolic pH (Fig. 1-1). Excessive alkalization by acid extruders such as NHE can be compensated for by acid loaders. The antagonistic actions of cytosolic acid loaders and acid extruders allow the cell to finely tune cytoplasmic pH. Some pH regulatory proteins such as NHE1 (see below) and AE2 contain intrinsic pH sensors which cause auto-activation upon cytosolic acidification (NHE1) or alkalization (AE2) ensuring that cytosolic pH remains near optimum (Alper et al., 2002; Aronson et al., 1982; Wakabayashi et al., 2003). Such mechanisms ensure that dangerous acid or base loads on the cell can be handled quickly, minimizing the damaging effects of extended exposure to extreme pH. Alternatively, simultaneous activation of NHE and AE results in no net pH change but the net uptake of NaCl into the cell, a mechanism used to protect the cell from shrinkage during hyperosmotic stress.
**Fig. 1-1. pH regulatory mechanisms.** (A) Model of cytosolic pH regulation by the acid extrusion mechanisms of Na⁺/H⁺ exchange (NHE), Na⁺/HCO₃⁻ cotransport (NBC), or Na⁺-driven Cl⁻/HCO₃⁻ exchange (NDCBE) or the acid loading mechanism of anion exchange (AE). Buffering is facilitated by carbonic anhydrase (CA) and Na⁺-dependent transporters are facilitated by the Na⁺/K⁺ ATPase (NKA). (B) Model of organelle pH regulation. Organelles are acidified primarily by the vATPase or H⁺-pump. Counter ions are provided through Cl⁻ channels (CIC). NHEs can act as acid loaders or extruders by transporting Na⁺ or K⁺ respectively.
This NaCl influx mechanism is also used by certain epithelial cells for the trans-epithelial secretion or resorption of salt and water (Bianchini et al., 1995; Mason et al., 1989; Rotin and Grinstein, 1989).

1.1.5 Organellar pH

The organelle compartments within eukaryotic cells have differing pH environments within their lumen. It is believed that organelles arose in eukaryotes to compartmentalize different biochemical pathways making them more efficient. The unique pH environment in each organelle is believed to support these specific biochemical pathways as the resident enzymes of a given organelle will have unique pH optimum for their activity. Thus, in addition to the unique set of resident proteins in a given organelle, the luminal pH plays a role in maintaining the organelle identity. The organelles along the secretory pathway become progressively more acidic, starting with the endoplasmic reticulum (ER) at pH 7.2 (Kim et al., 1998; Wu et al., 2001). The cis-Golgi has a luminal pH of 6.7 which progresses to pH 6.0 in the trans-Golgi network (Demaurex et al., 1998; Kim et al., 1998; Seksek et al., 1995). Finally, secretory granules can have a pH as low as 5.2 (Paroutis et al., 2004; Urbe et al., 1997; Wu et al., 2001). The organelles of the endocytic pathway have a similar trend of progressive acidification. Early endosomes have an approximate luminal pH of 6.3 which drops to 6.0 in late endosomes and as low as 4.5-4.7 in lysosomes. Recycling endosomes typically have a luminal pH of 6.5 (Yamashiro and Maxfield, 1984; Casey et al., 2010).

Protons in acidic organelles are predominantly transported into the organelle lumen by the v-ATPase (Fig. 1-1) (Forgac, 2007; Paroutis et al., 2004; Nelson and Harvey, 1999). However, how the differing luminal pH among organelles is regulated is not completely understood. Using the chemical energy released by ATP hydrolysis, the
v-ATPase could theoretically drive luminal pH to as low as 3.0 (Steinberg et al., 2010; Casey et al., 2010). Thus, it is clear that control of the v-ATPase or other mechanisms is required for the fine control of organelar pH. In part, luminal pH may be regulated by controlling the density of v-ATPase pumps, or by controlling their assembly or redox state (Brown et al., 2009; Forgac, 2007). As the v-ATPase is electrogenic (movement of net positive charge into the lumen with every cycle), continued organelle acidification requires the simultaneous flux of counter ions across the organelle membrane. The flux of anions (Cl\(^-\)) through members of the chloride channel (CIC) family and the cystic fibrosis transmembrane conductance regulator (CFTR) and cations (Na\(^+\), K\(^+\)) have been shown to play a role in organelle pH as well as organelle trafficking (Barasch and Awqati, 1993; Jentsch, 2008; Scheel et al., 2005; Steinberg et al., 2010). In lysosomes this counter ion flux is predominantly composed of a cation flux (Steinberg et al., 2010). The Na\(^+\)/K\(^+\) ATPase is primarily attributed to controlling cytoplasmic Na\(^+\) and K\(^+\) homeostasis and in controlling resting plasma membrane potential. However, this electrogenic ion pump can also enter early and recycling endosomes via clathrin-dependent endocytosis and has been shown to participate in regulating the luminal pH of these organelles (Feldmann et al., 2007; Fuchs et al., 1989). The Na\(^+\)/K\(^+\) ATPase transports 3 Na\(^+\) ions into the organelle lumen in exchange for 2 K\(^+\) ions resulting in the movement of net positive charge into the organelle lumen. This positive charge movement may antagonize the action of the v-ATPase which is sensitive to voltage across the organelle membrane (Feldmann et al., 2007).

The action of v-ATPase pumps can be inhibited rapidly and reversibly by macrolide antibiotics such as Concanamycin or Bafilomycin (Forgac, 2007). Acute inhibition of v-ATPase pumps and simultaneous measurement of organelle lumen pH revealed the existence of a profound proton leak pathway that could dissipate luminal pH gradients within minutes (Paroutis et al., 2004; Wu et al., 2001). It is believed that
controlling the magnitude of this proton leak pathway may in fact be the key parameter to control organelle pH and allow for differences in the pH environment such as pH 4.5 in lysosomes and pH 6.0 in early endosomes. The molecular basis of this proton leak is not clear but is likely to include members of the CIC family and NHE family. At least some members of the CIC family function as electrogenic $2\text{Cl}^-/1\text{H}^+$ exchangers and not solely as $\text{Cl}^-$ channels as originally thought (Scheel et al., 2005). Some members of the NHE family may have a higher preference for $K^+$ over $Na^+$ (see below) and thereby participate in proton leak by transporting $K^+$ from the cytosol into the organelle lumen in exchange for luminal protons (Brett et al., 2005b; Numata and Orlowski, 2001). Other isoforms of NHE may transport cytoplasmic protons into the organelle lumen in exchange for luminal $Na^+$, providing an alternate means of organelle acidification (D'Souza et al., 1998; Gekle et al., 2001) (Fig. 1-1).

Unlike the lumen of organelles along the secretory and endocytic pathways, the lumen (matrix) of mitochondria is alkaline (pH 7.8-8.0) due to active proton extrusion from the mitochondria by components of the electron-transport chain (Llopis et al., 1998). Mitochondria also have an inner-membrane potential (negative inside) which, together with a membrane pH gradient, provides a strong inward driving force for protons (proton motive force) that is harnessed by the mitochondrial ATP synthase complex to generate ATP from ADP and inorganic phosphate. Mitochondria contain additional proteins to regulate matrix pH and ion homeostasis, such as mitochondrial NHEs and $Na^+/Ca^{++}$ exchangers (Battaglino et al., 2008; Crompton and Heid, 1978).

Importantly, regulators of luminal pH have been shown in some cases to participate in controlling organelle trafficking by binding to components of the vesicular trafficking machinery in the cytoplasm. The yeast $Na^+/H^+$ exchanger, NHX1 has been shown to bind to Gyp6, a Rab GTPase-activating protein (GAP), to control traffic between endosomes and the yeast vacuole (Ali et al., 2004). Components of the v-
ATPase have been shown to interact with the small GTPase, Arf6 and the Arf6-guanine nucleotide exchange factor (GEF) ARNO to control traffic from the early endosomes to the degradative pathway (Hurtado-Lorenzo et al., 2006). This interaction was dependent on the luminal pH of the endosome, demonstrating that organelle pH could be sensed from the cytoplasm to affect organelle traffic and dynamics. While the regulation of organelle pH plays an important role in multiple aspects of organelle biology, the precise molecular basis of organelle pH homeostasis and pH-sensing are very poorly understood.

1.2 Na⁺/H⁺ exchangers

1.2.1 Introduction to NHEs

Na⁺/H⁺ Exchangers (NHEs or NHXs), also known as Na⁺/H⁺ antiporters (NHA), are integral membrane transport proteins found in some form in all cells across all kingdoms of life. NHEs perform diverse physiological functions related to cellular pH, salt and volume homeostasis (Brett et al., 2005a; Orlowski and Grinstein, 2004). These proteins belong to the monovalent cation proton antiporter (CPA) superfamily which includes the bacterial ancestors NhaA (electrogenic), and NhaP (electroneutral). In mammals, nine isoforms (NHE1-9) which share 25-70% identity with each other have been described and are related to the electroneutral ancestral NhaP from bacteria and NHX1 from yeast. There are an additional three poorly characterized genes NHA1 and NHA2, related to the ancestral electrogenic NhaA gene, and NHE10 which is only distantly related to other NHE proteins and has unclear origins (Battaglino et al., 2008; Brett et al., 2005a; Lee et al., 2008; Wang et al., 2003). NHE1-9 share a common predicted structure comprised of 12 conserved membrane spanning segments at the amino terminus of 450-500 amino acids in length followed by a more variable
cytoplasmic carboxy-terminal tail of 130-450 amino acids in length. The conserved amino terminal membrane domain has been shown to confer the ion translocation function of NHE proteins while the cytoplasmic C-terminus confers regulatory functions through protein-protein interactions and post-translational modifications (Brett et al., 2005a; Orlowski and Grinstein, 2004). NHE1-5 commonly exhibit transporter activity across the plasma membrane, while NHE6-9 are mostly found in organelle membranes and are believed to regulate organelar pH in most cell types at steady state (Brett et al., 2005a; Orlowski and Grinstein, 2007). Thus, the mammalian NHEs have been classified as plasmalemmal or organellar-type. This distinction is also reflected in the protein sequences where plasmalemmal NHE1-5 are more closely related to each other than to the organellar NHE6-9. Plasmalemmal-type NHEs can be further divided into resident plasma membrane NHEs (NHE1, 2, and 4), which show almost exclusive localization to the cell-surface, and recycling plasma membrane NHEs (NHE3 and 5), which traffic between the plasma membrane and recycling endosomes (Brett et al., 2005a; D'Souza et al., 1998; Szaszi et al., 2002). Organellar NHE6-9 have all been shown to be present on the cell surface in small amounts or in certain cell-types (Goyal et al., 2005; Hill et al., 2006; Lin et al., 2007; Ohgaki et al., 2008). Therefore, while it is useful to make the distinction between plasmalemmal- and organellar-type NHEs, this classification is not entirely accurate.

It is believed that the organelle-type NHEs (NHE6-9) evolved first as these proteins have homologues in all eukaryotes. The recycling plasma membrane NHEs (NHE3 and 5) are found in metazoans and appeared simultaneously with the Na⁺/K⁺-ATPase which provides the inward electrochemical Na⁺ gradient that powers plasmalemmal-type NHEs. Resident plasma membrane NHEs (NHE1, 2, and 4) are found only in vertebrates and hence are believed to have appeared the most recently in evolution (Brett et al., 2005a).
1.2.2 Physiological role of NHEs

All nine mammalian NHEs perform the electroneutral exchange of Na\(^+\) or K\(^+\) for H\(^+\) down their respective concentration gradients. Plasmalemmal-type NHEs have all been shown to preferentially transport Na\(^+\), consistent with their co-evolution with the Na\(^+\)/K\(^+\)-ATPase and localization to the plasma membrane. Organellar-type NHEs have been shown to preferentially transport K\(^+\) (Numata et al., 2001; Nakamura et al., 2005). This is consistent with an intracellular localization and function as K\(^+\) is the predominant intracellular cation. Despite a similarity in ion transport activity, different NHE proteins perform diverse physiological functions ranging from fine control of cellular/organellar pH and volume to systemic electrolyte, acid/base, and fluid volume homeostasis. Several other gene products and cellular processes are pH sensitive. Therefore, NHEs additionally participate in many other cellular events such as adhesion, migration, macropinocytosis and neurite growth by regulating the activity of various pH-sensitive proteins (Tominaga et al., 1998; Denker et al., 2002; Koivusalo et al., 2010; Sin et al., 2009). The unique physiological function of each mammalian NHE is dictated by the differences in tissue and sub-cellular distribution as well as differences in the regulation of ion transport activity. By far the best studied isoforms are NHE1 and NHE3.

NHE activity was first described by Pouyssegur and colleagues in 1982 as an ionic flux in hamster fibroblasts activated by growth factors (Pouyssegur et al., 1982). Subsequently, an H\(^+\)-suicide technique was developed to generate NHE-deficient cell lines from the parental hamster fibroblasts. For this strategy, the endogenous NHE activity was manipulated to act in reverse, resulting in a large influx of H\(^+\) into the cytosol which then killed the cell. The minority of cells which survived were isolated and found to be deficient in NHE activity (Pouyssegur et al., 1984). The responsible NHE gene, later termed NHE1, was cloned with a complementation approach using these NHE-
deficient hamster fibroblasts (Sardet et al., 1989). Since the initial discovery and cloning, NHE1 has become the most well studied isoform of NHE and can be considered in many ways to be the prototypical NHE. NHE1 is found on the plasma membrane of nearly all cells in the body; in epithelial cells NHE1 is found on the basolateral membrane (Fliegel, 2008; Orlowski and Grinstein, 2004). NHE1 has been shown to act as the principle alkalinizing mechanism to protect cells from excess acid production and hence is a key regulator of cellular acid-base homeostasis. For these reasons, NHE1 is believed to play a housekeeping function. Second, in conjunction with Cl⁻/HCO₃⁻ exchangers, NHE1 provides a major conduit for NaCl influx and H₂O uptake to protect cells from shrinkage during osmotic stress (Bianchini et al., 1995; Rotin and Grinstein, 1989). In addition to these house-keeping functions, NHE1 has been shown to participate in actin-cytoskeleton rearrangements important during cell attachment (Tominaga and Barber, 1998), cell migration (Bourguignon et al., 2004; Denker et al., 2000; Denker and Barber, 2002; Meima et al., 2009), neurite growth (Sin et al., 2009), and macropinocytosis (Koivusalo et al., 2010). pH regulation by NHE1 may also participate in cell proliferation (Denker et al., 2000; Putney and Barber, 2003). Last, extracellular acidification by localized NHE1 activity in invadopodia may promote extracellular matrix degradation and cancer cell invasion (Bourguignon et al., 2004; Busco et al., 2010). Thus, while NHE1 may principally act to protect cells from cytosolic acidification and cell shrinkage, its activity has been co-opted to participate in multiple aspects of cell physiology. NHE1 knock-out mice are viable suggesting that some of the house-keeping functions of NHE1 may be redundant with other pH-regulatory molecules, at least during development. Remarkably, these NHE1⁻/⁻ mice display distinct neurological symptoms such as ataxia and epileptic-like seizures leading to premature death suggesting that NHE1 may play an especially important role in the brain (Bell et al., 1999; Cox et al., 1997). Another interpretation of these results is that cells of the
central nervous system may have an especially strict requirement for proper pH regulation and cannot tolerate loss of NHE1 as well as other tissues.

Following NHE1, NHE3 is the next most well studied and characterized isoform. NHE3 is more restricted in its expression and is found primarily on the apical surface of epithelial cells of the gastrointestinal tract and proximal tubule in the kidney. Even though NHE3 performs a similar Na\(^+\)/H\(^+\) exchange reaction as NHE1, NHE3’s function is not strictly related to pH regulation but rather in systemic electrolyte balance, by regulating the uptake of Na\(^+\) from the intestinal lumen and kidney filtrate. NHE3 functions in conjunction with the Na\(^+\)/K\(^+\) ATPase, found on the basolateral membrane of the epithelial cell, to perform trans-epithelial flux of Na\(^+\) (Cai et al., 2008; Gomes and Soares-da-Silva, 2006). Further, by controlling Na\(^+\) uptake, NHE3 is also critical for H\(_2\)O absorption and fluid homeostasis. These functions are indeed confirmed by the NHE3\(^-\) mice. These mice exhibit diarrhea, alkalinization of the intestinal lumen, acidosis and reduced blood pressure (Schultheis et al., 1998b). In addition, these mice die on a low Na\(^+\) diet indicative of reduced Na\(^+\) absorption and systemic electrolyte defects. NHE3 has also been shown to be functional in endosomes following endocytosis from the apical plasma membrane. Once internalized, NHE3 is able to export luminal Na\(^+\) from the endosome in exchange for an H\(^+\) to promote endosome acidification (D’Souza et al., 1998; Gekle et al., 2001). Regulation of endosomal pH by NHE3 allows NHE3 to participate in receptor mediated endocytosis of albumin in kidney epithelial cells (Gekle et al., 1999; Gekle et al., 2001). NHE2 and NHE4 are also present in epithelial cells of the gastrointestinal tract and mutation of these genes in mice also reveals a predominant function of NHE2 and NHE4 in pH and salt balance in the gut lumen (Gawenis et al., 2005; Schultheis et al., 1998a). NHE2, 3, and 4 are all found in the central nervous system in low amounts, where NHE3 is only expressed in the cerebellum and brain stem (Ma and Haddad, 1997). However, due to the lack of neurological phenotypes in NHE2,
3, or 4-null mice the role of these isoforms in brain is not clear (Gawenis et al., 2005; Schultheis et al., 1998a; Schultheis et al., 1998b).

The organellar NHEs NHE6, 7 and 9 are closely related to each other, with 60-70% amino acid identity (Nakamura et al., 2005), and are homologous to NHX1 from yeast (Brett et al., 2005a), while NHE8 is more divergent with approximately 25% amino acid identity with the other mammalian NHEs. NHE6-9 have been shown to be expressed in all mammalian tissues tested thus far (Numata et al, 2001; Nakamura et al., 2005). Studies on NHX1 in yeast have shown that this protein plays an important role in endosomal pH and ion homeostasis, which impacts on vesicle trafficking and vesicle formation (Ali et al., 2004; Bowers et al., 2000; Brett et al., 2005b; Mukherjee et al., 2006). As organellar NHEs are ubiquitously expressed and evolutionarily conserved, organellar NHEs likely play important house-keeping functions, involving organelle ion and volume homeostasis and vesicular trafficking. NHE6 and NHE9 are predominantly localized to early and recycling endosomes and have been shown to regulate endosomal pH by contributing to a proton leak pathway (Brett et al., 2002; Nakamura et al., 2005; Ohgaki et al., 2008; Ohgaki et al., 2010). NHE7 is predominantly localized to the trans-Golgi network and is similarly believed to regulate the pH of this organelle (Numata and Orlowski, 2001). NHE8 was first suggested to localize to the medial Golgi compartment but has also been suggested to be present on the apical surface of renal epithelial cells and more recently in late endosomes and multivesicular bodies (Goyal et al., 2005; Lawrence et al., 2010; Nakamura et al., 2005). Though NHE6-9 are all believed to be ubiquitously expressed and to play a house-keeping role in maintaining organellar pH and volume homeostasis, evidence exists for more specialized functions of these isoforms in some tissues. NHE6 and NHE9 have been shown to be localized on the cell-surface and to regulate cytosolic pH in cochlear hair cells (Hill et al., 2006). These cells are bathed in a high K+ endolymph in the inner ear and hence exploit the
K⁺/H⁺ exchange activity of NHE6 and NHE9 to regulate their cytosolic pH rather than NHE1. NHE6 has also been shown to be important to maintain the apical membrane polarity of bile producing epithelia in the liver (Ohgaki et al., 2010). In addition, mutations in NHE6 are associated with the development of Angelman syndrome-like X-linked mental retardation syndrome (Gilfillan et al., 2008), and mutations in NHE9 are linked with attention-deficit and hyperactivity disorder and autism (Morrow et al., 2008). These latter findings suggest that NHE6 and NHE9 may play a specialized role in brain function.

1.2.3 Regulation of NHE transport activity and cellular trafficking

NHEs can be regulated by several unique mechanisms that include: allosteric regulation by cellular pH, alteration in ion translocation activity through post-translation modifications or protein-protein interactions, changes in cellular trafficking, or through changes in cell shape by linkage to the cytoskeleton. As mentioned above, the regulation of NHE transport activity and trafficking is typically mediated by the divergent cytosolic C-terminal tails of NHE proteins. These divergent C-terminal tails allow for isoform specific protein-protein interactions and post-translational modifications, leading to unique physiological functions and regulation (Orlowski and Grinstein, 2004). NHE proteins are known to be regulated by a large number of different cellular stimuli such as growth factors, integrin engagement, and activation of G-protein-coupled receptors (GPCRs). In addition, certain NHEs such as NHE1 and NHE3 interact with the actin cytoskeleton through interaction with ezrin-radixin-moesin (ERM) proteins. This linkage to the cytoskeleton allows NHE1 and NHE3 to sense changes in cell shape which then regulates their ion translocation activity (Alexander et al., 2005; Meima et al., 2007; Orlowski and Grinstein, 2004). As mentioned above, some NHE proteins are known to
have a greater than first order dependence on intracellular \([H^+]\) indicating that these NHEs may have multiple binding sites for protons. The additional proton binding sites allow for allosteric regulation of NHE activity by cytoplasmic protons forming a pH-sensor region which can spontaneously activate NHE proteins in the event of cytosolic acidification (Otsu et al., 1992; Wakabayashi et al., 1997; Wakabayashi et al., 2003). The regulation of NHEs at the molecular level by specific post-translational modifications or protein-protein interactions is best described for NHE1 and NHE3. Some of the details of NHE1 and NHE3 regulation are described here to offer insight into the range of signaling pathways that impart their effect on NHE activity.

The molecular regulation of NHE1 ion translocation activity is the most well characterized of the NHE isoforms. Importantly, NHE1 is believed to be auto-regulated via a pH-sensor domain that increases NHE1 intrinsic activity at acidic pH and terminates NHE1 activity as cellular pH is restored to neutral values forming the basis of the pH set-point hypothesis (Fliegel, 2008; Wakabayashi et al., 2003). This mode of regulation of NHE1 is consistent with NHE1 playing a ubiquitous house-keeping role to guard cells against excessive acidification. NHE1 can be further activated by multiple cellular signaling pathways involving protein-protein interactions and post-translational modifications, some of which may activate NHE1 by modifying the pH-sensor region, adjusting the “pH set-point” resulting in a more alkaline cellular pH (Wakabayashi et al., 1994). Growth factors and GPCR ligands activate NHE1 through a common mitogen-activated kinase (MAPK) pathway involving direct phosphorylation of NHE1 by the downstream kinase p90-rsk (Meima et al., 2007; Moor and Fliegel, 1999) as well as Akt, downstream of phosphatidyl-inositol-3-kinase (PI3K) (Meima et al., 2009). NHE1 is also activated by direct phosphorylation by the Rho-activated kinase ROCK, downstream of integrin receptor engagement and RhoA activation. In addition, NHE1 is activated by elevated intracellular Ca\(^{++}\) through binding of Ca\(^{++}\)-Calmodulin (CaM). CaM binding is
believed to induce a conformational change that relieves a block on ion transport by an auto-inhibitory domain in the NHE1 C-terminus (Wakabayashi et al., 1994). NHE1 localization on the plasma membrane is not uniform but rather NHE1 accumulates in specific regions on the cell-surface. For instance, NHE1 has been shown to accumulate in lamelipodia in migrating fibroblasts (Denker and Barber, 2002), in invadopodia in invasive cancer cell lines (Busco et al., 2010), at the tips of extending neurites in PC12 cells and neurons (Sin et al., 2009), and at intercalated disks and transverse tubules in cardiac myocytes (Petrecca et al., 1999). This targeting of NHE1 to specific regions of the plasma membrane is due to cytoskeletal anchoring through binding of ERM proteins in the juxtamembrane region of the NHE1 C-terminus, and may be important for establishing a localized pH environment.

Unlike NHE1, which is principally localized on the plasma membrane, NHE3 is regulated both in terms of ion translocation activity and by endocytosis/exocytosis to control the number of functional transporters on the cell-surface. This additional mode of regulation through controlling protein trafficking allows for isoform specific regulation as well as the possibility of unique physiological roles. NHE3 gene expression, protein trafficking and ion-translocation are primarily controlled by various hormones which regulate blood pressure and fluid homeostasis such as parathyroid hormone (PTH) and angiotensin II. PTH activates protein kinase A (PKA) which directly phosphorylates NHE3, reducing its ion translocation activity and promoting its endocytosis to endosomes (Collazo et al., 2000). This requires the PSD95, Drosophila discs large tumor suppressor, Zonula occludens-1 (PDZ) domain protein NHE-regulatory factor (NHERF) and ezrin which both bind the NHE3 C-terminus (Donowitz et al., 2009; Murthy et al., 1998; Zizak et al., 1999). Direct binding to another PDZ protein, Shank2, prevents phosphorylation of NHE3 by PKA and stabilizes NHE3 on the cell-surface (Han et al., 2006; Lee et al., 2010). Dopamine also inhibits NHE3 through activation of the
dopamine receptor, a GPCR, and subsequent activation of PKA and PKC causing NHE3 endocytosis (Hu et al., 2001). By contrast, Angiotensin II, glucocorticoids, epinephrine/norepinephrine, and insulin increase NHE3 activity by promoting its exocytosis to the apical plasma membrane (du Cheyron et al., 2003; Fuster et al., 2007; Hall et al., 1998; Tsuganezawa et al., 1998). Targeting of NHE3 to the apical surface requires activation of c-Src and PI3K (du Cheyron et al., 2003; Tsuganezawa et al., 1998). At the cell apical cell-surface, NHE3 can exist in an immobile pool that is resistant to endocytosis by anchoring to the actin cytoskeleton (through interaction with ezrin), and in a more mobile pool which readily undergoes endocytosis and colocalizes with the endocytic cargo megalin and cubulin (Alexander et al., 2005).

NHE3 is most closely related to NHE5 (53% amino acid identity), the subject of this thesis. Therefore, some of the details elucidated for the regulation and physiological role of NHE3 may serve to illuminate our understanding of the poorly characterized isoform, NHE5. NHE1 is less related to NHE5 (39% amino acid identity), but as the prototypical NHE, studies on NHE1 may similarly be useful to understand the role of NHE5.

1.2.4 NHE5

NHE5 mRNA has been shown to be expressed almost exclusively in the brain and can be found particularly in neuron-rich regions in the brain such as the hippocampus and cerebral cortex, shown using Northern blot and in situ hybridization (Attaphitaya et al., 1999; Baird et al., 1999). Reverse transcriptase-PCR analysis of mRNA isolated from cultured cortical neurons or astrocytes revealed NHE5 message in neurons, but not astrocytes (Wada et al., 2005). Therefore, NHE5 has even been suggested to be neuron-specific, showing the most restricted distribution of any NHE.
isoform. However, NHE5 mRNA can also be detected in other tissues such as skeletal muscle, spleen and testes. It is not clear whether NHE5 mRNA transcripts from these tissues produce functional transporters due to the possible inclusion of intronic sequences, not found in the brain NHE5 mRNA (Baird et al., 1999). The congenital neurological disorders spinocerebellar ataxia type 4 and autosomal dominant cerebellar ataxia have been mapped to chromosome region 16q22.1, a region containing NHE5, amongst other candidate genes (Hellenbroich et al., 2005; Owada et al., 2005). This further implicates a role for NHE5 in neuronal function, but further studies will be needed to define the role of NHE5 in normal and pathological brain function.

NHE5 is the most closely related to NHE3 (53% amino acid identity, Baird et al., 1999) and despite distinct expression in the brain and kidney respectively, these proteins may share overlap in their regulation and trafficking behavior. Like NHE3, NHE5 is known to undergo dynamic trafficking between the plasma membrane and recycling endosomes when expressed in non-neuronal cell lines (Szaszi et al., 2002). This suggests that like NHE3, NHE5 activity may be regulated by controlling its abundance on the cell surface. The restricted expression in brain and dynamic trafficking behavior suggest that the physiological function of NHE5 may be unique, though a role for NHE5 in brain function has not yet been described. This is due in part to the lack of isoform specific antibodies and the difficulty of maintaining neuronal cells in culture. For other NHE isoforms, gene knock-out models have been useful to reveal isoform specific functions (Cox et al., 1997; Schultheis et al., 1998b). However, NHE5 knock-out mice are not yet available. Previous studies on NHE5 have therefore focused on exogenously expressed, epitope-tagged NHE5 in non-neuronal cell lines. This system has begun to shed light on the molecular regulation of NHE5 and its kinetic and pharmacological properties. NHE5 preferentially transports Na⁺ and is unable to transport Li⁺ or K⁺.

Compared to other NHE isoforms, NHE5 has intermediate sensitivity to amiloride and its
derivatives. Further, no NHE5-specific inhibitor comound has been identified. (Attaphitaya et al., 2001; Szabo et al., 2000). Interestingly, human NHE5 has a first order dependence on intracellular [H\(^+\)] indicative of a single proton binding site, the proton transport site (Szabo et al., 2000). This finding suggests that NHE5 is not allosterically regulated by intracellular pH, unlike NHE1 which can be auto-activated by a drop in intracellular pH (Wakabayashi et al., 2003). Like NHE3, NHE5 is inhibited by pharmacological treatments that activate PKA or PKC suggesting that these kinases negatively regulate NHE5 activity (Attaphitaya et al., 2001). Whether PKA or PKC phosphorylate NHE5 directly is not known. Lastly, NHE5 exocytosis to the plasma membrane was found to require PI3K activity, while endocytosis requires an intact actin cytoskeleton (Szaszi et al., 2002). These initial studies have begun to elucidate the cellular signaling systems that may impact on NHE5. However, the physiological consequences of NHE5 regulation by PKC, PKA, PI3K and the actin cytoskeleton remain to be determined.

More recent studies have attempted to gain insight into the regulation and function of NHE5 through identification and characterization of NHE5 binding proteins. Yeast two-hybrid analysis revealed that NHE5 could interact with \(\beta\)-arrestin and the signaling scaffold protein Receptor for Active C-Kinase (RACK1). Subsequent experiments determined that these interactions occurred directly with the NHE5 C-terminus. Binding of \(\beta\)-arrestin promoted the endocytosis of NHE5 via the clathrin-dependent pathway and reduced NHE5 transport activity at the plasma membrane (Szabo et al., 2005). \(\beta\)-arrestin has a well-established role in promoting the endocytosis of ligand stimulated GPCRs and receptor tyrosine kinases (RTKs). Interestingly, interaction of \(\beta\)-arrestin with NHE5 was the first demonstration that \(\beta\)-arrestin could interact with a membrane protein that was not a GPCR or RTK. \(\beta\)-arrestin typically
interacts with and promotes the endocytosis of phosphorylated targets previously stimulated by ligand-binding (Orsini et al., 1999). Whether β-arrestin binding and subsequent endocytosis of NHE5 is regulated by specific stimuli is not known. Overexpression of RACK1 could enhance the NHE5-RACK1 interaction which stimulated NHE5 transporter activity (Onishi et al., 2007). The NHE5-RACK1 interaction was also stimulated by engagement of integrin receptors with fibronectin. Interestingly, we showed that NHE5 could also interact with β1-integrin and that this interaction occurred constitutively though whether the interaction is direct is not known. Engagement of integrins caused the recruitment of RACK1 forming a putative NHE5-integrin-RACK1 triple complex which promoted NHE5 transporter activity (Onishi et al., 2007). This later finding suggested that NHE5 may play a role in providing a distinct ionic environment that promotes integrin attachment and the formation of focal adhesions. This will be further discussed in section 6.3.1.

In summary, NHE5 mRNA is almost exclusively expressed in brain and is therefore likely to play some specialized role in this organ. In addition, NHE5 may traffic between the cell surface and recycling endosomes. This trafficking behavior may serve as a regulatory mode to control NHE5 activity in distinct membrane domains on the cell surface. Alternatively, NHE5 may be active in regulating luminal pH of recycling endosomes. In neurons, it is possible that cell surface NHE5 may regulate pH at distinct structures such as synapses, and intracellular NHE5 could play a role in regulating luminal pH of specialized organelles such as synaptic vesicles. However, the precise localization of NHE5 protein in brain as well the physiological role of this ion transporter is not known. In this thesis work, I determined that in neurons, endogenous NHE5 protein can be targeted to synapses (see chapter 3) the sites where neurons contact each other and communicate via synaptic transmission. This finding led me to further
investigate the possibility that the physiological role of NHE5 in the brain may be related to regulating pH at synapses (chapter 4). Further aspects of the molecular regulation of NHE5 were also investigated (chapter 5).

1.3 Synapse physiology

1.3.1 Introduction to synapses

Synapses are sites where neurons of the central and peripheral nervous system (CNS and PNS respectively) contact each other and communicate via synaptic transmission. There are two major components of the synapse: the pre-synapse which contains neurotransmitter loaded synaptic vesicles, and the post-synapse which houses the neurotransmitter receptors. These two components are separated by a narrow space (20nm across in the CNS) referred to as the synaptic cleft (Rostaing et al., 2006; Zuber et al., 2005). Neurons are polarized cells with distinct structures, namely a single axon which houses the pre-synapse, and several dendrites where the post-synapses are predominantly found (Burns and Augustine, 1995; Horton and Ehlers, 2003; Tang, 2001). Depolarization of the cell-body, or soma, can initiate an action potential, an electrical current propagated down the axon by a series of voltage-gated ion channels. When the action potential reaches the pre-synapse, the subsequent depolarization will trigger the activation of voltage-gated Ca\(^{++}\) channels (VGCC). This Ca\(^{++}\) influx stimulates Ca\(^{++}\)-sensitive vesicle release machinery that promotes the fusion of synaptic vesicles with the plasma membrane releasing the neurotransmitter contents to the extracellular space. These transmitter molecules diffuse across the synaptic cleft where they bind and activate neurotransmitter receptors (Katz and Kuffler, 1946; Kuffler and Katz, 1946; Llinas et al., 1981; Llinas et al., 1982; Rostaing et al., 2006; Südhof, 2008). These receptors are either ligand-gated ion channels (ionotropic receptors) that are
gated by the binding of neurotransmitter or GPCRs (metabotropic receptors), that initiate
down-stream signaling through release of G-proteins.

Each neuron generally releases a single type of neurotransmitter from the
axon/pre-synapse. Each post-synapse is usually responsive to a single neurotransmitter
type, but post-synapses of different types can be found together on the same dendrites.
There are two major neurotransmitters in the CNS, glutamate and γ-amino butyric acid
(GABA). Ionotropic glutamate receptors are ligand-gated cation channels which, when
activated, cause depolarization of the post-synaptic cell (Chua et al., 2010; Hollmann et
al., 1989; Michaelis et al., 1981; Sakimura et al., 1990; Shapovalov et al., 1978). By
contrast, GABA-gated ion channels are anion channels that flux mostly Cl⁻, which cause
membrane hyperpolarization of the post-synaptic cell (Curtis et al., 1970; Fritschy and
Brunig, 2003; Levitan et al., 1988; Schofield et al., 1987; Watanabe et al., 2002).
Glutamate and GABA also act at metabotropic mGluRs and GABAᵦ receptors
respectively. These receptors can be found at either the pre- or post-synapse
depending on isoform. Several other neurotransmitters such as dopamine, serotonin
and acetylcholine are also found in the central nervous system. However, unlike
 glutamate and GABA which cause direct depolarization or hyperpolarization of the post-
synaptic neuron through ionotropic receptors, these other transmitters predominantly
play a modulatory role by activating downstream signaling pathways that regulate the
strength of glutamatergic or GABAergic transmission or threshold of synaptic plasticity
(see below). Coordinated activation of many glutamatergic synapses can cause
sufficient depolarization to trigger an action-potential and further neurotransmitter
release at downstream synapses. This is antagonized by the hyperpolarization caused
by GABAergic transmission (Chua et al., 2010; Cline, 2005; Fritschy and Brunig, 2003;
Katz and Kuffler, 1946; Kuffler and Katz, 1946). Glutamate and GABA are hence
generally referred to as excitatory and inhibitory transmitters respectively. Neurons in
the central nervous system typically contain several thousands of synapses of both excitatory and inhibitory transmitter types and the level of synaptic activity is governed by the balance of glutamatergic and GABAergic transmission (Cline, 2005; Fritschy and Brunig, 2003). Individual synapses transmit a single neurotransmitter type and are referred to as glutamatergic or excitatory, and GABAergic or inhibitory synapses. These distinct synapse types have specific subsets of proteins as well as different structures (Horton and Ehlers, 2003; Kessels and Malinow, 2009; Shepherd and Huganir, 2007; Steiner et al., 2008). At the majority of excitatory synapses, the post-synaptic machinery is found on small mushroom-shaped protrusions, 1-2μm in length, from the dendrite referred to as dendritic spines, while inhibitory synapses are typically formed directly on the shaft of the dendrite or even directly onto the cell body.

1.3.2 Synaptic plasticity

Following an initial stimulation, excitatory synapses can undergo long lasting changes in synaptic strength such as long-term potentiation (LTP) and long-term depression (LTD) (Bliss and Lomo, 1973; Lynch et al., 1977). LTP and LTD involve stable increases or decreases respectively, in the strength of the synaptic response as well as changes in synapse number and structure. These changes act at individual synapses thereby altering the relative strength of synapses on a given neuron. Further, LTP and LTD depend on the history of the synapse and are therefore believed to form the basis of learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Malenka and Bear, 2004). LTP and LTD are not single phenomena, but rather a collection of synaptic changes that can vary with different synapse or neuron types, with brain region, and with age (Malenka and Nicoll, 1999; Malenka and Bear, 2004). There are two major ionotropic glutamate receptors at the excitatory post synapse, the N-
methyl-D-aspartate (NMDA) receptor and the α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) receptor (Chua et al., 2010; Kessels and Malinow, 2009; Lau and Zukin, 2007; Michaelis et al., 1981; Shepherd and Huganir, 2007). The AMPA receptor mediates the majority of fast synaptic transmission in the brain. The NMDA receptor primarily plays a signaling function and has low activity under basal conditions due to a block of the channel pore by extracellular Mg²⁺ ions, which is relieved at depolarized membrane potentials (Espinosa and Kavalali, 2009; Kumamoto, 1996; Nowak et al., 1984). Most forms of LTP are initiated by activation of synaptic NMDA receptors (Bliss and Collingridge, 1993; Errington et al., 1987; Malenka and Bear, 2004). Activated NMDA receptors are permeable to Ca²⁺ and permit Ca²⁺-entry into the post synapse. This Ca²⁺ influx initiates a number of downstream signaling cascades that ultimately cause an increase in synaptic strength due to recruitment of post-synaptic AMPA receptors (Kessels and Malinow, 2009; Lu et al., 2001; Shepherd and Huganir, 2007). LTP also causes an increase in the number and size of dendritic spines (Engert and Bonhoeffer, 1999; Lang et al., 2004; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004), the small protrusions of the dendrites on which excitatory post-synaptic compartments are localized, and this requires coordinated remodeling of the actin-cytoskeleton and membrane composition (Okamoto et al., 2004; Park et al., 2004; Park et al., 2006; Wang et al., 2008b). Paradoxically, many forms of LTD are also initiated by activation of the NMDA receptor (Desmond et al., 1991; Lee et al., 1998; Malenka and Bear, 2004). The signaling program leading to LTD is also Ca²⁺-dependent but relies on a separate subset of signaling molecules. For instance, LTP has been shown in most circumstances to require Ca²⁺-Calmodulin dependent protein kinase II α (CaMKIIα) whereas LTD requires the Ca²⁺-dependent protein phosphatase Calcineurin (also called protein phosphatase 2B) (Malenka and Bear, 2004). During LTD, there is a net endocytosis and removal of
AMPA receptors from the synapse as well as a shrinkage or loss of dendritic spines. It is believed that the duration and extent of the Ca\(^{++}\)-influx through NMDA receptors determines whether the downstream signaling will result in initiation of LTP or LTD. After the initial Ca\(^{++}\) influx through NMDA receptors to initiate LTP or LTD, the propagation and maintenance of LTP and LTD involves a host of positive and negative feedback loops to maintain and stabilize these long term changes (Li et al., 2007; Steiner et al., 2008), which have been shown in vivo to last months to years (Kasai et al., 2010).

Several experimental protocols have been developed to induce LTP or LTD in vitro. Originally, LTP and LTD were induced and observed through electrophysiological means (Bliss and Lomo, 1973; Lynch et al., 1977). The precise stimulation needed to induce LTP or LTD depends on the particular tissue preparation, the brain region being studied, and the age of the animals. In general, LTP requires a high-frequency stimulation of neurotransmitter release from excited axons, whereas LTD can generally be induced by a prolonged, low-frequency stimulation (Bliss and Lomo, 1973; Bramham and Srebro, 1987; Lynch et al., 1977; Malenka and Bear, 2004). More recently, chemical stimulations have been developed for the induction of LTP and LTD, referred to as chemical LTP or LTD (cLTP/cLTD) (Lee et al., 1998; Lu et al., 2001; Musleh et al., 1997; Shahi et al., 1993). These chemical protocols typically involve a pharmacological manipulation of the NMDA receptor through the addition of NMDA or the co-agonist glycine, and have been shown to act by activating the same downstream signaling responses elicited by traditional electrophysiological stimulations.
1.3.3 pH sensitivity of synapse components

Many of the key components of the synaptic machinery are known to be sensitive to changes in pH within the physiological range (near neutral). These include several voltage-gated ion channels, neurotransmitter receptors, both ionotropic and metabotropic, and proton-gated ion channels, also called acid sensing ion channels (ASICs) (Fig. 1-2A). Collectively, the proton sensitivity of these proteins results in a sharp sensitivity of synaptic transmission to changes in pH within the physiological range. In general, synaptic transmission is stimulated or inhibited by an increase or decrease in extracellular pH respectively (Chesler, 2003; Hsu et al., 2000; Velisek, 1998). Indeed excessive acidosis can lead to coma while alkalinization can induce seizures (Schuchmann et al., 2006). The sharp sensitivity of synaptic transmission is due, in part, to modulation of voltage gated Ca\(^{++}\) channels and Na\(^{+}\) channels by protons. For both these channels, the ionic current is reversibly inhibited by extracellular acidification and enhanced by extracellular alkalinization (from a resting pH\(_{o}\) close to 7.3). The activation voltages are similarly shifted to more positive values or negative values by extracellular acidification or alkalinization respectively (Chen et al., 1996; Chesler, 2003; Klockner and Isenberg, 1994; Tombaugh and Somjen, 1996). High-voltage activated (L, N, P, Q, and R type) but not low-voltage activated (T type) Ca\(^{++}\) channels are also inhibited or stimulated by intracellular acidification and alkalinization respectively (Tombaugh and Somjen, 1997). The pK\(_{a}\) of these effects is close to 7.1 suggesting that deviations from a resting pH of 7.3 in brain will have immediate and notable effects on Na\(^{+}\) and Ca\(^{++}\) currents in both positive and negative directions, affecting the overall excitability of the neuron and the probability of neurotransmitter release.
Changes in pH can also affect the chemical component of synaptic transmission, that is, responsiveness of neurotransmitter receptors to ligand and subsequent ionic currents. The best example of this is the NMDA receptor which is particularly sensitive to extracellular protons with a pK_a of 7.3-7.5 (Banke et al., 2005; Tang et al., 1990; Traynelis and Cull-Candy, 1990). Unlike the classic Mg^{++} ion block, which reduces open channel current in a voltage dependent manner (Kumamoto, 1996; Nowak et al., 1984), protons reduce the probability of channel opening independently of membrane voltage by interacting at a unique proton binding site, distinct from other ligand binding sites (Banke et al., 2005; Tang et al., 1990; Traynelis and Cull-Candy, 1990). Therefore, at resting pH close to 7.3, a tonic proton block exists that maintains the activity of the NMDA receptor to about 50%. The implication of this property is that, similarly to the proton sensitivity of voltage-gated Na^+ and Ca^{++} channels, any deviation from resting pH will have immediate and profound effects on NMDA receptor activity. Given the importance of NMDA receptors for synaptic plasticity, this suggests that local pH in the vicinity of the synapse may be important for the induction and maintenance of LTP and LTD. Consistent with this notion, high-frequency stimulation of Schaffer collaterals (axon projections from the hippocampus CA3 region) failed to induce LTP in hippocampal CA1 pyramidal neurons when the cells were bathed in an acidified media, likely a result of NMDA receptor inhibition (Velisek, 1998). Similarly, epileptiform activity (an uncontrolled increase in electrical activity) induced by low-Mg^{++} was suppressed by acidified media (Velisek et al., 1994). Other glutamate receptors are also known to be sensitive to extracellular protons, including AMPA receptors, Kainate receptors, and the pre-synaptic group III metabotropic glutamate receptors (Ihle and Patneau, 2000; Levinthal et al., 2009; Mott et al., 2003). However, unlike the NMDA receptor which has a pK_a close to resting pH, AMPA and Kainate receptors are only inhibited by protons at more acidic pH values. Therefore, these proteins are less likely to be affected by changes in pH that
occur under normal physiological conditions. GABA receptors are also modulated by extracellular pH, dependent on the molecular composition of the receptor (Krishek et al., 1996). The activity of certain subunit combinations of GABA receptors is actually enhanced by acidic extracellular pH (Krishek et al., 1996), thus acting synergistically with the proton-inhibition of NMDA receptors and voltage-gated Ca\(^{++}\) and Na\(^{+}\) channels to suppress neuronal activity (Fig. 1-2A).

In addition to the voltage and ligand-gated ion channels mentioned, acid-sensing ion channels (ASICs) are another type of ion channel affected by pH. However, unlike the other channels that are modulated by changes in pH, ASICs are cation channels that are directly gated by an increase in extracellular protons. These channels are homomeric or heteromeric tetramers composed of the subunits ASIC1-4. The function of these proteins is not completely understood but several studies have shown that these proteins reside in the post-synapse and contribute to synaptic plasticity and learning and memory (Zha et al., 2006; Zha et al., 2009). Tissue acidosis in the amygdala resulting from increased levels of CO\(_2\) have been shown to illicit a strong fear response due to activation of synaptic ASICs (Ziemann et al., 2009). In the PNS ASICs may contribute to sensing tissue acidosis, inflammation and nociception (Gautam et al., 2010). Further, termination of seizures may occur in part through activation of ASICs after a substantial drop in brain pH (Ziemann et al., 2008). ASICs seem to be activated by very low pH, pH5-6, and activate and deactivate with very rapid kinetics (Zha et al., 2006; Zhang and Canessa, 2002). As these extreme pH values are rarely reached under physiological conditions, it is unclear how exactly these ion channels function in the cell. However, the existence of these proton-gated channels at synapses suggest that local pH shifts known to occur at the synapse (see below) may in fact be larger than previously appreciated. Some of the observed physiological consequences of ASIC action have been attributed to their possible permeability to Ca\(^{++}\) and activation of Ca\(^{++}\)-
Proton sensitive proteins of the synaptic cleft. (A) Cartoon showing proteins in the synaptic cleft that are inhibited (horizontal bars) or activated/stimulated by extracellular protons (arrows). Proteins inhibited by protons include voltage gated Na\(^+\) and Ca\(^{++}\) channels, ionotropic glutamate receptors of the kainate-type (KR), AMPA-type (AMPAR) or NMDA-type (NMDAR), or group-III metabotropic glutamate receptors (mGluR). Proteins activated or stimulated by extracellular protons include some forms of GABA\(_A\) receptor (GABAR), the ecto-ATPase, and acid sensing ion channels (ASIC). (B) Strong synaptic stimulation results in a series of extracellular pH shifts in the vicinity of the synapse. (see Chesler, 2003).
sensitive signaling pathways (Zha et al., 2006). Other studies have found that ASICs are completely impermeable to Ca$^{++}$ (Samways et al., 2009; Zhang and Canessa, 2002). Therefore, it is unclear what the precise physiological functions of these proton-gated channels are, though it seems likely that changes in brain pH do affect neuron physiology and synaptic transmission through ASICs in addition to the other ion channels mentioned.

Changes in pH have also been shown to affect the extracellular levels of the metabolites ATP and adenosine (Dulla et al., 2009; Dulla et al., 2005; Gourine et al., 2005). Inside the cell, these metabolites are essential for cellular energy homeostasis. However, in the extracellular space, these metabolites can modulate synaptic transmission through signaling pathways initiated by cell-surface ATP or adenosine receptors. ATP is loaded into synaptic vesicles by a vesicular purine transporter and is co-released with neurotransmitter (Meunier et al., 1975; Sawada et al., 2008). Additionally, ATP can be released in a regulated manner from astrocytes surrounding the synapse (Gourine et al., 2010). This released extracellular ATP acts at synapses to enhance excitatory transmission through ionotropic P2X receptors (Rodrigues et al., 2005; Valera et al., 1994). Adenosine on the other hand, is not released through vesicle fusion but is rather transported to the extracellular space by purine transporters or generated locally from the hydrolysis of extracellular ATP by ecto-ATPase (Dulla et al., 2005; Dunwiddie et al., 1997; Dunwiddie and Masino, 2001; Gu et al., 1995). Extracellular adenosine acts to suppress excitatory transmission by acting through the GPCRs A1, A2A, A2B, and A3 (Dunwiddie and Masino, 2001). Extracellular and intracellular acidification in the hippocampus stimulated a rise in extracellular adenosine (Dulla et al., 2005; Dulla et al., 2009). In part, this rise in adenosine resulted from a stimulation of ecto-ATPase activity, increasing extracellular adenosine at the expense of ATP, reducing the excitatory action of ATP at P2X receptors and promoting the inhibitory
action of adenosine at A1 receptors (Dulla et al., 2005; Dulla et al., 2009). It is therefore likely that changes in purinergic signaling will act in a highly coordinated way with the changes in ion channel activities in response to pH shifts in the brain. In summary, multiple pH sensitive proteins and pathways at the synapse have been identified. The profound effects of pH shifts in the brain on synaptic transmission are likely to result from a complex interplay of protons acting simultaneously on several pH-sensitive targets.

1.3.4 pH modulation at the synapse

Significant drops in extracellular pH ($\text{pH}_o$) have been observed during pathological states. During stroke and following seizure activity both intracellular and extracellular pH becomes highly acidic. This pH drop has been shown to strongly inhibit synaptic transmission (Chesler, 2003; Li and Siesjo, 1997; Siesjo et al., 1985; Somjen, 1984). By using pH-sensitive microelectrodes or optical measurements of pH-sensitive dyes it has been found that more subtle and localized changes in $\text{pH}_o$ also occur under non-pathological states within the brain, specifically associated with synaptic transmission. Strong synaptic stimulation has been shown to cause a series of changes in $\text{pH}_o$ in the vicinity of the synapse beginning with an immediate acidification lasting a few milliseconds followed by a slower, alkaline transient lasting several hundred milliseconds. Intense neural activity ultimately leads to extracellular acidosis beginning minutes after the activity and persisting for several minutes or longer (Chesler, 2003) (Fig. 1-2B).

The sources of these pH shifts are not clear in all cases but they have begun to be identified. The initial acidification occurring within milliseconds of synaptic stimulation occurs in conjunction with neurotransmitter release and subsequent excitatory post-synaptic potentials (EPSPs) (Krishtal et al., 1987). Synaptic vesicles are highly acidic
with a luminal pH of 5.5-5.7 (Reimer et al., 1998; Tabb et al., 1992; Van der Kloot, 1987). The initial acid transient is therefore attributed to the release of the acidic contents of synaptic vesicles into the synaptic cleft (Krishtal et al., 1987; DeVries, 2001). Following the immediate acidification is a slower alkaline transient of 0.1-0.2 pH units, the source of which has been difficult to determine (Chesler, 2003; Fedirko et al., 2007; Krishtal et al., 1987; Makani and Chesler, 2007). A recent study has provided good evidence that the source of the alkaline transient involves the activity of the plasma membrane Ca\(^{++}\)-ATPase (PMCA), an obligatory Ca\(^{++}\)/2H\(^{+}\) exchanger, during restoration of Ca\(^{++}\) homeostasis (Makani and Chesler, 2010). Ca\(^{++}\)-influx into both the pre- and post-synaptic compartments activates the PMCA which then exports Ca\(^{++}\) back to the extracellular space in exchange for extracellular protons generating a local proton sink that causes the alkaline transient in pH\(_{o}\) (Makani and Chesler, 2010). The source of the final acidosis beginning seconds to minutes after activity is likely to be complex, and may include combined metabolic sources and active acid transport (Chesler, 2003).

As discussed in the previous section, several components of the synaptic machinery are known to be pH-sensitive. Therefore, it has been suggested that these experimentally observed pH shifts that occur during synaptic transmission may feedback onto these pH-sensitive components and modulate further synaptic transmission. The initial acid transient resulting from the release of the acidic contents of synaptic vesicles has been shown to be sufficiently large to transiently inhibit voltage-gated Ca\(^{++}\) channels positioned adjacent to vesicle release sites (DeVries, 2001). This localized inhibition was suggested to act as a mechanism to suppress further vesicle release. Based on the proton-sensitivity of Ca\(^{++}\) channels (Chen et al., 1996; Klockner and Isenberg, 1994), DeVries (2001) estimated that the synaptic cleft pH would drop by approximately 0.2 units pH during this acid transient. Importantly, the alkaline transient lasting a few hundred milliseconds has been shown to be sufficiently large to enhance the activity of
NMDA receptors and increase Ca\textsuperscript{++}-influx (Fedirko et al., 2007; Makani and Chesler, 2007). Thus the alkaline transient may be an important step in NMDA receptor dependent Ca\textsuperscript{++}-influx leading to the induction of LTP. This final point awaits further experimental support. Nevertheless, there is evidence to suggest that local pH does indeed play a role in the induction and maintenance of LTP (Ronicke et al., 2009; Velisek, 1998).

Thus, the pH environment in the vicinity of the synapse is known to undergo a series of changes under normal physiological conditions associated with synaptic transmission. Further, a precedent for feedback of protons onto pH-sensitive components of the synaptic machinery has been established. The precise physiological consequences of these localized pH changes that occur normally in the brain will require continued investigation to be completely understood. The role of pH modulations in controlling or contributing to long-lasting plasticity of the synapse will also require further investigation. Due to the pH-sensitive nature of the NMDA receptor and other synaptic components, it is likely that local pH changes may influence the induction and maintenance of LTP and LTD.

1.3.5 Regulation of pH at the synapse

Nerve terminals, consisting of both pre- and post-synaptic components, are highly stable structures that can be isolated biochemically. These purified nerve terminals, referred to as synaptosomes, recapitulate several physiological aspects of intact synapses (Dahlstrom et al., 1966; Witzmann et al., 2005). Recovery from acute acidification in synaptosomes was found to require external Na\textsuperscript{+} and was sensitive to NHE inhibitor drugs. The recovery did not require HCO\textsubscript{3}\textsuperscript{-}, nor was it sensitive to bicarbonate transporter inhibitors. Thus, it was concluded that the predominant mode of
pH regulation at synapses was through Na\(^+\)/H\(^+\) exchange, with little or no contribution from HCO\(_3\)\(^-\) transporters (Chesler, 2003; Nachshen and Drapeau, 1988; Sauvaigo et al., 1984). Na\(^+\)/H\(^+\) exchange has also been implicated in the regulation of synaptic transmission at glutamatergic, GABAergic and dopaminergic synapses (Jang et al., 2006; Rocha et al., 2008; Trudeau et al., 1999). These findings strongly suggest that an NHE isoform is present at synapses, but as yet the synaptic Na\(^+\)/H\(^+\) exchanger in nerve terminals has not been identified. All nine mammalian NHEs are expressed in the brain (Brett et al., 2005a; Ma and Haddad, 1997; Orlowski and Grinstein, 2004). NHE1 is ubiquitously expressed, and is predominantly localized to the plasma membrane, and is therefore a candidate for a synaptic NHE. In accordance, NHE1 null-mice display distinct neurological phenotypes including epileptic-like seizures resulting from enhanced neuronal excitability (Bell et al., 1999; Cox et al., 1997; Gu et al., 2001). Although Ma and Haddad (1997) detected low level expression of NHE2, 3 and 4 in the brain their role in this organ remains controversial. Indeed, mice with knock-out mutations of plasmalemmal type-NHE2, 3, and 4 show gastrointestinal and renal phenotypes with no obvious neurological symptoms (Gawenis et al., 2005; Schultheis et al., 1998a; Schultheis et al., 1998b), arguing against an obvious synaptic role for these isoforms, though subtle functions of these isoforms in the brain cannot be discounted. NHE5 mRNA is almost exclusively expressed in the brain, and has been suggested to be neuron-specific (Attaphitaya et al., 1999; Baird et al., 1999). Therefore, NHE5 is an excellent candidate for a synaptic NHE. However, the localization of NHE5 protein and the physiological function of NHE5 are unknown.
1.4 Objectives

Modulation of pH in the brain has been shown to play an important role in synaptic transmission through modulation of a large number of pH-sensitive components of the synaptic machinery. However, the molecular identity of pH regulatory molecules at the synapse has not been determined. My central hypothesis is that NHE5 plays an important role in the brain by regulating pH in the vicinity of the synapse and that NHE5 participates in synaptic transmission through a proton-based feedback onto pH-sensitive synaptic components. In order to address this question I proposed the following objectives:

Objective 1: Where is NHE5 protein expressed?
Objective 2: What is the role of NHE5 at synapses?
Objective 3: How is NHE5 transporter activity and cellular trafficking regulated?

1.4.1 Objective 1: where is NHE5 protein localized?

NHE5 mRNA is known to be abundant in brain but the expression of NHE5 protein has not been previously investigated due in part to the lack of NHE5-specific antibodies. In order to understand the physiological function of a protein it is essential to determine in which cells the protein of interest is expressed, when in development the protein is expressed, and where in the cell the protein is localized. The research described in chapter 3 entitled NHE5 Protein is Expressed in Brain aims to address these questions, with the hope that a detailed knowledge of the expression and localization of NHE5 protein will reveal strong clues as to the physiological function of this protein. Towards this goal I performed immunohistochemistry, immunocytochemistry and Western blot using a newly-developed NHE5-specific
antibody to examine NHE5 distribution with the notion that this information would reveal in part the physiological function of NHE5.

1.4.2 Objective 2: what is the role of NHE5 at synapses?

I next investigated the physiological function of NHE5 in the brain. Many aspects of brain physiology are expected to be pH-sensitive. In particular, the chemical and electrical aspects of synaptic transmission are altered by subtle changes in pH due to the sensitivity of voltage and ligand-gated ion channels to protons. Thus, I hypothesized that brain-enriched NHE5 may modulate synaptic transmission through local regulation of pH at the synapse. The research described in chapter 4 entitled NHE5 Synaptic Function was motivated principally by the discovery that NHE5 is present at synapses, determined in chapter 3. In order to understand the role of NHE5 at synapses I first used a combination of biochemistry and cell biology approaches to examine how NHE5 distribution was affected by synaptic activity. Following this, NHE5 activity was manipulated by overexpression of NHE5, or by expression of an NHE5 dominant-negative mutant or knock-down by expression of a short-hairpin RNA specifically targeting rat NHE5, and the effects on synapse structure were investigated.

1.4.3 Objective 3: how is NHE5 transporter activity and cellular trafficking regulated?

In a parallel approach to understand the physiological function of NHE5, I attempted to identify and characterize novel NHE5-interacting proteins. Very few, if any proteins act in isolation. Therefore, the identification of novel-binding proteins can often reveal aspects of the biology of a protein in question. In chapter 5 entitled Molecular
Regulation of NHE5 I describe the identification and characterization of a novel NHE5 binding partner that controls NHE5 trafficking between intracellular recycling endosomes and the cell surface. With the knowledge described in chapters 3 and 4, that NHE5 may be active at synapses, the work described in chapter 5 begins to unveil the molecular basis for how NHE5 is able to perform its function at synapses and how this may be regulated.
2. Materials and methods

2.1 Tissue culture

2.1.1 Neuronal cell culture

Hippocampi were obtained from E-18 rat embryos, rinsed three times in 10 ml of Hank’s Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY, USA) and incubated for 18 minutes at 37°C in a solution of 2 mg/ml trypsin in HBSS. 0.1% DNase was then added to the solution for the last 2 minutes of this incubation. The hippocampi were then rinsed three times in 10 ml of HBSS. Cells were then dissociated by trituration and distributed to 12-well plates containing poly-L-lysine coated glass coverslips or 35 mm, polyethylenimine-coated, plastic culture dishes, each containing 1 ml of Plating Medium (10% heat-inactivated Fetal Bovine Serum (FBS), 0.45% glucose, 1% sodium pyruvate (Invitrogen, Burlington, ON, Canada), 1% GlutaMAX (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen) in Minimum Essential Medium (MEM) (Gibco/Invitrogen)). The dissociated neurons were plated at a density of approximately 130 neurons/mm² of growth substrate, and incubated at 37°C in a humidified 6%CO₂/94% room air atmosphere. After 4 h incubation, Plating Medium was replaced with 1 mL Maintenance Medium (2% B-27 supplement (Stem Cell Technologies, Vancouver, BC, Canada), 1% GlutaMAX (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen) in Neurobasal Medium (Gibco).

2.1.2 Cell lines

Chinese hamster ovary (CHO), AP-1 and AP-1 based cell lines were maintained in α-MEM with 10% FBS, and PC12 and PC12 stably expressing 1D4-tagged NHE5 (PC12/NHE5₁D4) or NHE5-shRNA cells were maintained in D-MEM supplemented with
5% FBS and 10% horse serum. AP-1 cells are an NHE-deficient cell line derived from CHO cells produced previously by chemical mutagenesis (Rotin and Grinstein, 1989). AP-1 cells stably expressing NHE5 with a triple HA-tag inserted after amino acid residue 36 (AP-1/NHE5_{HA} cells) or NHE1 with an influenza hemagglutinin (HA) tag at the C-terminus (AP-1/NHE1_{HA}) were produced previously (Szaszi et al., 2002). AP-1 cells expressing HA-tagged NHE5 E209I were produced by transfecting parental AP-1 cells with pcDNA3 containing NHE5_{HA}-E209I using the conventional calcium phosphate method (Chen and Okayama, 1987). Stable transfectants were selected in growth media containing G418 (200μg/ml) and individual clones were screened by western blot and immunofluorescence microscopy using mouse anti-HA antibody. NHE5_{1D4} or NHE5-shRNA (A, B or C) was transfected into PC12 cells using the conventional calcium phosphate method and cells stably expressing NHE5_{1D4} or NHE5-shRNA were selected in media containing G418 (200μg/ml).

### 2.1.3 Lipofectamin 2000 transfection

Cultured neurons grown on glass coverslips in standard 12-well tissue culture plates (75,000 cells/well) or on glass-bottom 35mm dishes (100,000 cells/dish) were transfected using lipofectamine 2000 (Invitrogen) with a modified protocol from the manufacturer’s recommendation. Each 35mm dish or single well of a 12-well plate was transfected using 1μl of lipofectamine 2000 (Invitrogen), 2μg plasmid DNA in a total volume of 50μl OptiMem transfection media (Invitrogen). For experiments using transient transfection of tissue culture cell lines the following protocol was used for most experiments. 300,000 cells were plated into 35mm tissue culture dishes. Following cell attachment, cells were transfected using 2.4μl of lipofectamine 2000 (Invitrogen), 1.2μg plasmid DNA in a total volume of 120μl of serum-free growth media.
2.2 Antibodies

Rabbit polyclonal anti-NHE5 antibodies were raised against the epitope EEPTQEPGPLGEPP from rat NHE5 (amino acid residues 21-34) corresponding to the first extracellular loop of NHE5 and purified by GenScript (Piscataway, NJ, USA).

Affinity-purified anti-NHE5 rabbit polyclonal antibody raised against human NHE5 [G674-L896] that cross-reacts with rat NHE5 (Onishi et al., 2007) was used for endogenous co-immunoprecipitation experiments. The purified mouse monoclonal antibody rho 1D4 against a nine amino acid TETSQVAPA C-terminal epitope (Hodges et al., 1988; MacKenzie and Molday, 1982) was obtained from National Cell Culture Center (Minneapolis, MN, USA). The antibody was coupled to CNBr-activated Sepharose beads as previously described (Oprian et al., 1987).

The following additional primary antibodies were used: mouse monoclonal: anti-Tau (Millipore, Billerica, MA, USA), anti-NHE1 (BD biosciences, Mississauga, ON, Canada), anti-MAP2 (Sigma, Oakville, ON, Canada), anti-α and βTubulin (Sigma), anti-Gephyrin (Synaptic Systems, Goettingen, Germany), anti-green fluorescent protein (GFP) (Roche, Laval, QU, Canada), anti-HA (Covance, Montreal, QU, Canada), anti-PSD95 (Abcam, Cambridge, MA, USA), anti-SCAMP2 (8C10; Santa Cruz, Santa Cruz, CA, USA), and anti-Rab4 (StressGen Victoria, BC, Canada); rabbit polyclonal: anti-actin (Sigma), anti-myc (A-14, Santa Cruz), anti-HA (Y-11, Santa Cruz), anti-SCAMP1, anti-SCAMP2, anti-SCAMP5 (Affinity BioReagents, Golden, CO, USA), and anti-Rab11 (Zymed Laboratories, South San Francisco, CA); guinea pig polyclonal: anti-VGlut1 (Millipore), and anti-VGAT (Synaptic Systems).

The following secondary antibodies were used: AlexaFluor488 or AlexaFluor568-conjugated goat anti-rabbit, AlexaFluor568 or AlexaFluor647-conjugated goat anti-
mouse (Molecular Probes, Eugene, OR, USA), Cy5-conjugated donkey anti-guinea pig (Jackson ImmunoResearch, Westgrove, PA, USA), hoarse radish peroxidise-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (Jackson ImmunoResearch).

2.3 Drug treatments

2.3.1 Glycine treatment

Neurons were incubated in chemical LTP buffer (140mM NaCl, 5mM KCl, 1.5mM CaCl$_2$, 25mM HEPES pH 7.4, 33mM Glucose, 500nM tetrodotoxin (TTX), 20µM bicucculine, 1µM strychnine for 30 minutes at 37°C. Neurons were then treated with chemical LTP buffer containing 200µM glycine for 3 minutes at 37°C, followed by a return to buffer without glycine for the remainder of the experiment. The entire glycine treatment protocol was performed in a CO$_2$-free incubator. As a control for glycine treatment in some experiments, D-AP5 (100µM) was added to the chemical LTP buffer during the 30 minute pre-treatment and 3 minute glycine treatment steps.

2.4 Immunohistochemistry

Adult mice (P40-P60) were deeply anesthetized by intraperitoneal injection of 150 mg/kg ketamine and 15 mg/kg xylazine, and transcardially perfused with ice-cold phosphate-buffered saline (PBS) (pH 7.4), followed by 4% paraformaldehyde in PBS. The brains were quickly removed and post-fixed in 4% PFA for 1 hour before going through a series of sucrose-PBS solutions (10% to 30%). Whole brains were embedded in TissueTek (Sakura Finetek, Torrance, CA, USA), frozen in dry ice and stored at -80°C. 20µm thick coronal sections were cut by cryostat (Microm Instruments, San Marcos, CA, USA). For immunohistochemistry, sections were permeabilized in 0.1% Triton-X for 30 minutes,
blocked in 4% goat serum for 1 hour at room temperature, and incubated with PBS containing 1% goat serum and anti-NHE5 antibody overnight at 4°C. Following washes with PBS sections were then incubated with goat anti-rabbit AlexaFluor488-conjugated secondary antibody (1:500; Molecular Probes) in PBS containing 1% goat serum for 1 hour at room temperature. After final washes with PBS sections were then covered with glass coverslips using Prolong Gold anti-fade reagent (Invitrogen).

2.5 Immunocytochemistry microscopy

2.5.1 Cultured hippocampal neurons

Neurons were fixed by incubation with 4% paraformaldehyde in PBS for 10 minutes at room temperature and then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. Alternatively, cells were fixed/permeabilized by incubation with pre-chilled methanol at -20°C for 5 minutes. Cells were blocked by incubation with 10% goat-serum in PBS for 1 hour at room temperature followed by incubation with primary antibodies in PBS containing 1% goat-serum overnight at 4°C. Coverslips were washed and then incubated with fluorescently labeled secondary antibodies in PBS containing 1% goat-serum for 1 hour at room temperature. After final washes with PBS, coverslips were mounted onto glass slides using Pro-Long Gold Antifade Reagent (Invitrogen).

For experiments examining NHE5 surface expression, cells were fixed with 4% paraformaldehyde as above. Cells were blocked by incubation with 10% goat-serum in PBS for 30 minutes followed by incubation with rabbit anti-NHE5 antibody in PBS containing 1% goat-serum for 2 hours at room temperature. After washing with PBS cells were permeabilized with 0.1% Triton X-100 as above and incubated overnight at 4°C with mouse ant-PSD95 and guinea pig anti-VGlut1 in PBS containing 1% goat-serum. Finally, NHE5, PSD95 and VGlut1 were visualized by incubation with Alexa 488-
conjugated goat anti-rabbit, Alexa 568-conjugated goat anti-mouse, and Cy5-conjugated donkey anti-guinea pig secondary antibodies.

2.5.2 PC12 cells

PC12/NHE51D4 cells grown on glass coverslips coated with poly-L-lysine and laminin (10µg/mL, Sigma) were transfected with SCAMP2GFP or co-transfected with SCAMP2GFP and Arf6HA. 72 hours post-transfection, cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. Fixed cells were then treated with rabbit polyclonal anti-Rab4, anti-Rab11, or anti-HA antibodies and mouse monoclonal 1D4 antibodies followed by Alexa 647-conjugated goat anti-mouse IgG and Alexa 568-conjugated goat anti-rabbit IgG (Molecular Probes).

2.5.3 AP-1 based cells

To visualize recycling endosomes, AP-1/NHE5HA cells were serum-starved for 2 hours and then incubated with Alexa 568-conjugated transferrin (25µg/mL, Molecular Probes) for 30 minutes at 37°C. Cells were rinsed with PBS, fixed with pre-chilled methanol at -20°C for 5 minutes, permeabilized with 0.1% Triton X-100/PBS for 5 minutes, and internalized transferrin, NHE5HA, and endogenous SCAMP1 or SCAMP2 were visualized by immunofluorescence microscopy as described above using anti-SCAMP1 or anti-SCAMP2 rabbit polyclonal and anti-HA monoclonal primary antibodies, followed by Alexa 488-conjugated goat anti-rabbit IgG and Alexa 647-conjugated goat anti-mouse IgG secondary antibodies. Prepared coverslips were then analyzed by triple immunofluorescence confocal microscopy.
2.6 Image analysis

2.6.1 Quantification of synapse localization

Cells were immunolabelled using NHE5, VGlut1 and PSD-95, or VGAT and Gephyrin antibodies. Images were analyzed using ImageJ software (NIH, Washington, USA) and a colocalization plugin from the ImageJ website (http://rsb.info.nih.gov/ij/plugins/colocalization.html). Synapses were first identified as regions of colocalization greater than 10 pixels in size between pre- and post-synaptic markers; Vglut1 (threshold 1165) and PSD-95 (threshold 1290) for excitatory synapses, or VGAT (threshold 765) and Gephyrin (threshold 790) for inhibitory synapses. Thresholding levels were held constant between samples and experiments, randomly selected fields were imaged, and all the puncta were examined in a field. These regions of colocalization between pre- and post-synaptic markers were used to create a ‘mask’ and regions greater than 5 pixels where NHE5 puncta overlapped with the mask were considered synapses positive for NHE5. Numbers of synapses positive for NHE5 were expressed as a percentage of the total number of synapses identified, quantified using the same threshold and size criteria. The mean numbers of colocalized synaptic puncta quantified from 30 or greater fields in each treatment group were analyzed for statistical significance using the Student’s t test and are expressed ±s.e.m.

2.6.2 Dendritic spine length and width measurements

Hippocampal neurons were transfected at 9DIV using Lipofectamine 2000 (Invitrogen) and at 14DIV neurons were fixed and dendritic spine density and head width was measured. For all experiments examining spine number and morphology a GFP cell-fill was used. Serial z-section confocal images were compiled into a single image. Regions of dendrite within 100µm from the cell body where dendritic spines were clearly visible
(without excessive crossing of multiple dendrites or axons) were imaged. Spines were identified as protrusions < 3μm in length with or without a head from the dendrite shaft. The density and width of spines were measured using ImageJ software (National Institutes of Health). Statistical analysis and graph plotting were performed using Excel software (Microsoft). In some experiments transfected neurons were subjected to glycine treatment followed by a 60 minute chase period prior to fixation. As a control for glycine application some neurons were pre-treated (30 minutes) with the NMDA receptor antagonist D-AP5 (100μM). In separate experiments (without glycine treatment) transfected neurons were treated with or without D-AP5 (100μM) for 48 hours in normal growth media prior to fixation. Neuronal images were acquired using a laser scanning confocal microscope (Fluoview FV1000, Olympus, Markham, ON, Canada) with a 60X oil immersion objective.

2.7 Live-cell imaging

2.7.1 Microscopy of NHE5-GFP fusion protein

Hippocampal neurons were grown on gridded glass-bottom dishes (MatTech, Westlake Village, CA, USA) coated with poly-L-Lysine. Cells were transfected at 13DIV with NHE5-GFP together with RFP and imaging experiments were performed at 14DIV. Neurons were visualized using a laser-scanning confocal microscope (Olympus) in a temperature controlled chamber held at 37°C. Cells of interest were identified prior to any treatments and the coordinates were marked. For glycine treatment (see above) the gridded dishes were removed from the microscope for the treatment period. The dishes were then replaced in the microscope and cells of interest were further monitored. Individual spines were first identified from the RFP cell-fill and then manually scored for the presence of NHE5-GFP either contained within the spine or within 1μm of the spine.
base. NHE5-GFP location relative to spines was categorized according to the presence of NHE5-GFP at the base of the spine (in the dendritic shaft within 1µm of the spine base), in the spine neck (between the spine base and spine head), or in the spine head (at the spine tip). Spines containing NHE5-GFP in the head or neck and extending to the spine base were counted as positive for spine head or neck and not as spine base.

2.7.2 Monitoring dendritic spine pH using the pH-sensitive GFP variant de4GFP

Hippocampal neurons, grown on gridded glass-bottom dishes (MatTech), were transfected with de4GFP alone or together with NHE5 shRNA A (see section 2.8). The pH-sensitive de4GFP fluorescence from individual spines was captured using a confocal microscope by excitation with 488nm laser and emission at 510nm. Changes in fluorescence (ΔF) were calculated for each dendritic spine by normalizing the measured fluorescence intensity (F) of dendritic spines to the fluorescence intensity (Fo) of the same spine immediately prior to glycine treatment as described previously (Park et al., 2006). To calibrate pH-sensitive de4GFP fluorescence to pH, CHO cells were transfected with de4GFP and subjected to pH clamp using the high-[K⁺]/nigericin technique (140mM KCl, 2mM CaCl₂, 1mM MgCl₂, 10mM glucose, 10µM nigericin, 20mM MES/HEPES/Tris pH 5.0-9.0) (Baxter and Church, 1996; Rotin and Grinstein, 1989). In the calibration experiments, fluorescence intensity of de4GFP displayed sigmoidal relationship to pH between pH 5.0 and pH 9.0, and pKₐ was calculated to be 7.45, similar to the previously reported value (Hanson et al., 2002). Between pH 6.75 and 8.0, the relationship between pH and de4GFP fluorescence intensity normalized to that measured at pH 7.0 was linear (Vilas et al., 2009). Experimentally measured ΔF for
each spine was converted to $\Delta$pH using the linear relationship between relative fluorescence and pH determined in de4GFP transfected CHO cells under pH-clamp.

2.8 Molecular biology

Human NHE5 cDNA was cloned previously (Baird et al., 1999). HA-tagged human NHE5 was produced previously by introducing an artificial NotI restriction endonuclease site into the coding sequence corresponding to leucine 36 (contained in the first predicted exomembrane loop) followed by insertion of triple HA epitope (Szaszi et al., 2002). The E209I variant of NHE5 was produced by site-directed mutagenesis using quick-change mutagenesis kit (Stratagene, La Jolla, CA, USA) using human NHE5HA as a template. EGFP coding sequence was amplified by polymerase chain reaction (PCR) with primers containing an artificial NotI restriction site at their 5’ end using the pEGFPN1 vector (Clontech, Mountain View, CA, USA) as a template. This PCR product was then ligated into the NotI restriction site at the L36 of NHE5 in frame to make the GFP-NHE5 fusion construct. For short-hairpin RNA (shRNA A, B or C) constructs targeting rat NHE5 the following complementary synthetic oligonucleotides were used, 5’-

GATCCGTGCTTTGGTGAACAGATGTTATCTGATACGTAACATCTGTTCCAC
CAAGAGCAAAATTATTCCAAA-3’ (sense) and 5’-
AGCTTTTTGAAAAAATTTGCTTGTGAAAACAGATGTTACGGATATCAATAACATC
TGTTCACCAAGAGCAACGG-3’ (antisense, shRNA A); 5’-
GATCCCATAGTGGTGCCACAAAGTACGTCCTTTGATACGGACCATTTTGTGG
CCACCACATATTTTTCCAAA-3’ (sense) and 5’-
AGCTTTTTGGAAAAATAGTGGTGCCACAAAGTAGTCCTCGGATATCAAAAGGACT
ACTTTGTGGCCACACACTATGG-3’ (antisense, shRNA B); 5’-
GATCCGTTTGTAATCACTCTCTCTCACTTGTACGATATCCGGGAAGAGGAGTG
ATTACCACAAAATTTTTTCCAAA-3' (sense) and 5'-
AGCTTTTGGAATAAAATTTGTGGTAATCACTCTCTCTCTACCCTCGGATATCAGGTGAA
GAGGAGTGATTACCACAAA CGG-3' (antisense, shRNA C). Bold-type indicates sequences targeting rat NHE5. When annealed, duplexes contain 5' and 3' overhangs which allowed cloning into the multi-cloning site in pRNAT-H1 vector (GenScript). In order to clone human Arf6 and Rab11 first strand cDNA was synthesized from human brain RNA (Clontech) by using random hexamers and SuperScript II reverse transcriptase (Invitrogen), and subjected to PCR using Pfu-Turbo (Stratagene). The following primers were used: Arf6 forward 5'-ATG GGG AAG GTG CTA TCC AAA ATC TTC GG-3', and Arf6 reverse 5'-AGA TTT GTA GTT AGA GGT TAA CCA TGT G-3'; Rab11 forward 5'-ATG GGC ACC CGC GAC GAG TAC G-3′, and Rab11 reverse 5'-GAT GTT CTG ACA GCA CTG CAC CTT TGG-3'. The identities of the PCR fragments were verified by sequencing and subjected to a second round of PCR to introduce a myc- or HA-tag at the extreme C-terminus of the clone. The PCR fragment was ligated into mammalian expression vector pcDNA3 and the sequence of the myc-tagged constructs was verified subsequently. Arf6T27N and Rab11S25N dominant-negative mutants were generated by quick-change mutagenesis kit (Stratagene) using the HA-tagged Arf6/pcDNA3 or myc-tagged Rab11/pcDNA3 as a template. The myc-tagged human SCAMPs were described previously (Lin et al., 2005). The coding region for SCAMP2 and SCAMP5 was amplified by PCR and ligated into the pEGFP N1-vector in frame to make GFP-fusion constructs (SCAMP2GFP and SCAMP5GFP).
2.9 Biochemistry

2.9.1 Expression and purification of glutathione S-transferase (GST) fusion proteins

For producing GST fusion proteins, PCR fragments corresponding to different regions of the SCAMP2 cytoplasmic domains were inserted into a pGEX-2T bacterial expression vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in-frame with the N-terminal GST tag as described previously (Lin et al., 2005). Protein expression was induced by incubating transformed BL21 *Escherichia coli* cells with 0.2mM isopropyl-thio-β-galactopyranoside (IPTG) at 37ºC for 3 hours. *E. coli* cells were collected by centrifugation, resuspended in lysis buffer containing 1% Triton X-100 and protease inhibitor cocktail (Roche) in PBS. Cell lysates were then incubated for 30 minutes on ice and then sonicated four times for 30 seconds. After sonication, cell debris was cleared by centrifugation for 10 minutes at 16,000 x g at 4ºC. GST fusion proteins were purified by incubation with reduced form glutathione sepharose beads (Amersham Pharmacia Biotech) at 4ºC.

2.9.2 GST pull-down

35S-labeled NHE5 C-terminal domain (G491-L896) was produced by *in vitro* transcription-translation using the TnT-coupled reticulocyte lysate system (Promega, Madison, WI, USA) according to the manufacturer’s instructions. 35S-labeled *in vitro* translated protein was diluted to 1mL with cold PBS and then centrifuged at 16,000 x g for 5 minutes to remove insoluble materials. The supernatant was then further diluted to 6.2mL in cold PBS plus protease inhibitor cocktail (Roche). 750µL of this diluted solution was incubated with 2µg of GST fusion protein immobilized to the reduced form
glutathione sepharose beads for 90 minutes at room temperature. After extensive washing, $^{35}$S-labeled *in vitro* translated protein bound to the GST fusion protein was eluted with SDS sample buffer, resolved by SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and bound $^{35}$S-labeled NHE5 C-terminus was detected by phosphorimaging. Equal input of the different GST-fusion proteins was confirmed by resolving these proteins on SDS-PAGE followed by visualization using Coomassie blue protein stain.

### 2.9.3 Co-immunoprecipitation

AP-1/NHE5$_{HA}$ cells were transfected with SCAMP2$_{myc}$, SCAMP2$\Delta$NPF$_{myc}$ (deletion of amino acids 1-55), SCAMP2$\Delta$C$_{myc}$, or SCAMP2 1-154$_{myc}$, and the cells were lysed in PBS containing 1% CHAPS and protease inhibitor cocktail (Roche) on ice for 30 minutes. Lysates were cleared by centrifugation at 16,000 x g for 10 minutes (two times) at 4$^\circ$C. Cell lysates were then incubated with anti-HA monoclonal antibody or pre-immune serum at 4$^\circ$C for 4 hours, followed by overnight incubation with protein G sepharose beads (Amersham Pharmacia Biotech). After extensive washing, eluted samples were resolved in SDS-PAGE and the proteins present in the immunoprecipitate were detected by western blot. To isolate membrane fractions, cells were resuspended in sonication buffer (250 mM sucrose, 10 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, with protease inhibitor cocktail (Roche)) and homogenized by mild disruption through a 26.5-gauge needle. Insoluble cellular debris was removed by centrifugation at 800 x g for 10 minutes at 4$^\circ$C, and membrane fractions were isolated by ultra-centrifugation at 100,000 x g at 4$^\circ$C. The membrane fraction was then solubilized in PBS containing 1% CHAPS and protease inhibitor cocktail (Roche) and debris was removed by centrifugation at 16,000 x g for 10 minutes (two times) at 4$^\circ$C. NHE5$_{1D4}$ was immunoprecipitated from the
solubilized membrane fraction with 1D4 mouse monoclonal antibody coupled to sepharose beads and, after washing seven times, bound endogenous SCAMPs were resolved by SDS-PAGE, transferred to a PVDF membrane, and detected with anti-SCAMP1, -2, or -5 rabbit polyclonal antibodies. An association of endogenous SCAMP2 with endogenous NHE5 in brain tissues was assessed as follows. Rat brain was homogenized in sonication buffer using a glass homogenizer followed by mixing the homogenate with an equal volume of PBS containing 2% CHAPS and protease inhibitor cocktail (Roche). The lysate was cleared, as above, and then incubated with anti-NHE5 rabbit polyclonal antibody or pre-immune serum at 4ºC for 4 hours, followed by overnight incubation with protein A sepharose beads (Amersham Pharmacia Biotech). SCAMP2 found in the immunoprecipitate was detected by western blot using an anti-SCAMP2 mouse monoclonal antibody.

2.9.4 Surface biotin labeling

Cultured hippocampal neurons (14DIV) were first treated with glycine to induce chemical LTP as described above. Forty five minutes after washout of glycine, neurons were rinsed with ice-cold PBSCM (phosphate buffered saline containing 1mM MgCl₂ and 0.1mM CaCl₂, pH 8.0) and then incubated in PBSCM containing a protein reactive biotinylation reagent (NHS-SS-Biotin, 0.5mg/mL, Thermo Scientific, Waltham, MA, USA) for 30 minutes at 4ºC to label surface proteins. Cells were then rinsed with ice-cold PBSCM and excess biotinylation reagent was quenched by incubation with PBSCM containing 20mM glycine (2 times for 7 minutes each). Finally cells were lysed in PBS containing 1%CHAPS and protease inhibitor cocktail (Roche). Protein concentration of cell lysates was quantified using Bradford assay. Equal amounts of protein were added to Neutravidin agarose beads (Thermo Scientific) and incubated overnight at 4ºC. After
extensive washing of the beads with ice-cold lysis buffer, biotinylated proteins were eluted using SDS-sample buffer containing 100mM dithiothreitol. Proteins were resolved by SDS-PAGE and detected by Western blot using anti-NHE5 or anti-NHE1 antibodies. 10% of the total whole cell lysate not subjected to Neutravidin beads was visualized by Western blot using anti-NHE5, anti-NHE1 or anti-tubulin antibodies for normalization.

2.9.5 Surface biotin labeling and internalization

AP-1/NHE5HA cells were transiently transfected with SCAMP2myc, SCAMP5myc, Arf6HA, Rab11myc (wild-type or dominant-negative) or empty vector using Lipofectamine 2000. Cells were biotinylated for 30 minutes followed by quenching with glycine as above. For internalization experiments, labeled cells were subjected to chase incubation at 37°C in culture media. Cells were treated, or left untreated, with glutathione cleavage buffer (50mM glutathione, 90mM NaCl, 1mM MgCl₂, 0.1mM CaCl₂, 60mM NaOH, 0.2% BSA, pH 8.6) for 20 minutes (two times) at 4°C and then solubilized in PBS containing 1% CHAPS plus protease inhibitor cocktail (Roche). Insoluble debris was removed from the lysate by centrifugation at 16,000 x g for 10 minutes two times at 4°C. Protein concentration was determined using Bradford assay and an equal amount of protein was collected from each sample for analysis. A small amount (5%) of lysate was removed and represents the total fraction; the remaining lysate was then incubated with Neutravidin-Agarose beads (Thermo Scientific) overnight to extract biotinylated proteins. Following washing of the beads, biotinylated proteins were then eluted with SDS-sample buffer containing 100mM dithiothreitol, resolved in SDS-PAGE and detected by western blotting. The intensity of the bands was analyzed by densitometry of films exposed in the linear range.
2.10 NHE activity assays

2.10.1 $^{22}\text{Na}^+$ influx assay

Sub-confluent AP-1/NHE5$_{\text{HA}}$ cells were plated into 24-well plates and transfected with SCAMP2$_{\text{myc}}$, SCAMP5$_{\text{myc}}$, or empty parental pcDNA3 vector. Transfection efficiency exceeded 50%, as determined by immunofluorescence microscopy. Forty-eight hours post-transfection, cells were acidified using the NH$_4$Cl pre-pulse technique (Szaszi et al., 2002). In brief, cells were treated with ammonium-choline solution (50mM NH$_4$Cl, 80mM choline chloride, 1mM MgCl$_2$, 2mM CaCl$_2$, 5mM glucose, 20mM HEPES-Tris, pH 7.4) for 20 minutes at 37°C followed by a rapid washout with isotonic choline chloride solution (130mM choline chloride, 1mM MgCl$_2$, 2mM CaCl$_2$, 5mM glucose, 20mM HEPES-Tris, pH 7.4) to acutely acidify the cytosol. Assays were immediately initiated by adding radioactive-$^{22}\text{Na}^+$ (1 $\mu$Ci/ml $^{22}\text{Na}$Cl in choline chloride solution) to each well in the absence or presence of 1 mM amiloride. After 5 minutes, the influx of $^{22}\text{Na}^+$ was terminated by rapidly washing each well three times with ice-cold NaCl-saline solution (130mM NaCl, 5mM KCl, 1mM MgCl$_2$, 2mM CaCl$_2$, 5mM glucose, 20mM HEPES-NaOH, pH 7.4). Cells were then lysed in 0.5N NaOH to extract the radio-label. Lysates were neutralized by the addition of an equal volume of 0.5N HCl, and the radioactivity was counted by liquid scintillation spectroscopy. Influx values obtained in the presence of amiloride were subtracted from those in the absence of amiloride. The difference represents the "amiloride-sensitive" NHE-dependent $^{22}\text{Na}^+$ influx. Each experiment was conducted in quadruplicate, and three independent experiments were performed.

2.10.2 pH$_i$ measurements

AP-1/NHE5$_{\text{HA}}$ or AP-1/NHE5$_{\text{HA}}$E209I cells were used for pH$_i$ measurement experiments. In some experiments AP-1/NHE5$_{\text{HA}}$ were transfected with GFP-tagged SCAMP2,
SCAMP5, SCAMP2ΔNPF, SCAMP2ΔC, SCAMP2 1-154, or empty parental pEGFP expression vector (Clontech); co-transfected with Rab11myc or Arf6HA (either wild-type or dominant-negative) and SCAMP2GFP or pEGFP vector, or cotransfected with pEGFP vector and NHE5HA E209I. Cells were then plated onto glass coverslips and grown for 48 hours prior to pHi measurements. Coverslips with cells attached were mounted in a temperature-controlled recording chamber filled with NaCl-saline solution, placed on the microscope stage, and GFP-expressing cells were identified by viewing GFP fluorescence during excitation at 488 nm. Subsequently, cells were loaded with BCECF by adding 2µM BCECF acetoxymethyl ester to the NaCl-saline solution for 10 minutes at room temperature and were then superfused at 2mL/minute with NaCl-saline solution (without dye) at 34°C for the remainder of the experiment. BCECF-derived fluorescence emission intensities during excitation at 488 nm and 452 nm were at least 20-fold higher than the original GFP fluorescence signal. The dual excitation ratio method was used to estimate pHi employing a fluorescence ratio-imaging system (Atto Biosciences, Rockville, MD, USA); full details of the methods employed have been presented previously (Baxter and Church, 1996; Smith et al., 1998). The high-[K+]/nigericin technique was employed to convert background-corrected BCECF emission intensity ratios into pHi values. Intracellular acid loads were imposed by exposing the cells for 2 minutes to NH4+-choline solution. The recovery of pHi following an NH4+-pre-pulse was fitted to a single exponential function and the first derivative of this function was used to determine the rate of change of pHi (dPHi/dt) at 0.05 pHi unit increments from the point of maximum acidification (Baxter and Church, 1996; Smith et al., 1998). Proton efflux was calculated by multiplying the measured dPHi/dt at a given pHi value by the intrinsic intracellular buffering capacity (βi) at the same pHi value. We calculated βi in AP-1/NHE5HA cells by measuring the changes in pHi elicited by changing the extracellular
concentration of NH₄Cl as described previously (Boyarsky et al., 1988; Roos and Boron, 1981). Instantaneous proton efflux was then plotted against absolute pHᵢ values and results from different experiments were compared statistically (Student’s unpaired two-tailed t test) at corresponding values of pHᵢ. To confirm the identity of the acid-extrusion mechanism, the NHE inhibitor 5-((N-ethyl, N-isopropyl)-amiloride (EIPA, 10μM) was added to the perfusion solution for 2.5 minutes during the pHᵢ recovery phase followed by a return to NaCl-saline solution. The compositions of the NaCl-saline and NH₄⁺-choline solutions were the same as those used for the ^{22}Na⁺-influx assays.

2.11 Endosome function

2.11.1 Transferrin uptake

Parental AP-1 cells or AP-1 cells expressing HA-tagged NHE1, NHE5, or NHE5 E209I were grown 96-well plates. Cells were serum-starved for at least 2 hours and then incubated in serum-free media containing AlexaFluor568-conjugated transferrin (Molecular Probes, 10μg/mL) for 0-30 minutes at 37°C. Cells were rinsed three times with PBS and then fixed with 4% paraformaldehyde containing the DNA stain Hoechst (500ng/mL) for 15 minutes. Cells were then imaged using a high content screening automated confocal microscope (Cellomics system, Thermo Scientific). Individual cells were identified from the Hoechst stain and the average fluorescent transferrin content/cell/well was measured (expressed in arbitrary units). Each experiment was performed with 9 duplicate wells which were pooled. Averages from three independent experiments were compared statistically using Student’s t-test.
2.11.2 Endosome pH measurement

AP-1 based cells were grown in 96-well plates and serum-starved as above. Cells were then incubated in serum-free media containing Hoechst (500ng/mL), pH-sensitive fluorescein-conjugated transferrin (30µg/mL, Molecular Probes) and pH-insensitive AlexaFluor568-conjugated transferrin (10µg/mL, Molecular Probes) for 30 minutes minutes at 37°C. Cells were rinsed twice with Na+-saline and then chased for 30 minutes in Na+-saline before image acquisition using the Cellomics automated confocal microscope (Thermo Scientific). In some experiments the v-ATPase inhibitor bafilomycin (500nM) was added to the cells 5 minutes prior to imaging. The average fluorescence ratio/cell/well between fluorescein-transferrin and AlexaFluor568-transferrin was calculated and converted to pH using a standard. To generate the standard, cells incubated with fluorescein and AlexaFluor568-conjugated transferrin were subjected to pH-clamp by incubation with high K+/10µM Nigericin (140mM KCl, 2mM CaCl₂, 1mM MgCl₂, 10mM glucose, 10µM nigericin, 20mM MES/HEPES pH 5.0-7.0) (Baxter and Church, 1996; Feldmann et al., 2007; Ohgaki et al., 2008; Rotin and Grinstein, 1989).
3. NHE5 protein is expressed in brain

3.1 Introduction

The goal of this work was to determine the physiological function of NHE5 in brain. The first step towards this goal was to determine the distribution of NHE5 protein within the brain, within individual cells and also during development. A detailed knowledge of the expression of NHE5 in space and time could yield strong clues as to the possible physiological function of NHE5. In previous reports, Northern blot and in situ hybridization analysis demonstrated robust NHE5 mRNA expression in neuron-rich structures of the brain, such as the hippocampus and cerebral cortex, but not in glia-rich structures such as the corpus callosum (Attaphitaya et al., 1999; Baird et al., 1999) suggesting that NHE5 may be neuron-specific.

In this chapter I describe my research demonstrating for the first time NHE5 protein expression in the brain. In particular, NHE5 protein could be detected in neurons from the hippocampus and cerebral cortex, as well as in epithelial cells lining the brain ventricles. In cultured hippocampal neurons NHE5 protein could be found distributed in axons and dendrites, and between endosomes and synapses. Last, NHE5 protein was found to be expressed in the brain of both embryonic and adult animals.

3.2 Results

3.2.1 Characterization of the anti-NHE5 polyclonal antibody

The distribution of NHE5 protein in brain has never before been examined due to the lack of isoform specific antibodies. Therefore, to begin analysis of NHE5 protein expression patterns in brain we developed a polyclonal antibody against the peptide EEPTQEPGPLGEPP corresponding to amino acids 21-34 in the first extracellular loop of
rat NHE5. The antibody recognized a single band of the expected size for NHE5 in rat brain lysates probed by Western blot. This signal could be completely removed by a neutralizing peptide (to which the antibody was raised, rat NHE5 21-34) while detection of NHE1 was not altered by the presence of NHE5 peptide, demonstrating the specificity of the antibody (Fig. 3-1A). Next, I transfected cultured hippocampal neurons with rat NHE5 or HA-tagged rat NHE1 and tested the anti-NHE5 antibody by immunofluorescence microscopy. In addition to detecting endogenous NHE5 protein, the anti-NHE5 antibody was able to detect exogenously expressed rat NHE5 but not rat NHE1 (Fig. 3-1B, 1C). These experiments demonstrate the specificity and effectiveness of the anti-NHE5 antibody.

3.2.1 NHE5 protein expression in brain

Using the newly developed anti-NHE5 antibody I first examined the distribution of NHE5 protein in brain by immunohistochemistry on coronal sections of adult mouse brain. Similarly to what was observed previously at the mRNA level NHE5 protein expression was observed in neuron-rich structures including the hippocampus, cerebral cortex and amygdala. Focusing on the hippocampus which has a very well defined morphology, I clearly observed NHE5 protein expression in CA1 - CA3 hippocampal pyramidal neurons, and dentate gyrus granule neurons (Fig. 3-2A, 2A’), as well as within the stratum radiatum layer where CA3 neurons synapse onto CA1 pyramidal cells (Fig. 3-2A’’). This result shows for the first time that NHE5 protein is expressed in neurons. NHE5 protein was not highly expressed in glia-rich structures such as the corpus callosum (Fig. 3-2B). Preliminary immunohistochemistry results on sagittal sections of adult rat brain using the anti-NHE5 antibody showed NHE5 protein expression in neurons of the hippocampus and cerebral cortex. This preparation also
Fig. 3-1. Characterization of the anti-NHE5 antibody. An anti-NHE5 antibody was raised against a peptide (rat NHE5 21-34) corresponding to the first extracellular loop of NHE5. 

(A) Rat brain lysate was probed by Western blot using anti-NHE5 or anti-NHE1 in the presence or absence of peptide (NHE5 21-34, 100ng/mL) to which the anti-NHE5 antibody was raised. The peptide competed with anti-NHE5 but not anti-NHE1 indicating the specificity of the antibody. 

(B,C) Cultured hippocampal neurons were transfected with rat NHE5 together with GFP (B), or with HA-tagged rat NHE1 (NHE1_{HA}, C). 

(B) Neurons were labelled with antibodies against NHE5. The NHE5 antibody is able to detect exogenously expressed NHE5 protein in transfected neurons which were identified by GFP expression and marked by an asterisk in addition to the endogenous NHE5 protein in the surrounding untransfected neurons. 

(C) Exogenously expressed NHE1_{HA} and endogenous NHE5 were detected with anti-HA and anti-NHE5 antibodies respectively. Transfected cells were identified by positive staining for NHE1_{HA}, and are marked by an asterisk. The NHE5 antibody does not detect NHE1 as non-transfected and transfected neurons have the same NHE5 staining intensity. Scale bar =20µm.
allowed us to visualize NHE5 protein in the cerebellum which was highly expressed in
cerebellar Purkinje cells (not shown). In addition to expression in neurons, we also
observed NHE5 protein expression in ependymal cells (Fig. 3-2C). These are epithelial
cells which make up the ependyma, an epithelial membrane that lines the brain
ventricular system. These cells are responsible for the production of cerebral spinal fluid
and the maintenance of brain volume. More specifically, NHE5 protein appeared to be
localized to the apical membrane of these cells, facing the lumen of the brain ventricles
(Fig. 3-2C'). These findings suggest that in brain, NHE5 may play specific functions in
neurons and ependymal cells.

3.2.2 NHE5 subcellular distribution in neurons

Now that NHE5 protein has been confirmed to be expressed in neurons my next
goal was to determine the subcellular distribution of NHE5 within neurons. Towards this
goal I visualized NHE5 by immunofluorescence microscopy and compared its
distribution to that of known markers of different subcellular compartments. NHE5
displayed a punctate staining pattern in the cell body and along neurite extensions.
NHE5 was expressed in neurites positive for either the dendrite marker MAP2 or the
axon marker tau protein, suggesting that NHE5 is present in both dendrites and axons
(Fig. 3-3). NHE5 puncta showed moderate overlap with the excitatory synapse markers
VGlut1 and PSD95 (Fig.3-4A), as well as the inhibitory synapse markers VGAT and
Gephyrin (Fig. 3-4B), suggesting that a portion of NHE5 protein in the neurites can be
targeted to synapses. Quantification of triple colocalization between NHE5 and pre- and
post-synaptic markers revealed that 39.8 ± 2.2% of excitatory synapses and 23.8 ± 1.3%
of inhibitory synapses were positive for NHE5 (Fig. 3-4C). While NHE5 is not a global
component of synapses, these results suggest that at steady state, NHE5 is targeted to
NHE5 protein is expressed in hippocampal neurons and other brain regions. (A-C) NHE5 protein was visualized in adult mouse brain coronal sections by immunohistochemistry. (A) Confocal images demonstrate NHE5 immunolabelling in the hippocampus CA1, CA3 and dentate gyrus; insets demonstrate NHE5 expression in CA1 pyramidal neurons of the hippocampus (A’) and stratum radiatum (A”). Scale bar =100μm, inset 10μm. (B) NHE5 expression is low in the glia-rich corpus callosum. Scale bar =100μm. (C) NHE5 is expressed in ependyma, inset highlights NHE5 localization to the apical membrane of ependymal cells, facing the brain ventricle lumen (C’). Scale bar =50μm, inset 10μm.
Fig. 3-3. NHE5 protein is localized to both axons and dendrites. (A,B) Confocal images of cultured rat hippocampal neurons immunolabelled for NHE5 and the dendrite marker MAP2 (A) or axon marker tau (B). Scale bar = 10 μm.
a subset of synapses. This finding is the first demonstration of NHE5 targeting to a specific specialized compartment and strongly suggests that NHE5 may play a function in controlling pH at the synapse.

Exogenously expressed NHE5 in non-neuronal cells is known to localize to recycling endosomes (Szaszi et al., 2002). Therefore it was of interest to determine whether endogenous NHE5 protein in neurons is also localized to endosomes. NHE5 protein partly overlapped with the recycling endosomal markers Rab11 and transferrin receptor as well as with early endosomal marker EEA1 (Fig. 3-5). This finding suggests that like ectopically-expressed NHE5 in non-neuronal cells, endogenous NHE5 protein in neurons traffics between the cell-surface and endosomes.

3.2.3 NHE5 expression during development

As a final experiment in this initial characterization of NHE5 expression I was interested in the possibility that NHE5 may play some role during development. Identifying a distinct expression pattern of NHE5 between embryonic and adult tissue may give further clues about the physiological role of NHE5. To address this, I examined the expression of NHE5 in rat brain lysate prepared from both embryonic (E18) and adult stages using Western blot. NHE5 protein was clearly detected in both embryonic and adult brain tissue (Fig. 3-6) suggesting that NHE5 protein is expressed during development and into adulthood. The anti-NHE5 antibody cross-reacts with mouse NHE5 and similar results were obtained from embryonic and adult mouse brain lysates (not shown). Curiously, NHE5 expression was approximately two-fold higher in adult brain compared to embryonic brain, raising the possibility that NHE5 may play a more important role in adult brain function. However, this data suggests that NHE5 function may not be specific to a certain developmental stage but rather NHE5 could
Fig. 3-4. NHE5 protein is targeted to synapses. (A,B) Confocal images of cultured rat hippocampal neurons immunolabelled for NHE5 and the excitatory pre- and postsynaptic markers VGlut1 and PSD95, respectively (A), or the inhibitory pre- and postsynaptic markers VGAT and Gephyrin (B). Scale bar =20\(\mu\)m, inset 5\(\mu\)m. Arrows indicate regions of triple co-localization between NHE5 and synapse markers suggesting synaptic localization of NHE5. (C) Synapses were defined as regions of colocalization between PSD95 and VGlut1 or Gephyrin and VGAT, and the percent of synapses positive for NHE5 was quantified. Data presented are the mean value (±s.e.m.) \(N=30\) randomly selected fields from 3 separate cultures for each synaptic subtype.
Fig. 3-5. Neuronal NHE5 protein is partly localized to endosomes. NHE5 was immunolabelled in cultured hippocampal neurons and its distribution was compared to that of the recycling endosome markers Rab11 (A) or transferrin receptor (TfnR, B), or the early endosome marker EEA1 (C). Arrows indicate regions of colocalization between NHE5 and endosome markers. Scale bar =5 μm.
**Fig. 3-6. NHE5 is expressed in developing and adult brain tissue.** Brain lysate was prepared from embryonic (E18) or adult rats. Equal amounts of brain protein were separated by SDS-PAGE and subjected to Western blot using antibodies to NHE5, CaMKIIα, α and β Tubulin, or actin. NHE5 shows a higher level of expression in adult brain tissue compared to embryonic tissue, similar to the expression pattern of CaMKIIα, shown previously to be expressed at higher levels in adults. Western blots shown are representative of three separate Western blots.
play a more broad function during development.

### 3.3 Discussion

NHE5 mRNA is known to be almost exclusively expressed in brain tissue. In addition, NHE5 mRNA was shown to be predominantly expressed in neuron-rich regions of the brain such as the hippocampus and cerebral cortex (Attaphitaya et al., 1999; Baird et al., 1999). However, the precise distribution of NHE5 protein in brain was unknown. In addition, due to lack of specific molecular probes the subcellular distribution of endogenous NHE5 protein within cells could not be determined in previous studies. Finally a physiological function for NHE5 in brain has never been described.

My first goal was to describe in detail the localization of NHE5 protein in brain and within cells, as well as to obtain some clue as to the developmental profile of NHE5 protein expression. These studies were enabled by the development of a specific, anti-NHE5 antibody. Using this tool it was determined that NHE5 protein is expressed in neurons from multiple brain regions including the hippocampus. In cultured hippocampal neurons NHE5 protein is present in punctate structures in the cell body, axons and dendrites. Subsets of these NHE5 puncta were contained within endosomes, both early and recycling endosomes. Importantly, a portion of NHE5 was targeted to synapses. Localization of NHE5 to synapses strongly suggests that the physiological function of NHE5 may be related to synapse physiology and synaptic transmission. This exciting possibility was explored through further experiments (chapter 4). NHE5 was found to be expressed both in the embryo and adult. This finding gives some clue that NHE5 function may include a role during embryogenesis such as synaptogenesis. This possibility will be discussed further in section 6.1.3 (NHE5 during development). Finally, NHE5 protein was also highly enriched in the apical membrane of ependymal cells, epithelial cells that line the brain ventricles. These cells are responsible for the
generation of cerebral spinal fluid (CSF), the medium which baths all cells in the central nervous system. This observation suggests that in addition to a function in neurons, NHE5 on the apical surface of brain ventricles may participate in the generation of CSF or in regulating the salt content and pH of CSF. Regulation of CSF pH and osmolarity has large implications for controlling neuronal excitability as well as volume regulation in the brain (Kempski, 2001; Jacobs et al., 2008). Such a role for NHE5 in salt and volume regulation is highly reminiscent of NHE3 function in the kidney where NHE3 is important for fluid and salt absorption. This will be discussed further in section 6.1.2 (NHE5 in ependymal cells).

In this work I pursued a possible synaptic function of NHE5. A role for NHE5 during development or in CSF production and ventricle volume regulation was not pursued further in this thesis work but may be an interesting and important avenue for future research.
4. NHE5 synaptic function

4.1 Introduction

Synapses are highly specialized structures and their molecular composition is tightly regulated. In chapter 3, I described the novel finding that a portion of endogenous NHE5 protein in cultured neurons could be targeted to synapses, suggesting the possibility that NHE5 functions as a regulator of synaptic pH. This finding may be the strongest clue to date as to the physiological function of NHE5.

In this chapter I describe my findings that modulation of pH by NHE5 can feedback on the synapse to control dendritic spine growth following synaptic activity. NHE5 is recruited to the synapse surface in dendritic spines in response to synaptic activity and raises the intracellular pH of spines. Activity-induced spine outgrowth, a hallmark of LTP (Engert and Bonhoeffer, 1999; Lang et al., 2004; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004), was prevented upon overexpression of NHE5. Conversely knockdown of NHE5 or expression of a transport-defective, dominant-negative, NHE5 mutant resulted in spontaneous exuberant spine outgrowth which could not be enhanced further following heightened synaptic activity. Treatment of cells with the NMDA receptor antagonist, D-AP5, blocked the increase in spine density following NHE5 knockdown or mutant expression suggesting that the effects of NHE5 on spines are mediated through NMDA receptors. By controlling local pH at the synapse, I propose that NHE5 is a negative regulator of spine growth which functions to stabilize spine growth after the induction of LTP by inhibiting pH-sensitive synaptic proteins such as NMDA receptors.
4.2 Results

4.2.1 NHE5 is recruited to excitatory synapses by synaptic activity

During LTP, synapses undergo extensive remodeling of both the actin cytoskeleton and membrane composition. The latter has been shown to require delivery of proteins and membrane from recycling endosomes in a Rab11-dependent pathway (Park et al., 2004; Park et al., 2006; Wang et al., 2008b). Previous studies have demonstrated that exogenously expressed NHE5 in non-neuronal cell lines traffics between the plasma membrane and recycling endosomes. I confirmed that in neurons, a portion of endogenous NHE5 is also found in recycling endosomes (Chapter 3, Fig. 3-5). Further, I show that NHE5 traffics between recycling endosomes and the plasma membrane via the Rab11-pathway (see Chapter 5). Therefore I speculated that NHE5 localization to synapses may be regulated by synaptic activity. To test this, synaptic activity was enhanced using a previously described cocktail of glycine, biccuculine and TTX (Lu et al., 2001). This paradigm has been used in hippocampal slices to induce chemical LTP (cLTP) (Musleh et al., 1997; Shahi et al., 1993) and glycine is used routinely to enhance activity in cultured neurons through activation of NMDA receptors (Lu et al., 2001; Park et al., 2006; Wang et al., 2008b). Hippocampal neurons cultured for 14DIV were treated with or without glycine (200µM) for 3 minutes to induce cLTP. 45 minutes after glycine stimulation, neurons were fixed and NHE5 localization at excitatory synapses was assessed. I noted a significant increase in the number of synapses positive for NHE5 following glycine treatment (45.1 ± 4.7% of synapses positive for NHE5 in untreated neurons; 73.3 ± 2.2% of synapses positive for NHE5 in glycine-treated neurons, p<0.01, Fig. 4-1).

To determine whether NHE5 was specifically targeted to the plasma membrane following activity, we took advantage of the fact that the anti-NHE5 antibody, raised
Fig. 4-1. NHE5 is recruited to excitatory synapses following glycine treatment. (A,B) Cultured hippocampal neurons (14DIV) were left untreated (A) or treated with 200μM glycine for 3 minutes (B). 45 minutes following glycine treatment neurons were fixed and immunostained using antibodies to NHE5, PSD95 and VGlut1. Scale bar =5μm. (C) The percent of excitatory synapses positive for NHE5 in neurons without or with glycine treatment was quantified. Data presented are the mean value from 30 randomly selected fields (±s.e.m.). ** indicates statistical significance, p<0.01, from untreated control, Student’s t-test.
against the first extracellular loop of NHE5, could specifically recognize surface NHE5 in fixed neurons by immunofluorescence microscopy under non-permeabilized conditions. I found that at steady state very little NHE5 is present at the cell-surface, and that surface NHE5 was almost exclusively localized to synapses as shown by colocalization of surface NHE5 with PSD95 and VGlut1 (Fig. 4-2A). Following glycine treatment, there was a dramatic increase in the number and intensity of NHE5 puncta at the cell-surface (Fig. 4-2B). Similar to that observed at steady state, surface NHE5 was almost exclusively localized to synapses (Fig. 4-2A, 2B). Indeed, there was a significant increase in the number of synapses positive for surface NHE5 following glycine treatment compared to untreated cells (Fig. 4-2C). To quantify surface NHE5 levels following glycine treatment, surface NHE5 was detected using a cell-surface biotinylation assay. There was a marked increase in surface NHE5 in neurons treated with glycine compared to untreated cultures (Fig. 4-2D, 2E). Notably, this increase was specific as no change in surface or total expression of NHE1 was observed following glycine treatment (Fig. 4-2D, 2E). These findings suggest that NHE5 traffics between intracellular endosomes and the surface of synapses in an activity-dependent manner.

4.2.2 NHE5 is delivered to dendritic spines following synaptic activity

To analyze the activity-dependent trafficking of NHE5 by live-cell imaging, an NHE5-GFP fusion construct was prepared (see Chapter 2) and expressed in primary neurons. Similarly to endogenous NHE5 protein, NHE5-GFP fusion protein could be found along dendrites and entering into dendritic spines (Fig. 4-3) demonstrating that the intracellular localization of NHE5-GFP is similar to that of endogenous NHE5 at steady
Fig. 4-2. NHE5 is recruited to synapses and to the plasma membrane following glycine treatment. (A,B) Confocal images of hippocampal cultures left untreated (A) or treated with glycine for 3 minutes (B), and immunolabelled for surface NHE5 and the pre- and postsynaptic markers VGlut1 and PSD95, respectively. NHE5 was immunolabelled with the anti-NHE5 antibody under non-permeable conditions, followed by permeabilization and immunolabelling of synaptic markers. Scale bar =20 μm, inset 5 μm. (C) Synapses were defined as regions of colocalization between PSD95 and VGlut1 and the percent of excitatory synapses positive for surface NHE5 was quantified. Data presented are the mean value (±s.e.m.). N= 30 randomly selected fields from 3 separate cultures **Student’s t-test, p<0.01. (D,E) Cultured hippocampal neurons were treated with or without glycine followed by cell surface biotinylation. There is a dramatic increase in biotinylated, surface NHE5, but not NHE1 following glycine treatment. (E)
Surface NHE1 and NHE5 from glycine treated neurons were normalized to untreated control lanes. N= 3 independent experiments from 3 separate cultures. ** indicates statistical significance, p<0.01, from untreated control, Student’s t-test.
Endogenous NHE5 is targeted to dendritic spines similar to exogenous NHE5-GFP. (A) Hippocampal neurons were transfected with GFP as a cell-fill and endogenous NHE5 protein was visualized by immunofluorescence microscopy. Alternatively, neurons were transfected with RFP together with an NHE5-GFP fusion. In resting cells endogenous NHE5 and exogenous NHE5-GFP were noted at the base of dendritic spines and entering the spine neck (examples are indicated by arrow heads) or in the dendritic spine head (arrows). Scale bar =5 μm. (B) Spines identified from GFP or RFP cell-fill were scored for content of endogenous NHE5 or NHE5-GFP respectively either at the spine base or in the spine neck or head (mean values of 20 transfected neurons ±s.e.m.). While more spines contain NHE5-GFP than endogenous NHE5, the overall distribution of endogenous NHE5 and exogenous NHE5-GFP is comparable showing that NHE5-GFP traffics to spines similarly to the endogenous protein.
Fig. 4-4. NHE5-GFP is recruited to dendritic spines following glycine treatment. (A) Time-lapse confocal images of 14DIV hippocampal neurons transfected with RFP plus NHE5-GFP and treated with glycine for 3 minutes. Three examples are provided (a1-a3). Dendrites and spines were visualized using the RFP cell fill and are depicted as a dashed outline for clarity. Before glycine treatment NHE5-GFP is localized primarily at the base of spines in dendrites. 20 and 45 minutes after glycine treatment NHE5-GFP accumulates in spines in a time dependent manner. Bar =1μm. (B) Localization of NHE5-GFP 5 minutes before or 20 and 45 minutes after glycine treatment. Spines were scored for content of NHE5-GFP at the base of the spine, in the spine neck, or in the spine head proper. N>100 spines from each of 3 neurons from 3 separate cultures. *p<0.05, Student's t-test.
state. By using NHE5-GFP and RFP as a cell fill, the distribution of NHE5-GFP was examined before and after glycine stimulation using time-lapse microscopy (Fig. 4-4A). Prior to glycine treatment, NHE5-GFP was distributed in the dendritic shaft, at the base of dendritic spines, in the spine neck, or in the spine head proper (Fig. 4-4B). 20 and 45 minutes following glycine treatment NHE5-GFP translocated from the base of the spine to the spine neck and spine head (Fig. 4-4A). Quantification of the distribution of NHE5-GFP at spines revealed a time-dependent decrease in the proportion of spines that displayed NHE5-GFP at their base or neck and a concomitant increase in the proportion of spines that displayed NHE5-GFP in the spine head (Fig. 4-4B). Together, these data strongly suggest that NHE5 is recruited to postsynaptic spines and particularly to the plasma membrane in an activity-dependent manner.

4.2.3 Perturbation of NHE5 by shRNA-mediated knock-down or by dominant-negative mutant expression

As NHE5 is recruited to dendritic spines following glycine stimulation, I next wished to test whether NHE5 plays a role in activity-induced dendritic spine growth. In order to test this possibility two strategies were developed to perturb the activity of endogenous NHE5, either by knocking down NHE5 using short-hairpin RNA (shRNA), or by introducing a dominant-negative NHE5 mutant that lacks ion transport activity. Three independent shRNA constructs (shA, B and C) specifically targeting rat NHE5 were generated and their effectiveness was tested by stably expressing these constructs in rat neuroendocrine PC12 cells. Compared to untransfected PC12 cells, cells expressing shA-C showed up to 80% knock-down of endogenous NHE5 but no change in endogenous NHE1 (Fig. 4-5), showing that these shRNA constructs are able to effectively and specifically deplete NHE5.
Fig. 4-5. Characterization of NHE5 knock-down by shRNA. (A) Lysates from untransfected PC12 cells or PC12 cells stably expressing NHE5 shRNA constructs A, B or C were collected, and Western blot was used to measure the amount of NHE5 and NHE1 and Actin as a loading control. Despite an approximately 80% decrease in NHE5 protein levels in shRNA A-C lysates (B), NHE1 levels remain unchanged, demonstrating the specificity of the shRNA constructs. (B) Western blots in A) were analyzed by densitometry and normalized to untransfected PC12 cells and represent the mean of three independent western blots (±s.e.m.).
NHE proteins from various species contain highly conserved acidic amino acid residues in their transmembrane domains that are believed to be essential to perform their ion translocation activity (Bowers et al., 2000) (Fig. 4-6A). Mutation of a highly conserved glutamate in human NHE1 (E266) to isoleucine was shown to abolish its ion transport activity and cause dominant-negative effects (Busco et al., 2010; Hisamitsu et al., 2006). The corresponding mutation of highly conserved glutamate 209 to isoleucine was generated in HA-tagged human NHE5 (NHE5<sub>HA</sub>E209I). When expressed in NHE-deficient AP-1 cells, NHE5<sub>HA</sub>E209I showed comparable levels of protein expression as wild-type NHE5<sub>HA</sub> (NHE5<sub>HA</sub>WT) or NHE1<sub>HA</sub>. Further, there was no evidence that the mutant protein was being degraded at an accelerated rate suggesting that the E209I mutation does not render the protein unstable (Fig. 4-6B). To confirm that the E209I mutation did not impair normal NHE5 trafficking, I compared the distribution of wild-type and mutant NHE5. AP-1 cells expressing NHE5<sub>HA</sub>WT or NHE5<sub>HA</sub>E209I were incubated with anti-HA antibody, which can access the extracellular HA-tag, together with fluorescein-conjugated transferrin to label recycling endosomes, for 30 minutes at 37°C. Cells were then fixed and labeled with fluorescent secondary antibodies. Positive HA-staining confirmed that both NHE5<sub>HA</sub>WT and NHE5<sub>HA</sub>E209I were targeted to the cell surface where they could access extracellular anti-HA antibody, and subsequently underwent endocytosis to enter transferrin-positive recycling endosomes (Fig. 4-6C). These results show that NHE5 E209I is stable and undergoes normal cellular trafficking. For comparison, NHE1<sub>HA</sub> appears restricted to the cell surface and does not enter recycling endosomes (Fig. 4-6C). Next, I tested the effect of the E209I mutation on NHE5 ion-transport activity. AP-1 cells expressing wild-type or mutant NHE5 were loaded with the pH-sensitive dye BCECF and subsequently subjected to a cytosolic acid load by a brief treatment with ammonium followed by washout with Na<sup>+</sup>-
Fig. 4-6. Characterization of the NHE5 E209I point mutant: expression and trafficking. (A) Amino acid sequences of predicted transmembrane domain 6 of NHE proteins from different species. A highly conserved glutamate residue E209 from human NHE5 is indicated. (B) Lysates from NHE-deficient AP-1 cells stably expressing HA-tagged NHE1, NHE5 WT or NHE5 E209I were collected and Western blots probed with an anti-HA antibody. (C) Live AP-1 cells expressing HA-tagged NHE5 WT, NHE5 E209I, or NHE1 were incubated with fluorescein-transferrin (50μg/mL) and anti-HA antibody followed by fixation and visualization using fluorescently conjugated secondary antibodies. Bar =10μm.
Fig. 4-7. Characterization of the NHE5 E209I point mutant: ion transport activity. (A) AP-1 cells expressing HA-tagged NHE5 WT or E209I were loaded with the pH-sensitive dye BCECF. Cells were acidified by a brief treatment with 50mM NH₄Cl and Na⁺/H⁺ exchange was subsequently induced by reintroducing Na⁺. Traces of pH shown are the mean values obtained from simultaneous measurements on a single coverslip of 56 and 49 cells expressing NHE5 WT and NHE5 E209I respectively, and are representative of at least four independent experiments. (B) To determine whether transport-deficient NHE5 E209I is a dominant negative protein Na⁺-dependent recovery from and acid load was measured in AP-1 cells stably expressing HA-tagged NHE5 WT transfected with GFP alone (NHE5 WT) or together with NHE5 E209I (NHE5 WT/E209I). Transfected cells identified from GFP were subjected to an acid load as in A, and subsequent Na⁺/H⁺ exchange was monitored. Instantaneous proton efflux was then plotted against absolute pHᵢ values and results from two groups were compared statistically (Student's t-test) at corresponding values of pHᵢ. Continuous lines represent the non-linear least-squares regression fits to the data points (mean ± s.e.m.). In each case, data points were obtained from 4 independent experiments. Compared to cells expressing NHE5 WT only, proton efflux was significantly reduced at all pHᵢ values (p<0.05) in cells co-expressing NHE5 E209I indicating that the transport deficient mutant has dominant negative properties.
free buffer. Reintroduction of Na\(^+\) to cells expressing NHE5\(_{HA}\)WT resulted in robust Na\(^+\)/H\(^+\) exchange that restored cytosolic pH to neutral levels (**Fig. 4-7A**). In contrast, cells expressing NHE5\(_{HA}\)E209I showed no recovery of cytosolic pH showing that the E209I mutation completely abolished NHE5 ion transport activity (**Fig. 4-7A**). More importantly, I found that co-expression of NHE5\(_{HA}\)E209I and NHE5\(_{HA}\)WT in AP-1 cells significantly reduced the Na\(^+\)-dependent H\(^+\)-efflux compared to cells expressing NHE5\(_{HA}\)WT alone (**Fig. 4-7B**) showing that NHE5\(_{HA}\)E209I impaired the activity of wild-type NHE5 and is therefore a dominant-negative protein. Upon demonstration of the effectiveness of the NHE5 shRNA and dominant-negative constructs it was then possible to proceed to test the role of NHE5 in activity-dependent dendritic spine growth.

**4.2.4 NHE5 is a negative regulator of dendritic spine growth**

In order to test the involvement of NHE5 in dendritic spine growth, cultured neurons were transfected with GFP alone, or together with NHE5 (WT or E209I) or the shA-C constructs at 9DIV and the number and size of dendritic spines was analyzed at 14DIV. Overexpression of NHE5 did not significantly alter the density or size of dendritic spines compared to GFP-transfected controls. Conversely, NHE5 shRNA expressing cells exhibited a significant increase in the density and average head width of dendritic spines (**Fig. 4-8A, 8B**). Expression of shRNA-resistant human NHE5 rescued these effects (**Fig. 4-8A, 8B**), suggesting the spine phenotypes observed in these NHE5 shRNA expressing cells are not due to off-target effects.

Similar to the effects following NHE5 knock-down, expression of dominant-negative NHE5 significantly enhanced dendritic spine density and head width. Moreover, the expression of either mutant NHE5 or wild-type NHE1 could not rescue the spine growth effects following NHE5 knock-down, (**Fig 4-8A, 8B**). Together these
findings suggest that NHE5 is a negative regulator of dendritic spine growth, and that this requires NHE5-specific transporter activity.

NMDA receptor activation is an important step leading to changes in dendritic spine morphology and number (Engert and Bonhoeffer, 1999; Lang et al., 2004; Malenka and Bear, 2004; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004). Since NMDA receptors are inhibited by an increase in extracellular protons (Banke et al., 2005; Tang et al., 1990; Traynelis and Cull-Candy, 1990), I postulated that NHE5 recruited to the spine surface during activity may act as a local proton source to regulate NMDA receptor activity via local Na+/H+ exchange. To test this possibility, I treated transfected neurons with or without the NMDA receptor antagonist D-AP5 (100μM) for 48 hours. Interestingly, treating cells with D-AP5 for 48 hours did not impact dendritic spine density or width in cells expressing GFP alone or cells overexpressing wild-type NHE5, suggesting that maintenance of dendritic spines at a basal level does not require continued signaling through NMDA receptors. However, D-AP5 treatment completely abolished the increase in spine density and width observed in cells expressing NHE5 shRNA or NHE5HA>E209I (Fig. 4-9A, 9B). This suggests that NHE5 typically limits exuberant spine growth by constraining NMDAR activity. NMDA receptor activity is typically low at rest due to the Mg2+ ion block (Nowak et al., 1984). However, recent studies have demonstrated signaling through NMDA receptors during spontaneous synaptic transmission (Espinosa and Kavalali, 2009; Jung et al., 2008; Sutton et al., 2004; Sutton et al., 2006). My findings suggest that abrogating NHE5 activity may reduce a local proton source at the synapse and increase this spontaneous NMDA receptor signaling leading to the observed NMDA receptor-dependent exuberant spine growth.
Fig. 4-8. NHE5 is a negative regulator of spine density and head width. (A) Confocal images of 14 DIV cultured hippocampal neurons transfected at 9DIV with GFP alone or together with HA-tagged human NHE5 wild type (NHE5\textsubscript{HA} WT) or transport-dead mutant (NHE5\textsubscript{HA} E209I), or shRNA constructs (shA-C). Scale bar =10\textmu m. (B) The mean spine density and head width (±s.e.m.) was measured in transfected neurons. N=24-37 neurons per condition from 3 separate cultures. ** indicates statistical significance, p<0.01, from GFP transfected control, Student’s t-test.
Fig. 4-9. Spine growth associated with reduced NHE5 activity requires active NMDA receptors. (A) Confocal images of 14 DIV cultured neurons transfected at 9DIV with GFP alone or together with the indicated construct. Transfected neurons were left untreated or treated with 100 μM D-AP5 (added to the culture medium) for 48 hours prior to fixation at 14DIV. Scale bar = 10 μm. (B) The mean dendritic spine density and head width (±s.e.m.) in untreated (grey bars) and D-AP5 treated neurons (black bars) were measured. N=19-46 neurons per condition from 3 separate cultures. **p<0.01 Student’s t-test.
4.2.5 NHE5 and activity-dependent spine growth

I next investigated the role of NHE5 in activity-induced spine growth. Hippocampal neurons were transfected with wild-type NHE5, dominant-negative mutant NHE5 (NHE5 E209I) or NHE5 shRNA. At 14DIV neurons were treated with glycine or control solution, and cells were fixed and analyzed 60 minutes later. In control GFP-expressing neurons, glycine treatment resulted in a significant increase in the density and head width of dendritic spines (Fig. 4-10A, 10B). This activity-induced enhancement of spine density and width was abolished in cells pre-treated for 30 minutes with the NMDA receptor antagonist, D-AP5 (100μM), confirming previous reports that the glycine treatment paradigm mediates its effects through activation of NMDA receptors (Lu et al., 2001; Park et al., 2006) (Fig. 4-10B). Overexpression of NHE5 completely abolished activity-induced enhancement of spine density but not activity-induced increases in dendritic spine head width (Fig. 4-10A, 10B). Expression of NHE5_HA E209I or NHE5 shRNA significantly increased the basal dendritic spine density and width as shown above, however no significant enhancement of spine density or head width was observed following glycine treatment (Fig. 4-10A, 10B). Pre-treatment with D-AP5 for 30 minutes, unlike prolonged 48 hour treatment, was not sufficient to cause retraction of spines formed in NHE5 shRNA or mutant-expressing cells. These findings suggest that NHE5 activity is important for limiting activity-dependent formation of dendritic spines and that abrogating NHE5 function is sufficient to trigger spontaneous dendritic spine growth that cannot be enhanced further by neuronal activity.
Fig. 4-10. NHE5 and activity-dependent spine growth. (A) Confocal images of 14 DIV cultured neurons transfected at 9DIV with GFP alone or together with the indicated construct. Neurons were either left untreated or treated with glycine for 3 minutes to enhance activity, and imaged 60 minutes post-glycine. As a negative control neurons were treated with D-AP5 (100μM) for 30 minutes before and during glycine treatment. Scale bar =10μm. (B) The mean dendritic spine density and head width (±s.e.m.) in untreated (grey bars), glycine treated (black bars) and glycine/D-AP5 treated neurons (white bars) were measured. N=17-28 neurons from 3 separate cultures. *p<0.05 and **p<0.01, Student’s t-test.
4.2.6 Activity- and NHE5-dependent changes in dendritic spine pH

My results demonstrating the activity-dependent recruitment of NHE5 to dendritic spines and to the synaptic surface suggest the possibility that there may be an associated activity-dependent change in spine pH. This idea is further supported by my finding that the increased spine growth caused by perturbing NHE5 requires an active NMDA receptor, a pH-sensitive protein. To test this possibility, I transfected neurons with a pH-sensitive variant of GFP (de4GFP) (Hanson et al., 2002; McAnaney et al., 2002; Vilas et al., 2009) alone or together with shRNA to knock-down NHE5 and monitored the fluorescence intensity of dendritic spines before and after glycine-stimulation. In control experiments Chinese hamster ovary (CHO) cells were transfected with de4GFP and the fluorescence was monitored under pH-clamp conditions using the protonophore Nigericin (high K⁺, 10μM Nigericin). I found a linear correlation between intracellular pH and de4GFP fluorescence (Fig. 4-11A, 11B). In transfected neurons, changes in the fluorescence intensity (ΔF) of dendritic spines were determined with time after glycine treatment by normalizing to the fluorescence intensity of the same spine immediately prior to glycine treatment. Using the pH-fluorescence correlation I was able to convert subsequent experimentally measured changes in fluorescence to changes in pH (ΔpH). In control de4GFP expressing neurons, I noted a time-dependent increase in dendritic spine pH beginning 5-10 minutes after glycine treatment (compared to pre-glycine levels, spine ΔpH control: 5 minutes, +0.01 ± 0.04; 10 minutes, +0.19 ± 0.06; 60 minutes, +0.35 ± 0.04, Fig. 4-11C, 11D). This activity-induced alkalinization is consistent with the recruitment of NHE5 to dendritic spines and induction of Na⁺/H⁺ exchange. In contrast, in NHE5 knock-down cells, there was an immediate drop in spine pH following glycine treatment which recovered to pre-glycine levels after 60 minutes (spine ΔpH shA: 5 minutes, -0.28 ± 0.07; 10 minutes, -0.23 ± 0.07; 60 minutes, +0.03 ±
Notably, the enhanced spine pH observed in control cells was not reached, suggesting that activity-dependent spine alkalinization requires NHE5. The transient acidification observed in glycine-treated shRNA expressing cells was similar to previously observed drops in intracellular pH in neurons treated with other depolarizing stimuli such as application of high K⁺ or NMDA (Zhan et al., 1998). In a control experiment, we monitored de4GFP fluorescence in dendritic spines in control and shRNA expressing neurons for 60 minutes and noted no substantial fluctuations (Fig. 4-11E), suggesting that spine pH is stable in the absence of activity, even when NHE5 is knocked-down. Altogether, these results suggest that synaptic activity results in the recruitment of NHE5 to the dendritic spine surface resulting in activity and NHE5-dependent changes in dendritic spine pH.
Fig. 4-11. NHE5 acutely regulates dendritic spine pH during synaptic activity. (A) Confocal images of CHO cells transfected with de4GFP and placed under pH-clamp conditions (high K⁺, 10µM Nigericin). Sample images of cells pseudocolored for fluorescence intensity under pH clamp: pH 7.0, 7.5, and 8.0 are shown. Scale bar =20µm. (B) Quantification of de4GFP fluorescence intensity versus intracellular pH from transfected CHO cells pH clamped from pH 6.75-8.0. Fluorescence was normalized to fluorescence intensity observed in cells pH-clamped to pH 7.0. N=132-273 cells for each pH value tested. (C-E) Neurons were transfected with pH-sensitive de4GFP alone (control) or together with NHE5 shRNA A (shA) and treated with glycine for 3 minutes to enhance activity. (C) Confocal images of hippocampal neurons pseudocolored for fluorescence intensity. Insets highlight spines with notable fluorescence changes. Scale bar =5µm; insets 1µm. (D) Fluorescence intensity from each dendritic spine was monitored with time after glycine treatment and normalized to pre-glycine levels. The change in fluorescence intensity was converted to ΔpH using the linear relation between
relative fluorescence and pH determined in (B). The dashed line represents the pre-glycine dendritic spine resting pH. N= >50 spines each from 8-9 neurons per condition from 3 separate cultures. (E) As a control for normal fluctuations in spine pH over time, cells were imaged for 60 minutes without glycine treatment. No substantial fluctuations were observed. N= >75 spines each from 3-4 neurons per condition from 2 cultures.
4.3 Discussion

Most forms of LTP require activation of synaptic NMDA receptors (Malenka and Bear, 2004) and subsequent calcium influx. This initiates a signaling program resulting in AMPA receptor recruitment to synapses (Shepherd and Huganir, 2007) and formation of new dendritic spines and spine growth (Engert and Bonhoeffer, 1999; Lang et al., 2004; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004). As several studies have highlighted the pH-sensitive nature of the NMDA receptor, local pH is likely to play an important function in NMDA receptor-dependent synaptic plasticity. Unlike the classic magnesium ion block which reduces open channel current (Nowak et al., 1984; Kumamoto, 1996), protons have been shown to reduce the probability of channel opening by interaction at a unique proton-binding site, completely distinct from other ligand binding sites (Banke et al., 2005; Tang et al., 1990; Traynelis and Cull-Candy, 1990). The sensitivity of the NMDA receptor to protons has a pKa of 7.3-7.5, such that at resting pH (close to 7.3) a tonic proton block exists, which maintains NMDA receptor activity to about 50% (Banke et al., 2005; Tang et al., 1990; Traynelis and Cull-Candy, 1990). The implication of this is that any deviation in local pH will have an immediate and profound impact on NMDA receptor activity and possibly synaptic plasticity. However, NMDA receptor modulation by protons in the context of synaptic plasticity has not been well studied. Further, the mechanisms that control local pH at the synapse and the identity of synaptic pH-regulating proteins have not been determined.

In chapter 3 I described the finding that at steady state NHE5 can be targeted to a subset of synapses. In this chapter I show that following glycine treatment to enhance synaptic activity through NMDA receptor activation, NHE5 is targeted to the cell surface and recruited to synapses where NHE5 initiates a local pH change through Na⁺/H⁺ exchange. Further, my data suggest that NHE5 is a negative regulator of activity-
induced dendritic spine growth. Based on these findings, I propose a model in which NHE5 is recruited to the synaptic plasma membrane in dendritic spines following synaptic activity. Once at the plasma membrane, NHE5 Na⁺/H⁺ exchanger activity results in alkalinization of the dendritic spine and concomitant acidification of the synaptic cleft. This local acidification serves as a negative feedback mechanism that regulates pH-sensitive proteins in the synaptic cleft (Fig. 4-12). The NMDA receptor is of particular interest given its sensitivity to protons and important role in dendritic spine remodeling. Overexpression of NHE5 may enhance the tonic proton block of the NMDA receptor preventing activity-dependent formation of new spines. In support of this, a previous study found that high frequency stimulation of Schaffer collaterals failed to induce hippocampal CA1 LTP when brain slices were incubated in acidified media, likely a result of NMDA receptor inhibition (Velisek, 1998). Conversely, reducing the local proton-source by NHE5 knock-down or expression of a dominant-negative mutant may lead to hyper-activation of the NMDA receptor. This could effectively lower the threshold needed to induce LTP such that spontaneous network activity is sufficient to drive dendritic spine expansion to an LTP-like state. Consistent with this idea, perturbation of NHE5 function led to a significant and spontaneous increase in dendritic spine density and width, which was not enhanced further by glycine treatment, but was completely reversed by prolonged treatment with the chemical NMDA receptor antagonist, D-AP5. In addition, spine alkalization induced by glycine treatment was also prevented by knock-down of NHE5. Therefore, in my model NHE5 acts as part of a negative feedback loop to stabilize dendritic spine growth following NMDA receptor activation through a local pH shift at the synapse (Fig. 4-12). Interestingly, we showed previously that NHE5 could directly interact with the signaling scaffold molecule Receptor for Active C-Kinase (RACK1) and that this interaction enhanced NHE5 transporter activity (Onishi et al., 2007). RACK1 is also known to bind to the C-terminus of the NR2B subunit of the
NMDA receptor, an interaction shown to inhibit the NMDA receptor (Yaka et al., 2002). One possibility is that RACK1 may tether NHE5 adjacent to the NMDA receptor, through the NR2B subunit, making NHE5-transported protons a highly potent NMDA receptor inhibitor.

A recent study by Ronicke et al. (2009) showed that application of EIPA, an NHE-inhibitor drug during tetanus induction of LTP in hippocampal CA1 neurons resulted in enhanced LTP, suggesting that an NHE protein is a negative-regulator of synapse plasticity. While that study did not identify the specific NHE isoform involved, the concentration of EIPA used is sufficient to block NHE5. Our current findings together with the work of Ronicke et al. (Ronicke et al., 2009) suggest that NHE5 is a negative regulator of synaptic plasticity.

Here I present evidence that NHE5 is a novel regulatory molecule involved in synaptic plasticity and dendritic spine remodeling. Proton-sensitivity of key components of the synaptic machinery has been established. However, the identity of pH-regulating proteins at the synapse remains largely undetermined. NHE5 may be the very first example of a synaptic pH-regulating molecule whose synapse and cell-surface targeting is controlled in an activity-dependent manner. As several components of the synaptic machinery are proton-sensitive, local pH regulation at the synapse and NHE5 activity may impact on multiple aspects of synapse physiology and warrants continued investigation. Further implications of local regulation of pH at the synapse by NHE5 will be discussed in Chapter 6.
Fig. 4-12. Model for how NHE5 acts as a negative feedback molecule to limit activity-mediated spine growth.
5. Molecular regulation of NHE5

5.1 Introduction

In the previous chapters, I describe the novel findings that NHE5 can be targeted to synapses and plays a role in regulating the growth of dendritic spines in response to synaptic activity. This later finding is likely a result of an NHE5-dependent shift in local pH that can feedback onto nearby pH-sensitive components of the synaptic machinery. One of the major questions arising from these findings regards the molecular regulation of NHE5. How is NHE5 delivered to the synaptic surface in response to synapse stimulation? What regulates NHE5 transporter activity, and how is NHE5 organized in space with respect to other synaptic proteins such as the NMDA receptor?

Very few if any proteins work in isolation. Therefore identification and characterization of binding proteins often reveal novel functions and regulation mechanisms of the protein of interest. To begin to address the molecular regulation of NHE5, I have focused on the identification and characterization of novel NHE5-binding proteins. Previously β-arrestins, multifunctional scaffold proteins that play a key role in desensitization of G-protein coupled receptors (GPCRs), were shown to directly bind to NHE5 and promote its endocytosis (Szabo et al., 2005). This study demonstrated that NHE5-trafficking between endosomes and the plasma membrane is regulated by protein-protein interactions with scaffold proteins. More recently, we demonstrated that Receptor for Activated C-Kinase 1 (RACK1), a scaffold protein that links signaling molecules such as activated C-kinase, integrins and Src kinase (Sklan et al., 2006), directly interacts with and activates NHE5 via integrin-dependent and independent

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1 A version of this chapter has been published

pathways (Onishi et al., 2007). These results further indicate that NHE5 is partly associated with focal adhesions and that it’s targeting to a specialized microdomain of the plasma membrane may be regulated by various signaling pathways.

Secretory Carrier Membrane Proteins (SCAMPs) are a family of evolutionarily conserved tetra-spanning integral membrane proteins. SCAMPs are found in multiple organelles such as the Golgi apparatus, trans-Golgi network (TGN), recycling endosomes, synaptic vesicles, and the plasma membrane (Castle and Castle, 2005; Fernandez-Chacon and Südhof, 2000) and have been shown to play a role in exocytosis (Fernandez-Chacon et al., 1999; Guo et al., 2002; Liu et al., 2002; Liu et al., 2005) and endocytosis (Fernandez-Chacon et al., 2000). Currently, five isoforms of SCAMP have been identified in mammals. The extended N-terminus of SCAMP1-3 contain multiple Asn-Pro-Phe (NPF) repeats which may allow these isoforms to participate in clathrin coat assembly and vesicle budding by binding to Epsin homology (EH)-domain proteins (Montesinos et al., 2005; Polo et al., 2003). Further, SCAMP2 was shown recently to bind to a small GTPase Arf6 (Liu et al., 2005), which is believed to participate in traffic between the recycling endosomes and the cell surface (D’Souza-Schorey and Chavrier, 2006; Jones et al., 2006). More recent studies have suggested that SCAMPs bind to organellar membrane type NHE7 (Lin et al., 2005) and the serotonin transporter SERT (Muller et al., 2006) and facilitate targeting of these integral membrane proteins to specific intracellular compartments. I show here in chapter 5 that SCAMP2 binds to NHE5, facilitates the cell surface targeting of NHE5 and elevates Na⁺/H⁺ exchange activity at the plasma membrane, whereas expression of a SCAMP2 deletion mutant lacking the N-terminal domain containing the NPF repeats suppresses the effect. Further I show that this activity of SCAMP2 requires an active form of a small GTPase Arf6, but not Rab11. Rab11 can also promote the delivery of NHE5 to the cell-surface, independently of SCAMP2. I propose a model in which SCAMPs bind to NHE5 in the
endosomal compartment and control its cell surface abundance via an Arf6-dependent pathway.

5.2 Results

5.2.1 SCAMPs are novel binding partners of NHE5

Dr. Paulo Lin, a former member of the Numata lab, showed previously that SCAMPs directly bind the cytosolic C-terminal extension of the organelle-enriched NHE7 isoform and govern its intracellular trafficking between the TGN and recycling endosomes (Lin et al., 2005). NHE7 shuttles between the TGN, plasma membrane and recycling endosomes via the clathrin-dependent pathway (Lin et al., 2007). Similarly, NHE5 is internalized through the clathrin-dependent pathway and is predominantly associated with recycling endosomes following endocytosis (Szaszi et al., 2002). Thus, I reasoned that SCAMPs might also bind to NHE5 and regulate its targeting. To test this possibility, I first carried out co-immunoprecipitation using rat PC12 cells stably expressing 1D4-tagged NHE5 (PC12/NHE51D4). PC12 cells are widely used as a neuronal model system and endogenous expression of SCAMP1, 2 and 5 was observed by western blot (Fig. 5-1A; Lin et al., 2005). A membrane enriched fraction was prepared from PC12 or PC12/NHE51D4 cells and subjected to immunoprecipitation using 1D4 antibody coupled to sepharose beads. SCAMP1 and SCAMP2, but not SCAMP5, were readily detectable in the immunoprecipitate from PC12/NHE51D4 cells (Fig. 5-1A). Although equivalent levels of SCAMP expression were seen in lysates from both PC12 and PC12/NHE51D4 cells, SCAMPs were undetectable in the immunoprecipitated samples from untransfected PC12 cells.

Next I examined whether the SCAMP-NHE5 interaction was also found in brain tissue. Endogenous expression of NHE5 and SCAMP2 protein in rat brain was first
confirmed by western blot (Fig. 5-1B). Rat brain lysate was then subjected to immunoprecipitation using anti-NHE5 antibodies or pre-immune serum. A distinct band of approximately 37 kDa in size corresponding to SCAMP2 was detected by western blot in the lysate immunoprecipitated with anti-NHE5 antibody (IP, Fig. 5-1C), but not the lysate incubated with pre-immune serum (Con, Fig 5-1C) suggesting the existence of a SCAMP2-NHE5 complex in brain.

5.2.2 Determination of the NHE5-binding site of SCAMP2

The C-terminal cytosolic extension of NHE proteins serves as a major protein-protein interaction domain and most of the previously identified NHE-binding proteins were shown to bind to this domain (Orlowski and Grinstein, 2004). SCAMPs contain possible protein-protein interaction interfaces in the N-terminal and C-terminal cytosolic extensions as well as the cytoplasmic loop between the second and third transmembrane domains (Guo et al., 2002; Hubbard et al., 2000). To test whether these cytosolic domains of SCAMP2 and NHE5 directly interact, I performed in vitro GST pull-down protein-binding experiments. Immobilized GST alone or GST-SCAMP2 fusion proteins were incubated with in vitro transcribed/translated 35S-labeled NHE5 C-terminus (amino acids 492-896). NHE5 bound to the immobilized GST fusion proteins was eluted, resolved by SDS-PAGE and detected by phosphorimager. Radiolabeled NHE5 protein exhibited a specific association with the GST-tagged SCAMP2 N-terminus and C-terminus but not the cytosolic loop or GST alone (Fig. 5-2A). Further GST pull-down experiments revealed strong interactions between NHE5 and GST-SCAMP2[1-154], GST-SCAMP2[45-154], GST-SCAMP2[75-134] and GST-SCAMP2[75-154], weaker interactions with GST-SCAMP2[1-88] and GST-SCAMP2[45-88] and no interaction with GST-SCAMP2[75-117], GST-SCAMP2[1-44], GST-SCAMP2[134-154] or GST alone.
Fig. 5.1. **NHE5 interacts with SCAMPs.** (A) Membrane fractions from control PC12 cells or PC12 cells stably expressing NHE5<sub>1D4</sub> (PC12/NHE5<sub>1D4</sub>) were immunoprecipitated with 1D4 antibody conjugated to sepharose beads. Bound endogenous SCAMP1, SCAMP2 and SCAMP5 found in the immunoprecipitate fraction (IP) were detected by western blot using SCAMP-specific antibodies. Five percent of the membrane lysate (Lys.) was resolved as a positive control. (B) 2.5, 5, and 10 μg of protein from rat brain lysate was probed by western blot in order to assess the endogenous expression of NHE5 and SCAMP2 protein in brain tissue. (C) NHE5 was immunoprecipitated from rat brain lysate using an anti-NHE5 antibody (IP) or pre-immune serum control (Con.) and bound endogenous SCAMP2 was detected by western blot. One percent of the rat brain lysate (Lys.) was probed as a positive control. Western blots shown in A-C are representative of three independent experiments. (Figure originally published by Diering et al., 2009. Copyright American Society for Biochemistry and Molecular Biology).
Fig. 5-2. The cytosolic C-terminus of NHE5 interacts directly with SCAMP2. (A) Glutathione S-transferase (GST) or GST-fusion proteins containing either the cytoplasmic N-terminus (amino acids 1-154), C-terminus (amino acids 284-329) or the cytosolic loop (CL, amino acids 201-215) between the second and third trans-membrane domains of SCAMP2 were immobilized on reduced glutathione sepharose beads. The beads were then incubated with \(^{35}\text{S}\)-labeled \textit{in vitro} transcribed/translated NHE5 C-terminus (amino acids 492-896). After washing the beads, bound \(^{35}\text{S}\)-labeled NHE5 C-terminus was eluted, resolved by SDS-PAGE and detected by phosphorimager. A small amount of the NHE5 C-terminus input (3%), not subjected to pull-down, was also included as a control. (B,C) Two additional GST pull-down experiments were performed using GST fused to fragments of the SCAMP2 N-terminus (amino acids 1-154, 1-88, 45-88, 45-154, 75-117, 75-134 and 75-154 in B, or amino acids 1-154, 1-44 and 134-154 in C) in order to determine the minimum NHE5-binding sites within the SCAMP2 N-terminal tail. Each pull-down experiment was performed three times; representative results are shown. (D) A schematic representation showing the membrane topology of SCAMP2. NHE5-binding sites are highlighted with black rectangles and the N-terminal NPF repeats are labeled with black circles. Numbers indicate amino acid residues. (Figure originally published by Diering et al., 2009. Copyright American Society for Biochemistry and Molecular Biology).
Together, these results indicate that the NHE5 C-terminus binds to the cytosolic C-terminus, and amino acids 45-75 and 117-134 within the cytosolic N-terminus, of SCAMP2 (Fig. 5-2D).

5.2.3 Heterologous expression of SCAMP2 affects NHE5 surface localization, but not NHE5 internalization

SCAMPs have been suggested to play roles in both secretion (Guo et al., 2002) and endocytosis (Fernandez-Chacon et al., 2000). I postulated that SCAMP2 might modulate the targeting of NHE5 between endosomes and the plasma membrane, and thereby regulate transporter activity across the plasma membrane. To address the functional significance of the SCAMP-NHE5 interaction, I used Chinese hamster ovary AP-1 cells devoid of intrinsic NHE-activity (Rotin and Grinstein, 1989) stably expressing HA-tagged NHE5 (AP-1/NHE5\(_{\text{HA}}\), see Chapter 2). This is a widely used model system to measure the activity of different NHE isoforms in the absence of intrinsic NHE-activity (Onishi et al., 2007; Szabo et al., 2000; Szabo et al., 2005; Szaszi et al., 2002). It was previously shown that NHE5 cycles between the plasma membrane and recycling endosomes (Szaszi et al., 2002). SCAMPs are also localized to the eukaryotic cell surface recycling system (Castle and Castle, 2005). Therefore, I next tested whether NHE5 and SCAMPs colocalize in recycling endosomes. AP-1/NHE5\(_{\text{HA}}\) cells grown on glass coverslips were incubated at 37°C for 30 minutes in media containing fluorescently labeled transferrin to visualize recycling endosomes. In agreement with the previous study, NHE5\(_{\text{HA}}\) was associated with internalized transferrin in a perinuclear location (Fig. 5-3). Some of the NHE5\(_{\text{HA}}\) signal appeared dispersed. This is likely due to partial localization to the endoplasmic reticulum resulting from heterologous overexpression as noted earlier (Szaszi et al., 2002). As observed in the three color overlay picture, both
SCAMP1 and SCAMP2 colocalized with NHE5 predominantly in the perinuclear location positive for fluorescently labeled transferrin (Fig. 5-3), suggesting that recycling endosomes are the site of the SCAMP-NHE5 interaction.

To investigate whether SCAMPs regulate the subcellular distribution of NHE5, I performed biotin-labeling and internalization assays to monitor the trafficking of NHE5. AP-1/NHE5_HA cells were transiently transfected with either SCAMP2_myc or SCAMP5_myc, or empty pcDNA3 vector as a control. Cell-surface exposed proteins were labeled with a membrane impermeable protein-reactive biotinylation reagent containing a cleavable disulfide bond at 4°C. Labeled cells were then incubated in culture media at 37°C for 0-30 minutes to facilitate internalization of labeled proteins through endocytosis. Cells were then treated with reduced glutathione (cleavage buffer) to cleave the remaining surface biotin tags and allow for evaluation of the remaining internalized, biotinylated NHE5 population, or left untreated to assess the total surface exposed and biotinylated NHE5 population. Biotinylated proteins were affinity-purified with avidin-coupled agarose beads, and analyzed by SDS-PAGE and western blot. NHE5 was efficiently biotinylated on the cell surface (Surface NHE5_HA, Fig. 5-4A, 4B). The cell surface biotin tags of NHE5 were efficiently removed by incubation with cleavage buffer (time 0 in Fig. 5-4A, 4B) while internalized NHE5 protected from cleavage after chase incubation was detectable by western blot (time 15 and 30 minutes in Fig. 5-4A, 4B). The rates of NHE5 endocytosis appeared to be unaffected by overexpression of either SCAMP2_myc or SCAMP5_myc as compared to vector transfected controls (Fig. 5-4C). However, SCAMP2_myc, but not SCAMP5_myc, increased the surface abundance of NHE5_HA by approximately 50% relative to the control (p<0.01, Fig. 5-4D). To further define whether SCAMP2 regulates the surface targeting of NHE5, we measured cell surface Na+/H+ exchange activity in transfected AP-1/NHE5_HA cells using the 22Na+-influx assay. Forced expression of SCAMP2 into these cells increased the amiloride-sensitive, acidic H+-
**Fig. 5-3. The SCAMP-NHE5 complex is found in recycling endosomes.** AP-1/NHE5_{HA} cells were incubated with Alexa 568-conjugated transferrin at 37°C for 30 minutes. Cells were then fixed, permeabilized and SCAMP1 or SCAMP2, and NHE5_{HA} were visualized with anti-SCAMP and anti-HA antibodies respectively followed by fluorescently-labeled secondary antibodies. Images were acquired using confocal microscopy. Shown in the figure are fluorescent images of NHE5_{HA}, SCAMP1 or SCAMP2 and internalized transferrin (Tfn). Bar =10 μm. (Figure originally published by Diering et al., 2009. Copyright American Society for Biochemistry and Molecular Biology).
Fig. 5-4. SCAMP2 controls NHE5 cell surface abundance. (A,B) AP-1/NHE5<sub>HA</sub> cells were transiently transfected with myc-tagged SCAMP2 (SCAMP2<sub>myc</sub>, A) or myc-tagged SCAMP5 (SCAMP5<sub>myc</sub>, B), or with empty pcDNA3 vector control. Transfected cells were incubated with a biotinylation reagent followed by chase incubation in the culture media for 0, 15 or 30 minutes (Chase) to permit endocytosis of labeled proteins. Surface biotin was then removed by incubation with a cleavage reagent allowing visualization of internalized protein or left uncleaved (Cleavage: + or −). Cells were then lysed, biotinylated proteins were purified by incubation with avidin-conjugated agarose beads, and surface labeled and internalized NHE5<sub>HA</sub> was detected by Western blot (Surface NHE5<sub>HA</sub>). A small amount of total lysate (5%) was analyzed as a loading
control and probed for SCAMP2\textsubscript{myc} or SCAMP5\textsubscript{myc} and NHE5\textsubscript{HA} (Total NHE5\textsubscript{HA}, SCAMP2\textsubscript{myc} or SCAMP5\textsubscript{myc}). The western blots shown are representative of three independent experiments. (C) The percentage of labeled NHE5\textsubscript{HA} internalized after 15 or 30 minutes of chase was calculated by comparing the signal in the cleaved samples (Cleavage: +) to the corresponding uncleaved samples (Cleavage: −). The amount of NHE5\textsubscript{HA} internalized at each time point in SCAMP2\textsubscript{myc} or SCAMP5\textsubscript{myc} transfected cells is expressed relative to control cells (pcDNA3) and are averaged from three independent experiments ± s.d. (D) Densitometric analysis of the biotinylated samples without chase (time 0 minutes), representing total surface labeled protein, was used to measure the relative surface abundance of NHE5\textsubscript{HA}. Total surface NHE5\textsubscript{HA} in SCAMP2\textsubscript{myc} or SCAMP5\textsubscript{myc} transfected cells was compared directly to pcDNA3 transfected control cells from the same experiment and is expressed as a percentage relative to pcDNA3 transfected control. Values represent the averaged result from three independent experiments ± s.d. **, p<0.01 (unpaired Student’s t-test). (E) AP-1/NHE5\textsubscript{HA} cells were transfected with SCAMP2\textsubscript{myc}, SCAMP5\textsubscript{myc} or empty pcDNA3 vector and NHE activity was measured by the amiloride-inhibitable \(^{22}\)Na\(^{+}\) influx technique. Results are expressed as a percentage relative to pcDNA3 transfected control. Data from a representative of three independent experiments are shown here ± s.d. Each experiment was performed in quadruplicate. **, p<0.01 (unpaired Student’s t-test). (Figure originally published by Diering et al., 2009. Copyright American Society for Biochemistry and Molecular Biology).
activated influx of $^{22}\text{Na}^+$ typically by 50% or greater ($p<0.01$) while expression of SCAMP5 caused only a slight increase which was not statistically significant (Fig. 5-4E).

5.2.4 Heterologous expression of SCAMPs affects NHE5 activity at the cell surface

The results of cell-surface biotinylation experiments and $^{22}\text{NaCl}$ influx assays suggest the involvement of SCAMP2, but not SCAMP5, in controlling NHE5 surface expression. However, these assays involve measurements from a pool of transiently transfected cells, and the data could be affected by experimental conditions such as transfection efficiency. To address this possibility, $\text{pH}_i$ measurements were employed to assess Na$^+$/H$^+$ exchange activity in individual AP-1/NHE5$_{HA}$ cells. AP-1/NHE5$_{HA}$ cells were loaded with BCECF, the cytosol was acutely acidified by the ammonium pre-pulse technique, and rates of Na$^+$-dependent $\text{pH}_i$ recovery were measured. $\text{pH}_i$-recovery was undetectable when cells were superfused with a Na$^+$-free solution (not shown).

Similarly, treatment with the NHE inhibitors EIPA (10 $\mu$M) (Fig. 5-5A) or amiloride (1 mM) (not shown) effectively blocked $\text{pH}_i$ recovery in a reversible manner. No recovery of $\text{pH}_i$ was observed in parental AP-1 cells following an intracellular acid load under HEPES-buffered conditions (not shown). Altogether, these results indicate that the recovery of $\text{pH}_i$ in AP-1/NHE5$_{HA}$ cells is mediated by NHE5. In order to determine NHE5-dependent proton efflux, I measured the intrinsic intracellular buffering capacity ($\beta_i$) of AP-1/NHE5$_{HA}$ cells over the $\text{pH}_i$ range studied in the present experiments (see Methods). Consistent with previous reports in AP-1 cell transfectants (Bianchini et al., 1995; Szaszi et al., 2002), $\beta_i$ in AP-1/NHE5$_{HA}$ cells was 28.4 ±4.6 mM/pH unit and was not significantly altered in cells transfected with GFP-tagged SCAMP2, SCAMP5, SCAMP2ΔC, SCAMP2 1-154, or SCAMP2ΔNPF (see below).
To define the role of SCAMPs on NHE5 activity across the plasma membrane, I expressed GFP or GFP-tagged SCAMP2 (SCAMP2\textsubscript{GFP}) or GFP-tagged SCAMP5 (SCAMP5\textsubscript{GFP}) into AP-1/NHE5\textsubscript{HA} cells and Na\textsuperscript{+}-dependent, EIPA-sensitive pH\textsubscript{i} recovery following an ammonium pre-pulse was examined using single cell imaging. Cells transfected with SCAMP2\textsubscript{GFP} exhibited significantly faster (p<0.05 at all absolute values of pH\textsubscript{i}) proton efflux than GFP-transfected controls (Figs. 5-5B, 5C). In contrast, SCAMP5\textsubscript{GFP} expression failed to significantly affect (p>0.05 at all absolute values of pH\textsubscript{i}) proton efflux, suggesting the specificity of SCAMP2\textsubscript{GFP} overexpression.

In vitro protein-protein interaction assays indicated that both the N-terminal and C-terminal cytosolic extensions of SCAMP2 contribute to NHE5 binding (Fig. 5-2). To investigate the involvement of these domains of SCAMP2 in NHE5 targeting in the cell, we generated serial N-terminal and C-terminal deletion mutants. As some of the mutants were either poorly expressed or exhibited cell toxicity during pH\textsubscript{i} measurements, the following three mutants were further characterized: SCAMP2\textsubscript{AC} which lacks the cytosolic C-terminal tail, SCAMP2 1-154, the soluble SCAMP2 N-terminus alone, and SCAMP2\textsubscript{NPF} lacking the N-terminal 55 amino acids containing multiple Asn-Pro-Phe (NPF) repeats. We first tested whether the mutants bind to NHE5 in a cellular context. When expressed in AP-1/NHE5\textsubscript{HA} cells, myc-tagged SCAMP2 was co-immunoprecipitated with HA-tagged NHE5 while SCAMP2\textsubscript{AC} and SCAMP2 1-154 showed little or no binding (Fig. 5-5D). In contrast, SCAMP2\textsubscript{NPF} was co-immunoprecipitated with NHE5 as efficiently as full-length SCAMP2 (Fig. 5-5D). If SCAMP2 serves as a scaffold protein then some of the mutants lacking either NHE5 binding domains or binding motifs with other molecules might show dominant-negative effects by competing with intrinsic protein-protein interactions. To test this possibility, we transfected GFP-tagged SCAMP2 mutants and assessed their effects on NHE5 activity.
AP1/NHE5\textsubscript{HA} cells transfected with SCAMP2\textsubscript{ΔC\textsubscript{GFP}} recovered from an imposed internal acid load (Fig. 5-5E); proton efflux values were not significantly different (p>0.05 at all absolute values of pH\textsubscript{i}) to those observed in control (pEGFP-expressing) cells (Fig. 5-5F). In contrast, proton efflux in AP1/NHE5\textsubscript{HA} cells transfected with SCAMP2 1-154\textsubscript{GFP} was significantly slower than in GFP-transfected control cells (Fig. 5-5E, 5F). Finally, cells transfected with SCAMP2\textsubscript{ΔNPF\textsubscript{GFP}} exhibited very little pH\textsubscript{i} recovery from imposed internal acid loads (Fig. 5-5E). Thus, the SCAMP2 N-terminal domain, and in particular the N-terminal NPF repeats, seem to play an important role in targeting NHE5 to the cell surface.

5.2.5 Small GTPases Arf6 and Rab11 colocalize with NHE5 and SCAMP2 in juxtanuclear regions

The small GTPases Arf6 and Rab11 have been shown to facilitate the recycling of membrane proteins from recycling endosomes to the plasma membrane (D'Souza-Schorey and Chavrier, 2006; Donaldson, 2003; Jones et al., 2006; Ren et al., 1998; Schlierf et al., 2000). Another small GTPase Rab4 also controls the recycling process of certain membrane proteins from early endosomes to the plasma membrane (Jones et al., 2006). I next asked whether these small GTPases are involved in SCAMP-mediated NHE5 recycling. NHE5 showed considerable co-localization with both transiently transfected SCAMP2\textsubscript{GFP} and Arf6\textsubscript{HA} in PC12/NHE5\textsubscript{1D4} cells in a perinuclear structure. Similarly, the localization of SCAMP2\textsubscript{GFP} and NHE5 coincided with that of endogenous Rab11 (white foci, Fig. 5-6). In contrast, the distribution of endogenous Rab4 was clearly distinct from that of NHE5 and SCAMP2.
**Fig. 5-5. Effects of overexpression of SCAMPs on rates of pH$_i$ recovery from cytosolic acid loads.** (A) AP-1/NHE$_{5\text{HA}}$ cells grown on a glass coverslip were loaded with the pH-sensitive dye BCECF and acidified by exposure to 50 mM NH$_4$Cl for 2 minutes. The recovery of intracellular pH (pH$_i$) following the wash-out of NH$_4$Cl was monitored in individual cells. Partway through the recovery phase of the experiment, cells were exposed to the NHE inhibitor EIPA (10 μM) for 2.5 minutes. The record is the mean of data obtained simultaneously from 17 cells on a single coverslip and is representative of four independent experiments. (B) AP-1/NHE$_{5\text{HA}}$ cells were transfected with pEGFP, or GFP-tagged SCAMP2 or SCAMP5. pH$_i$ recoveries in the transfected cells were monitored 48 h following transfection. Records are means of data obtained simultaneously from 15, 8 and 8 cells transfected with pEGFP, GFP-tagged SCAMP2 or GFP-tagged SCAMP5, respectively, which exhibited similar peak acidifications. Each experiment was performed on a separate coverslip and each record is representative of 4-6 independent experiments in each case. (C) The pH$_i$-dependencies of H$^+$ efflux in cells transfected with pEGFP, GFP-tagged SCAMP2 or GFP-tagged SCAMP5. Continuous lines represent the weighted non-linear least-squares regression fits to the data points (mean ± s.e.m.) indicated for each experimental condition. In each case, data points were obtained from at least 4 experiments of the type illustrated in B. (D) AP-1/NHE$_{5\text{HA}}$ cells were transfected with myc-tagged SCAMP2, SCAMP2ΔC, SCAMP2 1-154, or SCAMP2ΔNPF. Transfected cells were lysed and subjected to immunoprecipitation with mouse anti-HA antibody (IP) or pre-immune serum (Con.). SCAMP2-constructs bound to the immunoprecipitate were detected by western blotting using rabbit anti-myc antibodies. Five percent volume of the cell lysate was analyzed as a positive control (Lys.). A non-specific doublet of approximately 24kDa detected in both Con and IP lanes is indicated by asterisks. (E) AP-1/NHE$_{5\text{HA}}$ cells were transfected with pEGFP, or GFP-tagged SCAMP2ΔC, SCAMP2 1-154, or SCAMP2ΔNPF and pH$_i$ measurements were conducted as in B. Records are means of data obtained simultaneously from 10, 7, 7 and 4 cells transfected with pEGFP or GFP-tagged SCAMP2ΔC, SCAMP2 1-154 or SCAMP2ΔNPF, respectively, which exhibited similar peak acidifications. Each experiment was performed on a separate
coverslip and each record is representative of 3 - 6 independent experiments in each case. (F) The pH$_i$-dependencies of H$^+$ efflux in cells transfected with pEGFP, GFP-tagged SCAMP2ΔC or GFP-tagged SCAMP2 1-154. Continuous lines represent the weighted non-linear least-squares regression fits to the data points (mean ± s.e.m.) indicated for each experimental condition. In each case, data points were obtained from at least 3 experiments of the type illustrated in E. Cells transfected with GFP-tagged SCAMP2ΔNPF failed to exhibit measurable pH$_i$ recoveries (see E) and the recovery of pH$_i$ could not be fitted to a single exponential function to accurately determine dpH/dt and thus proton efflux. (Figure originally published by Diering et al., 2009. Copyright American Society for Biochemistry and Molecular Biology).
Fig. 5-6. NHE5 and SCAMP2 colocalize with the small GTPases Arf6 and Rab11. PC12 cells stably expressing 1D4-tagged NHE5 (PC12/NHE5$_{1D4}$) grown on glass coverslips were transfected with GFP-tagged SCAMP2 (SCAMP2$_{GFP}$) together with HA-tagged Arf6 (Arf6$_{HA}$), and the localization of SCAMP2$_{GFP}$, NHE5$_{1D4}$ and Arf6$_{HA}$ was assessed by immunofluorescence confocal microscopy. Alternatively, cells were transfected with SCAMP2$_{GFP}$ and the localization of SCAMP2$_{GFP}$, NHE5$_{1D4}$ and endogenous Rab11 or Rab4 was assessed. White foci in the merged images result from co-localization of the three proteins. Bar =10 μm. (Figure originally published by Diering et al., 2009. Copyright American Society for Biochemistry and Molecular Biology).
5.2.6 Arf6 and Rab11 control the cell surface abundance and activity of NHE5

The immunofluorescence microscopic results showing that Arf6 and Rab11, but not Rab4, associate with NHE5 and SCAMP2 prompted me to test whether Arf6 and Rab11 influence the endosome-plasma membrane targeting of NHE5. AP-1/NHE5_HA cells were transfected with wild-type or GTP-binding deficient dominant-negative HA-tagged Arf6 (Arf6_T27N) or myc-tagged Rab11 (Rab11_S25N), and changes in the surface abundance of NHE5 in transfected cells were then determined by surface biotin labeling followed by Western blot. Transfection with wild-type Arf6 or Rab11 each increased the cell surface expression of NHE5 by ~30% compared to vector transfected control (p<0.05, Fig. 5-7A, 7B). Transfection of dominant-negative Arf6_T27N or Rab11_S25N caused no significant alterations in NHE5 surface abundance (p>0.05), whereas co-expression of both Arf6_T27N and Rab11_S25N led to a significant (p<0.05) reduction of approximately 35% in surface NHE5 abundance (Fig. 5-7A, 7B). To further define the role of Arf6 and Rab11 in NHE5 surface targeting, NHE5 activity across the plasma membrane was determined by single cell pH_i measurements. AP-1/NHE5_HA cells transfected with either wild-type Arf6 (Fig. 5-7C, 7E) or wild-type Rab11 (Fig. 5-7D, 7F) exhibited significantly faster proton efflux than GFP-transfected control cells (p<0.05 at all absolute values of pH_i in each case). Proton efflux values in Arf6_T27N (Fig. 5-7C, 7E) or Rab11_S25N (Fig. 5-7D, 7F) expressing cells were not significantly different (p>0.05 at all absolute values of pH_i) to those observed in control cells. However, co-expression of Arf6_T27N and Rab11_S25N significantly reduced proton efflux (p<0.05 at all absolute values of pH_i; Fig. 5-7C, 7E). The results of the pH_i-recovery assay and the biotin labeling assays suggest that NHE5 abundance and activity at the cell-surface are regulated by both Arf6 and Rab11 GTPases.
Fig. 5-7. Arf6 and Rab11 upregulate NHE5 cell-surface targeting and activity.

(A,B) AP-1/NHE5\textsubscript{HA} cells transfected with empty vector (pcDNA3), wild-type Arf6\textsubscript{HA} (Arf6\textsubscript{WT}), Arf6T27N\textsubscript{HA} (Arf6\textsubscript{T27N}), Arf6T27N\textsubscript{HA} + Rab11S25N\textsubscript{myc} (Arf6\textsubscript{T27N}/Rab11\textsubscript{S25N}), wild-type Rab11\textsubscript{myc} (Rab11\textsubscript{WT}) or Rab11S25N\textsubscript{myc} (Rab11\textsubscript{S25N}) were subjected to surface labeling using a protein reactive biotinylation reagent. Labeled proteins were isolated from cell lysates by incubation with avidin-coupled agarose beads and biotinylated NHE5\textsubscript{HA} was analyzed by Western blot (Surface NHE5\textsubscript{HA}). Lysate (5%), not subjected to avidin-coupled beads, was analyzed by Western blot as a loading control (Total NHE5\textsubscript{HA}, Rab11\textsubscript{myc}, and Arf6\textsubscript{HA}). (A) Representative western blots. (B) Surface NHE5\textsubscript{HA} from the different transfection conditions was measured by densitometry and is expressed
relative to pcDNA3 transfected control ± s.d. Data are averaged from five independent experiments, asterisks represent statistical significance, p<0.05 (Student’s unpaired two-tailed t-test). (C,D) AP-1/NHE5HA cells grown on glass coverslips were transfected with pEGFP vector alone or together with wild-type Arf6HA (Arf6WT), Arf6T27NHA (Arf6T27N), Rab11S25Nmyc + Arf6T27NHA (Arf6T27N/Rab11S25N), wild-type Rab11myc (Rab11WT) or Rab11S25Nmyc (Rab11S25N). Transfected cells were loaded with BCECF and pH recoveries from NH4+-induced internal acid loads were examined 48 h following transfection. Records are means of data obtained simultaneously from 12, 12, 19, 10, 7 and 20 cells transfected with pEGFP, Arf6WT, Arf6T27N, Arf6T27N/Rab11S25N, Rab11WT or Rab11S25N, respectively, which exhibited similar peak acidifications. Each experiment was performed on a separate coverslip and each record is representative of 4 independent experiments in each case. (E,F) The mean pH-dependent proton efflux was calculated based on four independent experiments ± s.e.m. The pH-dependencies of H+ efflux in cells transfected with pEGFP, Arf6WT, Arf6T27N, Arf6T27N/Rab11S25N, Rab11WT or Rab11S25N. Continuous lines represent the weighted non-linear least-squares regression fits to the data points (mean ± s.e.m.) indicated for each experimental condition. In each case, data points were obtained from 4 experiments of the types illustrated in C and D. (Figure originally published by Diering et al., 2009. Copyright American Society for Biochemistry and Molecular Biology).
5.2.7 NHE5-activation by SCAMP2 is Arf6-dependent

In order to test the involvement of Arf6 and Rab11 in SCAMP2-mediated trafficking of NHE5, AP-1/NHE5<sub>HA</sub> cells were co-transfected with SCAMP2<sub>GFP</sub> and either dominant-negative Arf6<sub>T27N</sub> or Rab11<sub>S25N</sub>, and NHE5 activity was measured using the single cell pH<sub>i</sub>-recovery assay. Cells expressing exogenous SCAMP2 exhibited robust recoveries from the induced acid load. Arf6<sub>T27N</sub> significantly reduced the ability of concomitantly transfected SCAMP2 to up-regulate NHE5 activity (p<0.05), while Rab11<sub>S25N</sub> did not influence the SCAMP2-mediated up-regulation of NHE5 (p>0.05, Figs. 5-8A, 8B). These results suggest that the activity of SCAMP2 in controlling NHE5 cell-surface targeting is Arf6-dependent and Rab11-independent.

5.3 Discussion

Secretory Carrier Membrane Proteins (SCAMPs) are a group of integral membrane proteins that cycle between multiple organelles and regulate membrane dynamics. In this chapter, I have shown that SCAMP2 directly binds to NHE5 and facilitates its cell-surface targeting. SCAMP2 contains an N-terminal cytosolic extension, four transmembrane spans, and a C-terminal cytosolic tail (Hubbard et al., 2000). Using an <em>in vitro</em> protein-binding assay, I have identified NHE5-binding sites within the cytosolic C-terminus, and amino acids 45-75 and 117-134 within the cytosolic N-terminus of SCAMP2. Further, I used a co-immunoprecipitation approach to show that NHE5 and SCAMP2 form a complex both in tissue culture cells and in brain tissue.

Exogenous expression of SCAMP2 increased both the cell surface abundance and the ion translocation activity of NHE5. The agreement between experiments examining cell surface NHE5 abundance and NHE5 activity suggest the predominant
Fig. 5-8. SCAMP2 facilitates cell-surface targeting of NHE5 via Arf6. AP-1/NHE5_{HA} cells were transfected with SCAMP2_{GFP} (SCAMP2), SCAMP2_{GFP} + Rab11S25N_{myc} (Rab11S25N/SCAMP2) or SCAMP2_{GFP} + Arf6T27N_{HA} (Arf6T27N/SCAMP2). Transfected cells were loaded with BCECF and examined 48 h following transfection. (A) pH_{i} recoveries from NH_{4}^{+}-induced internal acid loads. Records are means of data obtained simultaneously from 8, 5 and 5 cells transfected with SCAMP2, Rab11S25N/SCAMP2 or Arf6T27N/SCAMP2, respectively, which exhibited similar peak acidifications. Each experiment was performed on a separate coverslip and each record is representative of 3 independent experiments in each case. (B) The pH_{i}-dependencies of H^{+} efflux in cells transfected with SCAMP2, Rab11S25N/SCAMP2 or Arf6T27N/SCAMP2. Continuous lines represent the weighted non-linear least-squares regression fits to the data points (mean ± s.e.m.) indicated for each experimental condition. In each case, data points were obtained from 3 experiments of the type illustrated in A. (Figure originally published by Diering et al., 2009. Copyright American Society for Biochemistry and Molecular Biology).
action of SCAMP2 acts on membrane trafficking. Further, SCAMP2 appeared to have no effect on the rates of endocytosis of NHE5 from the plasma membrane. Thus, although we cannot rule out the possibility that NHE5 ion-translocation activity may be partially regulated through interaction with SCAMP2, SCAMP2 likely regulates the abundance of NHE5 at the cell surface by promoting its delivery from the perinuclear recycling endosomes. It is unlikely that the effect of SCAMP2 expression on NHE5 cell-surface targeting is an overexpression artifact because expressing comparable levels of SCAMP5 or deletion mutants of SCAMP2 did not cause the same change. Interestingly, among the SCAMP2 deletion mutants tested, the N-terminal deletion mutant lacking the NPF repeats (SCAMP2\textsubscript{NPF}) markedly suppressed NHE5 activity across the plasma membrane. Likewise, expression of a mini-gene encoding the N-terminal fragment of SCAMP2 (SCAMP2 1-154) caused a milder but significant decrease in NHE5 activity despite its weak binding affinity to NHE5. SCAMP2 1-154 may compete with endogenous SCAMP2 for binding to other molecules such as soluble EH-domain proteins. In contrast, the \(\triangle\)NPF mutant binds to NHE5 but may not be able to recruit necessary cytosolic factors to the NHE5-SCAMP2 complex. NPF-repeats commonly interact with the Epsin-homology (EH) domain and regulate endocytosis and endocytic recycling (Montesinos et al., 2005; Naslavsky et al., 2004; Naslavsky and Caplan, 2005; Shi et al., 2007). Furthermore, intersectins, EH domain containing proteins that were reported to bind to the NPF-repeats of SCAMP (Fernandez-Chacon et al., 2000), regulate recycling of synaptic vesicles in *Drosophila* and *C. elegans* (Koh et al., 2004; Marie et al., 2004; Rose et al., 2007; Wang et al., 2008a). Thus, we hypothesize that SCAMP2 recruits cytosolic EH-domain proteins to recycling endosomes via its N-terminal NPF repeats, and promotes vesicle formation and the plasma membrane targeting of NHE5. This proposed model is in agreement with a previous report showing
that newly formed transferrin-containing vesicles are SCAMP-deficient, which suggests that endocytosis of transferrin occurs independently of SCAMPs (Castle and Castle, 2005). The internalized transferrin-containing vesicles are fused with a pre-existing internal pool of SCAMP-positive membranes and then accumulate in the SCAMP-rich perinuclear region corresponding to the recycling endosomal compartment. Vesicles leaving this compartment, returning transferrin to the cell surface were again SCAMP-deficient, suggesting that the perinuclear recycling endosomes are a likely site of SCAMP function. Interestingly, SCAMP1 and 2 and to a lesser extent SCAMP3 showed considerably more overlap with trafficking transferrin than SCAMP4 that lacks a large part of the N-terminal cytosolic tail, including NPF repeats (Castle and Castle, 2005). Thus, these findings together with our own suggest that the N-terminal cytosolic extension of SCAMP2 is an important domain for cell-surface targeting through recycling endosomes.

The small GTPases Arf6 and Rab11 have both been implicated as master regulators of membrane traffic from recycling endosomes to the cell surface (Jones et al., 2006). Overexpression of either Arf6 or Rab11 significantly enhanced NHE5 abundance and activity at the cell surface, while expression of dominant-negative Arf6 and Rab11 alone had very little effect on NHE5 activity. Co-expression of both dominant-negative GTPases caused a substantial decrease in NHE5 activity and cell-surface abundance. When concomitantly expressed with SCAMP2, Arf6T27N but not Rab11S25N impaired the SCAMP2-mediated NHE5 translocation to the plasma membrane. These results indicate that while both Arf6 and Rab11 participate in controlling the membrane traffic of NHE5, SCAMP2-mediated trafficking is Arf6-dependent and Rab11-independent. Thus I propose that NHE5 accesses the cell surface from the recycling endosomes via at least two distinct pathways: a Rab11-dependent pathway and an Arf6/SCAMP2 pathway. It was previously shown that Arf6
binds to SCAMP2 and regulates fusion pore formation during dense-core vesicle exocytosis in PC12 cells (Liu et al., 2005). It is tempting to hypothesize that the SCAMP2-Arf6 complex targets NHE5 to vesicular docking sites on the plasma membrane and that the locally elevated NHE5-activity controls secretion of dense core vesicles.

We found previously that NHE5 was able to bind to integrin β1 subunits and could localize to focal adhesion complexes. We further showed that NHE5 could be activated by stimulating the integrin signaling pathway, in a process that required the Receptor for Activated C-Kinase 1 (RACK1) (Onishi et al., 2007). Integrin β1 has also been shown to traffic between the cell surface and the recycling endosomes, which was found to be sensitive to extra-cellular stimuli in a process termed “regulated recycling.” Further, the regulated traffic of integrin β1 was found to require the activity of both Rab11 and Arf6 (Powelka et al., 2004). As NHE5 is able to bind to integrin and traffics through recycling endosomes in a pathway that also involves Arf6 and Rab11, NHE5 may follow a similar “regulated recycling” pathway. In neurons integrin receptors are not found in large focal complexes but rather can be targeted to synapses (Chan et al., 2007; Cingolani et al., 2008; Huang et al., 2006). Thus, it is tempting to speculate that SCAMP2 promotes the targeting of NHE5 from endomembrane stores to the surface of synapses in response to NMDA receptor activation.

In summary, I have identified Secretory Carrier Membrane Proteins (SCAMPs) as novel NHE5-interacting proteins. I propose a model in which SCAMP2 binds to NHE5 in the recycling endosomes and promotes its cell surface targeting. This process is Arf6-dependent and Rab11-independent, and the N-terminal 55 amino acids of SCAMP2 containing the NPF-repeats represents a crucial domain. Many NHE isoforms contain a second H⁺ binding site in addition to the transport site, which contributes to allosteric regulation of the transporter activity, thus forming the basis of the pH-set point
concept (Aronson et al., 1982; Otsu et al., 1992; Wakabayashi et al., 1997; Wakabayashi et al., 2003). However, NHE5 does not appear to contain the second allosteric proton binding site (Attaphitaya et al., 2001; Szabo et al., 2000). Hence, regulation of NHE5 trafficking behavior rather than via allosteric regulation by cytosolic protonation may serve as a major mechanism in controlling NHE5 activity across the plasma membrane.

In chapter 4, I described how NHE5 is targeted to the neuronal cell surface in response to synaptic activity. It is likely that SCAMP2 will play a role in controlling NHE5 trafficking and ion-transport activity at the synapse, but further research is needed to address this. The implications of NHE5 regulation by SCAMP2 and other proteins at the synapse will be discussed in chapter 6.
6. Conclusions and future directions

Many components of the synaptic machinery are sensitive to changes in pH within the physiological range. Further, electrical activity in the brain is known to be enhanced or suppressed by increases or decreases in pH respectively. Given this sensitivity to pH it is likely that pH-regulatory proteins such as NHEs play an essential role in brain function. NHE5 is a unique member of the Na+/H+ exchanger (NHE) gene family that is highly enriched in brain. However, a physiological role for NHE5 has never been described. In addition, the mechanisms that regulate pH in the vicinity of the synapse are not known.

In this thesis work, I have shown that NHE5 protein is expressed in neurons, where it is found in both axons and dendrites. In these structures, NHE5 is distributed between the cell-surface and intracellular endosomes (Chapter 3). Importantly, NHE5 can be targeted to synapses, and this targeting is regulated by synaptic activity. I have also shown that dendritic spines, the location of the excitatory post-synapse, experience an activity and NHE5-dependent change in local pH. Thus, I propose that NHE5 is recruited to synapses during synaptic activity to regulate the activity of pH-sensitive components of the synaptic machinery (Chapter 4). I have also expanded on the current understanding of the molecular regulation of NHE5 by the identification and characterization of an NHE5-SCAMP2 interaction (Chapter 5). Therefore, I have uncovered part of the physiological function of NHE5 and also identified the very first molecule involved specifically in regulation of pH at the synapse.
6.1 NHE5 expression in brain, within neurons, and during development

My first goal in this thesis work was to describe the expression pattern of NHE5 protein in brain, to understand its localization within individual cells, and to obtain some information about the developmental profile of NHE5. This information is crucially important to begin to understand the physiological function of this protein. Using immunohistochemistry on mouse brain coronal sections and our newly developed anti-NHE5 antibody, I showed NHE5 protein expression in neurons of the hippocampus, cerebral cortex and amygdala. I also described NHE5 expression on the apical surface of ependymal cells; these are epithelial cells that line the brain ventricular system and are responsible for the production of CSF. NHE5 protein was poorly expressed in the corpus callosum, a region enriched in glia. These findings suggest that NHE5 may be important in neurons and ependymal cells. While informative, these findings should be supplemented with a more thorough characterization of NHE5 expression in brain.

6.1.1 NHE5 in neurons

The most striking finding of this initial characterization of the expression pattern of NHE5 was that NHE5 protein was abundantly expressed in neurons, the electrically excitable cells of the nervous system. This is consistent with previous studies that used in situ hybridization and Northern blot to demonstrate NHE5 expression at the mRNA level in neuron-rich structures within the brain (Attaphitaya et al., 1999; Baird et al., 1999). In cultured neurons, NHE5 was found in the cell body and in both axons and dendrites where it seemed to be distributed between endosomes and synapses. This data suggests that NHE5 may be active in neurons and play a role in electrical and
chemical signaling in the brain. While this is a tremendous advance in the understanding of the role of NHE5, continued characterization of NHE5 expression will be important to fully understand the role of this protein. To begin, immunohistochemistry of brain slices using the anti-NHE5 antibody should be repeated using both coronal and sagittal sections from mouse and rat. These different preparations allow for the examination of different brain regions, and the use of both mouse and rat brain will give a greater confidence in the experimental results. In particular, preliminary results show that NHE5 protein is highly expressed in cerebellar Purkinje and granule neurons (not shown). *In situ* hybridization performed by the Allen Institute for Brain Science suggested that in mouse, NHE5 mRNA expression may be highest in the cerebellum ([http://mouse.brain-map.org/brain/Slc9a5.html?ispopup=1](http://mouse.brain-map.org/brain/Slc9a5.html?ispopup=1)). NHE5 mRNA was also detected in human cerebellum by Northern blot (Baird et al., 1999). Therefore, NHE5 expression in the cerebellum and other brain regions should be further characterized to gain a comprehensive understanding of NHE5 expression in the brain, further illuminating the physiological function of NHE5. In addition, further immunohistochemistry studies should be performed in combination with additional marker antibodies in order to determine precisely which neuronal sub-types express NHE5. For instance, it was clear that NHE5 was expressed in glutamatergic pyramidal neurons of the hippocampus CA1 region, as these neurons are easily identified morphologically. NHE activity has been described at glutamatergic, GABAergic, and dopaminergic synapses (Jang et al., 2006; Rocha et al., 2008; Trudeau et al., 1999), though the identity of this synaptic NHE in these different neuron types was not determined. Therefore, it would be of interest to determine whether NHE5 is also expressed in other neurons such as GABAergic interneurons, or dopaminergic or cholinergic neurons by examining the overlap of NHE5 with markers such as glutamate decarboxylase, tyrosine hydroxylase or choline acetyltransferase respectively. Further
implications of NHE5 expression and activity in neurons are discussed below in section 6.2.

6.1.2 NHE5 in ependymal cells

The detection of NHE5 on the apical surface of ependymal cells is of great interest given the role of these cells in the production of CSF and in controlling brain volume (Jacobs et al., 2008; Kempski, 2001). CSF plays an important role in controlling neuronal excitability and changes in CSF pH can alter the severity of seizure (Jacobs et al., 2008). In addition, this apical targeting of NHE5 is highly reminiscent of the apical targeting of the highly related NHE3 in renal epithelium. Like NHE3, NHE5 undergoes trafficking between the recycling endosomes and the plasma membrane (Szaszi et al., 2002). Further, these two proteins are similarly affected by pharmacological manipulations that alter the activity of PKA, PKC, and PI3K (Attaphitaya et al., 2001; Szaszi et al., 2002), suggesting that NHE3 and NHE5 share a strong overlap in their mechanisms of regulation. As NHE3 plays a very important role in Na\(^{+}\) and water absorption in the renal proximal tubule (Schultheis et al., 1998b), NHE5 may play a homologous role in brain ventricles to regulate ventricle volume, ion, and pH homeostasis. To begin to address these possibilities, it will be important to confirm this apical targeting of NHE5 in polarized epithelia. A simple experiment would be to express NHE5 in a suitable polarized epithelial model cell line such as Madin-Darby Canine Kidney (MDCK) cells and compare the localization of NHE5 to other proteins with a well characterized apical or basolateral distribution. To test the role of NHE5 in brain ventricles and CSF production would almost certainly require an in vivo system. This may require the generation of an NHE5 knock-out mouse model. Ventricle volume, and CSF pH and salt content could then be compared between wild-type and NHE5-null mice. For comparison, mice with knock-out mutations for the Na\(^{+}\)-coupled Cl\(^{-}\)/HCO\(_3\)^{-}
exchanger (NCBE, also called Slc4a10) showed strikingly reduced brain ventricle volume and reduced sensitivity to invoked seizures (Jacobs et al., 2008), showing the usefulness of a transgenic approach to study pH regulation in brain ventricles. Alternatively, viruses containing appropriate NHE5 shRNA constructs, or an NHE5 inhibitor compound, could be injected directly into the brain ventricle to knock-down or block epithelial NHE5 and changes in CSF pH and salt content could then be monitored.

6.1.3 NHE5 during development

The last objective of this preliminary characterization was to obtain some information about the developmental profile of NHE5. Western blot analysis revealed that NHE5 protein was expressed in both embryonic (E18) and adult rat brain (Fig. 3-6). These data suggest that NHE5 may function during development and in the adult brain. In this thesis work, I pursued a role for NHE5 in synaptic plasticity and synapse pH regulation, which may play a more important function in the adult brain. Preliminary results indicate that NHE5 may also play a role in neuritogenesis, the formation of axons and dendrites in neurons of the developing nervous system (not shown), suggesting that NHE5 may indeed play a role during development. To strengthen these preliminary results, a more careful characterization of NHE5 expression during development will need to be performed. Western blot analysis could be repeated using brain samples from multiple developmental stages to better characterize the onset and extent of NHE5 expression during development. Immunohistochemistry of tissue from different developmental stages could also indicate the location of NHE5 expression. As mentioned above, NHE5 was found to be expressed in the cells lining the brain ventricles in adult tissue. From a developmental point of view, it would be of interest to determine whether NHE5 is expressed at the brain ventricles in the embryo as this is
believed to be the location of the neuronal stem cells (Tramontin et al., 2003). Neural stem cells persist in the ventricular zone into adulthood as well (Tramontin et al., 2003). NHE5 may therefore play a role during development, and into adulthood, in maintaining the local environment of the neuronal stem cells or in the stem cells themselves. Examination of the development of an NHE5-null mouse may be one way to address the possible role of NHE5 in brain development. For comparison, the NHE1-null mice develop normally and are viable until two-weeks after birth when they begin to show neurological symptoms (Bell et al., 1999; Cox et al., 1997). Therefore, it is possible that different NHE isoforms play distinct roles during development and that this will likely prove an interesting avenue for future research.

6.2 Synaptic function of NHE5

The second objective of this thesis work was to examine the role of NHE5 in physiological processes related to brain function. My hope was that this task would be guided heavily by my observations from objective 1. Indeed, I showed that NHE5 can be targeted to synapses. This finding strongly suggested that a major part of the physiological function of NHE5 may be as a regulator of pH at the synapse. In neurons, NHE5 seems to undergo trafficking between intracellular endosomes and the surface of synapses. Activation of NMDA receptors through our glycine stimulation protocol (Lu et al., 2001) results in the recruitment of NHE5 to the cell-surface and synapses, and the concomitant local alkalinization of dendritic spines was observed. I have also shown that NHE5 acts as a negative regulator of dendritic spine growth. As dendritic spines are known to increase in size and number following NMDA receptor activation (Engert and Bonhoeffer, 1999; Lang et al., 2004; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004) and NMDA receptor activation also causes recruitment of NHE5 to the synapse, I
speculate that NHE5 must function as part of a negative feed-back mechanism to control or stabilize activity-induced dendritic spine growth. As several components of the synaptic machinery, including the NMDA receptor, are expected to be negatively regulated by extracellular acidification (see below), it is highly likely that NHE5 acts to modulate synaptic activity through localized regulation of pH at the synapse. Because so many synaptic proteins are pH sensitive, the activity-dependent targeting of NHE5 likely has an impact on many aspects of synapse physiology. Thus, my work opens up many new avenues of investigation.

6.2.1 Targets of local pH regulation by NHE5

Following synaptic activity, NHE5 is recruited to the dendritic spine and to the synaptic plasma membrane where it initiates Na⁺/H⁺ exchange to raise the pH inside the dendritic spine. This exchange activity will necessarily cause a concomitant drop in extracellular pH which may be rapidly buffered, however, in the confined space of the synaptic cleft NHE5-transported protons may be significant. The most outstanding question relating to this local synaptic pH regulation by NHE5 is which are the pH-sensitive proteins most influenced by NHE5 activity? It is likely that local pH changes caused by NHE5 cannot have a specific target, but rather it is a question of which proteins are the most pH-sensitive and how these proteins are organized in space with respect to NHE5. Potential targets for modulation by NHE5 activity could reside inside the dendritic spine, or in the synaptic cleft on the cell surface. Due to the small size of the synaptic cleft these targets may reside on the pre- or the post-synapse.
6.2.2 Post-synaptic targets of NHE5 activity: NMDA receptor

In this work I implicated the NMDA receptor as a post-synaptic target of NHE5 activity. The NMDA receptor is highly sensitive to changes in extracellular pH (Tang et al., 1990; Traynelis and Cull-Candy, 1990), and is well known to play an essential role in controlling synaptic plasticity (Malenka and Bear, 2004). Blocking NHE5 activity by knock-down or dominant-negative mutant expression resulted in spontaneous spine outgrowth that required an active NMDA receptor, suggesting that NHE5 may normally function to control NMDA receptor activity through local regulation of pH in the synaptic cleft. Therefore, NMDA receptor modulation by NHE5 is the most likely mechanism to explain the phenotypes observed upon perturbing NHE5 activity (see Fig. 4-12). However, other pH-sensitive proteins at the synapse could also contribute to the observed results.

In addition to the morphological analysis described in chapter 4, further functional analysis using electrophysiology should be performed to fully characterize the physiological actions of NHE5 at synapses. Such analysis may also further address the question of which pH-sensitive proteins are most affected by local NHE5 activity. To characterize NHE5-dependent modulation of the NMDA receptor, NHE5 could be overexpressed or knocked-down in cultured neurons or brain slices and miniature excitatory post-synaptic currents (mEPSCs, also called “minis”) could be monitored. These events correspond to the spontaneous release of a single synaptic vesicle at individual synapses, and are used to gauge overall efficacy of synaptic transmission. By using electrophysiological manipulations and/or the use of specific pharmacological inhibitors, minis can be separated into an AMPA receptor and an NMDA receptor mediated component. If changes in local pH at the synapse following manipulation of NHE5 affect NMDA receptor activity, this would be reflected as changes in NMDA
receptor mini amplitude or AMPA/NMDA receptor ratio. NHE5-dependent modulation of NMDA receptors may also be observed as a change in the sensitivity to NMDA application which could be monitored using electrophysiological recordings. As NMDA receptors pass Ca\(^{++}\) ions, Ca\(^{++}\)-sensitive fluorescent dyes could also be used to monitor NMDA receptor responses to NMDA application. Modulation of NMDA receptors by NHE5 could be observed as a change in the amount of NMDA-induced Ca\(^{++}\)-influx following NHE5 manipulation. NMDA receptor modulation by NHE5 activity may ultimately influence the induction or maintenance of NMDA receptor-dependent long lasting forms of synaptic plasticity such as LTP and LTD. These profound implications will be discussed below.

6.2.3 Pre-synaptic targets of NHE5 activity: VGCC

In addition to affecting proteins of the post-synapse, NHE5 activity may also modulate pre-synapse proteins such as voltage-gated Ca\(^{++}\)-channels (VGCCs). This modulation could occur through NHE5 transporters inserted into the post-synaptic membrane as well as through insertion of NHE5 into the pre-synapse membrane (see section 6.2.6 for more discussion). Indeed NHE5 protein was detected in both axons and dendrites (Fig. 3-3). The current amplitude and activation curve of VGCCs are both negatively regulated by extracellular protons with a pK\(_a\) of close to 7.1 (Chen et al., 1996; Klockner and Isenberg, 1994). While VGCCs are found at the post-synapse where they contribute to dendritic Ca\(^{++}\)-signaling (Bloodgood and Sabatini, 2008), the best known function of synaptic VGCC is to promote the evoked release of synaptic vesicles from the pre-synapse (Südhof, 2008; Llinas et al., 1981; Llinas et al., 1982). NHE5 recruited to the synaptic cleft following activity may therefore act to reduce VGCC activity and increase the threshold needed for synaptic vesicle release or reduce the
probability of vesicle release. Reducing NHE5 activity may thereby enhance VGCC activity, increasing neurotransmitter release leading to the LTP-like expansion of dendritic spines that I described in chapter 4. A similar proton-based feedback mechanism controlling VGCC has been described previously in retina where the acidic contents of released synaptic vesicles inhibited VGCC adjacent to the release cites, possibly to limit further vesicle release (DeVries, 2001).

Paired-pulse facilitation (PPF), or the rate of run-down of the reserve synaptic vesicle pool are two parameters commonly used to measure VGCC activity and synaptic vesicle turnover using electrophysiological recordings. For PPF, an axon is stimulated with two pulses to evoke synaptic vesicle release with a very short interval in between. During this short interval, Ca^{++} that entered the pre-synapse resulting from the first pulse is still present when the second stimulation is delivered. This persistent Ca^{++} acts synergistically with the second pulse resulting in a larger amount of synaptic vesicle release. Knock-down of NHE5 could diminish a local proton source in the synaptic cleft which may result in enhanced Ca^{++}-flux through VGCC. If this were indeed the case, NHE5 knock-down may result in enhanced PPF, while NHE5 overexpression may inhibit VGCC activity and diminish PPF. Such results would suggest that VGCC activity is normally regulated by NHE5. A change in VGCC activity upon manipulation of NHE5 activity may also lead to changes in mEPSC frequency (a reflection of vesicle release probability).

6.2.4 Intracellular targets of NHE5 activity: cofilin

Lastly, the acute alkalinization of dendritic spines mediated by NHE5 could have an impact on intracellular pH-sensitive proteins in addition to the synaptic proteins sensitive to extracellular pH already mentioned. There are likely to be many pH-
sensitive proteins in the intracellular compartment performing a wide range of functions from protein synthesis to glycolysis. However, for the purposes of this discussion I plan to focus on the actin cytoskeleton regulating protein cofilin. Cofilin is an actin severing molecule which, when activated, leads to the formation of free barbed ends promoting actin-cytoskeleton rearrangements. Importantly, cofilin is highly pH sensitive being inhibited by cellular acidification and activated by cellular alkanization (Frantz et al., 2008). This modulation of cofilin by pH occurs in conjunction with the well defined regulation by phosphorylation at serine 3 (active in the dephosphorylated state) (Frantz et al., 2008; Shi et al., 2009; Zhou et al., 2007). Cofilin is known to play a role at synapses and in the morphology of dendritic spines. Inhibition of cofilin by phosphorylation has been shown to be important for maintenance of mature dendritic spines and expression of a hyper-active non-phosphorylatable cofilin (S3A) was shown to reduce dendritic spine head size, suggesting cofilin is a negative regulator of spine growth (Shi et al., 2009; Zhou et al., 2007). Loss of NHE1 and cellular acidification results in reduced cell-migration, which was recently shown to be partly a result of impaired cofilin-dependent actin dynamics (Choi et al., 2010). Like in migrating cells, cofilin activity in dendritic spines is likely regulated by cellular pH in addition to phosphorylation-status. I propose here that NHE5 is also a negative-regulator of spine growth (See chapter 4). NHE5-dependent dendritic spine alkanization (Fig. 4-11) may enhance cofilin activity, acting to limit spine growth. Conversely, exuberant dendritic spine growth upon perturbing NHE5 may in part have resulted from sustained acidification of the dendritic spine causing inhibition of cofilin and supporting spine growth.

These possibilities could be addressed by examining the combined effects of NHE5 overexpression or knock-down with the expression of dominant-negative (S3E) or hyper-active (S3A) cofilin mutants. Therefore the pH-sensitive nature of the NMDA
receptor, VGCC and cofilin could each, in part, explain the phenotype associated with NHE5 manipulation. NHE5 action likely includes a combined effect of these and other pH-sensitive proteins, with their specific contributions relying on their precise sensitivity to changes in pH and their proximity to NHE5.

### 6.2.5 NHE5 and synaptic plasticity

Based on the pH-sensitive nature of many synaptic components, in particular the NMDA receptor, it would be of great interest to further characterize the role of NHE5 in synaptic plasticity. The most studied forms of synaptic plasticity, LTP and LTD, are best characterized in the hippocampus CA1 region where LTP can be induced by stimulating the CA3 region axon projections (Schaffer collaterals) by high frequency stimulation (HFS) and LTD can be induced by prolonged low-frequency stimulation (LFS) (Bramham and Srebro, 1987; Malenka and Bear, 2004). Previously it was found that inhibition of NHE activity by application of EIPA could enhance LTP (Ronicke et al., 2009) suggesting that a synaptic NHE was a negative-regulator of synaptic plasticity. The responsible isoform was not determined. However, in this thesis work I have shown that NHE5 is a novel synapse component and that NHE5 is a negative-regulator of dendritic spine growth (chapter 4), raising the possibility that NHE5 may be the NHE isoform shown to participate in LTP (Ronike et al., 2009). To my knowledge, the role of extracellular pH or NHEs in LTD has never been examined. LTD involves a sustained reduction in synaptic strength as well as a shrinkage or loss of dendritic spines (Kasai et al., 2010; Malenka and Bear, 2004). NHE5 may participate in LTD by inhibition of synaptic components through acidification of the synaptic cleft and by promoting the shrinkage of dendritic spines. Indeed in chapter 4, I present evidence that NHE5 is a negative-regulator of dendritic spine growth. In order to test these exciting possibilities,
NHE5 could be overexpressed or knocked-down in CA1 pyramidal neurons using virus-mediated transfection in cultured organotypic hippocampal slices or by direct injection of virus into the CA1 region of the brain and changes in LTP or LTD could be measured following HFS or LFS stimulation respectively. As an alternative strategy to examine the role of NHE5 in synaptic plasticity, LTP and LTD could be examined in brain slices prepared from wild-type and NHE5-null mice. My model predicts that knock-down or knock-out of NHE5 may reduce or inhibit the onset or maintenance of LTD and may enhance the induction of LTP. Finally, as LTP and LTD are believed to be the cellular correlate of learning and memory, a functional role for NHE5 in synaptic plasticity may be revealed by a learning and memory deficit in NHE5 knock-out mice in behavioral tests.

6.2.6 Organization of NHE5 at the synapse

The composition and structure of the synapse is tightly regulated by multiple levels of scaffolding, cytoskeleton rearrangements and control of membrane trafficking. In particular, the post-synapse contains a striking structure known as the post-synaptic density (PSD), an electron dense structure easily observed in transmission electron microscopy. The PSD is believed to be made up of a dense network of scaffolding proteins that organize and anchor essential post-synaptic constituents. The PSD is also believed to be essential for controlling synaptic strength by regulating the post-synaptic content of neurotransmitter receptors (Kessels and Malinow, 2009). As mentioned above, the precise nature of the NHE5-mediated proton feedback mechanism may be dictated by the localization of NHE5 in space and assembly into macromolecular complexes. Interestingly, we showed previously that NHE5 could directly interact with RACK1 and that this interaction enhanced NHE5 transporter activity (Onishi et al.,
RACK1 is also known to bind to the C-terminus of the NR2B subunit of the NMDA receptor, an interaction which was shown to inhibit the NMDA receptor (Yaka et al., 2002). This action of RACK1 on the NMDA receptor may act preferentially in certain brain regions such as the hippocampus (Yaka et al., 2003). A possible NHE5-RACK1-NR2B triple complex would mean that NHE5 would only have to affect the pH in an area covering a protein-protein interaction to have a meaningful effect on NMDA receptor activity. NHE3, an epithelial isoform related to NHE5 (Attaphitaya et al., 1999; Baird et al., 1999), has been shown to directly interact with the PDZ-domain containing protein Shank2 and this interaction stabilizes NHE3 on the cell surface (Han et al., 2006; Lee et al., 2010). In neurons, Shank2 and the related proteins Shank1 and 3 make up a major component of the PSD and are known to anchor several different proteins at the synapse (Naisbitt et al., 1999; Tu et al., 1999). NHE5 is highly similar to NHE3 raising the possibility that NHE5 may also bind Shank proteins. Further, Shank proteins are known to interact with NMDA receptors through the additional scaffold proteins PSD95 and guanylate kinase associated protein (GKAP) (Naisbitt et al., 1999; Tu et al., 1999). Therefore, interaction with Shank could also position NHE5 in the PSD adjacent to the NMDA receptor. Indeed preliminary results show that Shank, the NR2B subunit of the NMDA receptor, and RACK1 co-immunoprecipitate with NHE5 in neurons (Fig. 6-1A). Thus, NHE5 positioning at the synapse may be highly regulated by interaction with RACK1 and/or Shank to allow for tight spatial regulation of pH-sensitive synaptic proteins such as the NMDA receptor (Fig. 6-1B, 1C). The precise nature of NHE5 scaffolding will require further biochemical analysis, possibly through in vitro protein-binding assays to test which components of the NHE5 interactome bind to NHE5 directly, and which through intermediate proteins.

An additional strategy to examine the organization of NHE5 at the synapse would be through visualization of NHE5 using immuno-gold electron microscopy (EM). Using
**Fig. 6-1 NHE5 organization at the synapse.** (A) Cultured neurons (14DIV) were lysed and subjected to immunoprecipitation (IP) using anti-NHE5 antibody (NHE5) or pre-immune serum (Con.). Western blot was used to detect bound Shank, using a pan-Shank antibody, NMDA receptor subunit NR2B, or RACK1. Input control equals 2% of the protein used for IP. (B and C) Models for possible complexes linking NHE5 to the NMDA receptor (NMDAR) through NHE5 binding to RACK1 (B) or Shank, GKAP and PSD95 (C).
this strategy it may be possible to determine the proportion of NHE5 which is found on the plasma membrane in or adjacent to the PSD as opposed to on the dendritic spine plasma membrane but outside of the PSD. Immuno-gold EM may allow us to address further questions about the intracellular compartment that delivers NHE5 to the cell surface in response to synaptic activity such as: How many NHE5 molecules are delivered to each synapse? Is NHE5 content at synapses scaled with synapse size?

Lastly, in this work I determined that NHE5 was present in both axons and dendrites (Fig. 3-2), and that NHE5 could be targeted to synapses. However, only NHE5 targeting to the post-synapse was investigated (Fig 4-4). It would therefore be of interest to determine whether NHE5 is also present on the pre-synapse plasma membrane or possibly in pre-synapse intracellular compartments such as synaptic vesicles, and this is best determined through EM.

6.3 Molecular regulation of NHE5

6.3.1 NHE5 binding partners: β-arrestin, RACK1, and SCAMP2

The final objective of this thesis was to further the understanding of the molecular regulation of NHE5, with a focus on the identification and characterization of novel NHE5 interacting proteins. Understanding how NHE5 may be regulated is a complementary strategy to understand the role of NHE5 in the brain. In this work I identified SCAMP2 as a novel NHE5-interacting protein. NHE5 and SCAMP2 directly interact in the recycling endosomal compartment where SCAMP2 acts to promote the delivery of NHE5 to the cell surface in a pathway that requires the small GTPase Arf6. I also show that NHE5 can access the cell-surface in a separate pathway controlled by Rab11 and independent of SCAMP2/Arf6. These findings add to a growing list of proteins and signaling pathways that regulate NHE5 (Fig. 6-2). The work presented here on the
NHE5-SCAMP2 interaction and previous work showing interaction with β-arrestin (Szabo et al., 2005) and RACK1 (Onishi et al., 2007) were all performed in non-neuronal tissue culture cell lines. Therefore, an important question remaining is whether NHE5 interacts with these proteins in neurons and whether and how these NHE5-interacting proteins regulate NHE5 trafficking and localization to synapses. NHE5 is delivered to the plasma membrane and to synapses in response to synaptic activity where it acts to limit dendritic spine growth (Chapter 4). Previously it was found that β-arrestins could directly bind NHE5 and promote its endocytosis. One possibility is that β-arrestins may act to reverse the action of NHE5 by enhancing its endocytosis and resetting NHE5 surface abundance to basal levels. As mentioned above, RACK1 may tether NHE5 to the NMDA receptor through the formation of a triple-complex with the NR2B subunit (Onishi et al., 2007; Yaka et al., 2002). We showed in our previous work that NHE5 was activated by interaction with RACK1 after integrin receptor engagement. We also showed that NHE5 could be targeted to focal adhesion complexes in a fibroblast cell line where NHE5 interacts with integrinβ1 (Onishi et al., 2007). Integrin receptors in neurons are not found in large focal adhesion complexes but rather can be targeted to synapses where they mediate synapse adhesion and play a role in synaptic plasticity (Chan et al., 2007; Cingolani et al., 2008; Huang et al., 2006). NHE5 may therefore interact with integrin at synapses and be regulated by integrin signaling pathways, including RACK1, as we found in fibroblasts.

In chapter 5, I show that NHE5 targeting to the cell surface could be enhanced by binding to SCAMP2. SCAMP1 also binds NHE5 (by CoIP, Fig. 5-1) and colocalizes with NHE5 in recycling endosomes (Fig. 5-3). SCAMP1 and to a lesser extent SCAMP2 are highly expressed in the brain, but their role in brain function is only beginning to be addressed (Castermans et al., 2010; Kurian et al., 2009; Zhao et al., 2009). In neurons,
Fig. 6-2. Summary of mechanisms of NHE5 regulation. This cartoon summarizes all the known regulators of NHE5 trafficking (green arrows) and transporter activity (red arrows). The known NHE5 binding partners are listed. Black arrows and plungers represent pathways shown to activate or inhibit NHE5 activity at the cell surface respectively.
SCAMPs may bind NHE5 in endosomes and promote NHE5 targeting to synapses during synaptic activity. These possibilities could be addressed by first examining the localization of NHE5 and SCAMPs in neurons, in conjunction with endosome or synapse markers. Second it would be of interest to examine the effects of the SCAMP2 mutants SCAMP2ΔNPF and SCAMP2 1-154 used in chapter 5, on synaptic targeting of NHE5 and dendritic spine morphology. These mutant constructs impaired NHE5 cell surface targeting in fibroblasts and may similarly impair NHE5 targeting to synapses in response to NMDA receptor activation. SCAMPs have also been shown to be present in synaptic vesicles (Fernandez-Chacon and Südhof, 2000). Further, I have shown that NHE5 is present in axons, the site of the pre-synapse. Therefore, NHE5 could bind to SCAMPs at the pre-synapse and in synaptic vesicles, where NHE5 may play a role in controlling the luminal pH of synaptic vesicles. SCAMPs also appeared as candidates in a screen looking for proteins which were palmitoylated in a synaptic activity dependent manner (Kang et al., 2008), and SCAMP palmitoylation was subsequently verified. The precise role of SCAMP palmitoylation on SCAMP activity is not clear at this time but it is possible that activity-dependent palmitoylation of SCAMPs may constitute part of the mechanism by which SCAMPs deliver NHE5 to the synaptic surface during synaptic activity.

6.3.2 NHE5 targeting by Rab11 and Arf6

In chapter 5, I also describe how NHE5 targeting to the cell-surface can be mediated by the small GTPases Arf6 and Rab11. Arf6 and its GEF ARNO bind to SCAMP2 (Liu et al., 2005) and SCAMP2 mediated targeting of NHE5 to the cell-surface required an active Arf6 (Fig. 5-8). Rab11 could promote NHE5 surface targeting presumably through a separate pathway, independent of SCAMP. Both Rab11 and Arf6 are known to be active at synapses and to control the formation and plasticity of
dendritic spines. Rab11 supports dendritic spine growth and is important for spine expansion during LTP (Park et al., 2006; Wang et al., 2008b), and has also been shown to be crucial for the activity-dependent targeting of AMPA receptors and recycling endosomes to the post synapse during LTP (Brown et al., 2007; Park et al., 2004; Wang et al., 2008b). Arf6 on the other hand, has been shown to regulate AMPA receptor removal from the synapse (Scholz et al., 2010). Further, expression of a dominant-negative Arf6 resulted in increased dendritic spine formation suggesting that Arf6 may be a negative regulator of dendritic spine growth (Miyazaki et al., 2005). However some of these findings have been questioned (see Choi et al., 2006). One possibility is that expression of dominant-negative Arf6 results in reduced NHE5 trafficking to the synapse leading to increased dendritic spine growth, similar to the phenotype observed upon NHE5 knock-down. Whether the actions of Rab11 and Arf6 on dendritic spine formation and synaptic membrane traffic are coordinated or separate is not known, but it seems likely that NHE5 trafficking to the synapse will involve either or both of these GTPases, perhaps under different stimulation conditions or in different steps in the trafficking cycle.

A hallmark of LTP is the delivery of AMPA receptors to the cell-surface via recycling endosomes and their subsequent insertion into the synapse. Several investigations have suggested that in addition to AMPA receptors, recycling endosomes delivered to the synapse during synaptic activity may contain a “plasticity package” a collection of different proteins or complexes delivered to synapses in the same endosomes. Components of this package act coordinately to support and stabilize synaptic strengthening and dendritic spine expansion (Li et al., 2007; Park et al., 2004; Park et al., 2006). NHE5 is also recruited to synapses during synaptic activity and is present in recycling endosomes. If NHE5, like AMPA receptors, is also recruited to synapses by Rab11, then it is highly likely that NHE5 will be one component of this...
plasticity package, further supporting the notion that local activity-dependent pH shifts may be an important part of synapse remodeling and synaptic plasticity.

6.3.3 NHE5 phosphorylation during synaptic plasticity

Though not addressed in this thesis work, an important possibility regarding the molecular regulation of NHE5 is that NHE5 activity and trafficking may be regulated by direct phosphorylation. Indeed other NHEs are regulated by direct phosphorylation (Alexander and Grinstein, 2009; Meima et al., 2007; Meima et al., 2009). NHE5 expressed in fibroblasts was shown to be inhibited by manipulations that activated PKA or PKC, suggesting regulation of NHE5 by kinases (Attaphitaya et al., 2001). However, it is not known whether these kinases directly phosphorylate NHE5. NHE5 trafficking was also dependent on the activity of PI3K, suggesting indirect regulation of NHE5 by the abundance of phosphatidylinositol phosphates (PIPs) (Fig. 6-2). Several protein and lipid kinases and phosphatases are implicated in synaptic plasticity. For instance both PKA and CaMKII are known to be activated during LTP (Malenka and Bear, 2004). Both these kinases directly phosphorylate AMPA receptor subunit GluR1 that promotes GluR1 containing AMPA receptor targeting and retention at synapses (Kessels and Malinow, 2009; Shepherd and Huganir, 2007). Similarly, phosphorylation of NHE5 may be important for targeting to synapses during activity. One way to test this possibility would be to examine activity-induced NHE5 cell-surface targeting in the presence of various pharmacological inhibitors such as H-89, to inhibit PKA, or KN-93, to inhibit CaMKII. In vitro kinase assays may also be useful to test whether different kinases are able to phosphorylate NHE5. It is interesting to note that PKA activation is important during induction of LTP (Malenka and Bear, 2004), and that during LTP NHE5 surface targeting and activity are enhanced. Yet in fibroblasts PKA activation was found to
inhibit NHE5. PKA is also known to inhibit NHE3 (Hu et al., 2001; Zizak et al., 1999). However, when NHE3 was in complex with Shank2, NHE3 was stabilized on the cell surface and protected from inhibition by PKA (Han et al., 2006; Lee et al., 2010). Similarly, it is possible that NHE5 targeted to synapses during LTP forms a complex with Shank and is protected from inhibition by PKA, allowing NHE5 to participate in synaptic plasticity through local Na+/H+ exchange.

6.3.4 Other NHE5-protein interactions

An additional strategy to learn more about the regulation and function of NHE5 would be to attempt to identify and characterize additional protein-protein interactions. Already in our lab we have performed a yeast two-hybrid screen that identified several interesting candidates for NHE5 binding in addition to RACK1 (Onishi et al., 2007). Moreover, we, in collaboration with Dr. Leonard Foster (Biochemistry and Molecular Biology, UBC), performed a pull-down assay by incubating different GST-tagged regions of the NHE5 C-terminus with rat brain lysate. Proteins bound to the immobilized NHE5 fragments were then identified using mass-spectrometry. Candidate binding proteins identified from this approach and the yeast two-hybrid screen will need to be validated through additional protein-binding studies. Indeed, a few candidate binding partners have been confirmed by preliminary co-immunoprecipitation experiments (not shown). One of these candidates is synaptotagmin1 (syt1), a synaptic vesicle component which is important for triggering the Ca++-induced fusion of synaptic vesicles with the plasma membrane (Südhof, 2008). NHE3 has also been shown to directly interact with syt1 in intestinal epithelium where syt1 plays a role in facilitating NHE3 endocytosis (Musch et al., 2007; Musch et al., 2010). The NHE5/NHE3 similarity together with our preliminary results, suggest that the NHE5-syt1 interaction is highly likely. Intriguingly, syt1 is
believed to interact with VGCCs in order to position the synaptic vesicle release machinery in close proximity to the Ca"++-source necessary for vesicle release (Sheng et al., 1997). As discussed above VGCCs are pH-sensitive. Therefore, interaction with syt1 may position NHE5 adjacent to VGCC allowing NHE5 to tightly regulate synaptic vesicle release through proton-mediated feed-back of VGCC activity.

Other putative NHE5-binding partners of interest include N-ethylmaleimide sensitive factor (NSF), all the subunits of the clathrin adaptor protein complex 2 (AP2), and the neuronal isoform of erythrocyte membrane protein band 4.1 (4.1N). NSF, AP2, and 4.1N are all known to bind post-synaptic AMPA receptor subunits and to regulate AMPA receptor targeting to the synapse (Kessels and Malinow, 2009; Shepherd and Huganir, 2007). This raises the possibility that NHE5 trafficking at the synapse may also be regulated by binding to these proteins, again suggesting that NHE5 and AMPA receptors may form a synaptic “plasticity package” to coordinately regulate synaptic transmission. Further experiments will need to be performed to confirm NHE5 interaction with NSF, AP2, and 4.1N and to characterize their role in controlling NHE5 trafficking.

6.4 NHE5 in endosomes

In my results and subsequent discussion, I have focused on the activity of NHE5 on the plasma membrane at synapses and on local regulation of pH-sensitive proteins at the synaptic cleft. However, based on microscopy analysis and cell-surface biotinylation assays it is apparent that a substantial proportion of NHE5 is found intracellularly in recycling endosomes, consistent with a previous study of ectopically expressed NHE5 in non-neuronal cells. This observation raises the possibility that part of NHE5’s function may be to regulate the pH of the endosome lumen. Indeed, the
related isoform NHE3 has been shown to regulate recycling endosome pH, which in turn affects endocytosis and vesicle trafficking (D'Souza et al., 1998; Gekle et al., 1999; Gekle et al., 2001). In order to address this possibility, I measured endocytosis and endosome pH in AP-1 based cell lines. Cells stably expressing NHE5 had significantly increased rates of transferrin uptake and acidic endosomes compared to untransfected control cells (Fig. 6-3). Conversely, expression of transport-dead NHE5 E209I resulted in the opposite trend of reduced transferrin uptake and alkaline endosomes (Fig. 6-3). These results indicate that NHE5 is active in recycling endosomes, acting in a similar fashion as NHE3 to acidify the endosome lumen and accelerate endocytosis. While preliminary, these findings suggest that NHE5 may have multiple physiological functions, similar to the related isoform NHE3. At the synapse surface, NHE5 may control synaptic cleft pH and therefore regulate the activity of nearby pH-sensitive proteins. While in endosomes, NHE5 may play a role in controlling membrane turnover and endocytosis through the regulation of organelle lumen pH. In fact, the observed role of NHE5 on dendritic spine growth may actually result from coordinated cell-surface and endosomal actions of NHE5. With this in mind, it would be interesting to examine in future experiments whether overexpression or knock-down of NHE5 affects endocytosis and membrane turnover in neurons.
**Fig. 6-3.** NHE5 is functional in endosomes. (A) Parental untransfected AP-1 cell or AP-1 cells expressing HA-tagged NHE1, wild-type NHE5, or transport-dead NHE5 E209I were incubated with Alexafluor568-conjugated transferrin for the indicated times. Cells were rinsed, fixed, and the average amount of fluorescence uptake per cell was measured using an automated confocal microscope. Asterisks indicate statistical significance (p<0.05 Student’s t-test) from parental AP-1 cells at the corresponding time points. (B) AP-1 based cell lines were incubated with pH-sensitive fluorescein-conjugated transferrin together with pH-insensitive Alexafluor568-conjugated transferrin to form a ratiometric endosome pH reporter. Live cells were imaged using an automated confocal microscope and the average fluorescence ratio was calculated. Standards were prepared by incubating transferrin loaded cells with the protonophore Nigericin (10μM) and high K⁺ at pH 6-7. Fluorescence ratios were converted to absolute pH. Asterisks indicate statistical significance (p<0.05 Student’s t-test) from the untransfected AP-1 cells.
6.5 Concluding remarks

The goal of this thesis was to elucidate the physiological function and regulation of NHE5, a unique member of the NHE gene family highly enriched in brain. This work is of additional significance given the profound sensitivity of electrical activity and synaptic transmission to fluctuations in pH in the brain. Further, while many components of the synaptic machinery are pH sensitive, and pH fluctuations are known to occur in conjunction with synaptic transmission, the molecules responsible for controlling pH at the synapse have not been identified. This thesis work is the very first demonstration of a pH-regulatory protein present and active at the synapse. I have shown that local control of pH at the synapse by NHE5 is a novel regulatory mode to control dendritic spine growth during synaptic plasticity.

My work has raised many exciting questions about the physiological function of NHE5. First, I have implicated the NMDA receptor as a pH-sensitive protein modulated by NHE5. This proton-based feedback mechanism is indeed a novel mode of regulation of the NMDA receptor, not previously described. However, it is likely that other pH-sensitive synaptic proteins may also be regulated by NHE5. Second, I have shown that NHE5 is a negative-regulator of dendritic spine growth, a hallmark of LTP. This is the first demonstration of NHE5’s physiological function in the brain. NHE5 may regulate other aspects of synapse physiology such as synaptic vesicle release. Future experiments regarding NHE5 would benefit from the use of systems such as brain slices to more easily examine the role of NHE5 in LTP, LTD, and other synapse parameters such as AMPA/NMDA ratio and paired-pulse facilitation. A role for NHE5 during development, such as in neuritogenesis and synaptogenesis should also be of great interest. These questions will all be greatly aided by the development of additional
materials, such as an NHE5-null mouse, virus-mediated transfection reagents, and NHE5-specific inhibitor compounds.

Lastly, an understanding of the role of NHE5 in brain function will continue to be aided by a greater knowledge of the regulation of NHE5 at the molecular level. This will be brought about by the identification and characterization of novel NHE5-interacting proteins, and phosphorylation events, as well as a better understanding of the localization of NHE5 at the pre and post-synapse.

A great deal of effort has been spent to understand the molecular basis of synaptic transmission, synaptic plasticity, and learning and memory. Several key themes have emerged such as the central importance of the NMDA receptor and downstream Ca\(^{++}\)-sensitive signaling cascades such as those involving CaMKII and calcineurin. In contrast, the role of synaptic protons and the molecules responsible for regulation of pH at the synapse have received far less attention. Given the pH-sensitive nature of synaptic transmission, the study of proteins like NHE5 that act to regulate pH at the synapse will give a greater understanding into the molecular basis of synaptic transmission, synaptic plasticity, and information processing in the brain. Modulation of synaptic activity by localized changes in pH may in fact be an additional layer of signaling that occurs in parallel with Ca\(^{++}\)-sensitive signaling pathways. Finally, it is possible that NHE5 may offer a new therapeutic window for pharmacological manipulation of synaptic efficacy and synaptic plasticity which may be important for the treatment of neurological disease that affects these aspects of nervous system function.
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