

A Distinct Role for Endosomal Na⁺-H⁺ Exchanger 9 (NHE9) from NHE6 in Neuronal Development

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A dissertation submitted in partial fulfillment of the requirements for the degree of
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This dissertation by Laura Nathalie Sciarra is accepted in its present form by the
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Gamsiz ED*, **Sciarra LN***, Maguire AM, Pescosolido MF, van Dyck LI, and Morrow EM. (2015) Discovery of Rare Mutations in Autism: Elucidating Neurodevelopmental Mechanisms. *Neurotherapeutics* **12**(3): 553-571.

ABSTRACTS

Bonaccorsi LN, Hammond RS, Henderson CS, and Healy A. STX107, a Novel mGluR5 Negative Allosteric Modulator, Reduces Marble Burying Behavior in a Mouse Model of Fragile X Syndrome [abstract]. Program No. 757.16. San Diego, CA: Society for Neuroscience, 2010.

Preface

Autism constitutes a diverse set of disorders thought to affect neuronal connectivity and circuit development. Its etiology is heterogeneous, but certain rare genetic mutations have been implicated in autism. In this thesis, I focus on endosomal Na^+ - H^+ exchangers, NHE6 and NHE9, and their roles in the process of neuronal development. I first describe the development of unique reagents crucial to our studies, after which I utilize those reagents to answer fundamental research questions. First, where within the endosomal pathway of neurons are Nhe6 and Nhe9 localized, and how do these localization patterns relate to one another? Second, what is the effect of Nhe9 loss on endosomal pH within neurons? And third, given the contrasting results in pH measurement with Nhe6 and Nhe9 loss, what is the effect of Nhe6 and Nhe9 on the trafficking of cargo throughout the endosomal system?

More specifically, Chapter 3 demonstrates that, in contrast to previous studies of mammalian cell lines, Nhe6 and Nhe9 localize to all endosomal compartments within primary hippocampal neurons. The localization patterns of Nhe6 and Nhe9 overlap to some extent; however, in large measure, their localizations are distinct. In Chapter 4, I show that loss of Nhe9 results in an increase in more alkaline endosomes in neurons, a surprising yet intriguing result, based on prior knowledge that Nhe6 loss overacidifies the endosome. I then proceed to investigate the effect of Nhe6 and Nhe9 loss on the trafficking of cargo through endosomes, as a model to explain the unexpected pH result. Data

from these studies suggest that there is a back-up of cargo in more alkaline early endosomes in *Nhe9*-null neurons, whereas *Nhe6*-null neurons may represent an acceleration of cargo to more acidic late endosomes. Thus, the work described within this thesis demonstrates a distinct role for NHE9 from NHE6 in neuronal development and informs the field as to the relationship between these two proteins.

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CHAPTER 1:

INTRODUCTION

Section on Christianson syndrome excerpted with permission of Springer from:
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Morrow EM. (2015) Discovery of Rare Mutations in Autism: Elucidating
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Developmental Disability and Identification of Na⁺-H⁺ Exchanger (NHE)

Mutations

Developmental disability (DD) is defined as a chronic condition manifesting during childhood, resulting in impairments to physical, behavioral, and/or cognitive functions (Centers for Disease Control and Prevention). Examples of DD include autism, cerebral palsy, fetal alcohol syndrome, fragile X syndrome, and intellectual disability (ID). Many forms of DD have an identified cause. For example, a mutation of the *MECP2* gene on the X chromosome leads to Rett syndrome (Amir et al., 1999), whereas possession of three copies of chromosome 21 results in Down syndrome (Lejeune et al., 1959). Other forms of DD, particularly autism, are currently less well understood, specifically with regard to etiology (An and Claudianos, 2016; De Rubeis and Buxbaum, 2015; Gamsiz et al., 2015). It is clear that there is not always a single genetic or environmental insult leading to a given disorder, and although many contributing factors have been identified, some cases of DD remain idiopathic. Through continued studies of mechanisms and functions, researchers aim to classify additional origins so that prevention and treatment may become a possibility.

Fortunately, the advent of advanced techniques, such as genome-wide microarray studies, has augmented the ability of scientists and physicians to investigate genetic contributors to DD (Sun et al., 2015; Weiss, 2009). One group of genes repeatedly identified in DD patients is solute carrier family 9 (*SLC9*), encoding multiple distinct sodium-proton (Na⁺-H⁺) exchangers (NHEs) (Zhao et al.,

2016). Gilfillan *et al.* (2008) published findings linking *SLC9A6* (also known as *NHE6*) mutations to the occurrence of a syndrome mimicking that of Angelman. Affected individuals presented with ID, ataxia, seizures, microcephaly, and speech deficits. As these patients were males, the gene responsible was presumptively localized to the X chromosome. By analyzing family pedigrees, performing linkage analyses, and sequencing DNA samples, researchers implicated a mutated portion of the q arm of chromosome X, which included a genetic sequence coding for the NHE6 protein (Gilfillan *et al.*, 2008).

Similarly, de Silva *et al.* (2003) identified a family in which members displayed features of attention-deficit/hyperactivity disorder (ADHD) and ID. Prior cytogenetic analysis had revealed a pericentric inversion on chromosome 3 segregating with a developmental phenotype (Efron *et al.*, 2003). Disrupted within this region were *DOCK3* (dedicator of cytokinesis 3, a member of the guanine exchange factor family), as well as *SLC9A9* (also known as *NHE9*), a novel member of solute carrier family 9 (de Silva *et al.*, 2003).

NHE9 has also been implicated in cases of autism with comorbid epilepsy. In a study searching for heritable causes of autism spectrum disorders (ASDs), Morrow *et al.* (2008) analyzed multiple related and unrelated pedigrees before performing homozygosity mapping. Results once again indicated mutations on chromosome 3, mapped to the *SLC9A9* locus (Morrow *et al.*, 2008).

NHE6 in Neurodevelopmental Disorders

Christianson Syndrome

Christianson syndrome (CS), first noted in 1999 in a 5-generation South African pedigree, is an X-linked recessive neurodevelopmental disorder found in males (Christianson et al., 1999). Although the exact prevalence is not known, CS is estimated to affect between 1 in 16,000 and 1 in 100,000 individuals (Pescosolido et al., 2014). A majority of CS individuals are nonverbal and present with epilepsy, ID, and ataxia. Additional core symptoms are postnatal microcephaly and hyperkinetic behavior. Other symptoms may include eye movement dysfunction, gastro-esophageal reflux disease, low muscle tone, cerebellar atrophy, and phenotypic regression (Christianson et al., 1999; Gilfillan et al., 2008; Pescosolido et al., 2014). In one study of phenotypic diversity within CS, 43% of affected individuals received a clinical diagnosis of autism, and a majority of patients present with autistic symptoms (Pescosolido et al., 2014).

CS is the result of likely loss-of-function mutations in *SLC9A6*, encoding the NHE6 protein. The first connection between CS and *SLC9A6* mutations was published in 2008 by Gilfillan *et al.* Researchers identified four distinct *SLC9A6* mutations in individuals with a CS phenotype within different families: a 6-base pair deletion resulting in the loss of a glutamic acid and a serine, a premature stop codon resulting in loss of the C-terminus of the protein, a splice-site mutation skipping over exon 3 and deleting the fourth transmembrane domain of the protein,

and a 2-base pair deletion resulting in a frameshift and protein truncation (Gilfillan et al., 2008).

Since that first report, additional novel mutations in *SLC9A6* have been characterized. These include: a point mutation resulting in a premature stop codon (Schroer et al., 2010); a single nucleotide deletion resulting in a frame shift (Takahashi et al., 2011); a single nucleotide insertion resulting in a frame shift; and a point mutation at the junction of exon 4 and intron 4 (Riess et al., 2013). Overall, the disease-associated genetic changes present in *SLC9A6* primarily represent loss-of-function mutations.

Other Central Nervous System (CNS) Pathology

In addition to ID and developmental dysfunction, *SLC9A6* has been implicated in further brain pathology. Garbern *et al.* (2010) published a study describing a family with severe ID, autistic traits, ataxia, and epilepsy. The phenotypic similarity to CS as well as linkage mapping of the X chromosome led researchers to suspect an *SLC9A6* mutation. Sequencing revealed a 9-base pair deletion in *SLC9A6* that segregated with the X-linked ID phenotype throughout the pedigree. Unique to this particular study, however, was a postmortem analysis of two male family members showing neuronal loss and tau deposition, suggesting that NHE6 may also play a role in neurodegeneration (Garbern et al., 2010).

An additional degenerative phenotype was reported by Mignot *et al.* in 2013. A young adult male was described as having CS, due to a cytosine (C) to thymine

(T) substitution, resulting in a novel nonsense mutation in *SLC9A6*. In accordance with other reports, cerebellar atrophy was evident on a magnetic resonance imaging (MRI) scan, but this case also included evidence of retinal degeneration, in the form of retinitis pigmentosum (Mignot et al., 2013).

Finally, a brief report by Zanni *et al.* (2014) described a young boy with classic CS symptoms, due to a novel splice-acceptor-site mutation (guanine [G] to adenine [A]) in *SLC9A6*. In addition to absence seizures during wakefulness, this patient exhibited increased epileptiform activity while asleep. Continuous spikes and waves throughout the entire sleep period revealed electrical status epilepticus during slow-wave sleep (ESES) (Zanni et al., 2014).

NHE9 in Neurodevelopmental Disorders

ADHD

The first connection between NHE9, then a novel NHE family member, and a brain disorder occurred in 2003. de Silva *et al.* identified a family in which members displayed features of ADHD and ID. Karyotype analysis of the proband revealed one abnormal copy of chromosome 3, with a pericentric inversion resulting in the loss of five base pairs. This inversion segregated with an ADHD/ID phenotype throughout the pedigree. Inverse and long-range polymerase chain reaction (PCR) techniques were used to amplify the breakpoint region on the q arm of chromosome 3, and the mutation was ultimately localized to the intron between exons 13 and 14 within the *SLC9A9* gene (de Silva et al., 2003). Seven

years later, Markunas *et al.* (2010) published a report of a family-based association study that identified two single nucleotide polymorphisms (SNPs) within *SLC9A9* that demonstrated significant association with ADHD symptoms. Interestingly, the other gene candidate disrupted by the pericentric inversion detailed by de Silva *et al.* (2003), *DOCK3*, showed no SNPs significantly associated with any of the ADHD traits (Markunas et al., 2010).

The connection between *SLC9A9* and ADHD has since been bolstered by multiple genome-wide association studies (GWAS). Lasky-Su *et al.* (2008) analyzed approximately 430,000 autosomal SNPs in over 900 proband-parent trios. From a list of 37 genes previously identified as ADHD candidates, *SLC9A9* was shown to have the strongest overall association with the ADHD phenotype, although this association did not reach significance when corrected for multiple comparisons across analyses (Lasky-Su et al., 2008).

An additional GWAS performed by Mick *et al.* (2010) confirmed the result of Lasky-Su *et al.* (2008). This study tested ADHD phenotype association in approximately 835,000 SNPs in 735 proband-parent trios. Once again, *SLC9A9* emerged with the strongest association of all candidate genes evaluated, and it was the only candidate with a SNP among the top 20 most significant (Mick et al., 2010).

Slc9a9 mutations have also been identified in animals demonstrating the ADHD phenotype. Outbred Wistar Kyoto (WKY/NCrl) rats have been established as an experimental model of inattentive ADHD (Sagvolden et al., 2008), and this

strain shows evidence of *Slc9a9* dysfunction. The WKY/NCrl rat possesses two nonsynonymous single-nucleotide changes in conserved regions on chromosome 8, resulting in two amino acid changes (V512G and K534R) and mapping to the *Slc9a9* gene locus (Zhang-James et al., 2011). In a further study, this rat strain was also shown to exhibit differential expression of *Slc9a9* mRNA in adult hippocampus when compared to a control strain (WKY/NHsd) (Zhang-James et al., 2012).

Autism with Epilepsy

The first report of *SLC9A9* as it relates to autism and epilepsy was published in 2008 by Morrow *et al.* Homozygosity mapping of consanguineous pedigrees was used to probe the heterogeneity of autism etiology. Results, confirmed by PCR, indicated the presence of a homozygous 886 kilobase pair deletion on chromosome 3, just upstream of the *SLC9A9* transcript initiation site, in a proband with autism and comorbid epilepsy. Furthermore, a nonsense mutation, which changes an arginine to a premature stop codon and is predicted to result in truncation of the protein, was identified in individuals with autism and epilepsy from non-consanguineous pedigrees (Morrow et al., 2008).

One additional case was recently reported by Cardon *et al.* (2016). Researchers used array comparative genomic hybridization to identify the loss of exon 2 of *SLC9A9* in a male diagnosed with autism and epilepsy, a deletion predicted to result in truncation of the protein (Cardon et al., 2016).

Interestingly, the nonsense mutation in *SLC9A9* reported by Morrow *et al.* (2008) occurs within a region similar to that which is spontaneously mutated in the *Nhe1* (*Slc9a1*) slow-wave epilepsy (*swe*) mouse. This animal demonstrates a neurological phenotype involving tonic-clonic and absence seizures, ataxia, and neuronal death within the cerebellum. Through the use of single-strand conformation polymorphism analysis and PCR amplification, the mutation in question was determined to be an A to T substitution, resulting in the change of a lysine to a stop codon upstream of the twelfth transmembrane domain. The *swe* mouse represents the first report of a disease-causing mutation in an *Nhe* gene (Cox *et al.*, 1997).

Autism

SLC9A9 expression is also misregulated in certain cases of idiopathic autism. Schwede *et al.* (2014) evaluated publically available gene expression data from postmortem cortical samples. When compared to controls, many samples from autism cortices demonstrated a significant increase in *SLC9A9* transcript level, coupled with a decrease in that of *SLC9A6*. In addition, both genes showed a strong association with *NRXN1* (neurexin 1), an established autism-related gene (Schwede *et al.*, 2014).

Multiple groups have since demonstrated upregulation of *SLC9A9* gene expression in postmortem autism brains, a result withstanding genome-wide corrections (Garbett *et al.*, 2008; Voineagu *et al.*, 2011). Kondapalli *et al.* (2013)

confirmed in yeast that missense mutations in autism found by Morrow *et al.* (2008) are indeed loss-of-function, and Vardarajan *et al.* (2013) have independently implicated NHE9 in autism (Kondapalli *et al.*, 2013; Vardarajan *et al.*, 2013).

Slc9a9 has also been implicated in autism through the use of animal models. Yang *et al.* (2016) hypothesized that *Slc9a9*-knockout (KO) mice would exhibit evidence of ASD-like traits. Female KO pups emitted significantly fewer ultrasonic vocalizations, suggestive of communication impairments. *Slc9a9* KO mice also exhibited deficits in a three-chamber social interaction test, spending less time with a novel animal than their wild-type counterparts, suggestive of the social deficits characteristic of autism. Additionally, KO mice showed increased grooming behavior when compared to controls, which may represent the restricted and repetitive interests/behaviors present in many autistic individuals (Yang *et al.*, 2016).

Sodium-Proton Exchangers

Mutations in sodium-proton exchanger genes thus have profound phenotypic impacts; what then is important about the expression and function of the proteins encoded by these genes? NHEs are a nine-member family of integral membrane proteins tasked with exchanging hydrogen ions for sodium ions in an electrically neutral manner along the ions' concentration gradients (Figure 1.1) (Casey *et al.*, 2010). They range in size from approximately 575 to approximately 890 amino acids. Although the individual structures have not been directly solved,

all NHE family members are predicted to possess 12 transmembrane domains, based on *in silico* modeling and the solved structure of the *Escherichia coli* Na⁺/H⁺ antiporter NhaA (Donowitz et al., 2013; Padan et al., 2009).

The N-terminus contains the domain responsible for ion translocation, which is thought to be mediated by the formation of a pore between adjacent transmembrane domains (Landau et al., 2007; Slepko et al., 2007). In contrast, the hydrophilic C-terminus contains the domain to which regulatory proteins can bind (Ohgaki et al., 2011). Different NHEs, both within and across species, exhibit a higher degree of sequence homology within their N-termini and a higher degree of sequence divergence within their C-termini, which suggests that the C-terminus may be specific to each family member (Ohgaki et al., 2008).

There are nine known NHEs present in vertebrates, encoded by the *SLC9A* gene family (Ohgaki et al., 2011). NHE1 through NHE5 localize to the cell surface, whereas NHE6 through NHE9 are primarily found in the membranes of intracellular organelles (Nakamura et al., 2005; Ohgaki et al., 2011).

NHEs and Endosomes

Multiple studies have demonstrated the important role of endosomes in nervous system development, including axon outgrowth and guidance (Sann et al., 2009; Yap and Winckler, 2012). Neurons are morphologically complex, polarized cells requiring transport of proteins to distinct cellular domains (Figure 1.3). Aiding in this process is the endosomal system, capable of internalizing,

recycling, and degrading proteins throughout the neuron (Lasiecka and Winckler, 2011).

The dynamic endosomal-lysosomal system comprises multiple membrane-bound intracellular organelles – early endosomes, recycling endosomes, late endosomes, and lysosomes. As cargoes move through the system, luminal pH progressively decreases (Figure 1.4), with lysosomes being the most acidic compartment (Mukherjee et al., 1997). Early endosomes have a pH of approximately 6.3, recycling endosomes have a pH of approximately 6.5, late endosomes have a pH of approximately 5.5 – 6.0, and lysosomes have a pH of approximately 4.7 – 5.5 (Casey et al., 2010; Ohgaki et al., 2011)

Once cargo has entered the early endosome of the neuron, it faces one of three pathways. Rapid recycling back to the initial site of cargo internalization at the plasma membrane can be achieved directly from early endosomes. In contrast, cargo can be targeted for return to multiple locations on the plasma membrane, moving through recycling endosomes. Finally, cargo can be targeted for degradation, trafficking through late endosomes to lysosomes (Yap and Winckler, 2012).

Evidence supports a role for endosomal recycling in neuronal outgrowth. Yap *et al.* (2008) reported that endocytosed NgCAM, a neuron-glia cell adhesion molecule known to function in outgrowth (Buchstaller et al., 1996), was transported out of the somatodendritic compartment to the axonal compartment, where it was recycled back to the plasma membrane (Yap et al., 2008). Further work from the

same group revealed that interruption of function through the expression of an antisense-NEEP21 (neuron-enriched endosomal protein, 21 kDa)-GFP resulted in a decrease in dendrite and axon lengths (Lasiecka et al., 2014).

NHEs are important for proper endosomal function, as loss of Nhe6 expression in mouse has been shown to result in endosomal and lysosomal dysfunction. Stromme *et al.* (2011) demonstrated that neurons within the amygdala, hippocampus, and cortex exhibited an increased accumulation of GM2 ganglioside and cholesterol in late endosomes and lysosomes, suggesting the compromise of degradative functions. Additionally, histochemical staining for lysosomal enzymes was undetectable in brain tissue, confirming the prior result. Intriguingly, upon behavioral examination, these mice recapitulated aspects of the CS phenotype, including hyperactivity and deficits in motor coordination, linked to cerebellar dysfunction (Stromme et al., 2011).

Yeast Nhx1

The study of endosomal NHEs began in *Saccharomyces cerevisiae*. Nass *et al.* (1997) described a gene present on yeast chromosome IV with strong homology to NHE1-4 in vertebrates, termed *Nhx1*. Mutations in the plasma membrane H⁺-ATPase (*pma*) in multiple yeast strains led to a decrease in sodium tolerance at an acidic pH, as well as loss of the ability to sequester sodium within an intracellular organelle, hypothesized to be the vacuole. This effect is mediated

through Nhx1, which contributes to the mutant *pma* Na⁺-tolerance phenotype (Nass et al., 1997).

A further study by the same group provided the first report that NHEs could localize to endosomes. Nass and Rao (1998) observed through cellular fractionation that HA (hemagglutinin)-tagged Nhx1 cofractionated with markers of both the yeast prevacuole (analogous to the mammalian late endosome) and the vacuole. Live imaging analysis confirmed this result, as GFP (green fluorescent protein)-tagged Nhx1 colocalized with both a vacuolar stain and a prevacuolar marker. In contrast, the Nhx1 signal was distinct from that of mitochondrial and Golgi markers (Nass and Rao, 1998).

In addition, studies of Nhx1 provided the first report that NHEs could help to mediate trafficking of proteins through the endosomal system. Site-directed mutagenesis of yeast *Nhx1* resulted in aberrant accumulation of various organellar markers near the vacuole. Additionally, the fluorescent styryl dye FM4-64 aberrantly accumulated in intracellular compartments in the *Nhx1* mutant. These results are suggestive of a defect in trafficking to the vacuole (Bowers et al., 2000). Re-expression of Nhx1 (as Nhx1-3GFP) rescued the defective trafficking of FM4-64 (Kojima et al., 2012).

Yeast *Nhx1* studies also linked Na⁺-H⁺ exchange to pH regulation. An *Nhx1*-null yeast strain exhibited an acidification in both the cytosol and the vacuole of 0.8 – 1 pH units as compared to wild-type, as measured through the use of ratiometric pHluorin (Brett et al., 2005). Similarly, when *Nhx1* activity is increased

through the loss of *gyp6* (GTPase-activating protein for Ypt6), a negative regulator of Nhx1 in yeast, vacuolar pH becomes less acidic (Ali et al., 2004).

NHEs in Mammalian Cell Lines

To date, NHE expression and function have been most extensively studied in mammalian cell lines. In an extension of the lab's work on *Nhx1* in yeast, Brett *et al.* (2002) described the localization of Nhe6 in Chinese hamster ovary and opossum kidney cells. Although a previous report had demonstrated overlap between NHE6 and a mitochondrial marker in HeLa cells (Numata et al., 1998), this result was not in accordance with phylogenetic predictions, nor was it repeated. Rather, NHE6-eGFP (enhanced green fluorescent protein) was shown to localize to early and recycling endosomes, as evidenced by colocalization with EEA1 (early endosome antigen 1, an early endosome marker) and Rab11 (a recycling endosome marker) (Brett et al., 2002).

Nakamura *et al.* (2005) expanded upon this observation, demonstrating that both NHE6 and NHE9 can localize to recycling endosomes. Nhe6 and Nhe9 were found within the recycling pathway of COS-7 simian kidney cells. Rhodamine-labeled transferrin was loaded into the cell; early after internalization, the signal overlapped with that of Nhe6, whereas later after internalization, the signal overlapped with that of Nhe9. This particular study also demonstrated the role of NHEs in pH regulation, as overexpression of the exchangers resulted in alkalization of the organelles (Nakamura et al., 2005). Interestingly, a further

study from the same group revealed that NHE6 can also transiently localize to the cell surface in HeLa cells, as evidenced by a surface biotinylation assay and an association with RACK1 (receptor for activated C kinase 1), a cytoplasmic scaffolding protein (Ohgaki et al., 2008).

NHE6 through NHE9 constitute the organellar NHEs, and although NHE6 and NHE9 primarily localize to endosomes, NHE7 and NHE8 show a different distribution, despite conserved amino acid sequences (Figure 1.2). NHE7 is predominantly found within the trans-Golgi network (Fukura et al., 2010; Nakamura et al., 2005), and NHE8 is found within the mid- to trans-Golgi (Nakamura et al., 2005).

Within non-neuronal cells, NHE6 has also been shown to play a role in endocytosis. Xinhan *et al.* (2011) knocked down NHE6 expression in HeLa cells, which resulted in a decrease in the cellular uptake of transferrin. Pulse-chase experiments revealed that NHE6 primarily affects the early stages of endocytosis, consistent with its localization to early endosomes. This study also discussed the role of NHE6 in endosomal pH regulation; luminal pH in the knockdown cells was consistently more acidic than that within the control cells (Xinhan et al., 2011).

NHEs and the Control of pH and Neuronal Circuitry

NHEs and pH

Organelle pH homeostasis is controlled through the function of the vacuolar H⁺-ATPase (V-ATPase). The V-ATPase uses energy to pump hydrogen ions into

the endosomal compartment, thereby acidifying it (Futai et al., 2000). NHEs are proposed to work in opposition to this function, allowing protons to leak out of the organelle, thereby alkalinizing it (Figure 1.5). This process is also aided by chloride (Cl⁻) transporters, which conduct Cl⁻ ions to counterbalance the internal positive charge generated by the V-ATPase, thus maintaining energetic favorability (Zifarelli, 2015).

Several research groups have reported the functional role of organellar NHEs in pH regulation, through the investigation of *Nhe*-null cells, as well as *Nhe* overexpression. Ouyang et al. (2013) used two independent live imaging methods to calculate endosomal pH in *Nhe6*-null mouse hippocampal neurons. In each case, loss of *Nhe6* resulted in endosomal overacidification; luminal pH within *Nhe6*-null cells was 0.5 pH points lower than that within wild-type cells, in both soma and processes (Ouyang et al., 2013).

Further evidence of the role of NHE6 in endosomal pH homeostasis was described in 2015. Prasad and Rao measured endosomal pH by a ratiometric method involving two fluorescently tagged transferrin reagents and FACS (fluorescence-activated cell sorting) analysis. Overexpression of NHE6-mCherry alkalinized endosomes in HeLa cells. In addition, expression of a patient-derived *NHE6* mutation (Δ WST³⁷², an in-frame deletion of three amino acids) failed to increase endosomal pH in HEK293 (human embryonic kidney) cells (Prasad and Rao, 2015).

NHE9 has also been shown to affect endosome pH in primary cells. Overexpression of NHE9 in mouse astrocytes resulted in endosomal alkalization (i.e. increased pH) as compared to wild-type cells. This same study also examined the effect of NHE9 knockdown on endosomal pH in human glioma cells, which demonstrated modest yet significant acidification (i.e. decreased pH) when compared to wild-type cells (Kondapalli et al., 2013).

Diering *et al.* (2013) reported a contrasting phenotype related to Nhe5 knockdown in rat pheochromocytoma (PC12) cells, mediated by short hairpin RNAs (shRNAs). Previous studies from the lab demonstrated that Nhe5 in PC12 cells is localized to recycling endosomes through association with Rab11, a recycling endosome marker (Diering et al., 2009). When Nhe5 expression is attenuated via shRNA knockdown, the pH of these endosomes does not become acidified. Instead, endosomal pH becomes alkalized (i.e. increased) as compared to wild-type PC12 cells (Diering et al., 2013).

NHEs and Neuronal Circuitry

NHEs are important for proper circuit function. Ouyang *et al.* (2013) demonstrated that loss of *Nhe6* in a mouse model results in impaired connectivity. Although paired-pulse ratios were as expected when compared to wild-type, synaptic field potentials were reduced in *Nhe6*-null mouse brain. Staining of SV2 (synaptic vesicle protein 2, a presynaptic marker) in *Nhe6*-null neurons revealed a decrease in synapse number within mouse hippocampal cultures, and *in vivo*

analysis of CA1 (cornu ammonis 1) sections via Golgi staining revealed both an increase in the number of immature dendritic spines and an overall decrease in spine density (Ouyang et al., 2013).

Deane *et al.* (2013) completed further studies of Nhe6 at the synapse. Within dendritic spines of mouse hippocampal pyramidal neurons, Nhe6 was shown to localize to both early and recycling endosomes. Additionally, Nhe6 costained with GluA1, an AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor subunit. Recycling of AMPA receptors to the synapse is known to mediate both synaptic strength and synaptic morphology. Furthermore, Nhe6 showed increased recruitment to dendritic spines following NMDA (N-methyl-D-aspartate) receptor-dependent long-term potentiation (Deane et al., 2013).

Nhe6 also plays a role in the neuronal morphology comprising the circuitry, as shown by Ouyang *et al.* (2013). Loss of Nhe6 expression leads to diminished neuronal arborization in both axons and dendrites of cultured mouse hippocampal neurons, a result that was replicated *in vivo* within brain tissue sections. Cell autonomous re-expression of Nhe6 through transient transfection rescued the arborization defect, returning branching measures to those of wild-type cells. Importantly, expression of ion-exchanger deficient Nhe6 in these neurons did not restore proper branching, suggesting that exchanger function is necessary for phenotypic rescue (Ouyang et al., 2013).

Ilie *et al.* (2014) made a similar observation regarding the effect of NHE6 expression level on neuronal arborization. Expression of the NHE6 Δ WST³⁷² mutation in hippocampal pyramidal neurons led to a decrease in neurite outgrowth. Wild-type neurons showed multiple first-order and higher processes, whereas the mutant cells possessed fewer primary neurites and lacked all higher-order branches (Ilie *et al.*, 2014).

Other NHE family members also play a role in neuronal arborization. Sin *et al.* (2009) showed that Nhe1 is expressed in the growth cones of mouse cortical neurons, suggestive of a role for Nhe1 in neurite outgrowth and branching. This result was extended through the use of primary cortical neurons derived from *Nhe1*-null mice, which exhibited reduced early neurite outgrowth at 3 days *in vitro* (DIV); neurites and their branches were both shorter and fewer in number in the mutant neurons (Sin *et al.*, 2009).

Additionally, Diering *et al.* (2013) studied the role of Nhe5 in neuronal arborization. Short hairpin (sh) RNA-mediated knockdown of *Nhe5* in primary rat cortical cultures resulted in a significant decrease in both neurite number and length as compared to neurons transfected with a control plasmid. This same result was also demonstrated in PC12 (rat pheochromocytoma) cells, as only 20% of shNhe5 cells showed outgrowth of neurites, and the neurites that were formed were significantly shorter (Diering *et al.*, 2013).

Summary

The study of endosomal NHEs within biological systems dates back only 20 years. Within this time, however, substantial work has been completed on their expression and function within yeast, mammalian cell lines, rodent disease models, and even humans. Although the family of NHEs serves a basic function within the cell, that of pH control through $\text{Na}^+\text{-H}^+$ exchange, loss of this function has profound phenotypic effects, specifically aberrant circuit development coupled to cognitive disorders.

In this thesis, I will describe and utilize unique reagents developed within the Morrow lab (Chapter 2) to answer fundamental questions about the specific role of the endosomal NHEs, NHE6 and NHE9, in neuronal development. I will begin by establishing the specific localization patterns of these proteins within neurons, both individually and in conjunction with one another (Chapter 3). Endosomal NHE expression is known in mammalian cell lines, but polarized neurons present a challenge to the endosomal system, and trafficking and transport must be precisely controlled within both the long- and short-range.

Results of the studies presented in Chapter 3 informed the design and interpretation of functional investigations (Chapter 4). The role of NHE6 in circuit development, through its effects on arborization and pH control, has been established, but this information is currently lacking for NHE9. Therefore, I investigated the role of NHE9 in circuit development and established how it compares to that of NHE6. In addition, elucidation of the role of these two NHEs

in the trafficking of endosomal cargo will be valuable for a deeper understanding of how NHE6 and NHE9 function within neuronal development.

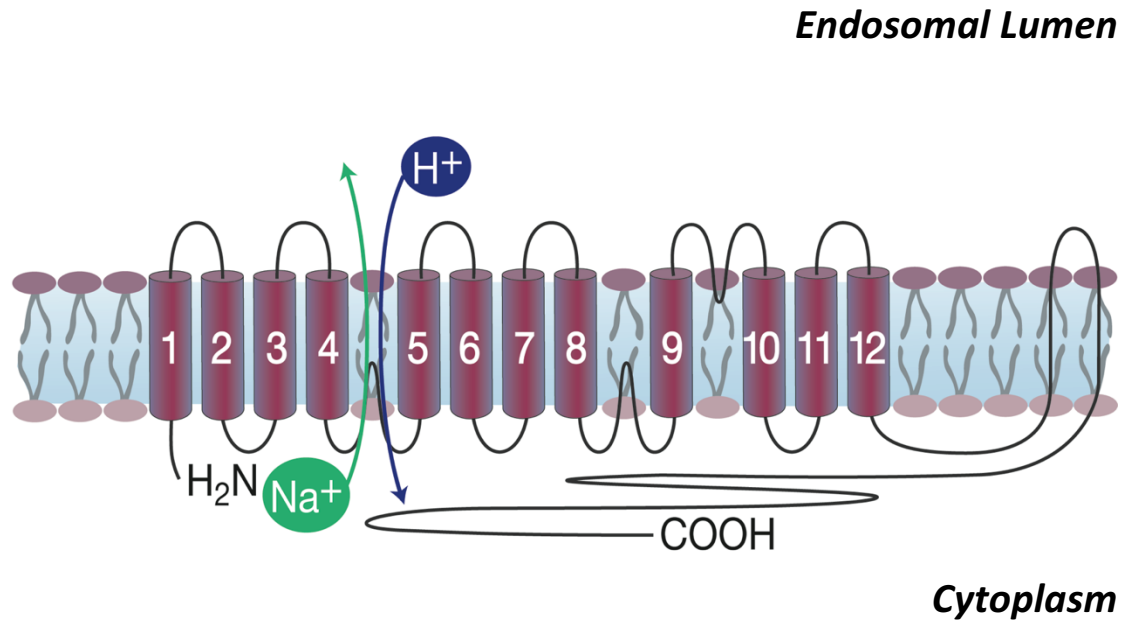


Figure 1.1. Representative NHE schematic. Each NHE contains 12 transmembrane domains and exchanges hydrogen ions (H⁺) for sodium ions (Na⁺) in an electroneutral fashion along the ions' respective concentration gradients. H₂N, N-terminus. COOH, C-terminus. (Adapted with permission from Pescosolido MF, Stein DM, Schmidt M, et al. (2014). Genetic and phenotypic diversity of NHE6 mutations in Christianson syndrome. *Annals of Neurology* 76, 581-593. Copyright 2014 John Wiley & Sons, Inc.)

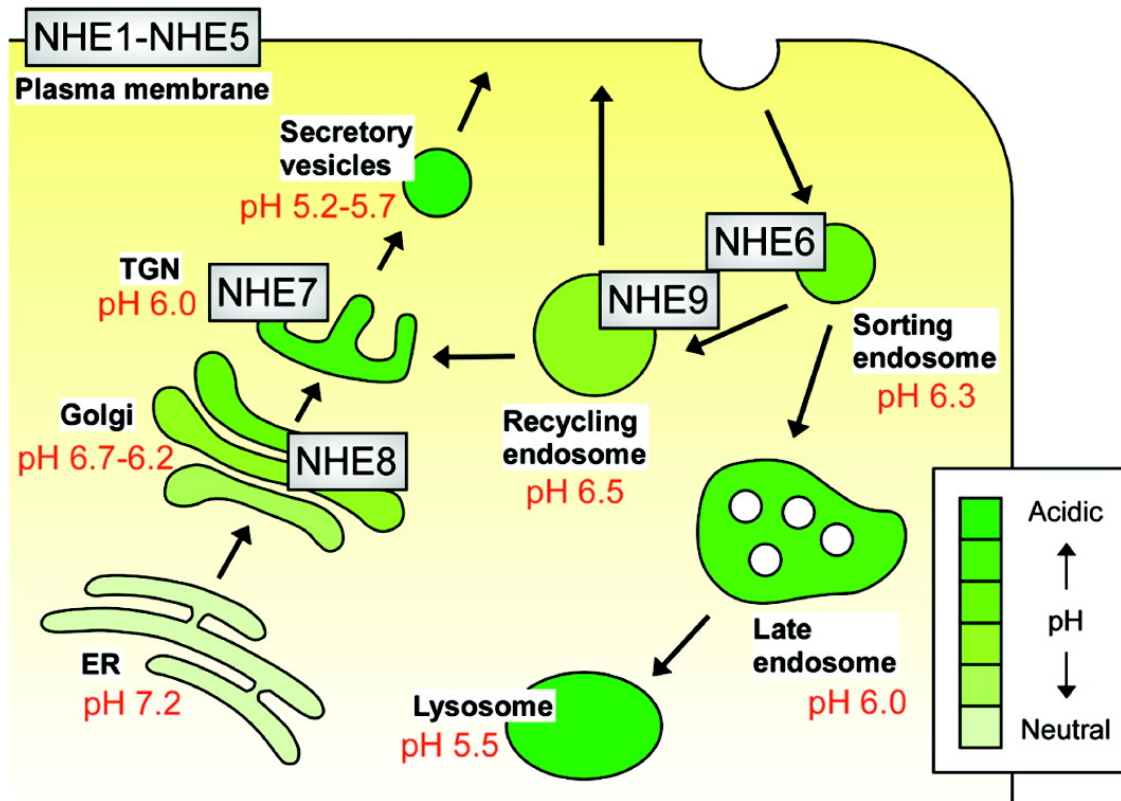


Figure 1.2. Localization of NHEs within non-neuronal mammalian cells. NHE1 - NHE5 are localized to the plasma membrane; NHE6 – NHE9 are localized to intracellular organelles. Specifically, NHE6 is localized to early (“sorting”) endosomes, NHE7 is localized to the trans-Golgi network (TGN), NHE8 is localized to the mid- to trans-Golgi, and NHE9 is localized to recycling endosomes. (Adapted with permission from Ohgaki, R., van IJzendoorn, S.C.D., Matsushita, M., Hoekstra, D., and Kanazawa, H. (2011). Organellar Na⁺/H⁺ Exchangers: Novel Players in Organelle pH Regulation and Their Emerging Functions. *Biochemistry* 50, 443-450. Copyright 2011 American Chemical Society.)

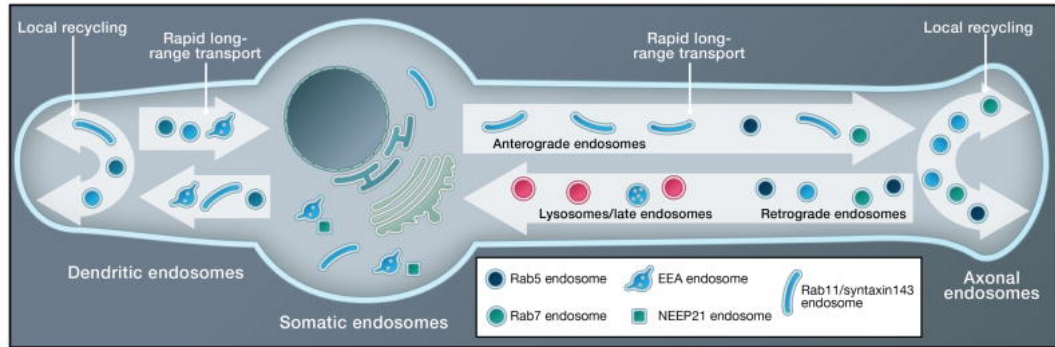
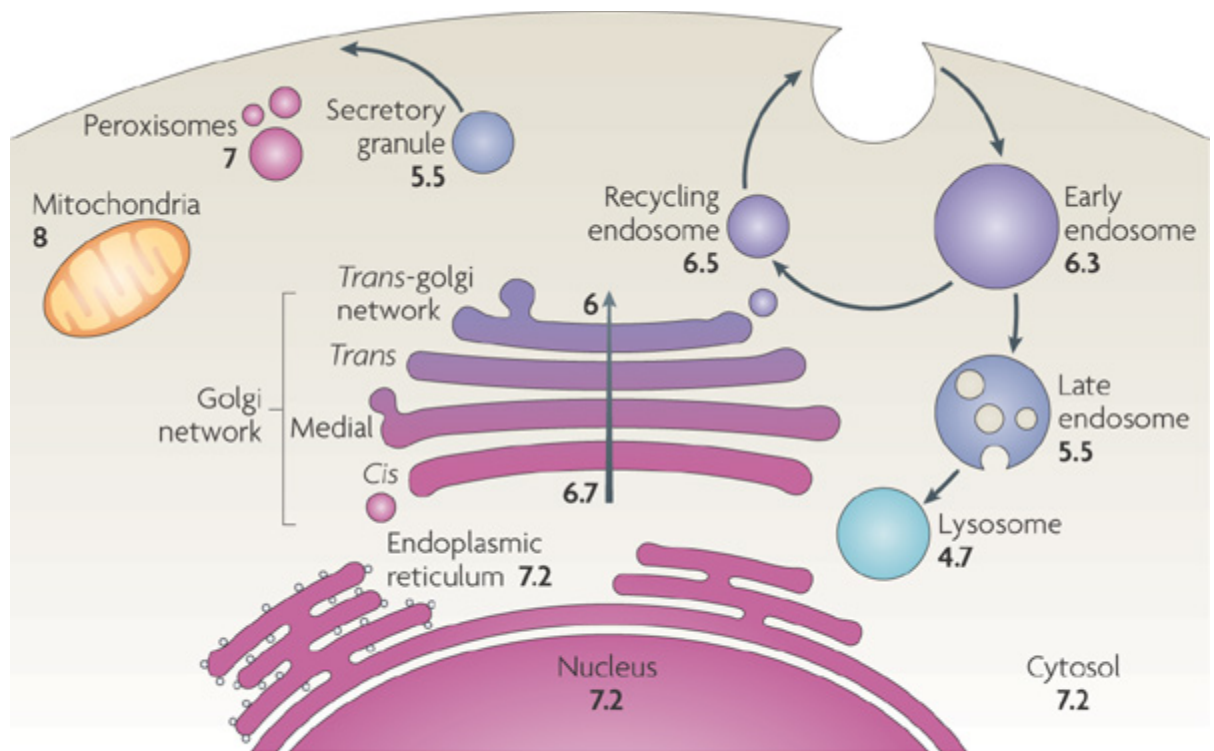


Figure 1.3. Schematic of the endosomal system of neurons, within the soma, axon, and dendrites. Neurons are morphologically complex, polarized cells requiring transport of proteins to distinct cellular domains, which is achieved through the endosomal system. (Reprinted from Yap CC and Winckler B. (2012) Harnessing the Power of the Endosome to Regulate Neural Development. *Neuron* 74, 440-451. Copyright 2012, with permission from Elsevier.)



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Figure 1.4. The pH of intracellular organelles in a model mammalian cell. As cargo moves through the endosomal system, luminal pH progressively decreases. Early endosomes have a pH of approximately 6.3, late endosomes have a pH of approximately 5.5, and lysosomes have a pH of approximately 4.7. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, Casey, J.R., Grinstein, S., and Orlowski, J. (2010). Sensors and regulators of intracellular pH. *Nature Reviews Molecular Cell Biology* 11, 50-61, Copyright 2010.)

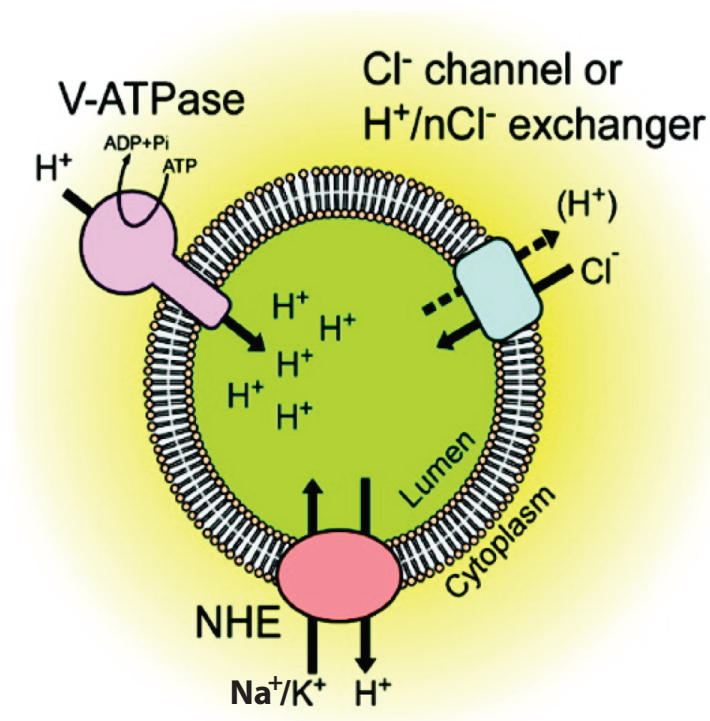


Figure 1.5. Schematic depiction of an intracellular organelle (such as an endosome), featuring proteins involved in pH control and homeostasis. The vacuolar H^+ -ATPase (V-ATPase) acidifies the endosome by pumping H^+ ions into the compartment. The Na^+ - H^+ exchanger (NHE) alkalinizes the endosomes by allowing H^+ ions to leak out of the compartment, in exchange for the inward flow of Na^+ or K^+ ions. In addition, chloride (Cl^-) transporters conduct Cl^- ions to counterbalance the internal positive charge generated by the V-ATPase, maintaining energetic favorability (Zifarelli, 2015). (Adapted with permission from Ohgaki, R., van IJzendoorn, S.C.D., Matsushita, M., Hoekstra, D., and Kanazawa, H. (2011). Organellar Na^+/H^+ Exchangers: Novel Players in Organelle pH Regulation and Their Emerging Functions. *Biochemistry* 50, 443-450. Copyright 2011 American Chemical Society.)

CHAPTER 2:

**VALIDATION OF NHE9 PRIMARY
ANTIBODY AND GENERATION OF
SLC9A9-NULL MOUSE LINE**

Introduction

The mouse possesses many characteristics making it a desirable model for biological study. Mice are proficient breeders, reaching sexual maturity at approximately six weeks of age, with a gestation period of 18-21 days for each litter of offspring. Mice are small in size, making it feasible to house and maintain large stocks of animals. Mice also share a large portion of their genome with humans - when the mouse genome was first sequenced in 2002, approximately 99% of murine genes were found to have a human orthologue (Waterston et al., 2002). This genetic similarity may not be the most attractive feature of the mouse as an experimental model, however; rather, it may be the variety of techniques that exist to manipulate these murine genes.

One such technique, gene trapping, is an approach to mutagenesis that both interrupts and reports endogenous gene expression. Gene trap vectors are promoterless constructs that integrate into introns and can be transcribed through the gene's endogenous promoter. They contain a reporter gene flanked by an upstream splice acceptor site and a downstream poly(A) tail (Schnutgen et al., 2005).

When inserted into an intron via retroviral vector, gene traps force incorrect splicing of the target gene, resulting in loss of expression of downstream exons. In this way, gene trap vectors are predicted to result in null alleles. Due to the potential for alternative splicing, however, a complete loss of function may not be

achieved. Rather, a low level of wild-type allele expression can persist (Lee et al., 2007).

Chapter 2 of this thesis details the generation and characterization of an *Slc9a9*-null mouse derived from a gene-trap line. The validation of an NHE9 primary antibody is also detailed herein. These two reagents, when used separately and in combination, are central to the elucidation of the role of NHE9 in neuronal development.

Results

Confirmation of Rabbit Anti-NHE9 Primary Antibody Specificity

Commercially available antibodies for the study of the endosomal NHEs, including NHE9, have historically been lacking, motivating the Morrow lab to generate its own primary antibody. A protein BLAST (basic local alignment search tool) query revealed 100% identity between *mus musculus* Nhe9 and the peptide antigen used to generate the rabbit anti-NHE9 antibody (SPSPSSPTTKLALDQKSSGKC), with no identity to any other Na⁺-H⁺ exchanger. These query results suggest that the new antibody would be specific, but that fact still needed to be confirmed biologically.

To validate experimentally the specificity of the rabbit anti-NHE9 primary antibody, I employed two different techniques. First, I transfected HeLa cells with a plasmid consisting of NHE9 tagged with eGFP, which emits fluorescence in the green channel. HeLa cells were chosen for their availability and ease of use, as

well as the fact that they express NHE9, allowing for antibody staining of endogenous protein for comparison to NHE9 overexpression. After allowing ample time post-transfection, I stained the same cells for endogenous NHE9 signal using the new anti-NHE9 primary antibody, coupled to a secondary antibody in the red channel. Red and green signal showed complete overlap throughout the cell (Figure 2.1A), providing qualitative evidence of antibody specificity.

Further validation was achieved through the use of wild-type and *Slc9a9*-null fibroblasts. Both lines of cells were stained with the anti-NHE9 primary antibody, but positive signal was predicted only in the wild-type cells. As expected, *Slc9a9*-null fibroblasts showed no evidence of staining with the anti-NHE9 antibody (Figure 2.1B), further demonstrating antibody specificity. The overlap of overexpressed and endogenous NHE9 signal in HeLa cells, as well as the absence of detectable Nhe9 expression in mutant fibroblasts, demonstrates that our new antibody is specific to NHE9.

Generation and Confirmation of *Slc9a9*-Null Mouse Model

An *Slc9a9* gene-trap mouse line (*Slc9a9*^{tm1a(KOMP)Wtsi}, Project ID CSD22519) was acquired from the Knockout Mouse Project (KOMP) repository at the University of California-Davis. A vector containing the promoter-driven cassette (HTGRS01001_A_F07) was electroporated into mouse embryonic stem cells of C57BL/6N origin (JM8A3.N1 subline), which were then injected into blastocysts. Mice were generated on a mixed B62DF1 x C57BL/6 background strain, and the

line was maintained through heterozygous female by heterozygous male breeding pairs.

The gene-trap cassette is located upstream of exon 5, and its transcription relies heavily on the presence of a strong splice acceptor site (Figure 2.2). To verify the resulting mutation, we performed a Western blot for Nhe9 in mouse tissue, which revealed unexpected Nhe9 protein expression in the supposed null brain (Figure 2.3). This evidence suggests that insertion and transcription of the gene-trap cassette did not generate a completely null mutation.

One functional feature of the *Slc9a9* gene-trap construct is the presence of loxP sites flanking exon 5 (Figure 2.2). Utilization of these sites upon Cre recombination enables excision of the intervening genetic material. In this way, we predicted that we would be able to delete exon 5 from the gene and prevent the transcription of downstream exons, thereby rendering the animal a true null.

I crossed the gene-trap mouse line to a Zp3-Cre mouse line, acquired from the Jackson Laboratory in Bar Harbor, ME. The mouse Zp3, or zona pellucida 3, promoter directs gene expression in growing germ cells. Progeny from this mating were both viable and fertile, allowing me to propagate the post-Cre recombination line. As pups were born, tail and brain samples from putative wild-type, heterozygous, and *Nhe9*-null mice were collected for PCR genotyping, RNA isolation, and protein analysis.

To begin the analysis of this mouse line, I isolated DNA from each of the tail tips and used these samples in two PCR reactions, one for each of the *Slc9a9*

alleles. I saw a strong wild-type allele band (441 base pairs) in both the wild-type and heterozygous lanes, as well as a strong mutant allele band (532 base pairs) in both the heterozygous and null lanes (Figure 2.4A).

To verify further the complete loss of *Nhe9* expression in this new mouse line, I designed RT-PCR primers around two different exon junctions - exon 1/2, upstream of the loxP sites flanking exon 5, and exon 15/16, downstream of the loxP sites. Following RNA isolation from postnatal day (P) 6 wild-type, heterozygous, and *Nhe9*-null mouse brain, I performed a reverse transcription reaction to generate complementary DNA (cDNA). This cDNA was then cycled 30 times and amplified via PCR, and the gene product was run out on an agarose gel. Figure 2.4B demonstrates the loss of exon 15/16 transcript expression in the null mouse brain, suggesting once again that I have generated a complete loss-of-function allele.

The final assessment of the post-Cre recombination mouse line took place at the protein level. I performed a Western blotting experiment similar to that shown in Figure 2.3, using hippocampal homogenates collected from P4 wild-type, heterozygous, and *Nhe9*-null mice. Figure 2.4C shows the image of a membrane probed first for *Nhe9* and second for tubulin. *Nhe9* protein is present only in the wild-type and heterozygous brain samples, whereas the tubulin loading control is present in approximately equal amounts in tissue of all three genotypes.

Description of *Slc9a9* Post-Cre Recombination Mouse Colony

The *Slc9a9* post-Cre recombination mouse colony was maintained through the use of female heterozygote by male heterozygote mating pairs. Within a time period of three years, 54 litters of mice on a >98% pure C57BL/6J background were born. These litters included a total of 357 animals with the following genotypic distribution: 86 wild-type (WT), 183 heterozygous (Het), and 88 null. This distribution reveals a WT:Het:Null ratio of 1:2.1:1, which is directly in line with expected Mendelian genetics.

Of all progeny born to C57BL/6 mating pairs, 52.3% are expected to be female (Verley et al., 1967). Out of 357 offspring, 161 were male, and 196 were female, equaling 54.9% female. Furthermore, for a C57BL/6 breeding pair of typical fertility, the average litter size is 6.23 (Verley et al., 1967). Dividing the total number of mice born by the total number of litters produced reveals an average litter size of 6.61 for the *Slc9a9* post-Cre recombination mouse colony.

Discussion

In Chapter 2, I have established the validity of an anti-NHE9 primary antibody and an *Slc9a9*-null mouse line, two unique reagents for use in the study of the role of NHE9 in neuronal development. The peptide antigen used in antibody production was shown to be specific to NHE9, and immunocytochemical verification using two different techniques in two different cell lines bore out the antibody's specificity. When applied to HeLa cells overexpressing NHE9 tagged

with GFP, the signal arising from the binding of the antibody to endogenous NHE9 completely overlapped with that of exogenous NHE9.

Application of the newly generated antibody also revealed the absence of staining in *Slc9a9*-null fibroblasts. If the antibody were to share sequence homology with a protein other than NHE9, positive signal may have been anticipated in the null cells. Instead, only the wild-type fibroblasts expressing Nhe9 showed a positive signal upon antibody staining, proving both the specificity of the anti-NHE9 antibody and its suitability as a central research reagent.

The other reagent central to my research is that of the *Slc9a9*-null mouse line. The original line obtained from KOMP revealed some Nhe9 protein expression in the null animal, perhaps a result of alternative splicing. Successful Cre recombination, however, generated a mouse line completely lacking Nhe9. This was true at both the transcript and protein levels, as indicated by RT-PCR and Western blotting.

I have also demonstrated that this *Slc9a9* post-Cre recombination mouse line is both viable and fertile. Pups of each genotype occur in ratios almost identical to those predicted by Mendelian genetics, and litter size and sex distribution are in line with published standards. Taken together, the complete loss of Nhe9 expression in the post-Cre recombination *Slc9a9*-null mouse, as well as its viability and fecundity, establish it as a model for further study of the role of NHE9 in neuronal development. Subsequent experiments detailed within this thesis utilize this particular mouse line.

Materials and Methods

Anti-NHE9 Primary Antibody Production

Through Covance, rabbits were immunized with an antigen corresponding to the following peptide, located within the C-terminal tail of NHE9: SPSPSSPTTKLALDQKSSGKC. Serum was then collected and affinity-purified to generate polyclonal rabbit anti-NHE9 IgG.

HeLa Cell Transfection and Immunocytochemistry

One day prior to transfection, HeLa cells were plated at a density of 20,000 cells/well in 8-well chamber slides, in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal bovine serum (FBS) + 1% GlutaMAX (all media components from Gibco). The following day, cells were transfected with a plasmid encoding NHE9-eGFP using Lipofectamine® 2000 (Invitrogen). DNA and Lipofectamine® reagent were diluted in Opti-MEM in a 1:2.5 ratio and allowed to incubate at room temperature for 20 minutes. The mixture was then applied to cells and incubated at 37°C for 4-6 hours, after which transfection medium was replaced with conditioned HeLa growth medium.

One day after transfection, cells were rinsed briefly 2 times with warm 1X phosphate-buffered saline (PBS) and fixed for 10 minutes at room temperature with cold 4% paraformaldehyde (PFA). After washing 3 times with 1X PBS, cells were permeabilized with 0.25% Triton-X for 15 minutes at room temperature,

rinsed with 1X PBS, and blocked with 10% goat serum/0.1% Triton-X in PBS for 1 hour at room temperature. Cells were then incubated with rabbit anti-NHE9 primary antibody diluted 1:500 in 2% goat serum/0.1% Triton-X in 1X PBS for 3 hours at room temperature. Cells were then washed with blocking buffer 3 times for 5 minutes each. After washing, secondary antibody (AlexaFluor® 594 goat anti-rabbit IgG, 1:1,000 in 2% goat serum/0.1% Triton-X) was applied for 1 hour at room temperature, and 4',6-diamidino-2-phenylindole (DAPI) at a 1:1,000 dilution was included with the secondary antibody to stain nuclei. Finally, slides were washed 3 times with 1X PBS for 5 minutes each, overlaid with coverslips using Fluoromount-G™ (SouthernBiotech, #0100-01), and allowed to dry overnight at room temperature, protected from light.

Generation of Fibroblast Cultures

Fibroblast cultures were established as previously described (Takashima, 2001). Skin samples were collected from neonatal P0 – P1 mice, minced, and incubated in a humidified 37°C/5% CO₂ incubator in complete growth medium, consisting of DMEM + 10% FBS + 1% GlutaMAX + 1% penicillin/streptomycin. Cells grew out, were passaged, and reached confluency after approximately one month, at which point several aliquots were frozen down in 90% FBS + 10% dimethyl sulfoxide (DMSO) for later use.

Fibroblast Immunocytochemistry

Fibroblasts derived from wild-type and *Slc9a9*-null mouse skin (Passage 5) were plated on 8-well chamber slides one day prior to staining. Slides were rinsed briefly with warm 1X PBS and fixed for 10 minutes at room temperature with cold 4% PFA. Slides were then washed 3 times with 1X PBS and permeabilized for 15 minutes at room temperature with 0.25% Triton-X. After a brief rinse with 1X PBS, fibroblasts were blocked overnight at 4°C with 10% goat serum/0.1% Triton-X in PBS. The following day, cells were incubated with rabbit anti-NHE9 primary antibody diluted 1:500 in 2% goat serum/0.1% Triton-X in 1X PBS for 3 hours at room temperature. Fibroblasts were then washed with blocking buffer 3 times for 5 minutes each. After washing, secondary antibody (AlexaFluor® 594 goat anti-rabbit IgG, 1:1,000 in 2% goat serum/0.1% Triton-X) was applied for 1 hour at room temperature, and DAPI at a 1:1,000 dilution was included with the secondary antibody to stain nuclei. Finally, slides were washed 3 times with 1X PBS for 5 minutes each, overlaid with coverslips using Fluoromount-G™ (SouthernBiotech), and allowed to dry overnight at room temperature, protected from light.

Microscopy

Images of single optical sections of HeLa cells and fibroblasts were collected with a Zeiss LSM710 confocal microscope. HeLa cells were imaged using a Plan-Apochromat 63X oil/1.4 numerical aperture (N.A.) objective, and fibroblasts were imaged using a Plan-Apochromat 20X dry/0.8 N.A. objective.

Mating and Colony Generation

To generate post-Cre recombination *Slc9a9*-null animals, *Slc9a9* heterozygous females from the gene-trap line were mated to a C57BL/6J male, expressing Cre recombinase under the control of a Zp3 promoter (C57BL/6-Tg(Zp3-cre)93Kwn/J, The Jackson Laboratory, #003651). Progeny were backcrossed for 7 generations onto an inbred C57BL/6J background prior to experimentation.

PCR Genotyping

Tail tips from each animal were collected between P17 and P28 and lysed overnight in a Mastercycler Pro (Eppendorf) in a buffer containing the following: 50 mM potassium chloride, 10 mM Tris (pH 8.3), 2.5 mM magnesium chloride, 1% gelatin, 0.45% NP-40, 0.45% Tween-20, and 1% proteinase-K. Using native Taq DNA Polymerase (Invitrogen), the DNA samples were cycled and amplified according to PCR protocols established by KOMP. Two primer sets were designed as follows, identifying the *Slc9a9* post-Cre recombination wild-type (441 bp) and mutant (532 bp) alleles – WT Forward (IF): 5'-GGC CAG ACT TTG GTT GGT CAT TCC-3'; WT Reverse (IR): 5'-GGC TAT GTA CCA TGC ATA TCC TTT TGG-3'; Mut Forward (IacF): 5'-GCT ACC ATT ACC AGT TGG TCT GGT GTC-3'; Mut Reverse (R): 5'-ATG GCA GTC CTC TTT TCT CTG GTC C-3'. All samples were

then electrophoresed on a 1.5% agarose gel containing 0.005% ethidium bromide (Promega) and visualized under ultraviolet light using the GelDoc-It Imager (UVP).

RNA Isolation, Reverse Transcription, and PCR

Brains were collected from P6 post-Cre recombination wild-type, heterozygous, and *Slc9a9*-null mice, snap-frozen in liquid nitrogen, and stored at -80°C. Total RNA was purified from the frozen animal tissue using the RNeasy Mini Kit (Qiagen). Briefly, tissue was homogenized in Buffer RLT prior to centrifugation for 3 minutes at 13,200 rpm. The supernatant was removed, combined with one volume of 70% ethanol, and run through an RNeasy spin column. After several wash steps with buffers RW1 and RPE, RNA was eluted from the column with RNase-free water. RNA concentration and purity ($260_{\text{Abs}}/280_{\text{Abs}}$) were then quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

Generation of cDNA via reverse transcription reaction was performed using the SuperScript® III First-Strand Synthesis SuperMix kit (Invitrogen). A total of 178 ng of RNA, isolated as above, was combined with 50 μM oligo(dT)₂₀ primer, annealing buffer, and RNase/DNase-free water. The mixture was incubated at 65°C for 5 minutes, placed on ice for 1 minute, and then combined with 2X First-Strand Reaction Mix and SuperScript® III/RNase OUT™ Enzyme Mix. Each sample was vortexed and centrifuged briefly, and then incubated in an Eppendorf Mastercycler Pro thermal cycler for 50 minutes at 50°C. The reaction was

terminated for 5 minutes at 85°C, after which the samples were placed on ice prior to PCR.

Samples were then cycled 30 times and amplified via PCR. Two primer sets were designed as follows, spanning the *Slc9a9* exon 1/2 (337 base pairs) and exon 15/16 (310 base pairs) junctions – Exon 1/2 Forward: 5'-TTC GGT TTA CGT CAG GGA AG-3'; Exon 1/2 Reverse: 5'-GGG CTG ATA TTG TGC TGG TT-3'; Exon 15/16 Forward: 5'-CCT GAA ACC CAT CCT AAC ACA-3'; Exon 15/16 Reverse: 5'-CCG AGT CTG CTC CAG TTT GA-3'. All samples were then electrophoresed on a 1.5% agarose gel containing 0.005% ethidium bromide (Promega) and visualized under ultraviolet light using the GelDoc-It Imager (UVP).

Western Blotting

Hippocampi were collected from P4 wild-type, heterozygous, and *Slc9a9*-null mice, and protein was isolated via homogenization with cold RIPA (radioimmunoprecipitation assay) buffer, consisting of 50 mM Tris, 150 mM sodium chloride, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, and 1% NP-40. Protease inhibitor cocktail (cOmplete ULTRA Tablets, Roche, #05-892-970-001) and phosphatase inhibitor cocktail (PhosSTOP, Roche, #04-906-837-001) were added to the buffer immediately prior to use. Protein concentration in each homogenized sample was then measured using the Pierce™ BCA (bicinchoninic acid) Protein Assay Kit (ThermoFisher Scientific), and 10 µg of each sample was combined with 4X NuPAGE® LDS Sample Buffer and 10X NuPAGE® Reducing

Agent and loaded onto a NuPAGE® 4-12% Bis-Tris precast gel (Invitrogen). After electrophoresis, proteins from the gel were transferred onto a nitrocellulose membrane.

Membranes were blocked for 1 hour at room temperature in 5% nonfat milk in 1X Tris-buffered saline + 0.05% Tween-20 (TBS-T). They were then incubated in rabbit anti-NHE9 primary antibody, diluted 1:500 in 5% nonfat milk, overnight at 4°C. After several washes with TBS-T, membranes were incubated with a peroxidase IgG fraction mouse anti-rabbit secondary (Jackson ImmunoResearch Laboratories, 211-032-171) at a 1:20,000 dilution for 1 hour at room temperature. Following several more TBS-T washes, membranes were rinsed briefly with Milli-Q water and incubated in a 1:1 ratio of SuperSignal® West Pico Chemiluminescent Substrates (ThermoFisher Scientific) for 5 minutes. Membranes were exposed to film for 35 minutes and developed with a Konica Minolta Medical Imaging SRX-101A tabletop processor.

Membranes were then stripped with 1X NewBlot™ Nitro Stripping Buffer (LI-COR) and the above process was repeated, probing for mouse anti-tubulin (Sigma, T6074) at a 1:8,000 dilution with a peroxidase AffiniPure goat anti-mouse IgG secondary (Jackson ImmunoResearch Laboratories, 115-035-174) at a 1:20,000 dilution.

Animal Use Statement

All procedures involving live mice were carried out in accordance with policies outlined in the *Guide for the Care and Use of Laboratory Animals* (The National Academies Press), as well as protocols approved by the Brown University Institutional Animal Care and Use Committee (#1212990041 and #1509000162).

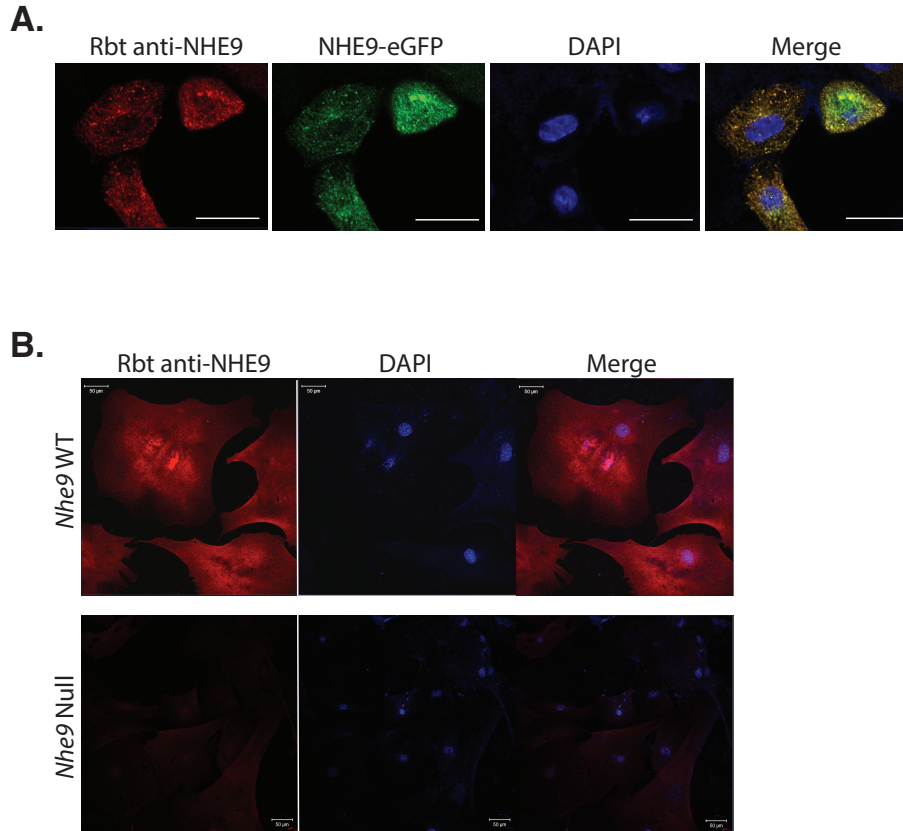


Figure 2.1. Rabbit anti-NHE9 antibody is specific. (A) HeLa cells were transfected with a plasmid encoding NHE9-eGFP (green), fixed with 4% PFA, and stained for endogenous NHE9 expression (red). DAPI stain was included to visualize nuclei. Merged panel shows complete overlap (yellow) between the two signals (overexpressed and endogenous NHE9). Scale bar, 20 μ m. (B) *Nhe9* staining in wild-type (WT) and *Nhe9*-null fibroblasts derived from neonatal mouse skin. Fibroblasts were fixed with 4% PFA and stained with the rabbit anti-NHE9 primary antibody. This antibody produces a strong *Nhe9* signal in WT cells, yet no positive signal in *Nhe9*-null cells. Scale bar, 50 μ m. All images represent single optical sections.

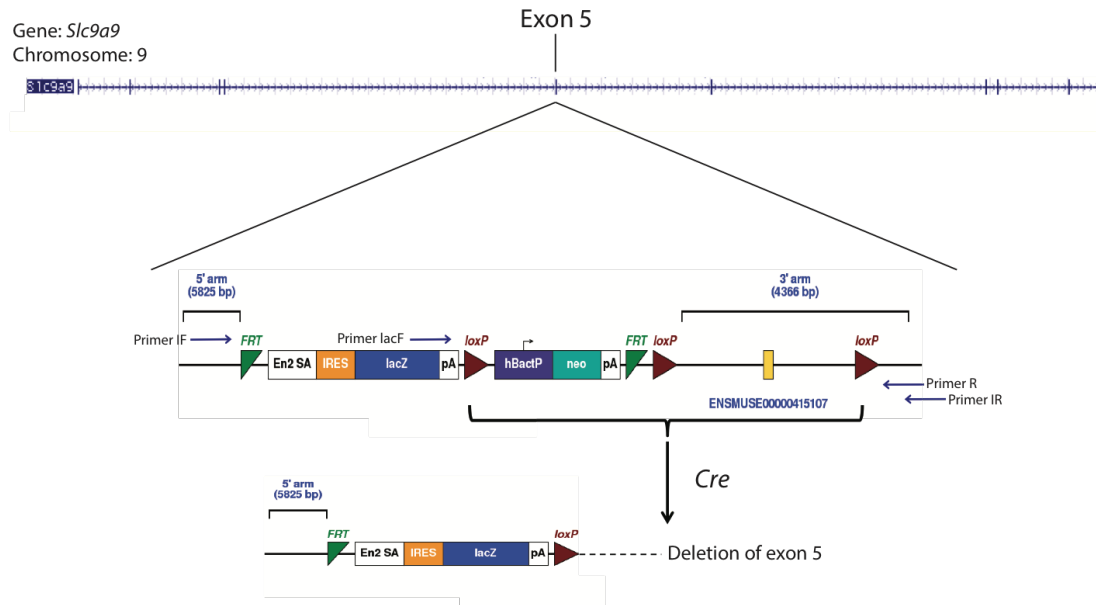


Figure 2.2. Diagram of targeted disruption of *Slc9a9* gene in mouse. A gene-trap cassette was inserted with arms of homology flanking exon 5. Crossing of the gene-trap mouse line to a Cre mouse line was predicted to result in excision of the region between loxP sites, including exon 5. FRT, flippase recombination target. En2 SA, engrailed-2 splice acceptor. IRES, internal ribosome entry site. pA, poly(A) tail. hBactP, human beta-actin promoter. neo, neomycin resistance gene.

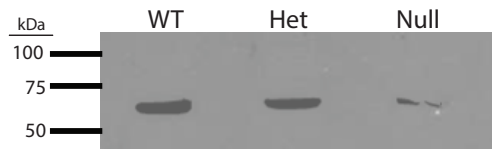


Figure 2.3. *Slc9a9* pre-Cre recombination gene-trap mouse is not a complete null. Western blot probing for protein expression shows a low level of Nhe9 (approximately 70 kDa in size) in *Slc9a9*-null brain, as well as the expected Nhe9 protein in wild-type (WT) and heterozygous (Het) brains. Antibody was used at a 1:1000 dilution, 10 μ g of protein was loaded per lane, and film was exposed for 30 minutes.

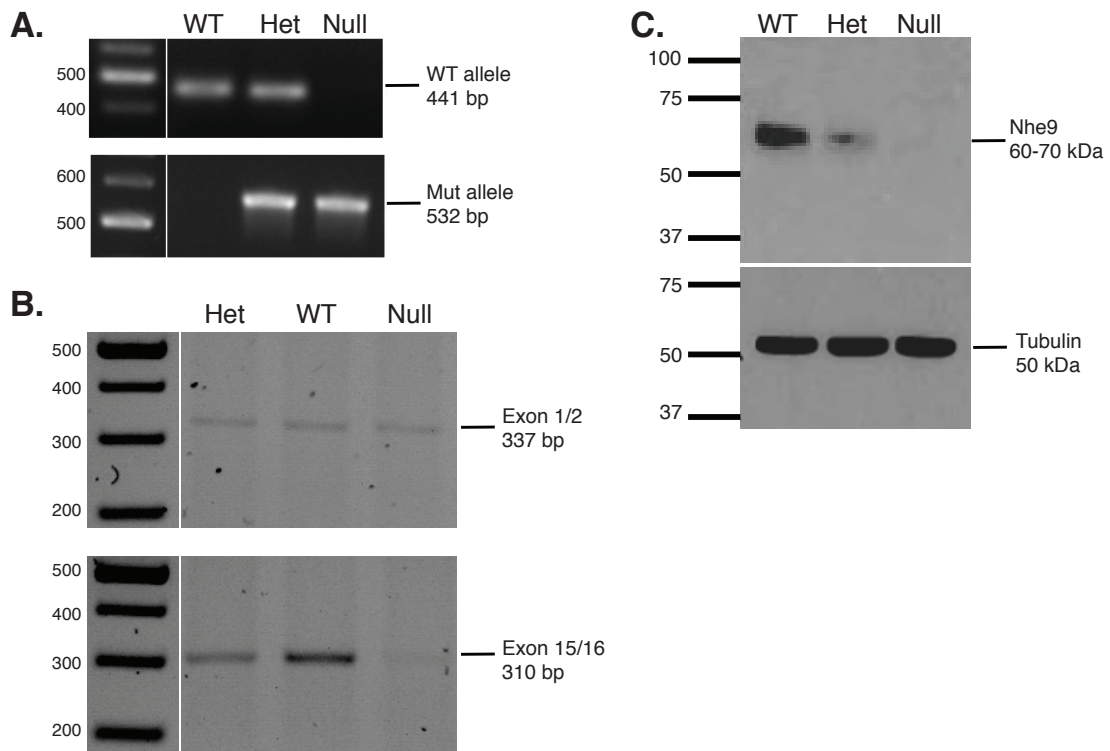


Figure 2.4. *Slc9a9* post-Cre recombination mouse is a complete null, with loss of expression at both the transcript and protein levels. (A) PCR genotyping of tail lysate DNA from wild-type (WT), heterozygous (Het), and *Slc9a9*-null mice. Tail tips were collected from individual mice and lysed overnight, prior to PCR amplification the following day. Band indicating the WT allele (441 base pairs) is present in both WT and Het lanes; band indicating the mutant (Mut) allele (532 base pairs) is present in both Het and Null lanes. (B) RT-PCR gel shows loss of expression of transcript downstream of *Slc9a9* exon 5 in the null. Total RNA was collected from mouse brains, cDNA was generated via reverse transcription, and samples were cycled 30 times and amplified. (C) Western blot probing for Nhe9 shows no protein expression in *Nhe9*-null mouse hippocampus. The expected protein expression (approximately 70 kDa) is present in WT and Het samples. For Nhe9 expression analysis, rabbit anti-NHE9 antibody was used at a 1:500 dilution, 10 μ g of protein was loaded per lane, and film was exposed for 35 minutes. For tubulin expression analysis after membrane stripping, mouse anti-tubulin antibody was used at a 1:8000 dilution, and film was exposed for 10 seconds.

CHAPTER 3:

**LOCALIZATION OF NHEs WITHIN
THE ENDOSOMAL PATHWAY OF
NEURONS**

Introduction

In Chapter 2, I described and characterized a rabbit anti-NHE9 primary antibody. In Chapter 3, I will be detailing studies making use of that antibody, as well as the rabbit anti-NHE6 antibody, also developed by the Morrow lab (Ouyang et al., 2013). My overall question is, where within the endosomal pathway of neurons are NHE6 and NHE9 localized, both individually and in relation to one another?

Individual localization patterns of NHE6 and NHE9 have been previously characterized in non-neuronal mammalian cell lines. In opossum kidney cells, overexpressed NHE6-eGFP was shown to colocalize with markers of early (EEA1, early endosome antigen 1) and recycling (Rab11) endosomes, but not markers of late endosomes (mannose-6-phosphate receptor) or lysosomes (LAMP2, lysosome-associated membrane protein 2) (Brett et al., 2002). This result was replicated in COS7 simian kidney cells, as NHE6 localization overlapped with both EEA1 and rhodamine-conjugated transferrin, a marker of recycling (Nakamura et al., 2005).

The comparative localizations of NHE6 and NHE9 within the recycling pathway have also been described. COS7 cells were loaded with rhodamine-conjugated transferrin for up to 30 minutes prior to fixation and immunostaining for NHE6 and NHE9. NHE6 colocalized with rhodamine-conjugated transferrin within 10 minutes after uptake, suggesting that NHE6 resides on compartments early within the recycling pathway. In contrast, NHE9 signal did not overlap with that of

transferrin until 15 to 30 minutes after uptake, suggesting that NHE9 resides on compartments later within the recycling pathway (Nakamura et al., 2005).

The distribution of NHEs within neuronal endosomes has yet to be fully described. Ouyang *et al.* described the localization of Nhe6 within mouse neurons, with approximately 35% of Nhe6 localized to early endosomes, 40% localized to recycling endosomes, and 35% localized to late endosomes (Ouyang et al., 2013). Similarly, Kondapalli et al. (2013) demonstrated the endosomal localization of overexpressed NHE9-eGFP in primary mouse astrocytes. In immunostaining experiments, NHE9-eGFP localization showed modest overlap with the early endosome marker EEA1 and extensive overlap with the recycling endosome marker Rab11, yet no overlap with the late endosome marker lysobisphosphatidic acid (Kondapalli et al., 2013). Nhe9 localization within neurons, however, remains to be determined, as does a comparison of its distribution with that of Nhe6.

In this chapter, I present immunocytochemical evidence of the localization patterns of Nhe6 and Nhe9 throughout the endosomal system of neurons. Through the use of costaining with antibodies against Rab proteins, I was able to localize both Nhe6 and Nhe9 within endosomes. Rab proteins are members of the Ras family of small GTPases (guanosine triphosphatases), and they function as regulators of trafficking within the cell (Bhuin and Roy, 2014; Stenmark and Olkkonen, 2001). Each component of the endosomal pathway is characterized by a different Rab. Rab5 is associated with early endosomes (Chavrier et al., 1990),

Rab11 is associated with recycling endosomes (Ullrich et al., 1996), and Rab7 is associated with late endosomes (Chavrier et al., 1990).

In addition, I describe how the localization patterns of Nhe6 and Nhe9 relate to one another within cultured mouse hippocampal neurons. As these two proteins are proposed to have the same function within neurons, as endosomal $\text{Na}^+\text{-H}^+$ exchangers, and thus regulators of endosomal pH homeostasis, it would be informative to know if they are found on the same endosomes.

Thus, the goal of this chapter is to determine the localization patterns of Nhe6 and Nhe9, both separately and in relation to one another, within the endosomal system of cultured mouse hippocampal neurons. The results of these foundational studies have informed our functional studies, detailed in Chapter 4.

Results

Nhe6 Is More Abundant in Neonatal Mouse Hippocampus Than Nhe9

Nhe6 and Nhe9 are both expressed within developing hippocampus (Kondapalli et al., 2014). However, it is important to determine their levels of expression relative to one another, as differences in protein expression could be relevant to differences in mutant phenotypes. To determine these relative levels, I collected hippocampi from seven different neonatal (P0-P1) wild-type CD-1 mouse pups across two litters. I then homogenized the brain samples to collect protein and analyzed levels of Nhe6 and Nhe9 expression via Western blot.

Results demonstrate that Nhe6 expression level is 3.6 times that of Nhe9. When normalized to tubulin, Nhe6 has a relative expression level of 0.881 ± 0.26 (Figure 3.1A), whereas Nhe9 has a relative expression level of 0.245 ± 0.063 (mean $\pm$ standard error) (Figure 3.1B). These two levels differ significantly, with a p value equal to 0.036 (Figure 3.1C).

Nhe6 and Nhe9 Are Distributed Throughout Neuronal Endosomes

In mammalian cell lines, Nhe6 is reported to be localized to early and recycling endosomes (Brett et al., 2002; Nakamura et al., 2005), whereas Nhe9 is reportedly localized to a later stage within the recycling pathway (Nakamura et al., 2005). This information, however, has not yet been fully determined for the localizations of endogenous Nhe6 and Nhe9 within the neuron, a polarized cell whose endosomal system must function in both long- and short-range transport.

I assessed the localization of Nhe6 and Nhe9 within different populations of endosomes using wild-type primary hippocampal neurons derived from neonatal mice. After 3 days of growth *in vitro*, I fixed the neurons and used immunocytochemistry to stain for either Nhe6 or Nhe9, in conjunction with the endosomal markers Rab5, Rab11, and Rab7 (Figures 3.2 and 3.3).

To calculate the overlap between the two signals, I used the MetaMorph® software program's colocalization function (Figure 3.4). For analysis, I chose regions of interest corresponding to individual endosomes within neuronal processes. Cell bodies were excluded from analysis due to the high density of

staining present therein and thus the inability to distinguish individual puncta. To determine the relative distribution of Nhes throughout the endosomal system, I calculated a percentage value by dividing the (Total Number of Pixels Positive for Both Red and Green Signals) by the (Total Number of Pixels Positive for Red Signal), with red representing the Nhe and green representing the Rab marker. If the fraction of overlap between the two channels within each region of interest was greater than or equal to 25%, I counted that region (i.e. endosome) as having colocalization between the Nhe and the Rab marker.

Based on these colocalization values, I assessed the distribution of Nhe6 and Nhe9 throughout the different endosomal compartments. Of all Nhe6 staining within the neuron, $35.4 \pm 1.5\%$ (mean $\pm$ standard error) was found on Rab5-positive early endosomes, $34.4 \pm 1.5\%$ was found on Rab11-positive recycling endosomes, and $27.2 \pm 1.3\%$ was found on Rab7-positive late endosomes (Figure 3.5A). Of all Nhe9 staining within the neuron, $33.4 \pm 1.4\%$ was found on Rab5-positive early endosomes, $25.0 \pm 1.3\%$ was found on Rab11-positive recycling endosomes, and $22.9 \pm 1.2\%$ was found on Rab7-positive late endosomes (Figure 3.5B).

The proportions of each Nhe within each endosomal compartment thus varied. There was significantly less Nhe6 in Rab7-positive late endosomes as compared to Rab5-positive early endosomes ($p < 0.001$), and there was also significantly less Nhe6 in Rab7-positive late endosomes as compared to Rab11-

positive recycling endosomes ($p < 0.001$). The difference between Nhe6 localization to early and recycling endosomes was not significant ($p = 0.39$).

For Nhe9-positive staining, there was also significant variation in localization to different types of endosomes. Nhe9 localization to Rab5-positive early endosomes was greater than to both Rab11-positive recycling endosomes ($p < 0.001$) and Rab7-positive late endosomes ($p < 0.001$). Nhe9 localization to Rab11-positive recycling endosomes was also greater than that to Rab7-positive late endosomes ($p = 0.029$).

Overall, the localization patterns of Nhe6 and Nhe9 in cultured mouse hippocampal neurons are similar. For both proteins, staining was greatest within early endosomes, lowest within late endosomes, and roughly intermediate within recycling endosomes. Thus, Nhe6 and Nhe9 localize to all endosomal compartments, albeit to varying degrees.

Different Endosomal Compartments Contain Different Proportions of NHEs

Based on the data just presented, it appears that Nhe6 and Nhe9 are similarly distributed throughout neuronal endosome populations. When considering the inverse relationship, however, different trends emerge. Instead of analyzing the localization of total Nhe6 and total Nhe9 across endosomal populations, I instead analyzed the fraction of endosomes positive for Rab5, Rab11, or Rab7 that were also positive for Nhe6 or Nhe9 (Figure 3.5C). To do so, I utilized the same images as before, but I chose regions of interest in the green

(Rab) channel, and used total green fluorescence signal as the denominator within my comparison ($[\text{Total Number of Pixels Positive for Both Red and Green Signals}]/[\text{Total Number of Pixels Positive for Green Signal}]$).

Analyzing the total population of Rab5-positive early endosomes reveals that $42.3 \pm 1.6\%$ (mean $\pm$ standard error) of early endosomes contain Nhe6, whereas $37.2 \pm 1.5\%$ contain Nhe9. Analyzing the total population of Rab11-positive late endosomes reveals the opposite trend; more recycling endosomes contain Nhe9 ($45.3 \pm 1.6\%$) than contain Nhe6 ($35.7 \pm 1.4\%$). Similarly, more Rab7-positive late endosomes contain Nhe9 ($45.3 \pm 1.6\%$) than contain Nhe6 ($34.7 \pm 1.5\%$). All differences between Nhe6 and Nhe9 localization within a given endosomal compartment were significant (Rab5, $p = 0.024$; Rab11, $p < 0.001$; Rab7, $p < 0.001$).

Nhe6 and Nhe9 Have a Moderate Level of Overlapping Localization Within Neurons

Traditional multiplexed immunocytochemistry typically uses primary antibodies raised in different animal species, such as mouse and rabbit, to allow for concurrent staining. As described previously, our anti-NHE6 antibody (Ouyang et al., 2013) and our anti-NHE9 antibody (Chapter 2) were both raised in rabbit, negating the ability of the secondary antibodies to distinguish between the two reagents and making it impossible to use them for concurrent staining. To

overcome this challenge, I instead utilized a consecutive staining protocol involving Fab fragment blocking.

After fixing, permeabilizing, and blocking the neurons with normal goat serum, I incubated them with the first primary antibody, which was rabbit anti-NHE9, followed by the first secondary antibody, AlexaFluor® goat anti-rabbit 594. I then incubated the neurons with normal rabbit serum, which saturates the open binding sites on the first secondary antibody so that they cannot bind to the second primary antibody. Following this step, I incubated the neurons with an excess (100 µg/mL) of Fab fragment, overnight at 4°C. The Fab, or fragment antigen-binding, fragment covers the rabbit IgG so that it cannot interact with the second secondary antibody.

Once the Nhe9/antibody complex had been inactivated to prevent further binding, I applied the second primary antibody, rabbit anti-NHE6, followed by the second secondary antibody, AlexaFluor® goat anti-rabbit 488. Once coverslips had been mounted on the slides and dried, I collected Z-stack images, which were later collapsed into maximum intensity projections (Figure 3.6A).

Images were again analyzed using the MetaMorph® software program. Within the maximum intensity projection images, I counted a total of 976 Nhe6-positive endosomes and a total of 1,186 Nhe9-positive endosomes across 46 cells over three replicates of the experiment. Endosomes were selected throughout the processes; puncta within the soma were again excluded from analysis. Of these neuronal endosomes, 331 showed overlapping localization between Nhe6 and

Nhe9 (Figure 3.6B). Thus, 33.9% of Nhe6-positive endosomes are also positive for Nhe9, and 27.9% of Nhe9-positive endosomes are also positive for Nhe6. These results indicate that although there is some overlap between the two signals, the majority of the localization is distinct.

Discussion

The localization patterns of Nhe6 and Nhe9 were characterized in non-neuronal cell lines over a decade ago, and more recently, Ouyang et al. (2013) detailed the individual localization pattern of Nhe6 throughout the endosomal system of hippocampal neurons (Ouyang et al., 2013). Until this point, however, the comparative localizations of the two endogenous proteins within neurons had not been described. It is important to understand the distribution of these proteins within neuronal endosome populations, as the polarized nature of neurons poses a unique challenge to endosomal trafficking.

It is also important to understand relative expression levels of the two proteins within the experimental system (i.e. neonatal mouse hippocampus), as these results can inform the interpretation of foundational and functional studies. Through Western blotting, I have demonstrated that Nhe6 is 3.6 times more abundant than Nhe9 within wild-type neonatal mouse hippocampus. Studies reported by Kondapalli et al. (2014) demonstrate a similar result at the level of the transcript. *In situ* hybridization analyses from the Allen Brain Atlas (www.brain-map.org) (Lein et al., 2007) reveal much more robust expression of Nhe6 than

Nhe9 within the hippocampi of male mice at the age of 56 days (Kondapalli et al., 2014).

With regard to comparative localization patterns, the results presented within this chapter reveal that both Nhe6 and Nhe9 are localized across all endosomal compartments – early, recycling, and late. Their distributions within the three different compartments follow the same trends. Considering the inverse relationship, however, that of the number of endosomes positive for Nhe6 and Nhe9, instead of the number of Nhe6 and Nhe9 puncta positive for an endosomal marker, two differences emerge. More early endosomes are positive for Nhe6 than Nhe9, whereas more recycling and late endosomes are positive for Nhe9 than Nhe6. These results are consistent with those seen in non-neuronal mammalian cell lines; Nhe6 is over-represented early within the endosomal pathway, and Nhe9 is over-represented later within the endosomal pathway (Brett et al., 2002; Nakamura et al., 2005).

An additional study that had been missing from the field was that of the analysis of colocalization between endogenous Nhe6 and Nhe9 signals. In part due to a lack of suitable reagents, this immunostaining has never been reported. I was able to utilize a different type of staining protocol with consecutive instead of concurrent staining, enabling me to use both of the NHE antibodies developed by the Morrow lab. Staining for Nhe6 and Nhe9 in conjunction revealed some colocalized signal. Approximately 30% of total signal was overlapping between the two proteins; however, most of the staining was distinct.

The method used herein does not quantify how much of each protein is on each endosome when Nhe6 and Nhe9 are colocalized. Localization of these two proteins to the same endosomes may suggest similar protein functions, but if there are differential levels of localization, the effect of each protein's loss may differ in magnitude. It is also possible that when Nhe6 and Nhe9 are not colocalized, they may play non-redundant roles within the cell, separated from one another both spatially and functionally.

Overall, the results presented within Chapter 3 represent a detailed characterization of Nhe6 and Nhe9 localizations, both individually and in comparison to one another, throughout the endosomal pathway of neurons.

Materials and Methods

Reagents

Cells were dissociated using the Papain Dissociation System (LK003150) from Worthington Biochemical Corporation. All cell culture reagents were purchased from Gibco: Neurobasal®-A medium (10888-022), B-27® Supplement (17504-001), Penicillin-Streptomycin (15140-122), and GlutaMAX™ Supplement (35050-061). Rabbit anti-NHE6 (Ouyang et al., 2013) and rabbit anti-NHE9 (Chapter 2) antibodies were generated as previously described. Commercial antibodies were as follows: mouse anti-Rab5 (Abcam, ab50523), mouse anti-Rab7 (Sigma, R8779), and mouse anti-Rab11 (BD Transduction Laboratories, 610656). Normal goat serum (#005-000-121), normal rabbit serum (#011-000-120), and

unconjugated goat anti-rabbit IgG Fab fragment (#111-007-003) were purchased from Jackson ImmunoResearch Laboratories, Inc.

Hippocampal Homogenization and Western Blotting

Hippocampi were collected from P0-P1 wild-type CD-1 mice, and protein was isolated via homogenization with cold RIPA buffer, consisting of 50 mM Tris, 150 mM sodium chloride, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, and 1% NP-40. Protease inhibitor cocktail (cOmplete ULTRA Tablets, Roche, #05-892-970-001) and phosphatase inhibitor cocktail (PhosSTOP, Roche, #04-906-837-001) were added to the buffer immediately prior to use. Protein concentration in each homogenized sample was then measured using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific), and 10 µg of each sample was combined with 4X NuPAGE® LDS Sample Buffer and 10X NuPAGE® Reducing Agent and loaded onto a NuPAGE® 4-12% Bis-Tris precast gel (Invitrogen). After electrophoresis, proteins from the gel were transferred onto a nitrocellulose membrane.

Membranes were blocked for overnight at 4°C in 5% nonfat milk in 1X Tris-buffered saline + 0.05% Tween-20 (TBS-T). They were then incubated in rabbit anti-NHE9 or rabbit anti-NHE6 primary antibody, each diluted 1:500 in 5% nonfat milk, for 2.5 hours at room temperature. After several washes with TBS-T, membranes were incubated with a peroxidase IgG fraction mouse anti-rabbit secondary (Jackson ImmunoResearch Laboratories, 211-032-171) at a 1:20,000

dilution for 1 hour at room temperature. Following several more TBS-T washes, membranes were rinsed briefly with Milli-Q water and incubated in a 1:1 ratio of SuperSignal® West Pico Chemiluminescent Substrates (ThermoFisher Scientific) for 5 minutes. Membranes were exposed to film for 1 minute and developed with a Konica Minolta Medical Imaging SRX-101A tabletop processor.

Membranes were then stripped with 1X NewBlot™ Nitro Stripping Buffer (LI-COR) and the above process was repeated, probing for mouse anti-tubulin (Sigma, T6074) at a 1:8,000 dilution with a peroxidase AffiniPure goat anti-mouse IgG secondary (Jackson ImmunoResearch Laboratories, 115-035-174) at a 1:20,000 dilution. Band intensity was quantified through densitometry analysis using the ImageJ software program, and Nhe signal was normalized to that of tubulin.

Primary Neuronal Cultures

Wild-type CD-1 timed pregnant female mice (strain #022) were purchased from Charles River Laboratories. After birth (P0 to P1), 4 to 6 mice were collected and euthanized via swift decapitation. Hippocampi were dissected, and cells were dissociated with papain in Earle's balanced salt solution for 30 minutes at 37°C followed by trituration with a 1-mL pipette. Neurons were plated on either 8-well glass chamber slides (Lab-Tek®, 177402) coated with 1 mg/mL poly-D-lysine hydrobromide (Sigma, P6407) at a density of approximately 50,000 cells/well or on #1.5 coverslips pre-coated with poly-D-lysine (neuVibro, GG-12-1.5-pdl) at a

density of approximately 30,000 cells/cover slip. Neurons were grown in a 37°C/5% CO₂ incubator in medium consisting of Neurobasal®-A supplemented with 2% B-27®, 1% GlutaMAX™, and 1% penicillin-streptomycin (complete growth medium).

Nhe/Rab Immunocytochemistry

Wild-type primary hippocampal cultures were fixed at 3 DIV with cold 4% PFA for 10 minutes, washed 3 times with 1X PBS, and permeabilized for 15 minutes with 0.25% Triton-X. Cells were blocked with 10% goat serum/0.1% Triton-X for 1 hour at room temperature. Cells were then incubated with a combination of primary antibodies (one Nhe and one Rab), diluted in 2% goat serum/0.1% Triton-X, for 1.5 hours at room temperature. Primary antibody dilutions were as follows: NHE6, 1:500; NHE9, 1:500; Rab5, 1:250; Rab11, 1:250; and Rab7, 1:500.

After primary antibody incubation, cells were washed 3 times for 5 minutes each with blocking buffer, followed by incubation with the secondary antibodies (AlexaFluor® goat anti-mouse 488 and AlexaFluor® goat anti-rabbit 594), diluted 1:1000 in blocking buffer. DAPI was added to the secondary antibodies at a 1:1000 dilution to visualize nuclei. Cells were washed 3 final times with 1X PBS, and coverslips were mounted on slides using Fluoromount-G (SouthernBiotech, #0100-01).

Nhe6/Nhe9 Immunocytochemistry with Fab Fragment Blocking

Wild-type primary hippocampal neurons were fixed at 3 DIV with cold 4% PFA for 10 minutes, washed 3 times with 1X PBS, and permeabilized for 15 minutes with 0.25% Triton-X. Slides were then blocked with 2% goat serum/0.1% Triton-X in PBS overnight at 4°C. The following day, the first primary antibody (rabbit anti-NHE9) was diluted 1:250 in block and applied to cells for 1.5 hours at room temperature. Unbound antibody was removed by 3 washes (5 minutes each) with blocking buffer; the first secondary antibody (AlexaFluor® goat anti-rabbit 594) was then diluted 1:1000 in block and applied to the cells for 45 minutes at room temperature. DAPI was added to the first secondary antibody at a 1:1000 dilution to visualize nuclei. Cells were then washed 3 times with blocking buffer and incubated with 5% normal rabbit serum in 1X PBS for 1 hour at room temperature. Following 3 more washes with blocking buffer, cells were incubated with an excess (100 µg/mL) of unconjugated goat anti-rabbit Fab fragment overnight at 4°C.

The following day, cells were washed 3 times with blocking buffer and incubated in the second primary antibody (rabbit anti-NHE6), diluted 1:3000 in blocking buffer, for 1 hour at room temperature. Cells were then washed 3 times with blocking buffer and incubated in the second secondary antibody (AlexaFluor® goat anti-rabbit 488), diluted 1:1000 in blocking buffer, for 45 minutes at room temperature. After 3 final washes with 1X PBS, coverslips were mounted on slides with Fluoromount-G.

Confocal Imaging

Six Z-stack images of each condition within each replicate were collected with a Zeiss LSM800 confocal microscope, using a 63X oil/1.4 N.A. objective. Images were collapsed into maximum intensity projections using the Zen Black software and converted into single-channel TIFFs using ImageJ.

MetaMorph® Analysis

TIFF images were opened in the MetaMorph® program (Figure 3.4A), and a 3 x 3 median filter was applied to each image (Figure 3.4B) prior to thresholding (Figure 3.4C). Individual puncta were manually circled in each channel (Figure 3.4D), and the “Colocalization” app was used to calculate the percentage of overlap between the red and green channels.

Statistical Analysis

For analysis of Western blotting (data presented in Figure 3.1) and Nhe6/Nhe9 colocalization (data presented in Figure 3.6), values were averaged across replicates and compared using a Student's t-test. For analysis of colocalization between NHEs and Rab endosomal markers (data presented in Figure 3.5), values were averaged across individual endosomes and compared using a mixed effects logistic regression model. In all cases, a p-value of < 0.05

was considered to be significant. All data were expressed as mean $\pm$ standard error.

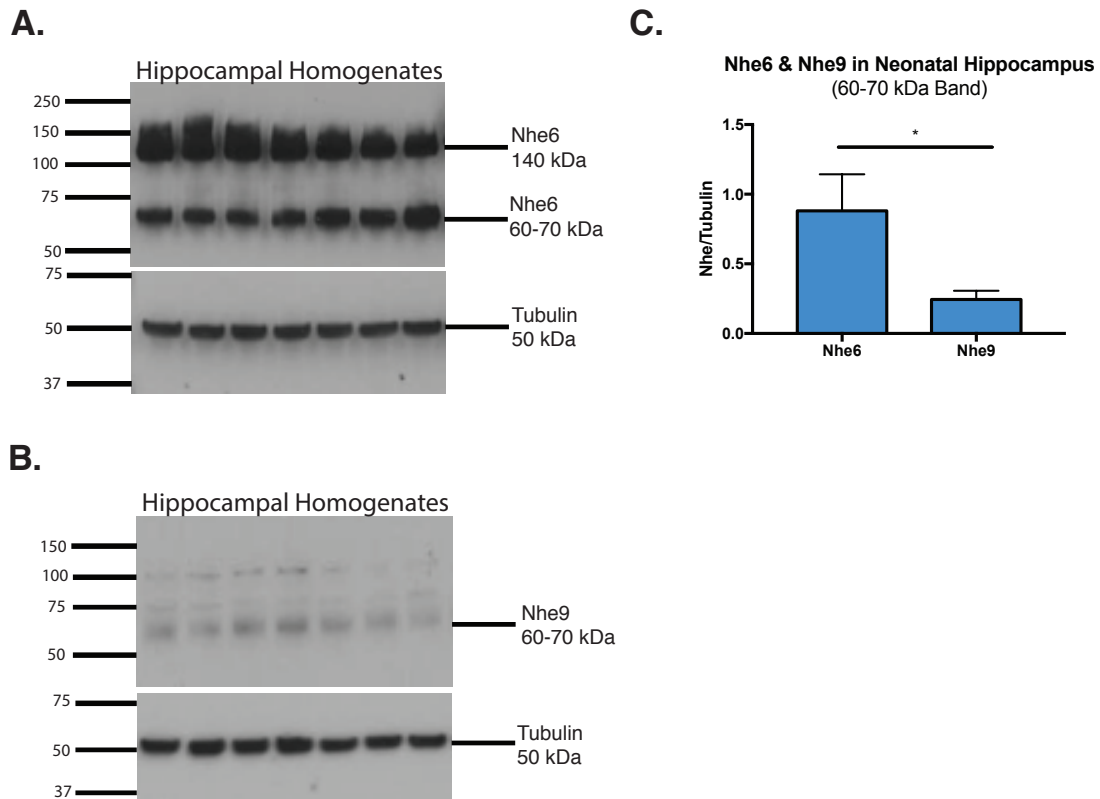


Figure 3.1. Nhe6 protein is more abundant than Nhe9 protein in neonatal mouse hippocampus. (A) Hippocampi from 7 different neonatal wild-type CD-1 mouse pups across 2 litters were collected and homogenized in cold RIPA buffer. Samples were run out on a polyacrylamide gel, transferred to a nitrocellulose membrane, and probed for both Nhe6 (1:500 antibody dilution) and tubulin (1:8,000 antibody dilution) expression. Membranes were exposed to film for 1 minute for Nhe6 and 2 seconds for tubulin, prior to development. Each lane represents the hippocampi from a different mouse pup. (B) The same 7 hippocampal samples from panel A were run out on a second gel and probed for Nhe9 (1:500 antibody dilution) and tubulin (1:8,000 antibody dilution) expression. Membrane exposure times were the same as above (1 minute for Nhe9 and 2 seconds for tubulin). Each lane represents hippocampi from a different mouse pup. (C) Quantification of relative expression level of Nhe6 and Nhe9 within neonatal mouse hippocampus. Band intensity was analyzed via the densitometry method using the ImageJ software package. Nhe6 and Nhe9 intensity values were normalized to those of tubulin. Data represent mean $\pm$ standard error. *, $p = 0.036$.

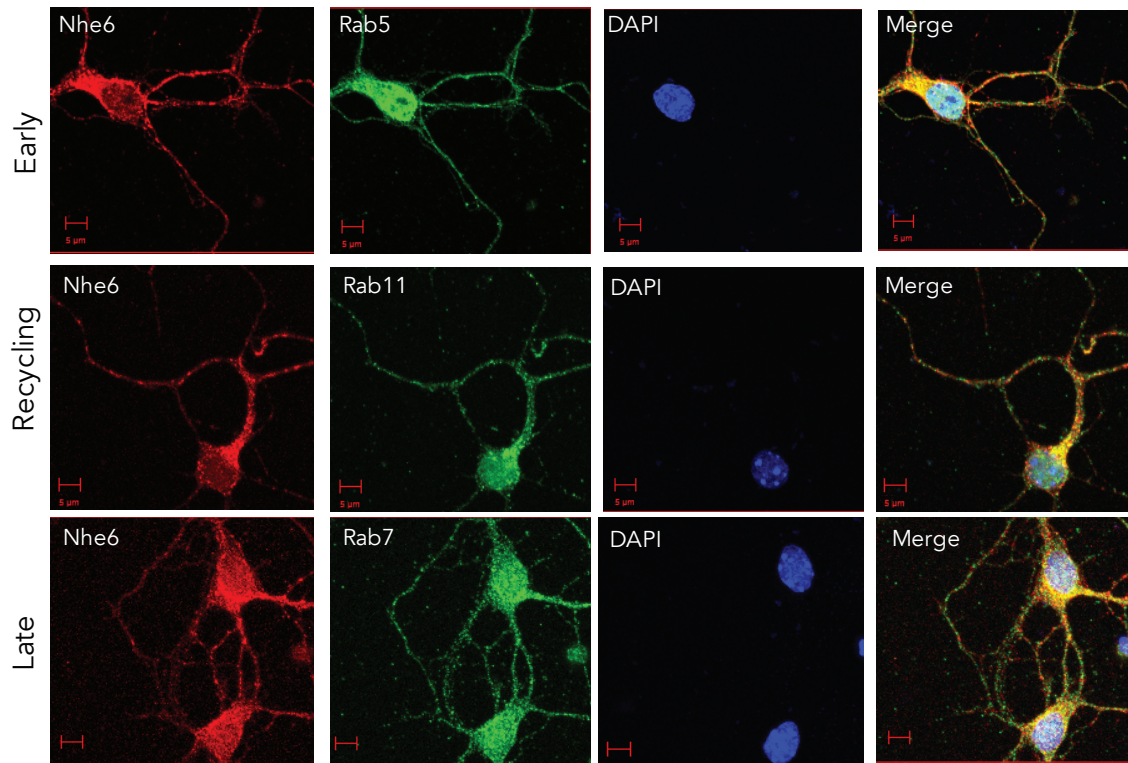


Figure 3.2. Colocalization of Nhe6 with endosomal markers in wild-type primary hippocampal neurons. Cells derived from neonatal mice were grown to 3 DIV prior to fixation and staining with both rabbit anti-NHE6 (in red, AlexaFluor® 594) and mouse anti-Rab5, 11, or 7 (in green, AlexaFluor® 488). Rab5 is a marker of early endosomes, Rab11 is a marker of recycling endosomes, and Rab7 is a marker of late endosomes. DAPI, a nuclear stain, is in blue. Overlapping signal is shown in yellow. Scale bars represent 5 μm.

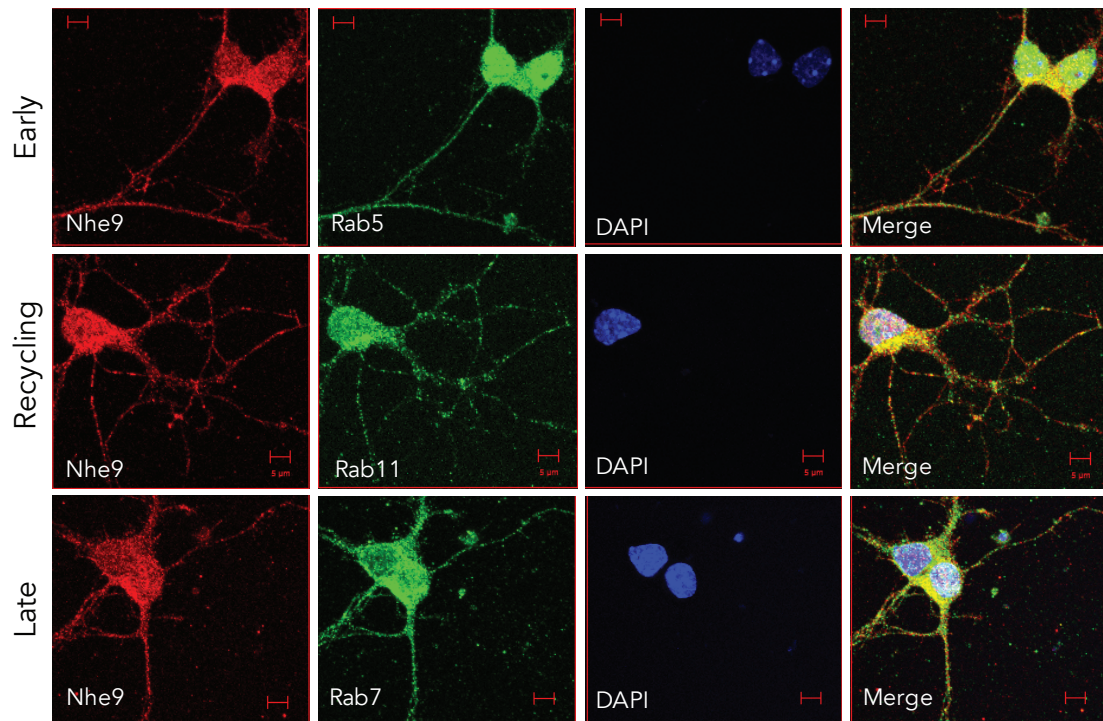


Figure 3.3. Colocalization of Nhe9 with endosomal markers in wild-type primary hippocampal neurons. Cells derived from neonatal mice were grown to 3 DIV prior to fixation and staining with both rabbit anti-NHE9 (in red, AlexaFluor® 594) and mouse anti-Rab5, 11, or 7 (in green, AlexaFluor® 488). Rab5 is a marker of early endosomes, Rab11 is a marker of recycling endosomes, and Rab7 is a marker of late endosomes. DAPI, a nuclear stain, is in blue. Overlapping signal is shown in yellow. Scale bars represent 5 μm.

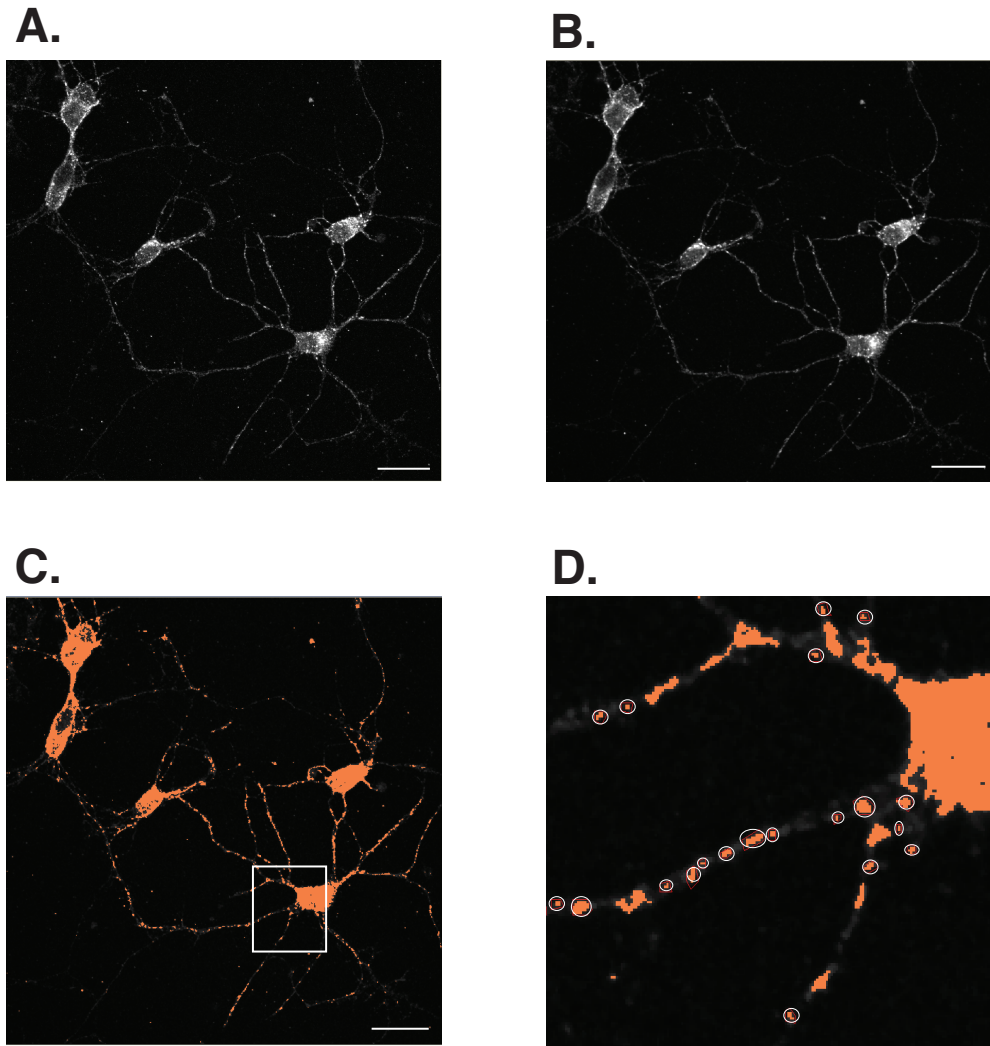


Figure 3.4. Use of MetaMorph® software program to analyze colocalization of proteins within neuronal endosomes. Shown are representative primary hippocampal neurons grown to 3 DIV, fixed with 4% PFA, and stained for Nhe6 expression. (A) A Z-stack image of each region of interest was collected, prior to being collapsed into a maximum intensity projection. Each image was then split by channel, converted into TIFFs, and opened in MetaMorph®. (B) A 3 pixel x 3 pixel median filter was applied to smooth each image and decrease nonspecific background staining. (C) An automatic threshold was applied to each image, with verification by the experimenter. Orange represents signal above threshold. (D) Magnified image of the region within the white box in panel C. Endosomes within neuronal processes were traced manually (depicted by white circles), and the “Colocalization” app was used to calculate the number of positive pixels within each channel for each region of interest. Scale bar represents 20 μm .

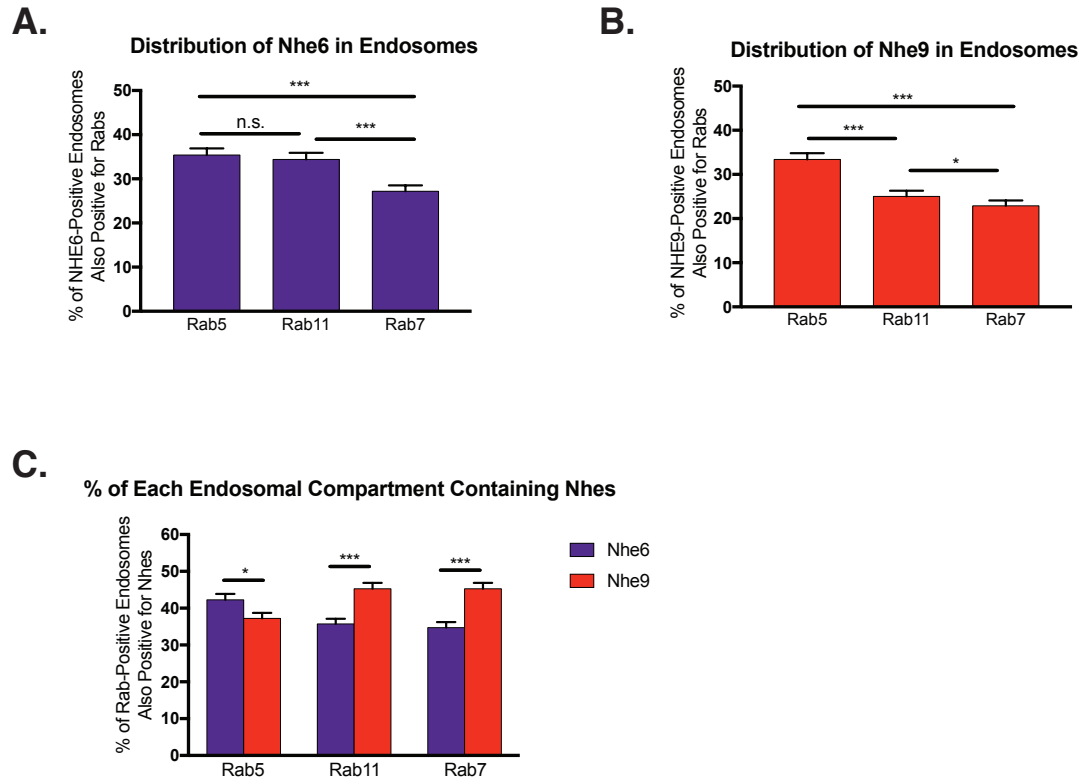


Figure 3.5. Quantification of colocalization between Nhes and Rab endosomal markers (staining in Figures 3.2 and 3.3). (A) Representation of the total population of Nhe6-positive puncta and their distribution throughout the endosomal pathway, as measured by % overlap with Rab markers. (B) Representation of the total population of Nhe9-positive puncta and their distribution throughout the endosomal pathway, as measured by % overlap with Rab markers. (C) Representation of the total population of Rab-positive endosomes and the % overlap with Nhe6 versus Nhe9. All overlap was quantified by defining individual endosomes as regions of interest using the MetaMorph® software program. For panels A and B, distribution of staining was calculated as (Overlapping Red and Green Signal)/(Total Red Signal). For panel C, distribution of staining was calculated as (Overlapping Red and Green Signal)/(Total Green Signal). Across 3 replicates of the experiment, a total of 12,979 Rab5-positive endosomes, 15,701 Rab11-positive endosomes, and 15,862 Rab7-positive endosomes were counted. Data represent mean $\pm$ standard error. n.s., not significant; *, $p < 0.05$; ***, $p < 0.001$.

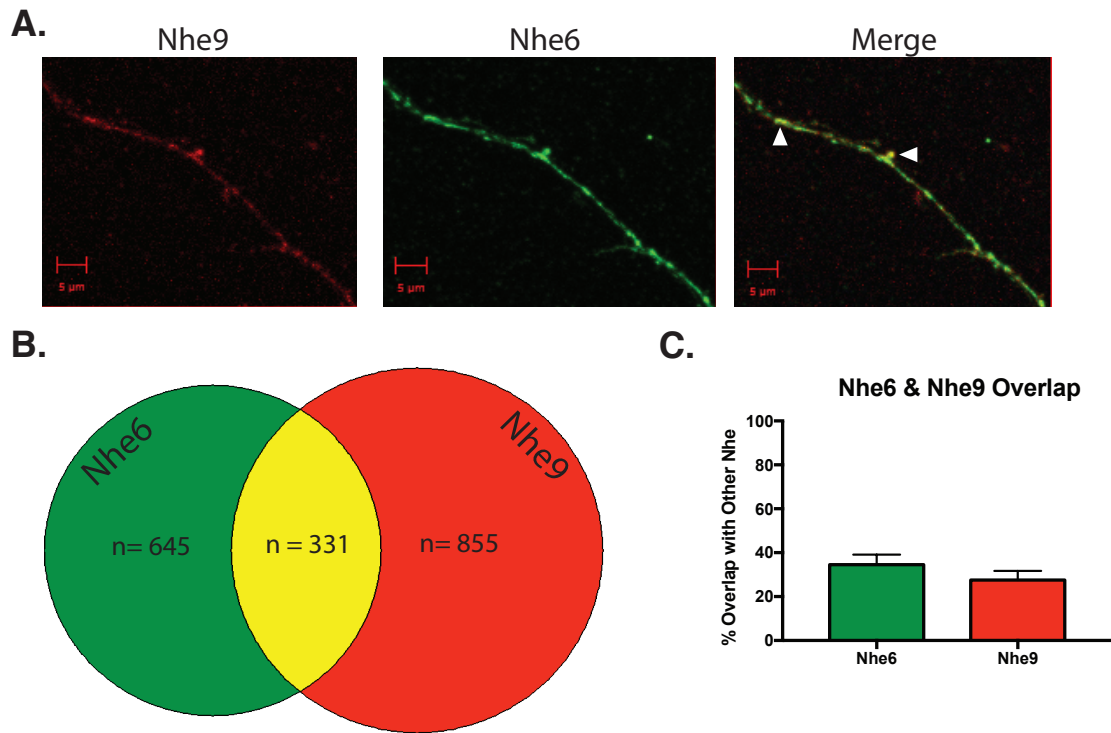


Figure 3.6. Colocalization between Nhe6 and Nhe9 in the processes of wild-type primary hippocampal neurons. (A) Neurons derived from neonatal mice were grown to 3 DIV and stained for Nhe9 and Nhe6 consecutively using the Fab fragment blocking method. Nhe9 is depicted in red (AlexaFluor® 594), Nhe6 is depicted in green (AlexaFluor® 488), and the overlap is depicted in yellow, with colocalized puncta denoted by the white arrowheads. Scale bar represents 5 μ m. (B) Quantification of Nhe6/Nhe9 costaining. The green circle represents the total population of Nhe6-positive endosomes ($n = 976$), whereas the red circle represents the total population of Nhe9-positive endosomes ($n = 1186$). Of these endosomes, 331 contained positive localization for both Nhe6 and Nhe9 (depicted in yellow). Total number of cells, $n = 46$. (C) Quantification of Nhe6/Nhe9 costaining, averaged across three replicates. Percentage of Nhe6-positive endosomes also positive for Nhe9 are depicted in green; percentage of Nhe9-positive endosomes also positive for Nhe6 are depicted in red. Data represent mean $\pm$ standard error.

CHAPTER 4:
FUNCTIONAL CONSEQUENCES
OF ENDOSOMAL NHE
MUTATIONS

Introduction

In Chapter 3, I described the localization patterns of Nhe6 and Nhe9 within neurons, both separately and in combination. In Chapter 4, I use information garnered from these experiments to inform the interpretation of functional studies of endosomal Nhe loss. Here, I am asking two central questions. First, what is the effect of Nhe9 loss on neuronal arborization and endosomal pH, and how do these results compare to the loss of Nhe6 phenotype? Second, how do the loss of Nhe6 and Nhe9 affect trafficking of cargo through the endosomal system of neurons?

NHE6 and NHE9 both play a role in cognitive disorders, the basis of which is proposed to be the aberrant development of neuronal connectivity and circuitry. A component of neuronal connectivity and circuit development is the outgrowth of arbors, or multiple processes emanating from the cell body. Ouyang *et al.* (2013) provided evidence of the role of NHE6 in axonal and dendritic arborization. Hippocampal neurons derived from *Nhe6*-null mice displayed markedly reduced arborization when compared to wild-type, in measures of length, branch point quantity, and branch quantity. This impoverished arborization was evident as early as 2 DIV. These results were also replicated *in vivo* in brain tissue sections, with both basal and apical dendrites of hippocampal CA1 and CA3 neurons exhibiting significantly reduced branching. Furthermore, exogenous application of BDNF (brain-derived neurotrophic factor) rescued the *in vitro* deficits.

In addition to their work on the relationship between NHE6 and neuronal arborization, Ouyang *et al.* (2013) established a direct connection between NHE6 expression level and endosomal pH. Through the use of two independent methods, Ouyang *et al.* (2013) demonstrated that luminal pH in *Nhe6*-null neurons is consistently half of a pH unit lower (i.e. more acidic) than that of wild-type neurons. This overacidification phenotype was coupled to an attenuation of BDNF/TrkB (brain-derived neurotrophic factor/tropomyosin receptor kinase B) signaling from the endosomes.

In addition, within the prior chapter, I demonstrated that the localization patterns of *Nhe6* and *Nhe9* within neuronal endosomes are similar, with some overlap of localization between the two proteins. Based on these results as well as those of Ouyang *et al.* (2013), we sought to test the role of *Nhe9* in neuronal development through its effect on arborization and pH control. Our initial hypothesis was that *Nhe9* would affect development in a manner similar to that of *Nhe6*. Unlike *Nhe6* loss, however, loss of *Nhe9* does not produce a profound difference in branching when compared to wild-type. Furthermore, whereas *Nhe6* loss overacidifies endosomes, *Nhe9* loss results in an increase in more alkaline endosomes.

Endosomal pH is a tightly regulated compartmental feature critical to proper cellular function. The moderate acidity level in early endosomes facilitates the dissociation of some ligand-receptor complexes, allowing the receptors to be cycled back to the cell surface and the ligands to be cycled through late

endosomes to lysosomes. The lysosomes, in contrast, have a very acidic pH, which facilitates their degradative function (Mellman, 1992). When control of endosomal pH is disrupted, impairments in trafficking may also result.

In this chapter, I demonstrate that Nhe9 loss has an effect on pH distinct from that of Nhe6. In *Nhe9*-null neurons, there is an increase in more alkaline endosomes when compared to wild-type. Nhe6 and Nhe9 also appear to have distinct effects on the trafficking of cargo through the endosomal system. In *Nhe6*-null neurons, there is a trend towards increased localization of transferrin cargo in more acidic late endosomes. In *Nhe9*-null neurons, however, there is a significant decrease in transferrin localization in late endosomes, accompanied by an apparent increase in cargo in more alkaline early endosomes.

Results

Neuronal Morphogenesis and Arborization in Nhe9-Null Neurons

The loss of NHE6 expression leads to defects in neuronal morphogenesis. *Nhe6*-null hippocampal neurons exhibit impoverished arborization, as demonstrated by a decrease in dendritic and axonal branching both *in vitro* in cultured cells and *in vivo* in brain slices (Ouyang et al., 2013). As Nhe6 and Nhe9 are thought to function similarly within the cell, namely, as proton “leaks” involved in endosomal pH homeostasis, we hypothesized that the loss of Nhe9 expression within hippocampal neurons would also lead to arborization deficits.

To assess neuronal morphogenesis and arborization, primary cultures were derived from P1 wild-type and *Nhe9*-null littermate mice. At 3-4 DIV, neurons were transiently transfected with a plasmid encoding eGFP. At 5 DIV, cells were fixed with 4% PFA, stained for Ankyrin-G to identify axons, and imaged with an LSM710 confocal microscope (Figure 4.1). Using the Neurolucida software package, neuronal processes were traced manually, and arborization was assessed separately for axons and dendrites.

Within axons, the mean length, mean number of branch points, and mean number of branches were measured. Wild-type ($1201.7 \pm 98.76 \mu\text{m}$) (mean $\pm$ standard error) and *Nhe9*-null ($1226.2 \pm 79.34 \mu\text{m}$) axons do not differ from one another in length ($p = 0.85$) (Figure 4.2A). Likewise, quantities of wild-type (41.1 ± 3.15) and null (35.3 ± 2.29) branch points are not significantly different ($p = 0.12$), nor are quantities of wild-type (44.6 ± 3.33) and null (38.2 ± 2.43) branches ($p = 0.11$) (Figure 4.2B).

Analysis of dendrite parameters yielded similar results. Both the mean total length of all dendrites per neuron and the mean length of each individual dendrite per neuron were quantified. In neither case is the difference between wild-type and *Nhe9*-null statistically significant. Wild-type neurons have a total dendrite length of $755.9 \pm 49.16 \mu\text{m}$, and null neurons have a total dendrite length of $696.8 \pm 32.90 \mu\text{m}$ ($p = 0.30$). Each individual wild-type dendrite has a mean length of $96.0 \pm 6.43 \mu\text{m}$, whereas each individual null dendrite has a mean length of $105.9 \pm 5.99 \mu\text{m}$ ($p = 0.27$) (Figure 4.3A).

Neither dendrite branch point quantity nor branch quantity differed with genotype. Each wild-type neuron has an average of 37.9 ± 2.57 dendritic branch points, and each *Nhe9*-null neuron has an average of 33.3 ± 1.90 dendritic branch points ($p = 0.15$). Similarly, each wild-type neuron has an average of 50.2 ± 3.04 dendritic branches, whereas each null neuron has an average of 43.7 ± 2.32 dendritic branches ($p = 0.090$) (Figure 4.3B).

The one parameter that does differ significantly, although modestly, between wild-type and *Nhe9*-null neurons is primary dendrite quantity. On average, each wild-type neuron possesses 8.4 ± 0.38 primary dendrites, whereas each null neuron possesses 7.4 ± 0.34 primary dendrites ($p = 0.042$) (Figure 4.3C). When considered in total, however, these results indicate that *Nhe9* loss has little effect on neuronal arborization and morphogenesis *in vitro*.

Measurement of Endosomal pH in *Nhe9* Neurons

Endosomal NHEs assist with pH regulation through the electroneutral exchange of hydrogen ions for sodium ions along the ions' respective concentration gradients. We hypothesized that in the absence of *Nhe9* expression, hydrogen ions would remain within the endosomal compartment, thereby decreasing endosomal pH. Thus, the loss of *Nhe9* expression in hippocampal neurons would lead to an increase in the acidity of endosomal compartments (i.e. decreased pH), similar to the effect of *Nhe6* loss (Ouyang et al., 2013).

To quantify endosomal pH, live wild-type, heterozygous, and *Nhe9*-null neurons were incubated with two fluorescently-conjugated transferrin reagents for 10 minutes at 37°C (Figure 4.4A). Fluorescein isothiocyanate (FITC)-conjugated transferrin is sensitive to changes in pH, such that the fluorophore is progressively quenched as pH decreases, whereas AlexaFluor® 546-conjugated transferrin is pH-stable. Additionally, wild-type neurons incubated with the transferrin reagent cocktail were buffered in a series of solutions of decreasing pH to generate a calibration curve. For quantification of acidity level, the ratio of FITC fluorescence intensity to AlexaFluor® 546 fluorescence intensity was determined, and pH was calculated according to the linear equation of the calibration curve (Figure 4.4B).

Quantitative analysis of individual endosomes revealed a significant effect of genotype on pH (Figure 4.4C). Within neuronal processes, wild-type endosomes have an average pH of 6.30 ± 0.06 , heterozygous endosomes have an average pH of 6.45 ± 0.06 , and *Nhe9*-null endosomes have an average pH of 6.51 ± 0.05 . The increase in endosomal pH was significant for null as compared to wild-type cells ($p < 0.0001$), as well as for heterozygous as compared to wild-type cells ($p = 0.006$).

Within cell bodies, wild-type endosomes have an average pH of 6.12 ± 0.07 , heterozygous endosomes have an average pH of 6.32 ± 0.06 , and *Nhe9*-null endosomes have an average pH of 6.43 ± 0.06 . The change in endosomal pH was again significant for wild-type versus null neurons ($p < 0.0001$), as well as for wild-type versus heterozygous neurons ($p = 0.006$). The pH of heterozygous and

null endosomes did not differ significantly from one another in either processes ($p = 0.485$) or soma ($p = 0.119$). *Nhe9*-null and heterozygous neurons thus exhibit a significant increase in endosomal pH when compared to wild-type neurons, in both processes and soma.

Endosomal Size in Nhe9 Neurons Does Not Vary with Genotype or pH

Based on qualitative observations of endosomal size in images of live neurons, we hypothesized that there would be an increase in larger endosomes in neurons derived from *Nhe9*-null mice, as compared to wild-type (see Figure 4.4A). Additionally, perturbing the flux of ions could result in changes in endosomal size (Scott and Gruenberg, 2011). Therefore, when endosomes were analyzed for fluorescence intensity, the area of each individual compartment was also measured.

Within neuronal cell bodies, wild-type endosomes have an average area of 0.62 ± 0.03 (mean $\pm$ standard error) square pixels, heterozygous endosomes have an average area of 0.67 ± 0.03 square pixels, and *Nhe9*-null endosomes have an average area of 0.66 ± 0.02 square pixels. Within processes, wild-type endosomes have an average area of 0.76 ± 0.03 square pixels, heterozygous endosomes have an average area of 0.83 ± 0.02 square pixels, and null endosomes have an average area of 0.77 ± 0.02 square pixels.

When endosomes from both soma and processes are considered in combination, the average wild-type endosome area is 0.70 ± 0.02 square pixels,

the average heterozygous endosome area is 0.77 ± 0.02 square pixels, and the average null endosome area is 0.73 ± 0.02 square pixels. There was no significant difference in endosome size among wild-type, heterozygous ($p = 0.094$), and null ($p = 0.84$) neurons, whether in soma, processes, or total (Figure 4.5A).

An additional hypothesis related to pH measurement within live neurons was that endosomal size would vary according to pH, whereby there would be an increase in larger, higher-pH endosomes within *Nhe9*-null neurons. When the data are graphed to show the correlation between endosomal pH and area across all three genotypes (wild-type, heterozygous, and *Nhe9*-null), a weak correlation emerges (correlation coefficient of 0.2) (Figure 4.5B). However, this association is not significant ($p = 0.379$). Therefore, endosomal size within neurons does not vary according to *Nhe9* genotype or pH level.

Trafficking of Transferrin Through the Endosomal Systems of *Nhe6* and *Nhe9* Neurons

Loss of *Nhe9* expression in neurons results in an increase in more alkaline endosomes. This result was unexpected, according to the proposed function of NHEs. Thus, we hypothesized that there may be a defect in the trafficking of transferrin through the endosomal system. If transferrin within *Nhe9*-null neurons fails to move from early endosomes and instead accumulates in these less acidic compartments, the overall pH as measured by the transferrin method will be more alkaline than in wild-type neurons.

In contrast, loss of Nhe6 expression in neurons results in endosomal overacidification. Although this observation is consistent with a role for Nhe6 as a proton leak, there also exists the possibility of a role in endosomal trafficking, opposite that proposed for Nhe9. Instead of cargo being retained in early endosomal compartments, perhaps cargo in *Nhe6*-null neurons is accelerated through the pathway, resulting in an accumulation in lower-pH late endosomal compartments.

To assess trafficking, hippocampal neurons at 5 DIV were incubated with pH-stable AlexaFluor® 546-conjugated transferrin, as described in pH measurement experiments above. After 10 minutes, cells were fixed and stained for markers of endosomal compartments: Rab5 (early endosomes), Rab11 (recycling endosomes), and Rab7 (late endosomes). Images were collected of several neurons per condition, and the colocalization of the transferrin signal with each of the endosomal markers was quantified using MetaMorph® (Figures 4.6 and 4.7).

In *Nhe9* neurons, the trend suggests that there is an increase in transferrin accumulation within Rab5-positive early endosomes in the null as compared to the wild-type (Figure 4.8A). In wild-type cells, $38.4 \pm 6.3\%$ (mean $\pm$ standard error) of transferrin colocalizes with Rab5 staining, whereas in *Nhe9*-null cells, $45.7 \pm 6.3\%$ of transferrin colocalizes with Rab5 staining, although this difference did not reach statistical significance ($p = 0.354$). Within recycling endosomes, $48.5 \pm 6.6\%$ of

transferrin colocalizes with Rab11 in wild-type neurons, and $41.8 \pm 2.8\%$ of transferrin colocalizes with Rab11 in null neurons ($p = 0.316$).

The effect of genotype on transferrin distribution is significant in Rab7-positive late endosomes. In wild-type neurons, $41.9 \pm 1.1\%$ of transferrin colocalizes with Rab7 staining. In *Nhe9*-null neurons, however, this value is significantly decreased, with $35.2 \pm 1.1\%$ of transferrin colocalizing with Rab7 staining ($p < 0.001$).

There is no significant effect of genotype on transferrin distribution in *Nhe6* neurons, although the trend in null cells suggests a bias towards accumulation of cargo in more acidic late endosomes, with less cargo being contained in more alkaline early endosomes (Figure 4.8B). In *Nhe6*-null neurons, $30.0 \pm 5.4\%$ of transferrin colocalizes with Rab5-positive early endosomes, $37.1 \pm 4.2\%$ colocalizes with Rab11-positive recycling endosomes, and $42.6 \pm 1.5\%$ colocalizes with Rab7-positive late endosomes. This result is in contrast to that of wild-type neurons, which demonstrate roughly equal distributions of transferrin within endosomal compartments. Overall, $33.7 \pm 3.3\%$ of transferrin colocalizes with Rab5-positive early endosomes, $39.7 \pm 1.6\%$ colocalizes with Rab11-positive recycling endosomes, and $37.9 \pm 7.6\%$ colocalizes with Rab7-positive late endosomes.

When compared directly to one another after normalization to the respective wild-type values, *Nhe6*- and *Nhe9*-null neurons exhibit distinct trends in the distribution of endosomal transferrin following a 10-minute incubation period

(Figure 4.8C). In the *Nhe6*-null cells, the normalized proportion of transferrin within each compartment increases as it moves through the endosomal pathway, from $89.2 \pm 9.2\%$ in early endosomes to $130.5 \pm 40.9\%$ in late endosomes. In the *Nhe9*-null cells, the normalized proportion of transferrin within each compartment decreases as it moves through the endosomal pathway, from $123.9 \pm 20.7\%$ in early endosomes to $81.7 \pm 5.5\%$ in late endosomes. Although the trends are distinct, the differences between transferrin distributions according to genotype do not reach statistical significance.

Discussion

In this chapter, I demonstrate that the functional consequences of *Nhe6* and *Nhe9* mutations in cultured hippocampal neurons are distinct. I analyzed neuronal arborization, endosomal pH, and transferrin trafficking, and in each case, I determined that *Nhe9* loss does not recapitulate the established effects of *Nhe6* loss.

Prior work from the Morrow lab demonstrated that *Nhe6* loss has a profound impact on neuronal arborization and morphogenesis. *Nhe6*-null neurons display significantly impoverished arbors, in both axons and dendrites. Across multiple measures of arborization, including axonal and dendritic branch point number, both *in vitro* in cultured hippocampal neurons and *in vivo* in hippocampal brain sections, the decrease in the *Nhe6*-null cells as compared to wild-type was as great as 50% (Ouyang et al., 2013).

These results are in stark contrast to the work presented here, demonstrating that *Nhe9* loss has little effect on neuronal arborization and morphogenesis. Axons at 5 DIV in *Nhe9*-null neurons do not differ from those in wild-type neurons in measures of mean length, branch point quantity, or branch quantity. Similarly, *Nhe9*-null and wild-type dendrites do not differ in measures of total length, mean length, mean branch point quantity, or mean branch quantity. The only significantly different parameter between genotypes was that of primary dendrite number, with the null neuron quantity being slightly decreased when compared to wild-type. Even so, this effect was modest (a decrease of approximately 12%), with p value of 0.042.

I also demonstrated an increase in more alkaline endosomes in *Nhe9*-null neurons, a result that was unexpected based on our initial hypothesis and the proposed role of NHEs. When *Nhe6* expression is lost in neurons, the proton exchange function is also lost, resulting in a failure of ion flux and an overacidification of endosomal compartments. Using a method identical to that used to measure endosomal pH in *Nhe6*-null neurons, my results reveal an opposing result in *Nhe9*-null neurons. Within the *Nhe9*-null cells, I observed an overall increase in endosomal pH when compared to the wild-type condition. In addition to being in the opposite direction, the effect of genotype in *Nhe9* neurons was smaller in magnitude than that of *Nhe6* neurons. Within *Nhe6* neurons, wild-type and null pH values differ by approximately 0.50 units, whereas within *Nhe9* neurons, wild-type and null pH values differ by approximately 0.25 units.

The transferrin method is a global measure of endosomal pH, in that it does not isolate specific types of endosomal compartments. To answer fully the question of the exact mechanism of endosomal pH changes in *Nhe*-null neurons, a method to target early versus late endosomes should be utilized. However, the observed increase in endosomal pH of *Nhe9*-null neurons described here could be due to one of several factors – an overalkalinization of individual endosomes, an increase in the number of more alkaline endosomes, or an over-representation of transferrin cargo in more alkaline endosomes.

We chose to assess the possibility of an over-representation of transferrin in early endosomes, potentially due to a trafficking defect. Utilizing techniques identical to those used to quantify endosomal pH, I incubated wild-type and *Nhe9*-null neurons with pH-stable transferrin and assessed its distribution throughout the endosomal system using Rab protein markers. The overall trend in the *Nhe9*-null neurons is one of increased Rab5-transferrin colocalization and decreased Rab7-transferrin colocalization, as compared to wild-type.

The decrease in transferrin colocalization with Rab7 in *Nhe9*-null neurons is consistent with the increase in endosomal pH in these same cells. With less transferrin being found in these lower-pH compartments, the contribution of the acidity of late endosomes to the overall endosomal pH will be diminished. This fact could make it appear as though endosomes in *Nhe9*-null neurons are overalkalinized, instead of overacidified.

The back-up of cargo within *Nhe9*-null early endosomes could potentially be explained by differential localization patterns. Although *Nhe9* is found on a large proportion of early endosomes, we cannot say what quantity of the protein is present. If *Nhe9* is low in quantity on early endosomes (i.e. a single molecule per endosome), its loss may not have a profound effect on function. If *Nhe9* is high in quantity on late endosomes (i.e. many molecules per endosome), however, loss in this location would have an effect of greater magnitude. Perturbation of late endosomal function could impair the overall maturation process, leading to an over-representation of cargo within early endosomes.

As established, when *Nhe6* expression is lost, endosomal overacidification occurs, presumably due to the extinction of outward flow of hydrogen ions. An additional possibility, however, could be the acceleration of cargo through the endosomal pathway to the more acidic late endosomes. If acceleration is occurring in conjunction with a loss of outward hydrogen ion flux, the combination of two factors could explain why the magnitude of the pH change is greater in the *Nhe6*-null neurons than in the *Nhe9*-null neurons.

The trend in *Nhe6*-null neurons suggests an over-representation of transferrin cargo in more acidic late endosomes. Although the differences between genotypes in each endosomal compartment do not reach statistical significance, the qualitative difference in transferrin distribution is evident. Less transferrin is present in *Nhe6*-null early endosomes than in *Nhe6*-null late endosomes.

When normalized to wild-type neurons and compared directly against one another, *Nhe6*- and *Nhe9*-null neurons show distinct trends in transferrin loading in endosomes. Transferrin trafficking within *Nhe6*-null neurons is biased towards late endosomes with a more acidic pH; transferrin trafficking within *Nhe9*-null neurons is biased towards early endosomes with a less acidic pH. This difference is consistent with the opposing effects these two mutations have on overall endosomal pH.

Chapter 4 thus details the alterations in neuronal biology and function arising from NHE mutations. *Nhe6* mutation profoundly decreases neuronal arborization; *Nhe9* mutation has little effect on branching. *Nhe6* mutation overacidifies endosomal compartments; *Nhe9* mutation results in an increase in more alkaline endosomes. *Nhe6* mutation biases transferrin trafficking to later endosomal compartments; *Nhe9* mutation biases transferrin trafficking to earlier endosomal compartments. Therefore, when considered in total, the results presented in Chapter 4 suggest distinct roles for endosomal Nhe6 and Nhe9 in neuronal development.

Materials and Methods

Reagents

Cells were dissociated using the Papain Dissociation System (LK003150) from Worthington Biochemical Corporation. FITC-conjugated transferrin (T2871) and AlexaFluor® 546-conjugated transferrin (T23364) were purchased from

Molecular Probes. All cell culture reagents were purchased from Gibco: Neurobasal®-A medium (10888-022), B-27® Supplement (17504-001), Penicillin-Streptomycin (15140-122), and GlutaMAX™ Supplement (35050-061).

Primary Neuronal Cultures

Hippocampi were dissected from P0 to P1 mice (euthanized by swift decapitation), and cells were dissociated with papain in Earle's balanced salt solution for 30 minutes at 37°C followed by trituration with a 1-mL pipette. Neurons were plated on either 8-well glass chamber slides (Lab-Tek®, 177402) coated with 1 mg/mL poly-D-lysine hydrobromide (Sigma, P6407) at a density of approximately 50,000 cells/well or on #1.5 coverslips pre-coated with poly-D-lysine (neuVibro, GG-12-1.5-pdl) at a density of approximately 30,000 cells/coverslip. Neurons were grown in a 37°C/5% CO₂ incubator in medium consisting of Neurobasal®-A supplemented with 2% B-27®, 1% GlutaMAX™, and 1% penicillin-streptomycin (complete growth medium).

GFP Transfection of Neurons

Primary neuronal cultures were derived from P1 wild-type and *Nhe9*-null mouse hippocampi and plated at approximately 60,000 cells/well on 8-well chamber slides. At 3-4 DIV, cells were transfected with a plasmid encoding eGFP using Lipofectamine® 2000 (Invitrogen). DNA and Lipofectamine® reagent were

diluted in Opti-MEM at a 1:2.5 ratio and allowed to incubate at room temperature for 20 minutes. The mixture was then applied to cells and incubated at 37°C for 4-6 hours, after which transfection medium was replaced with complete neuronal growth medium. At 5 DIV, cells were fixed with 4% PFA and stained for Ankyrin-G to visualize the axon initial segment and identify axonal projections.

Confocal Imaging and Neurolucida Tracing

Z-series images of one GFP-positive cell per field were collected using a C-Apochromat 40X water/1.2 N.A. objective on a Zeiss LSM710 confocal microscope. Neuronal morphology was traced manually using the Neurolucida neuron tracing software (MBF Bioscience), with axons and dendrites being assessed separately. For the wild-type condition, 56 cells across 4 animals were analyzed; for the *Nhe9*-null condition, 80 cells across 7 animals were analyzed. The experimenter was blind to genotype throughout the analysis.

Measurement of Endosomal pH Using Fluorescent-Conjugated Transferrin

Wild-type, heterozygous, and *Nhe9*-null hippocampal neurons grown to 5 DIV were starved in B27-free Neurobasal®-A medium (NBM-A) for 30 minutes at 37°C. Cells were then incubated in complete growth medium containing 66 µg/mL FITC-conjugated transferrin (pH-sensitive) and 33 µg/mL AlexaFluor® 546-conjugated transferrin (pH-stable) for 10 minutes at 37°C. After the 10-minute transferrin incubation, cells were washed 4 times with 1X PBS, and 4 to 5 images

were collected with a Plan-Apochromat 63X oil/1.4 N.A. objective on a Zeiss LSM710 confocal microscope over the span of 7-8 minutes.

In addition, a pH calibration curve was constructed by incubating wild-type cells in a series of solutions containing the following: 125 mM potassium chloride (KCl), 25 mM sodium chloride (NaCl), 10 μ M monensin, and either 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5 and 7.0) or 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.5, 6.0, 5.5, 5.0, and 4.5). Final pH was adjusted to desired level with 1N sodium hydroxide (NaOH). Four images of cells at each pH level were collected as above.

Fluorescence intensity of labeled endosomes was quantified with the ImageJ software package (National Institutes of Health) by tracing individual compartments in soma versus processes. The experimenter was blind to genotype throughout the analysis. Endosomal pH was calculated from the ratio of fluorescence intensity (FITC/AlexaFluor® 546), according to the linear equation of the standard curve.

Transferrin Incubation and Rab Immunocytochemistry

Primary hippocampal neurons derived from *Nhe6* or *Nhe9* wild-type and null mice were grown to 5 DIV. Cells were starved in B27-free NBM-A for 30 minutes in a 37°C/5% CO₂ incubator. Upon completion of the starvation period, existing medium was removed and replaced with complete growth medium plus 33 μ g/mL AlexaFluor® 546-conjugated transferrin. After 10 minutes of transferrin

application, cells were gently washed twice with warm (37°C) 1X PBS and immediately fixed with cold (4°C) 4% PFA for 10 minutes. Throughout the staining protocol, cells were protected from light to avoid bleaching of fluorescent transferrin signal. Cells were then washed 3 more times with 1X PBS and permeabilized for 15 minutes at room temperature with 0.25% Triton-X. Following a brief rinse with 1X PBS, cells were blocked overnight at 4°C in 10% goat serum/0.1% Triton-X in PBS.

The following day, cells were incubated in one of three Rab primary antibodies diluted in 2% goat serum/0.1% Triton-X for 90 minutes at room temperature. Antibodies and dilutions were as follows: mouse anti-Rab5, 1:250 (Abcam, ab50523); mouse anti-Rab7, 1:500 (Sigma, R8779); or mouse anti-Rab11, 1:250 (BD Transduction Laboratories, 610656). Cells were then washed 3 times with blocking buffer and incubated in secondary antibody (AlexaFluor® goat anti-mouse IgG 488, ThermoFisher Scientific, A-11029) for 45 minutes at room temperature. DAPI was added to secondary antibody at a 1:1000 dilution to visualize nuclei. Finally, cells were washed 3 times with 1X PBS, and coverslips were mounted on slides with Fluoromount-G® (Southern Biotech, 0100-01).

Image Acquisition and Colocalization Analysis

Z-series images were collected using a Plan-Apochromat 63X oil/1.4 N.A. objective on a Zeiss LSM800 upright confocal microscope. Six to 7 images per condition were collected for each experimental replicate, with 4 replicates in total.

Colocalization analysis was performed using the MetaMorph® software package (Molecular Devices). Each image was split into red and green channels prior to background smoothing through the application of a 3 pixel x 3 pixel median filter. Filtered image thresholds were then determined automatically, with visual verification by the experimenter. Using the red channel (AlexaFluor® 546-conjugated transferrin) as the reference, individual endosomes were circled and red/green overlap was calculated for each region of interest. Endosomal staining was considered “colocalized” if the fraction of overlapping red and green signal was greater than or equal to 25% of the total red signal. The experimenter was blind to genotype throughout the analysis.

Statistical Analysis

For analysis of neuronal arborization (data presented in Figures 4.2 and 4.3), values were averaged across cells and compared using a Student’s t-test. For analysis of endosomal pH (data presented in Figure 4.4) and endosomal size (data presented in Figure 4.5), values were averaged across individual endosomes and compared using a generalized linear mixed effects model. For analysis of transferrin distribution throughout endosomes (data presented in Figure 4.8), values were averaged across individual endosomes and compared using a mixed effects logistic regression model. In all cases, a p-value of < 0.05 was considered to be significant. All data were expressed as mean ± standard error.

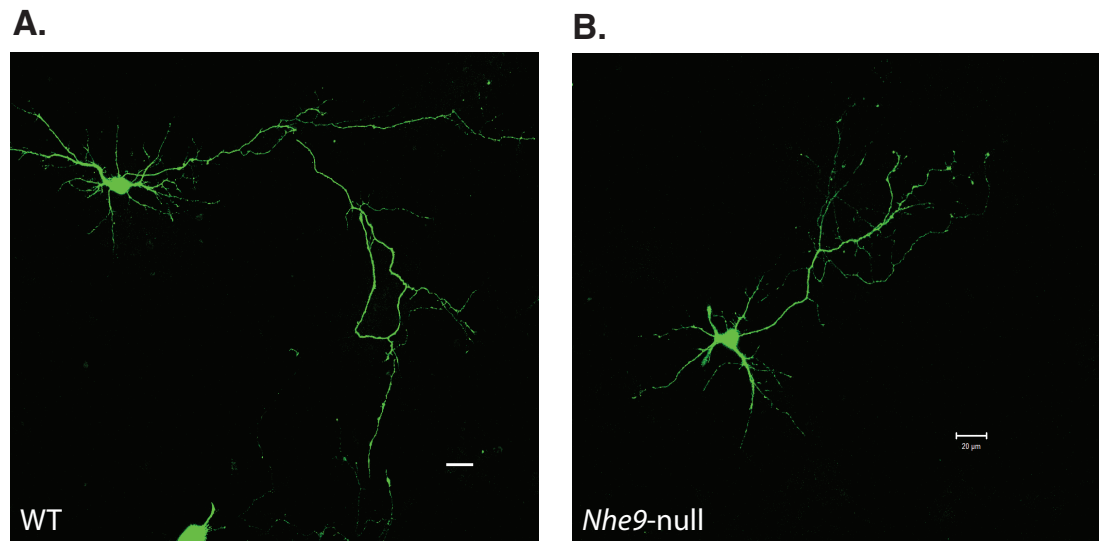


Figure 4.1. Arborization of wild-type and *Nhe9*-null neurons. Primary hippocampal neurons were transfected with a plasmid encoding eGFP (green) at 3-4 DIV. At 5 DIV, cells were fixed and arbors were traced and analyzed using the Neurolucida software package. (A) Representative image of an eGFP-transfected wild-type (WT) neuron. (B) Representative image of an eGFP-transfected *Nhe9*-null neuron. Scale bar represents 20 µm.

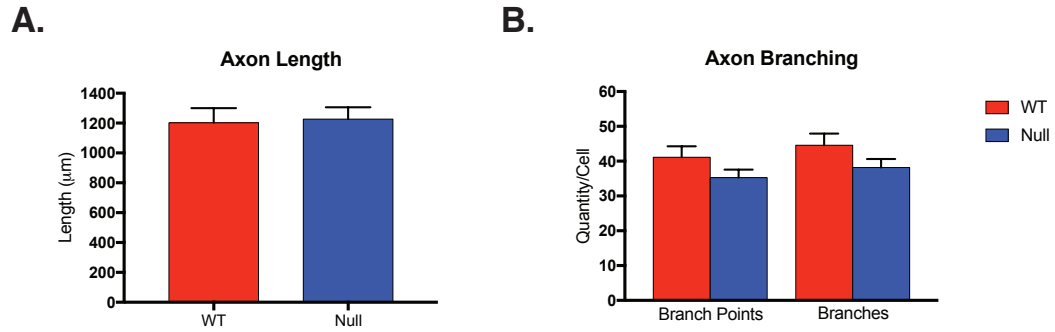


Figure 4.2. Quantification of axonal arborization in wild-type (WT) and *Nhe9*-null neurons. (A) Average axonal length (in μm) per neuron in WT versus *Nhe9*-null cells. Length does not vary by genotype. (B) Average number of axonal branch points and branches per neuron in WT versus *Nhe9*-null cells. Branching measures do not vary by genotype. Data represent mean $\pm$ standard error. $n = 56$ cells/4 mice (WT), 80 cells/7 mice (*Nhe9*-null).

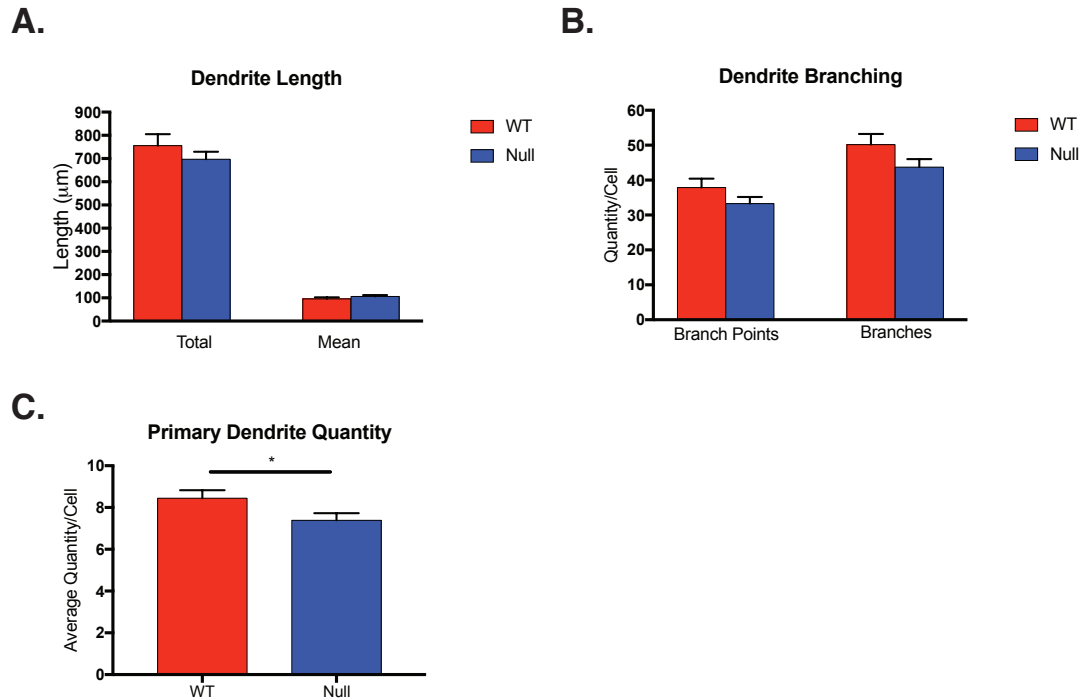


Figure 4.3. Quantification of dendritic arborization in wild-type (WT) and *Nhe9*-null neurons. (A) Average total and mean dendritic length (in μm) per neuron in wild-type versus *Nhe9*-null cells. Length does not vary by genotype. (B) Average number of dendritic branch points and branches per neuron in WT versus *Nhe9*-null cells. Branching measures do not vary by genotype. (C) Average number of primary dendrites per cell in WT versus *Nhe9*-null cells. WT neurons exhibit significantly more primary dendrites than *Nhe9*-null neurons. Data represent mean $\pm$ standard error. *, $p = 0.042$. $n = 56$ cells/4 mice (WT), 80 cells/7 mice (*Nhe9*-null).

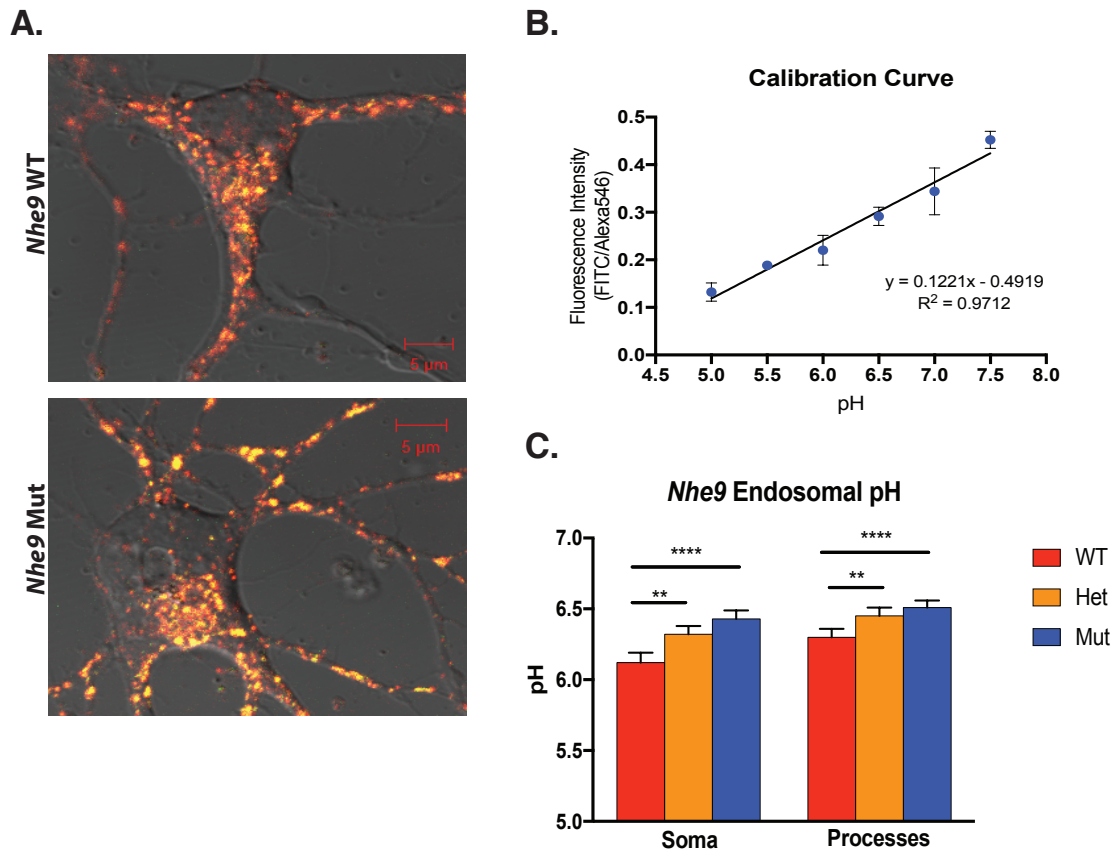


Figure 4.4. Loss of Nhe9 in neurons results in an increase in more alkaline endosomes. (A) Representative images of live hippocampal neurons at 5 DIV. Cells were incubated with both FITC-conjugated transferrin (green) and AlexaFluor® 546-conjugated transferrin (red) for 10 minutes at 37°C. Top, wild-type (WT) neuron. Bottom, *Nhe9*-null neuron. Scale bar represents 5 μ m. (B) Representative calibration curve used for pH quantification. Wild-type neurons were incubated with both FITC-conjugated transferrin (green) and AlexaFluor® 546-conjugated transferrin (red) for 10 minutes at 37°C, prior to buffering in a series of solutions of decreasing pH. (C) Endosomal pH in *Nhe9*-null and heterozygous (Het) neurons is greater than in wild-type (WT), in both soma and processes. $n = 2,029$ endosomes/4 mice (WT), 2,264 endosomes/5 mice (Het), and 3,353 endosomes/9 mice (*Nhe9*-null). **, $p < 0.01$. ****, $p < 0.0001$.

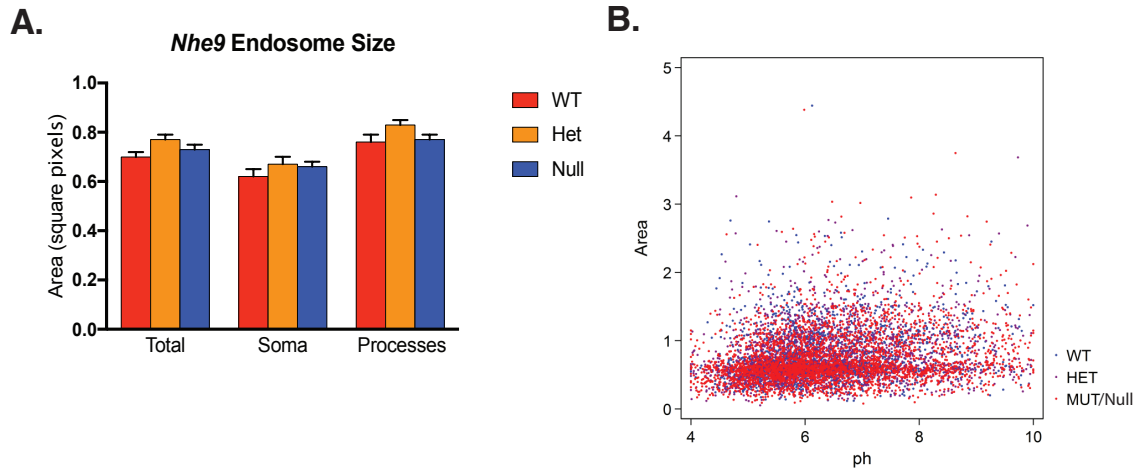


Figure 4.5. Endosomal size does not vary with genotype or pH level. (A) Area in square pixels was calculated for individual endosomes within soma, within processes, and in total. There is no significant difference in endosomal area among wild-type (WT), heterozygous (Het), and *Nhe9*-null neurons. (B) A weak correlation emerges between endosomal area and pH level (correlation coefficient = 0.2), but this association is not significant ($p = 0.379$). $n = 2,029$ endosomes/4 mice (WT), 2,264 endosomes/5 mice (Het), and 3,353 endosomes/9 mice (*Nhe9*-null).

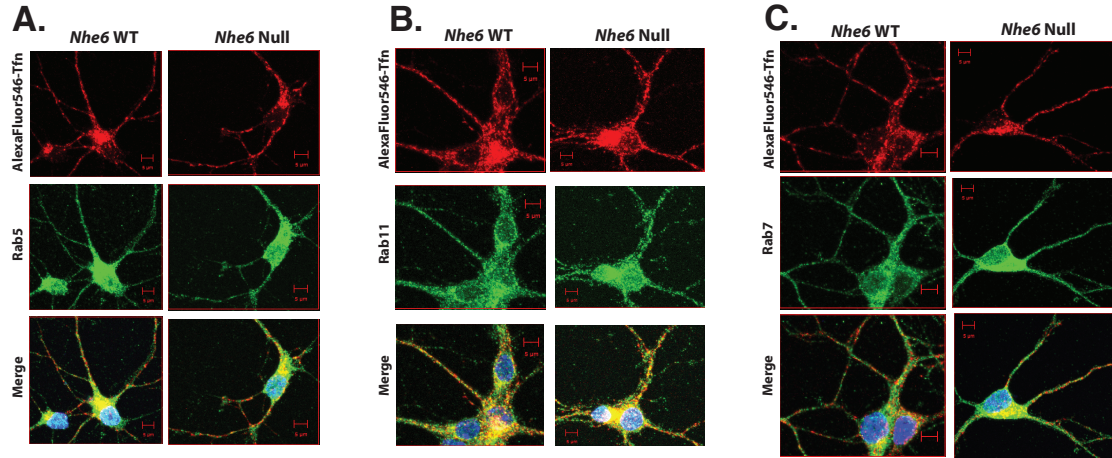


Figure 4.6. Transferrin incubation with Rab marker staining in *Nhe6* neurons. Neurons were grown to 5 DIV, incubated with pH-stable transferrin reagent for 10 minutes at 37°C, fixed with 4% PFA, and stained with antibodies for markers of different endosomal compartments. (A) Representative images of wild-type (WT) (left) and *Nhe6*-null (right) neurons incubated with AlexaFluor® 546-conjugated transferrin (red) and stained for Rab5 (green), a marker of early endosomes. (B) Representative images of WT (left) and *Nhe6*-null (right) neurons incubated with AlexaFluor® 546-conjugated transferrin (red) and stained for Rab11 (green), a marker of recycling endosomes. (C) Representative images of WT (left) and *Nhe6*-null (right) neurons incubated with AlexaFluor® 546-conjugated transferrin (red) and stained for Rab7 (green), a marker of late endosomes. Blue represents DAPI, a marker of nuclei. Per condition, $n = 1,482$ -1,860 endosomes across 34-57 cells. Scale bar represents 5 μ m.

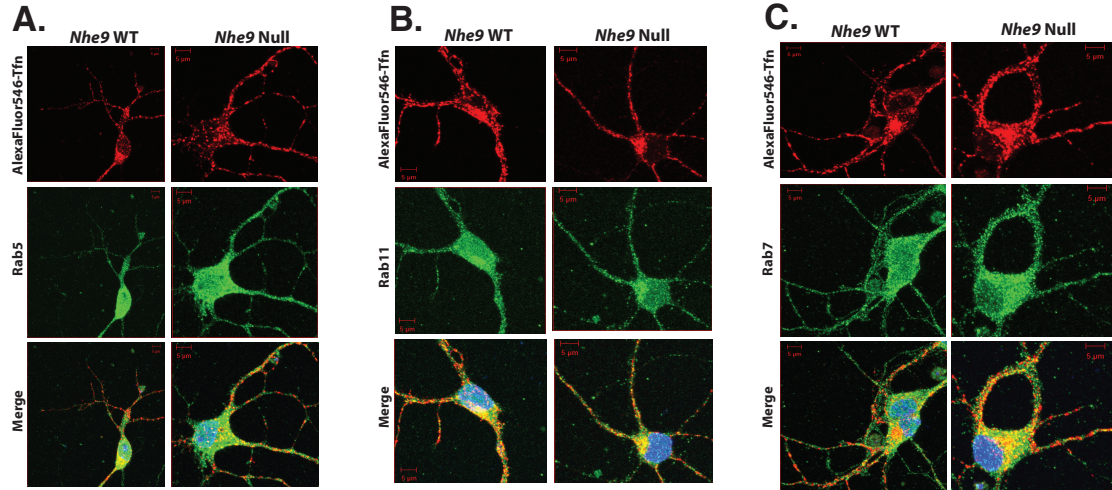


Figure 4.7. Transferrin incubation with Rab marker staining in *Nhe9* neurons. Neurons were grown to 5 DIV, incubated with pH-stable transferrin reagent for 10 minutes at 37°C, fixed with 4% PFA, and stained with antibodies for markers of different endosomal compartments. (A) Representative images of WT (left) and *Nhe9*-null (right) neurons incubated with AlexaFluor® 546-conjugated transferrin (red) and stained for Rab5 (green), a marker of early endosomes. (B) Representative images of WT (left) and *Nhe9*-null (right) neurons incubated with AlexaFluor® 546-conjugated transferrin (red) and stained for Rab11 (green), a marker of recycling endosomes. (C) Representative images of WT (left) and *Nhe9*-null (right) neurons incubated with AlexaFluor® 546-conjugated transferrin (red) and stained for Rab7 (green), a marker of late endosomes. Blue represents DAPI, a marker of nuclei. Per condition, $n = 1,686$ - $2,177$ endosomes across 43-52 cells. Scale bar represents 5 μ m.

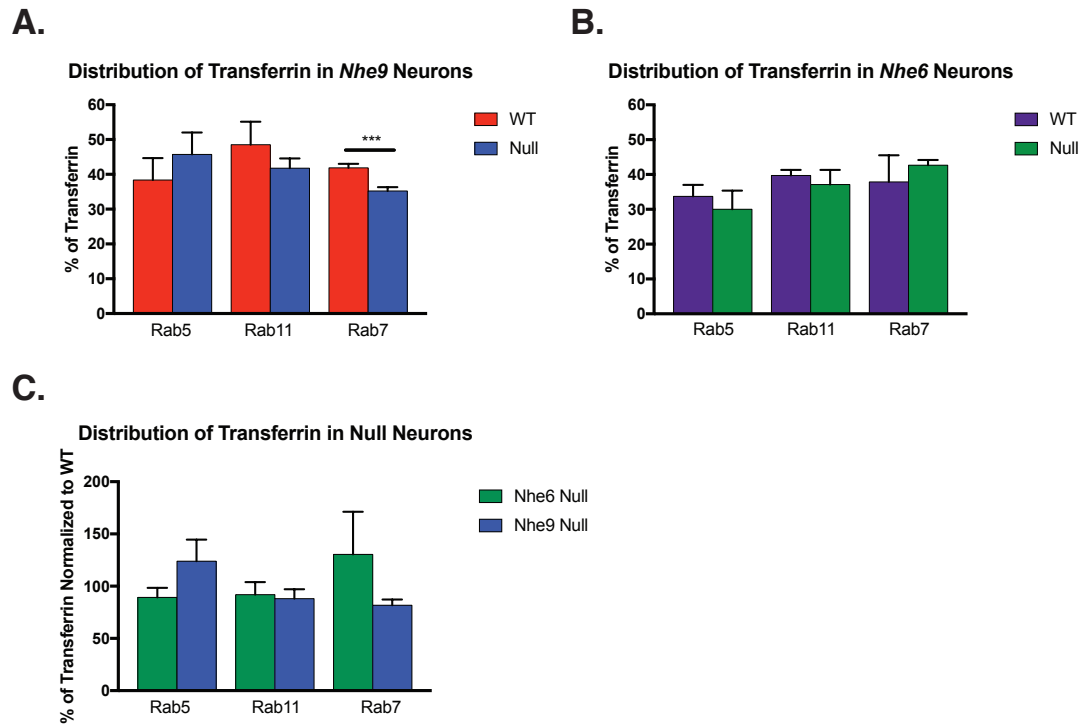


Figure 4.8. Quantification of transferrin localization within early, recycling, and late endosomal compartments, in both *Nhe9* and *Nhe6* neurons. (A) Endosomal distribution of transferrin in wild-type (WT) versus *Nhe9*-null neurons. (B) Endosomal distribution of transferrin in WT versus *Nhe6*-null neurons. (C) Endosomal distribution of transferrin in *Nhe6*-null versus *Nhe9*-null neurons. Each percentage was normalized to the respective wild-type prior to direct comparison.

CHAPTER 5:

DISCUSSION

Summary and Perspectives

In this thesis, I present data demonstrating a distinct role for NHE9 from NHE6 in neuronal development. Although both proteins are localized throughout the endosomal system of neurons, their specific localization patterns overlap by only approximately 30%. Their effects on endosomal pH and neuronal arborization also differ. Nhe6 loss overacidifies neuronal endosomes and leads to impoverished neuronal branching (Ouyang et al., 2013), whereas Nhe9 loss results in an increase in more alkaline endosomes and does not significantly decrease arborization. We propose that these distinct phenotypic effects may be due to opposite effects of Nhe6 and Nhe9 on endosomal trafficking, whereby Nhe9 loss results in a backup of cargo within less acidic early endosomes, and Nhe6 loss may represent an acceleration of cargo towards more acidic late endosomes.

In Chapter 2, I describe and validate two reagents unique to our studies. I first detail a rabbit anti-NHE9 primary antibody. This antibody, shown to be specific to the NHE9 protein, is heavily utilized in many of the experiments detailed herein. I also generate and characterize an *Nhe9*-null mouse, demonstrating complete loss of Nhe9 expression at both the transcript and the protein levels. Therefore, this mouse is suitable as a model central to my functional studies of endosomal pH and cargo trafficking.

In Chapter 3, I demonstrate the localization patterns of Nhe6 and Nhe9 throughout the endosomal system of neurons. Contrary to results garnered from studies of non-neuronal mammalian cell lines, isolating Nhe6 and Nhe9 to

restricted endosomal compartments (Brett et al., 2002; Nakamura et al., 2005), my data reveal that both Nhe6 and Nhe9 are present in all endosomal compartments – early, recycling, and late. Localization of both proteins is highest in the Rab5-positive early endosomes, lowest in the Rab7-positive late endosomes, and roughly intermediate in the Rab11-positive recycling endosomes. When considering each endosomal compartment separately, however, distinct patterns emerge. More early endosomes contain Nhe6 than contain Nhe9, whereas more recycling and late endosomes contain Nhe9 than contain Nhe6.

In addition, I demonstrate the pattern of colocalization of Nhe6 and Nhe9 within the processes of hippocampal neurons. Although there is some overlap between the two signals, the majority of the expression exhibits distinct localization. I also describe differential expression levels of Nhe6 and Nhe9 within neonatal mouse hippocampus, with Nhe6 protein being significantly more abundant than Nhe9 protein.

In Chapter 4, I determine the effect of Nhe9 loss on neuronal arborization and endosomal pH, as well as the roles of Nhe6 and Nhe9 in the process of endosomal trafficking. In *Nhe9*-null neurons, there is a significant increase in more alkaline endosomes as compared to wild-type neurons, in contrast to the overacidification of endosomes in *Nhe6*-null neurons (Ouyang et al., 2013). This Nhe9 pH result, although unexpected based on our knowledge of endosomal NHEs, is not entirely unprecedented. Loss of Nhe5 in PC12 cells via shRNA

expression has been shown to alkalinize the pH of recycling endosomes (Diering et al., 2013).

The *Nhe6* and *Nhe9* pH phenotypes are coupled to opposing trends in the trafficking of transferrin cargo. Following a 10-minute incubation, I find significantly less transferrin in more acidic late endosomes within *Nhe9*-null neurons, as well as a trend towards less transferrin in more alkaline early endosomes within *Nhe6*-null neurons.

An important question to consider is why the phenotypic changes seen in *Nhe6*-null versus *Nhe9*-null neurons are not only distinct but also different in magnitude. For example, the loss of *Nhe6* in neurons results in an overacidification of endosomes, with pH in *Nhe6*-null cells decreasing by approximately 0.5 pH units. In contrast, the loss of *Nhe9* results in an increase in more alkaline endosomes, although the pH in *Nhe9*-null cells only increases by approximately 0.25 pH units. In part, this distinction may be due to differing levels of *Nhe6* and *Nhe9* protein within the experimental system, that of neonatal mouse hippocampus. Data from multiple sources indicate that *Nhe6* expression is enriched in hippocampus relative to *Nhe9*, so it stands to reason that loss of *Nhe6* would have a greater phenotypic impact.

The effects of *Nhe6* and *Nhe9* loss on neuronal arborization also differ greatly. Whereas loss of *Nhe6* decreases dendritic and axonal branching by approximately 50% in cultured hippocampal neurons, *Nhe9* loss has a very modest effect, decreasing the number of primary dendrites by only 12% as compared to

wild-type. This difference may be due to distinct localization patterns. Only approximately 30% of Nhe6 and Nhe9 expression overlap within neurons, which means that their localizations are, in large part, distinct. The endosomal system helps to drive neuronal morphogenesis and arborization, and if localizations to specific endosomes are different, the effects of Nhe6 and Nhe9 loss may also be different.

Experimental Model

Taken together, the data presented herein suggest the following model (Figure 5.1). When Nhe9 expression within neurons is absent, there appears to be an over-representation of cargo in the less acidic early endosomes, with a significant decrease in the representation of cargo in more acidic late endosomes. This effect may be due to a “bottleneck,” whereby cargo is backing up within early endosomes, unable to be appropriately trafficked further through the endosomal system. If the cargo in question is transferrin, as in my live pH experiments, the resulting pH assessment would include an increase in less acidic early endosomes and a decrease in more acidic late endosomes, making the overall measured endosomal pH more alkaline.

The opposite effect is proposed to occur when Nhe6 expression within the neuron is absent. In this situation, there appears to be a decrease in the representation of cargo within less acidic early endosomes and an apparent over-representation of cargo in more acidic late endosomes, suggestive of an

acceleration in trafficking. It is thus possible that, in addition to the loss of Nhe6 proton exchanger function overacidifying endosomes, there is also an increase in the representation of more acidic late endosomes and a decrease in the representation of less acidic early endosomes within the pH measurement.

There is scientific precedent for the role of endosomal NHEs in protein trafficking. Bowers *et al.* (2000) studied the $\text{Na}^+\text{-H}^+$ exchanger Nhx1 in yeast and demonstrated that its expression is required for transport of proteins out of the prevacuolar compartment. *Nhx1*-mutant cells displayed aberrant accumulation of carboxypeptidase Y-related proteins in a prevacuolar structure, indicative of an impairment in trafficking from the prevacuole to the vacuole.

Future Directions

My work has focused on the study of endosomal NHEs within hippocampus, but protein expression within the brain is not limited to this area. An additional line of investigation to pursue could be the analysis of NHE localization and function throughout alternative brain regions, such as the cerebral cortex. Both Nhe6 and Nhe9 are expressed within cortex, but like hippocampus, Nhe6 expression as assessed by *in situ* hybridization is greater. Their spatial distributions also vary; Nhe6 is localized throughout the cortical structure, whereas Nhe9 is restricted to more superficial layers of the cortex (Kondapalli et al., 2014). As in hippocampus, differential levels and localization patterns may result in contrasting phenotypes in the absence of either Nhe6 or Nhe9 expression.

In addition, an important question to ask is the role of endosomal NHEs in glia. Astrocytes may play a critical role in autism and other developmental brain disorders (Molofsky et al., 2012), and it is known that both *Nhe6* and *Nhe9* are expressed within astrocytes (Kondapalli et al., 2013). Additionally, it has been reported that *NHE6* mutations in human patients result in glial dysfunction, characterized by widespread gliosis within the brain, as well as tau-positive inclusions within glial cells (Garbern et al., 2010).

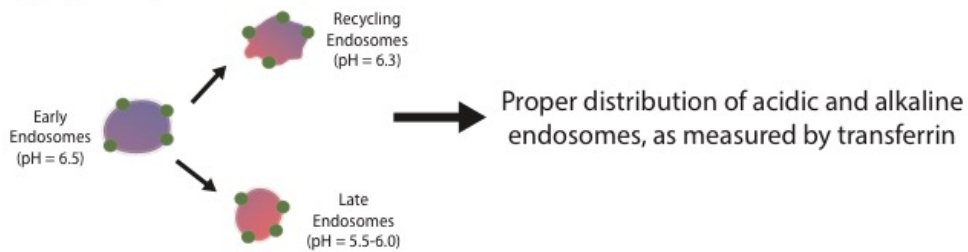
Finally, it would be informative to characterize the behavior of the *Nhe9*- and *Nhe6*-null mice. In humans, *NHE9* mutations can lead to ADHD (de Silva et al., 2003), but we have not yet examined the possibility of a hyperactive phenotype in the *Nhe9*-null mouse. Both *NHE9* and *NHE6* mutations also result in intellectual disability, and if null mice show deficits in learning and cognitive function as measured by behavioral assays, this could represent an important new model to recapitulate different aspects of the *Nhe6* and *Nhe9* phenotypes.

Conclusion

Endosomal trafficking has a role in neuronal circuit development, through the delivery of membrane materials to the cell surface, as well as the signaling of growth factors (i.e. BDNF) from within endosomes (Ouyang et al., 2013). When this process goes awry, developmental brain disorders, including autism and related conditions, can result. The ablation of *NHE6* versus *NHE9* expression affects circuit formation in different ways, as evidenced by results garnered from

studies of subcellular localization patterns, neuronal arborization, and endosomal acidification. Thus, we conclude that NHE9 has a distinct role from NHE6 in neuronal development.

A. Wild-Type (Mean pH = 6.1-6.2)



B. *Nhe9*-Null (Mean pH = 6.4-6.5)



C. *Nhe6*-Null (Mean pH = 5.8)

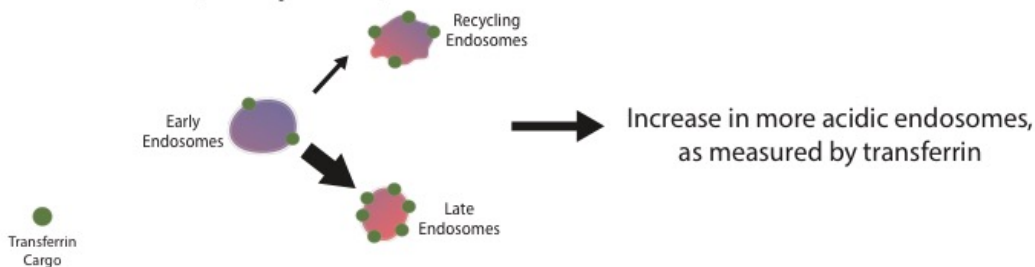


Figure 5.1. Proposed model of endosomal trafficking of cargo in both *Nhe9*- and *Nhe6*-null neurons, as compared to wild-type. (A) Trafficking within wild-type endosomes proceeds appropriately, with a continuous distribution of cargo throughout the endosomal system. (B) Trafficking within *Nhe9*-null endosomes is backed up within the early endosomal compartment, resulting in an increase in more alkaline endosomes, as measured by transferrin. (C) Trafficking within *Nhe6*-null endosomes is accelerated to the late endosomal compartment, resulting in an increase in more acidic endosomes, as measured by transferrin.

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