NHE6-Depletion Corrects ApoE4-Mediated Synaptic Impairments and Reduces Amyloid Plaque Load

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Impact Statement: Genetic disruption of sodium-hydrogen exchanger 6 (NHE6/*Slc9a6*) reduces amyloid plaques in humanized Alzheimer's disease mouse models and restores normal synaptic responses to neuromodulatory input in humanized ApoE4 expressing animals.

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ABSTRACT

Apolipoprotein E4 (ApoE4) is the most important and prevalent risk factor for late-onset Alzheimer's disease (AD). The isoelectric point of ApoE4 matches the pH of the early endosome (EE), causing its delayed dissociation from ApoE receptors and hence impaired endolysosomal trafficking, disruption of synaptic homeostasis and reduced amyloid clearance. We have shown that enhancing endosomal acidification by inhibiting the EE-specific sodium-hydrogen exchanger 6 (NHE6) restores vesicular trafficking and normalizes synaptic homeostasis. Remarkably and unexpectedly, loss of NHE6 (encoded by the gene *Slc9a6*) in mice effectively suppressed amyloid deposition even in the absence of ApoE4, suggesting that accelerated acidification of early endosomes caused by the absence of NHE6 occludes the effect of ApoE on amyloid plaque formation. NHE6 suppression or inhibition may thus be a universal, ApoE-independent approach to prevent amyloid buildup in the brain. These findings suggest a novel therapeutic approach for the prevention of AD by which partial NHE6 inhibition reverses the ApoE4 induced endolysosomal trafficking defect and reduces plaque load.

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INTRODUCTION

ApoE is the principal lipid transport protein in the brain. Three different ApoE isoforms are common in humans: ApoE2 (ε2), ApoE3 (ε3), and ApoE4 (ε4). Each ApoE4 allele reduces the age of Alzheimer's disease (AD) onset by approximately three to five years compared to ApoE3 homozygotes, which comprise ~80% of the human population (Roses, 1994; Sando et al., 2008). By contrast and by comparison, ApoE2 is protective against AD (Corder et al., 1994; Panza et al., 2000; West et al., 1994). ApoE is an arginine-rich protein and a major component of very-low density lipoproteins (Shore and Shore, 1973). The number of positively charged arginine residues differs between the three human isoforms due to two single nucleotide polymorphisms in the ApoE gene. The most common isoform, ApoE3, has a charge neutral cysteine at amino acid position 112 and an arginine at position 158. The second most common isoform, ApoE4, has two arginines, while the less frequent ApoE2 has two cysteines at these respective positions. The positively charged arginines raise the net charge and thus the isoelectric point (IEP) of the protein. (Eto et al., 1985; Warnick et al., 1979). The IEP of ApoE2 is the lowest (5.9), ApoE3 has an intermediate IEP of 6.1 and the IEP of ApoE4 is ~6.4 (Ordovas et al., 1987).

For cargo delivery, ApoE binds to lipoprotein receptors and undergoes endocytosis and recycling. Endocytic subcompartments become progressively more acidic, and the pH of these compartments is regulated by the opposing functions of vesicular ATP-dependent proton pumps (vATPase) and proton leakage channels (Na^+/H^+ exchangers, NHEs). Early endocytic vesicles are slightly acidic (pH, ~6.4), which facilitates ligand/receptor dissociation. Lysosomes are highly acidic (pH 4 to 5), which is required for the digestion of endocytosed biomolecules (**Figure 1A**) (Casey et al., 2010; Naslavsky and Caplan, 2018). For maturation of the early endosomes (EE) and entry into the next sorting stage, ligand/receptor dissociation is required. The pH-dependent release of ApoE from its receptor in the EE is important for endosomal maturation and cargo delivery (Yamamoto et al., 2008) for the ability of endosomal content to rapidly recycle to the cell surface (Heeren et al., 1999; Nixon, 2017). The early endosomal pH, which triggers ligand-receptor dissociation, closely matches the IEP of ApoE4 (Xian et al., 2018). Loss of net surface charge at the IEP is accompanied by reduced solubility in an aqueous environment, leading to impaired dissociation of ApoE4 from its receptors (Xian et al., 2018) and aided by a greater propensity of ApoE4 to form a molten globule configuration under acidic conditions (Morrow et

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71 al., 2002). Dysregulation of endolysosomal trafficking by ApoE4 causes an age dependent
72 increase in EE number and size (Nuriel et al., 2017).

73   Based on these observations, we have proposed a model in which destabilization of
74 ApoE4 in the acidic EE environment, combined with a greater propensity for self-association,
75 results in delayed detachment from its receptors (**Figure 1B**). Subsequent endosomal swelling
76 through K^+, Na^+ and H_2O influx further impairs cargo delivery, receptor recycling, and ligand re-
77 secretion. Importantly, in neurons, ApoE and its receptor Apoer2 travel together with glutamate
78 receptors through the endosomal recycling pathway (Chen et al., 2010; Xian et al., 2018). Rapid
79 endocytosis and subsequent recycling of synaptic receptors is triggered by the synaptic
80 homeostatic modulator and Apoer2 ligand Reelin (Hiesberger et al., 1999; Trommsdorff et al.,
81 1999). We previously showed that ApoE4, in contrast to ApoE3 and ApoE2, prevents Reelin-
82 mediated glutamate receptor insertion at the synapse, a state we refer to as ApoE4-mediated
83 Reelin resistance (Chen et al., 2010; Durakoglugil et al., 2009; Lane-Donovan and Herz, 2017;
84 Lane-Donovan et al., 2014; Xian et al., 2018). Reduction of endosomal pH and increasing the
85 differential to the ApoE4 IEP abolishes this effect *in vitro* (Xian et al., 2018).

86   The pH of EE compartments is controlled by the vATPase-dependent proton pump and
87 proton leakage through NHE6 (Nakamura et al., 2005; Basu et al., 1976; Davis et al., 1987;
88 Rudenko et al., 2002). NHE6 is encoded by the gene *Slc9a6*. We showed that EE acidification
89 by pharmaceutical pan-NHE inhibition or selective NHE6/*Slc9a6* knockdown in neurons
90 prevents the ApoE4-caused trafficking delay of ApoE and glutamate receptors (Xian et al.,
91 2018). NHE6-deficiency in humans causes neurodevelopmental defects, which result in
92 Christianson syndrome, an X-linked genetic disorder characterized by cognitive dysfunction,
93 autism, ataxia, and epilepsy. However, some *Slc9a6* mutant variants causing Christianson
94 syndrome in humans do not significantly alter the ion exchange properties of NHE6 (Ilie et al.,
95 2020; Ilie et al., 2019) suggesting that Christianson syndrome could be caused by loss of NHE6
96 scaffolding functions and not by loss of endosomal pH regulation. To investigate whether NHE6-
97 depletion can reverse ApoE4 pathology *in vivo*, we generated a conditional *Slc9a6* knockout
98 mouse line (*Slc9a6^fl;CAG-Cre^ERT2*) to avoid complications caused by neurodevelopmental
99 defects by temporally and spatially controlling NHE6-ablation. We show that genetic NHE6-
100 ablation attenuates both, the ApoE4 induced Reelin resistance and impaired synaptic plasticity
101 in ApoE4 targeted replacement (*Apoe^APOE4*) mice using hippocampal field recordings.

The pathological hallmarks of AD are extracellular aggregates of the amyloid β (Aβ) peptide and intracellular tangles of hyperphosphorylated tau protein. Processing of the transmembrane amyloid precursor protein (APP) at the β- and γ-sites leads to Aβ production. Aβ forms neurotoxic oligomers and accumulates in plaques. The β-site amyloid precursor protein cleaving enzyme 1 (BACE1) cleaves APP in its extracellular juxtamembrane domain to create a membrane-anchored C-terminal fragment (β-CTF) and a soluble extracellular APP domain (sAPPβ). βCTF is further cleaved by the γ-secretase complex, which leads to the release of the Aβ-peptide. APP and its secretases co-localize in endosomal compartments where cleavage can occur (Wang et al., 2018). It has further been reported that BACE1 activity is preferentially active in acidic environments (Shimizu et al., 2008). We therefore investigated whether NHE6-depletion alters BACE1 activity in neurons and whether NHE6-deficiency leads to changes in plaque deposition *in vivo*. We found that NHE6 inhibition or knockdown did not alter BACE1 activity, as judged by unchanged Aβ generation. By contrast, NHE6 ablation led to glial activation and decreased plaque load in $Apoe^{APOE4}$(Sullivan et al., 1997) and App^{NL-F} (Saito et al., 2014) double knockin mice.

RESULTS

NHE6 is Required for Postnatal Purkinje Cell Survival

NHE6 germline knockout mice ($Slc9a6^-$) and tamoxifen-inducible conditional NHE6 knockout mice ($Slc9a6^{fl}$;CAG-Cre^{ERT2}) were generated as described in Materials and Methods and **Figure 2A-C**. To validate early endosomal pH acidification by NHE6-deficiency we isolated mouse embryonic fibroblasts from the $Slc9a6^-$ line and infected them with a Vamp3-pHluorin2 lentivirus expressing a fusion protein consisting of the endosomal Vamp3 protein and the ratiometric pH indicator pHluorin2 (Stawicki et al., 2014). We found a significantly reduced number of vesicles with pH 6.4 and above in $Slc9a6^-$ fibroblasts when compared to control (**Figure 2D-F**).

To induce the conditional KO of $Slc9a6$, tamoxifen was administered to $Slc9a6^{fl}$;CAG-Cre^{ERT2} mice at two months (**Figure 3A**) and experiments were performed at the indicated time

points. *Slc9a6* knockout efficiency in the brains of tamoxifen injected *Slc9a6fl;CAG-CreERT2* mice was 65-82% and varied between brain regions (**Figure 3B**). To further investigate CreERT2 activity upon tamoxifen-injection, we bred the *CAG-CreERT2* line with a stop-tdTomato reporter line in which a floxed stop-codon precedes the tdTomato start-codon. After tamoxifen injection, brains were examined for tdTomato expression. Without tamoxifen injection, tdTomato expressing cells were almost absent in the hippocampus. Tamoxifen induced recombination led to a broad expression of tdTomato in the hippocampus **(Figure 2G**).

Individuals with Christianson Syndrome and mice lacking NHE6 present with motor deficits due to a dramatic progressive loss of cerebellar Purkinje cells (Ouyang et al., 2013). We have reproduced the Purkinje cell loss in our germline *Slc9a6-* mice (**Figure 3C**). Next, we investigated whether Purkinje cell loss is the consequence of neurodevelopmental or neurodegenerative effects caused by loss of NHE6. *Slc9a6*-deficiency was induced at 2 months, after Purkinje cells had developed and matured. One year after *Slc9a6*-ablation Purkinje cell loss was indistinguishable from that seen in the germline knockouts (**Figure 3D-F**). Therefore, Purkinje cell degeneration manifests itself postnatally and is not developmentally determined by the absence of NHE6. However, it is possible that loss of scaffolding functions and proper sorting, rather than dysregulation of endosomal pH, could be the main mechanism that causes Christianson syndrome, including Purkinje cell loss (Ilie et al., 2020; Ilie et al., 2019). If this could be substantiated by the development or discovery of *Slc9a6* mutants that selectively ablate its ion exchange capacity without affecting its subcellular sorting or interaction with cytoplasmic or luminal binding partners, this would further raise the potential of NHE6 as a novel drug target for neurodegenerative diseases.

Genetic Disruption of NHE6 Restores Trafficking of Apoer2, AMPA and NMDA Receptors in the Presence of ApoE4

As we reported previously, ApoE4 impairs the trafficking of synaptic surface receptors (Chen et al., 2010). To monitor receptor recycling in neurons we made use of an assay where Reelin is used to modulate receptor surface expression. Reelin is applied to primary neurons for 30 minutes in the presence or absence of naturally secreted, receptor binding-competent ApoE (**Figure 4**). Subsequently, surface biotinylation is performed and cells are harvested for

161 immunoblotting to quantify the amount of Apoer2 and glutamate receptors expressed on the
162 neuronal surface (Chen et al., 2010; Xian et al., 2018).

163   We have shown previously that in the presence of ApoE4, Apoer2 and glutamate
164 receptors recycle poorly to the neuronal surface. This recycling block could be resolved by
165 endosomal acidification induced by shRNA knockdown of *Slc9a6* or by applying the NHE
166 inhibitor EMD87580 (Xian et al., 2018). To further exclude a nonspecific effect caused by the
167 inhibition of other NHE family members or by shRNA off-target effects, we applied this assay on
168 neurons isolated from *Slc9a6⁻* embryos (**Figure 4B**). Apoer2 recycling was completely restored
169 in *Slc9a6⁻* neurons treated with ApoE4 (**Figure 4C**). We previously reported that the addition of
170 ApoE3 to neurons also affects Apoer2 trafficking to a small, but reproducible extent. This was
171 also abolished in *Slc9a6⁻* neurons (**Figure 4C**). In addition, genetic loss of NHE6 equally
172 restored the ApoE4-impaired surface expression of AMPA and NMDA receptor subunits (**Figure
173 4D-F**).

174

175 **Conditional Disruption of NHE6 Relieves Synaptic Reelin Resistance in *Apoe^APOE4* mice**

176   Reelin can enhance long term potentiation (LTP) in hippocampal field recordings of
177 *Apoe^APOE3* but not *Apoe^APOE4* acute brain slices (Chen et al., 2010). We previously showed that
178 this Reelin-resistance in *Apoe^APOE4* slices was attenuated by pharmacological NHE inhibition
179 (Xian et al., 2018). To investigate if endogenous loss of NHE6 also restores synaptic plasticity in
180 the presence of ApoE4 we performed hippocampal field recordings on *Slc9a6^fl;CAG-Cre^ERT2*
181 mice bred to *Apoe^APOE3* or *Apoe^APOE4* mice. To avoid potentially compounding effects of NHE6
182 deficiency during embryonic development (Ouyang et al., 2013), NHE6/*Slc9a6* gene disruption
183 was induced at 8 weeks by intraperitoneal tamoxifen injection (Lane-Donovan et al., 2015).
184 Electrophysiology was performed 3-4 weeks after NHE6-depletion in 3 months old mice
185 (*Slc9a6^fl;CAG-Cre^ERT2*, **Figure 5B, 5D, 5F, and 5H**). Tamoxifen-injected *Slc9a6^fl;CAG-Cre^ERT2*-
186 negative mice expressing human ApoE3 or ApoE4 served as controls (*Apoe^APOE3* and
187 *Apoe^APOE4*, **Figure 5A, 5C, 5E, and 5G**). For field recordings hippocampal slices were perfused
188 with Reelin as described (Beffert et al., 2005; Chen et al., 2010; Durakoglugil et al., 2009;
189 Weeber et al., 2002). Conditional genetic loss of NHE6 resulted in a moderate reduction of the
190 ability of Reelin to enhance LTP in *Apoe^APOE3* mice (comparing **Figure 5A and 5E to 5B and**

191 **5F**). By contrast, as reported previously (Chen et al., 2010), hippocampal slices from *Apoe*^*APOE4*^

192 mice were completely resistant to LTP enhancement by Reelin (**Figure 5C and 5G**). This

193 resistance was abolished when NHE6 was genetically disrupted in *Apoe*^*APOE4*^ mice: Reelin

194 application enhanced LTP (**Figure 5D and 5H**) in *Apoe*^*APOE4*^;*Slc9a6*^*fl*^;*CAG-Cre*^*ERT2*^ to a

195 comparable extent as in the *Apoe*^*APOE3*^;*Slc9a6*^*fl*^;*CAG-Cre*^*ERT2*^ mice (**Figure 5B**). Synaptic

196 transmission was monitored and input-output curves were generated by plotting the fiber volley

197 amplitude, measured at increasing stimulus intensities, against the fEPSP slope. No significant

198 differences were found (**Figure 5I and 5J**).

199

200 **NHE Inhibition or NHE6 Knockdown does not Alter β-CTF Generation *in vitro***

201 Cleavage of APP by γ-secretase and the β-site APP cleaving enzyme 1 (BACE1)

202 generates the short neurotoxic polypeptide Aβ. Cleavage by BACE1 results in a membrane

203 anchored fragment called βCTF, which is further processed by γ-secretase to yield the soluble

204 Aβ peptide. BACE1 processing of APP occurs in the Golgi complex, on the cell membrane, and

205 after endocytosis in endosomes (Caporaso et al., 1994; Vassar et al., 1999). It has been

206 reported that BACE1 activity increases with lower pH (Hook et al., 2002). In a recent *in vitro*

207 study in a HEK293 cell line overexpressing APP and BACE1, NHE6 overexpression reportedly

208 led to a reduction of Aβ production and conversely shRNA knockdown of NHE6/*Slc9a6* resulted

209 in an increase in Aβ production (Prasad and Rao, 2015). To investigate if NHE6/*Slc9a6*

210 deficiency contributes to APP processing by BACE1 in neurons, we used primary neurons

211 derived from *AppSwe* (*Tg2576*) mice, an Alzheimer's disease mouse model that overexpresses

212 human APP with the "Swedish" mutation (Hsiao et al., 1996). Neurons were treated with the

213 NHE inhibitor EMD87580 or transduced with lentiviral shRNA directed against NHE6/*Slc9a6* in

214 the presence or absence of the γ-secretase inhibitor L-685458. βCTF was detected using the

215 monoclonal antibody 6E10 directed against Aβ-residues 1-16 (**Figure 6**). Inhibition of γ-

216 secretase in ApoE4-treated *AppSwe* neurons strongly enhanced βCTF accumulation, however,

217 additional treatment with EMD87580 did not alter the amount of βCTF in the cell lysates (**Figure**

218 **6A**). NHE6 knockdown using lentiviral shRNA also had no effect on the amount of βCTF (**Figure**

219 **6B**). We conclude that NHE6 inhibition is unlikely to increase Aβ production under near-

220 physiological conditions.

221

NHE6 Deficiency Reduces Aβ Plaque Load in Human *App* Knockin Mice

To further study the effect of NHE6-deficiency on Aβ pathology *in vivo* we bred humanized *App^NL-F* mice (Saito et al., 2014) to our germline NHE6 knockout line (*Slc9a6^-*). In these *App^NL-F* mice, the Aβ-sequence has been completely humanized and the early onset AD Swedish mutation (5' located mutations encoding K670N and M671L = NL) and the Beyreuther/Iberian mutation (3' flanking mutation encoding I716F = F) were also introduced, resulting in increased Aβ production, but physiological regulation of APP expression. This allowed us to determine the effect of Aβ overproduction while keeping APP expression under the control of the endogenous promoter. *App^NL-F*;*Slc9a6^-* and control *App^NL-F* littermates were aged to one year. Perfusion-fixed brains were harvested and analyzed by H&E staining, Thioflavin S staining to visualize plaque load, and Aβ-immunohistochemistry. H&E staining did not reveal any obvious anatomical structural differences between genotypes, but brain size, cortical thickness, hippocampal area, and CA1 thickness were reduced, as described previously (Xu et al., 2017) (**Figure 7 – Figure Supplement 1**). Plaques were more frequent in *Slc9a6 wild type* than *Slc9a6^-* mice. To further investigate and quantify plaque load we analyzed the same brains after Thioflavin S staining (**Figure 7A**). We found an approximate 80% reduction of plaques in *Slc9a6^-* mice when compared to littermate controls (**Figure 7B**). Immunohistochemistry against Aβ showed the same reduction (**Figure 7 – Figure Supplement 3A-B**). In addition, we analyzed soluble (TBS fraction) and insoluble (GuHCl and 70% FA fractions) Aβ in cortical brain lysates of 1.5 year old *App^NL-F*;*Slc9a6^-* mice and their control littermates. The amount of insoluble Aβ (GuHCl and 70% FA fractions) was reduced in NHE6-depleted mice by approximately 71%, when compared to their control littermates (**Figure 7 – Figure Supplement 3C-E**). The ~50% reduction in soluble Aβ was statistically not significant in *Slc9a6^-* lysates (TBS fraction).

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NHE6/*Slc9a6* Deficiency Reduces Plaque Load in *App^NL-F*;*Apoe^APOE4*

To further investigate whether *Slc9a6*-deficiency also protects the brain from plaques in the presence of human ApoE4 instead of murine ApoE, we bred *Slc9a6^fl*;*CAG-Cre^ERT2*; *Apoe^APOE4* with *App^NL-F* mice. At two months of age we induced *Slc9a6*-ablation with tamoxifen

and aged the mice to 14 - 16 months. *Slc9a6*-deficiency on the background of human ApoE4 reduced plaque deposition, as shown by Thioflavin S staining (**Figure 7 – Figure Supplement 3F and 3G**) and 4G8 immunoreactivity **(Figure 7C and 7D)**. The age-dependent increase in plaque load in *Apoe*^{APOE4};*App*$^{NL-F}$ mice was abolished or delayed when *Slc9a6* was depleted after 2 months of age (**Figure 8**). *App*$^{NL-F}$ mice expressing murine ApoE developed plaques at 12 months (**Figure 7A and 7B**), compared to *Apoe*^{APOE4};*App*$^{NL-F}$ which showed a similar number of plaques at 15-16 months (**Figures 7C, 7D, 8, and Figure 7 – Figure Supplement 3F-G**). This delay of plaque deposition caused by the presence of human ApoE4 as opposed to murine ApoE is consistent with earlier findings by the Holtzman group (Liao et al., 2015). Importantly, NHE6/*Slc9a6* ablation induced at two months showed a comparable reduction of plaque load as germline NHE6/*Slc9a6* depletion. We conclude that plaque deposition is modulated by the presence of NHE6 postnatally and is not affected by NHE6 activity during development.

NHE6/*Slc9a6* Deficiency Does Not Affect Cortical Thickness and Hippocampus Size in *App*$^{NL-F}$;*Apoe*^{APOE4} cortices

Xu et al. (2017) reported reduced cortex thickness and hippocampus volume in *Slc9a6*$^-$ mice, which we were able to reproduce in our germline *Slc9a6*$^-$ model (**Figure 7 – Figure Supplement 1**). In addition, based on their findings Xu et al. (2017) concluded that the difference in brain size was a combined result of both neurodevelopmental and neurodegenerative effects caused by NHE6/*Slc9a6* deficiency. Since both, germline deficiency and adult-onset deficiency of NHE6 causes massive Purkinje cell loss in the cerebellum (**Figure 3C-E**), we next investigated the effect of conditional NHE6/*Slc9a6*-loss on hippocampal and cortical neuronal loss in our *App*$^{NL-F}$;*Apoe*^{APOE4} model (**Figure 7E-I**). We measured brain size, cortical thickness, hippocampal area, and CA1 thickness. In contrast to germline *Slc9a6*$^-$ mice (**Figure 7 – Figure Supplement 1**) none of the analyzed parameters differed significantly between *Slc9a6*^{fl};*CAG-Cre*^{ERT2} and controls (**Figure 7E-I**), and we specifically did not detect any reduction in brain size compared to *Slc9a6*^{fl};*App*$^{NL-F}$;*Apoe*^{APOE4} littermate controls. This is consistent with the undergrowth model proposed by Xu et al. (2017) as we induced conditional disruption of NHE6/*Slc9a6* in the adult after postnatal brain growth was completed.

10

NHE6 Deficiency Increases Iba1 and GFAP Expression in the Brain

Neuroprotective astrocytes and microglia have been described to reduce Aβ deposition in early stages of AD (Sarlus and Heneka, 2017). It has been reported that NHE6-deficiency leads to increased glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule (Iba1) immunoreactivity in different brain regions (Xu et al., 2017). To validate if plaque load correlated with Iba1 and/or GFAP immunoreactivity, we performed DAB-immunostaining for both marker proteins. We found that Iba1 and GFAP immunoreactivity is increased in the white matter and to a lesser extent in the cortex of App^{NL-F};$Slc9a6^-$ (**Figure 9 – Figure Supplement 1A, 1C, and 1D**) and App^{NL-F};$Apoe^{APOE4}$;$Slc9a6^{fl}$;$CAG-Cre^{ERT2}$ (**Figure 9 – Figure Supplement 1B, 1E, and 1F**) mice compared to their littermate controls. There was a non-significant trend towards increased immunoreactivity for both markers in the hippocampus of the $Slc9a6^-$ group. Taken together, these data are consistent with the findings of the Morrow group in germline $Slc9a6^-$ mice (Xu et al., 2017). Neurodegeneration can be a trigger for glial activation (Yanuck, 2019). However, our data on glial activation and cerebral volume in cKO mice, where NHE6/$Slc9a6$ was disrupted postnatally, suggest that neurodegeneration induced by NHE6/$Slc9a6$-deficiency is unlikely to be the trigger for the glial activation we observe.

Microglia and Astrocytes Surround Plaques in Both, $Slc9a6^-$ and Control App^{NL-F} mice

Conditional and germline $Slc9a6$-deficient App^{NL-F} mice have a reduced number of plaques in the brain and an increase of Iba1 and GFAP labeled glia. Others have shown that Aβ plaques levels are reduced with increased plaque-associated microglia detected with a co-stain for Iba1 and Aβ (Parhizkar et al., 2019; Zhong et al., 2019). In order to investigate the contribution of microglia and astrocytes to the observed plaque reduction we analyzed brain slices by costaining for Aβ using the 6E10 antibody (**Figure 9 and Figure 9 – Figure Supplement 2**). Whereas the total amount of plaques labeled by 6E10 was reduced in $Slc9a6^-$ as compared to control mice (**Figure 9C**), the amount of microglia and astrocytes surrounding plaques did not differ between genotypes (**Figure 9D and G**). In addition, there was no difference between genotypes in the amount of microglia co-labeled with 6E10 (**Figure 9E**) or the intensity of immunoreactivity for 6E10 within microglia (**Figure 9F**).

DISCUSSION

The prevalence of Alzheimer's disease (AD) is increasing with life-expectancy in all human populations. ApoE4 is the most important genetic risk factor. This makes it of paramount importance to understand the underlying mechanisms by which ApoE4 contributes to the pathology of the disease in order to devise effective targeted therapies that can be deployed on a global scale. Only small molecule drug therapies or, alternatively, immunization approaches, can satisfy this requirement. Biologics, including monoclonal antibodies and potential viral gene therapy approaches, are unlikely to be sufficiently scalable. We have previously reported a novel small molecule intervention that has the potential to neutralize ApoE4 risk (Xian et al., 2018) through prevention of the ApoE4-induced endosomal trafficking delay of synaptic receptors by the early endosomal sorting machinery. The mechanistic basis of this conceptually novel intervention is the acidification of the early endosomal compartment through inhibition of NHE6. Remarkably and unexpectedly, loss of NHE6/*Slc9a6* effectively reduced Aβ accumulation even in the absence of ApoE4, suggesting that hyperacidification of early endosomes prevents amyloid plaque formation independently of ApoE4. NHE6/*Slc9a6* suppression or inhibition may thus be a universal approach to prevent amyloid buildup in the brain, irrespective of ApoE genotype. Our previous (Xian et al. 2018) and current studies thus suggest a novel mechanism to prevent ApoE4-risk for Alzheimer's disease and delay plaque formation. In addition, genome wide association studies (GWAS) in conjunction with studies on cell culture and mouse models of AD show that various AD risk factors enhance endo-lysosomal dysfunction (Knopman et al., 2021; Small et al., 2017; Verheijen and Sleegers, 2018), which potentially could be corrected by NHE6 inhibition.

Upon endocytosis, endosomes undergo gradual acidification controlled by vATPases which actively pump protons into the vesicular lumen, and by the Na^+/H^+-exchanger NHE6 which functions as a regulatable proton leak channel. NHE6-depletion acidifies early and recycling endosomes (Lucien et al., 2017; Ohgaki et al., 2010; Ouyang et al., 2013; Xinhan et al., 2011). pH is an important regulator of the endolysosomal sorting machinery in which vesicles undergo multiple rounds of fusion. EEs undergo fusion and fission events in close proximity to the cell membrane. Recycling endosomes originate from EEs while they undergo early-to-late-endosomal maturation. In contrast to late endosomes, recycling endosomes do not

undergo further acidification (Jovic et al., 2010; Schmid, 2017). The pH of EEs and recycling endosomes is approximately 6.4-6.5 (Casey et al., 2010). This is normally sufficient to induce ligand receptor dissociation and enable cargo sorting. Our data, however, suggest that ApoE4 dramatically delays this fast recycling step in neurons, where ApoE, Apoer2, and glutamate receptors co-traffic through fast recycling compartments upon Reelin stimulation (Xian et al., 2018). We have proposed that ApoE4 impairs vesicle recycling due to isoelectric precipitation and structural unfolding at the physiological pH of the EE environment. This delays the dissociation of ApoE4 from its receptors, which in turn prolongs the entry of ApoE4 - along with Apoer2 and glutamate receptors in the same vesicle - into the recycling pathway (illustrated in **Figure 1**). We conclude that ApoE4 net-charge affects its endosomal trafficking. This is further supported by recent findings by Arboleda-Velasquez and colleagues (Arboleda-Velasquez et al., 2019), who reported the presence of the "Christchurch" R136S mutation in an E3/E3 PS1 mutation carrier without dementia. By neutralizing the positive charge of Arg136 in ApoE3, the IEP of this ApoE3 isoform is predicted to match that of ApoE2, which is protective against AD (Corder et al., 1994). ApoE2 homozygous carriers have an exceptionally low likelihood of developing AD (Reiman et al., 2020). Moreover, the Christchurch mutation is located within the heparin binding domain of ApoE, which reduces its affinity for cell surface heparan sulfate proteoglycans. That in turn would result in decreased uptake and thus depletion of ApoE in EEs. The net effect would be unimpeded trafficking of EE vesicles through the fast recycling compartment.

In dendritic spines, NHE6 co-localizes with markers of early and recycling endosomes and with the glutamate receptor subunit GluA1. In the hippocampus, NHE6 is highly expressed in the pyramidal cells of the CA and the granule cells of the dentate gyrus (Stromme et al., 2011). Apoer2 is present at the postsynaptic density of CA1 neurons (Beffert et al., 2005). During LTP induction, translocation of NHE6-containing vesicles to dendritic spine heads is enhanced (Deane et al., 2013) and glutamate receptors are recruited to the synaptic surface through fast recycling (Fernandez-Monreal et al., 2016). Our findings are consistent with a model where NHE6 serves as a pH-regulator of Reelin-controlled fast recycling endosomes containing Apoer2 and glutamate receptors. This mechanism possibly translates to other cell types and other ApoE receptors.

We previously showed that ApoE4 impairs Reelin-mediated receptor recruitment to the neuronal surface and this can be reversed by functionally disabling NHE6 in primary neurons, which results in the increased acidification of EEs to a level sufficiently different from the IEP of ApoE4, which then allows its efficient dissociation from its receptors. Conditional *Slc9a6* deletion accordingly alleviates the ApoE4-mediated resistance to Reelin-enhanced synaptic plasticity in hippocampal field recordings. (Gao et al., 2019; Xian et al., 2018)

Amyloid-β (Aβ) and tau, forming amyloid plaques and neurofibrillary tangles, are the defining features of AD pathology. As of today, it remains controversial how ApoE-isoforms interfere with Aβ and tau pathology. ApoE, which is primarily expressed by astrocytes, is the major lipid transporter in the brain and in an isoform-dependent manner affects inflammatory, endolysosomal and lipid-metabolic pathways (Gao et al., 2018; Minett et al., 2016; Van Acker et al., 2019; Xian et al., 2018). Most risk factors identified by genome-wide association studies, including but not limited to APOE, ABCA7, CLU, BIN1, TREM2, SORL1, PICALM, CR1 are members of one or more of these pathways (Kunkle et al., 2019). In recent years, endosomal dysfunction has increasingly gained acceptance as a causal mechanism for late-onset AD. Our findings now provide a mechanistic explanation how ApoE4 impairs endolysosomal trafficking and recycling, by interfering with vesicular sorting and maturation at a crucial bottleneck juncture of the endosomal trafficking machinery. This has far-reaching consequences for neuronal function, synaptic plasticity, and tau phosphorylation (Brich et al., 2003; Cataldo et al., 2000; Chen et al., 2005; Chen et al., 2010; Nuriel et al., 2017; Pensalfini et al., 2020). More specifically, ApoE4 causes abnormalities of Rab5-positive endosomes (Nuriel et al., 2017). Intriguingly, over-activation of the small guanosine triphosphatase (GTPase) Rab5, recapitulates neurodegenerative features of AD (Pensalfini et al., 2020).

ApoE4 alters APP processing and Aβ-degradation, (reviewed in (Benilova et al., 2012; Haass et al., 2012; Huynh et al., 2017; Lane-Donovan and Herz, 2017; Pohlkamp et al., 2017; Yamazaki et al., 2019)) and the ability of Reelin to protect the synapse from Aβ toxicity is impaired by ApoE4 (Durakoglugil et al., 2009). Aβ-oligomerization followed by plaque formation is one hallmark of AD. NHE6 controls endosomal pH, which can affect BACE1 activity, one of the two enzymes required to process APP to release the Aβ-peptide. Prasad and Rao (2015) reported that overexpression of NHE6 and full length APP in HEK293 cells, rather than its inhibition or knockdown, reduced Aβ generation, which conflicts with our findings in primary

cortical neurons (**Figure 6**). Although the cause of this discrepancy remains currently unresolved, it is possible that it is the result of the two fundamentally different experimental systems that were used in the respective studies, i.e. overexpression in immortalized kidney cells on one hand and primary cortical neuronal cultures on the other. Using a humanized App^{NL-F} knockin mouse model we show that NHE6/$Slc9a6$-deficiency in one-year old animals reduces plaque deposition by approximately 80%. In App^{NL-F} mice plaques can be identified as early as 9 months of age. Whereas plaque deposition only increases by less than twofold between 9 and 12 months of age, it increases tenfold between 12 and 18 months (Saito et al., 2014). Importantly, the reduction in plaque deposition by $Slc9a6$-deficiency persists from early (12 months) to later stages (18 months) of AD, as $Slc9a6$-deficient App^{NL-F} animals aged 18 months had a reduction in insoluble Aβ by approximately 71%. NHE6/$Slc9a6$ depletion in App^{NL-F};$Apoe^{APOE4}$ mice showed a comparable reduction in plaque load (**Figure 7 and Figure 7 – Figure Supplement 3**). Our data are consistent with previous findings by the Holtzman group (Fagan et al., 2002) that showed that mouse ApoE promotes plaque deposition more potently than human ApoE4.

Prevention of plaque formation in our $Slc9a6$ deficient model was likely caused by increased microglial activation and plaque phagocytosis (**Figure 7, Figure 7 – Figure Supplement 3, Figure 8, Figure 9, Figure 9 – Figure Supplement 1 and Figure 9 – Figure Supplement 2**). In the brains of AD patients and APP overexpressing mice, plaques are surrounded by reactive microglia and astrocytes (Meyer-Luehmann et al., 2008; Serrano-Pozo et al., 2013), but the pathological significance of this is incompletely understood. Beneficial or detrimental roles of reactive microglia and astrocytes in the degradation of Aβ have been reported, depending on the activation state of these cells (Meyer-Luehmann et al., 2008; Ziegler-Waldkirch and Meyer-Luehmann, 2018). We observed an increase in reactive microglia and astrocytes resulting from NHE6/$Slc9a6$-depletion, which correlated with reduced plaque deposition in App^{NL-F} mice, irrespective of the presence of either murine ApoE or human ApoE4. As murine ApoE exacerbates plaque deposition even more than ApoE4, the comparable plaque reduction in $Slc9a6$-deficient mice with murine $Apoe$ or $Apoe^{APOE4}$ might be the result of a maximally accelerated early endosomal maturation and cargo transport in the absence of NHE6. When compared to control App^{NL-F} mice, $Slc9a6$-deficient App^{NL-F} mice show an increase in Iba1 (microglia) and GFAP (astrocytes) immunoreactivity, but reduced Aβ immunoreactivity.

Surprisingly, the intensity of GFAP and Iba1 in plaque areas was comparable between the groups. Moreover, even though Aβ is reduced and Iba1 is increased in the *Slc9a6⁻*, the proportion of microglial structures containing Aβ (6E10 antibody) was comparable between *Slc9a6*-deficient and control *App^{NL-F}* mice, as was the intensity signal for 6E10. This suggests that microglia in the *Slc9a6⁻* may be more efficient in taking up and degrading Aβ. Whether the reduction in plaques is due to the presence of an increased number of microglia and astrocytes that actively phagocytose nascent plaques, or whether endosomal acidification in microglia and astrocytes improves their ability to degrade or export Aβ from the brain remains to be determined. It is tempting to speculate that the mechanism that leads to reduced plaque load in *Slc9a6*-deficient *App^{NL-F}* mice may involve an increased catabolic rate (Shi et al., 2021), brought about by the accelerated acidification and vesicular trafficking of early endosomes.

It is also possible that *Slc9a6*-deficiency alters the efficiency of astrocytes to lipidate ApoE. In a mouse model, improved ApoE lipidation by the overexpression of ATP-binding cassette transporter family member A1 (ABCA1) decreased plaque deposition (Wahrle et al., 2005; Wahrle et al., 2008; Wahrle et al., 2004). During HDL assembly, ABCA1 shuttles between EE and the plasma membrane, a process also referred to as retroendocytosis (Ouimet et al., 2019). Moreover, membrane trafficking of ABCA1 is altered by ApoE in an isoform dependent fashion (Rawat et al., 2019). The *App^{NL-F}* mouse model used in our study develops plaques at 12 months (Saito et al., 2014) in the presence of murine ApoE. However, onset of plaque deposition in human *Apoe^{APOE4}* mice was delayed by approximately three months. The effect of germline *Slc9a6*-deficiency and conditional *Slc9a6*-deficiency induced at two months had a comparable effect on plaque reduction in *App^{NL-F}* and *App^{NL-F}*;*Apoe^{APOE4}* mice.

Slc9a6⁻ and *Slc9a6^{fl}*;*CAG-Cre^{ERT2}* both show progressive Purkinje cell loss in the cerebellum, indicating that NHE6 requirement is cell-autonomous and not developmentally determined. *Slc9a6⁻* and *Slc9a6^{fl}*;*CAG-Cre^{ERT2}* show a comparable increase in immunoreactivity against Iba1 and GFAP. Increased glia reactivity can be a direct cell-autonomous effect of NHE6-loss or an indirect effect caused by NHE6-deficiency related neurodegeneration. As germline *Slc9a6⁻* mice present with a reduction in cortical thickness and hippocampal volume caused by both neurodevelopmental and neurodegenerative effects (Xu et al., 2017) (**Figure 7 – Figure Supplement 1**), it is possible that neurodegeneration gives rise to glia activation. However, the tamoxifen induced *Slc9a6^{fl}*;*CAG-Cre^{ERT2}* mice do not present with a reduction in

464   cortical or hippocampal volume (**Figure 7**), yet have comparable immunoreactivity for markers

465   of glial activation. Our study supports a temporally distinct function of NHE6 in the adult brain

466   where the cerebrum requires NHE6 for development, but not for postnatal neuronal survival,

467   whereas Purkinje cells in the cerebellum do. A similar dual function for NHE6 has been

468   described previously by Xu et al. (2017). Moreover, this further indicates that reduced plaque

469   load is also not an effect of neuronal loss. Therefore, our two mouse models together suggest

470   that the observed increased glial activation is not caused by neuronal cell loss, but rather is

471   likely a direct cell-autonomous effect of NHE6 loss of function. It thus remains to be determined

472   whether endosomal acidification in NHE6 deficient microglia alone is sufficient to induce Aβ

473   degradation and plaque reduction.

474   NHE6 is ubiquitously expressed in all cells of the body, however, in the CNS it is highly

475   expressed in neurons where abundant synaptic vesicles and neurotransmitter receptors recycle

476   in synapses (Lee et al., 2021a; Lee et al., 2021b) and to a lesser extent in glial cells (Zhang et

477   al., 2014). Neuronal NHE6 plays a direct role in both synaptic development and plasticity,

478   potentially through BDNF/TrkB (Deane et al., 2013; Ouyang et al., 2013) and other pathways.

479   The impact of NHE6 loss in microglia and astrocytes is still unknown. Global *Slc9a6*-deficiency

480   causes glial activation *in vivo*, which could be mediated through either a primary cell-

481   autonomous mechanism or a secondary mechanism induced by damaged neurons. In ApoE4-

482   expressing astrocytes, others have shown that overexpression of NHE6 increases LRP1 on the

483   surface, which correlated with an increase in Aβ uptake (Prasad and Rao, 2018). Microglia are

484   the primary glial cells that degrade Aβ; thus, it will be imperative to determine how NHE6 levels

485   alter Aβ degradation selectively in astrocytes and microglia, respectively.

486   Early endosomal pH balance in phagocytic cells plays an important role in viral and

487   bacterial infection response. In phagocytic cells, the deacidification of endosomes using

488   pharmacological inhibitors like chloroquine has been shown to reduce endosomal toll like

489   receptor (TLR) response (de Bouteiller et al., 2005; Fox, 1996; Kuznik et al., 2011; Wozniacka

490   et al., 2006; Yang et al., 2016), suggesting that NHE6 depletion in microglia might conversely

491   augment TLR response.

492   It would also be intriguing to test whether *Slc9a6*-deficiency increases cognitive

493   performance in AD mouse models. In the current study we used App^{NL-F} and $App^{NL-F};Apoe^{APOE4}$

494   mice as the most physiological currently available mouse models of AD that do not rely on

excessive amyloid overproduction. These mice, however, do not show cognitive impairments in spatial learning tests like Morris water maze (Saito et al., 2014) (own unpublished observations). Other neurobehavioral phenotypes have been described for $Slc9a6^-$ mice, which also recapitulate symptoms in Christianson syndrome patients, for example hyposensitivity to pain (Petitjean et al., 2020). Future studies on our novel tamoxifen inducible $Slc9a6^{fl};CAG\text{-}Cre^{ERT2}$ line will help to understand whether these symptoms are based on neurodevelopmental deficits caused by germline $Slc9a6$-deficiency or whether they can be reproduced by induced loss of NHE6 postnatally.

In conclusion, we have shown that both, the endosomal trafficking defect induced by ApoE4 in neurons as well as increased plaque deposition irrespective of ApoE genotype can be corrected by inhibition or genetic deletion of NHE6/$Slc9a6$, a key regulator of early endosomal pH. Accelerated acidification of early endosomes abolishes the ApoE4-induced Reelin resistance and restores normal synaptic plasticity in ApoE4 targeted replacement mice. The first FDA approved drug for AD treatment in 18 years is aducanumab (Sevigny et al., 2016), an antibody directed against Aβ, which clears amyloid from the brain. However, amyloid removal in individuals already afflicted with AD provides at best marginal benefits at this stage. Moreover, in excess of 1 billion people world-wide are ApoE4 carriers, making early treatment with a complex biologic such as aducanumab impractical on the global scale. Here we have presented a potential alternative approach which should be adaptable to large-scale prevention treatment using blood-brain-barrier penetrant NHE6-specific inhibitors. Taken together, our combined data suggest that endosomal acidification has considerable potential as a novel therapeutic approach for AD prevention and possibly also for the prevention of disease progression.

MATERIALS AND METHODS

Animals

All animal procedures were performed according to the approved guidelines (Animal Welfare Assurance Number D16-00296) for Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center at Dallas.

524   The mouse lines Rosa-stop-tdTomato *B6.Cg-Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/*J*
525   (Madisen et al., 2010) (JAX #007909) and CAG-Cre^{ERT2} *B6.Cg-Tg(CAG-cre/Esr1)5Amc/J* mice
526   (Hayashi and McMahon, 2002)(JAX #004682), were obtained from The Jackson Laboratories
527   (Bar Harbor, ME). ApoE3 and ApoE4 targeted replacement mice (*Apoe*^{APOE3}, *Apoe*^{APOE4})
528   (Knouff et al., 1999; Sullivan et al., 1997) were kind gifts of Dr. Nobuyo Maeda. *AppSwe*
529   (*Tg2576*) were generated by (Hsiao et al., 1996). The Meox-Cre *B6.129S4-Meox2tm1(cre)Sor/J*
530   mice (JAX 003755) were provided by Drs. M. Tallquist and P. Soriano (Tallquist and Soriano,
531   2000). Conditional NHE6 knockout (*Slc9a6*^{fl}*;CAG-Cre*^{ERT2}) and germline NHE6 knockout
532   (*Slc9a6*$^{-}$) mice were generated as described below. The human APP knockin line (*App*$^{NL-F}$)
533   (Saito et al., 2014) has been described earlier. Pregnant female SD (Sprague Dawley) rats were
534   obtained from Charles River (SC:400). Mice were group-housed in a standard 12-h light/dark
535   cycle and fed ad libitum standard mouse chow (Envigo, Teklad 2016 diet as standard and
536   Teklad 2018 diet for breeding cages).

537   To generate *Slc9a6* floxed (*Slc9a6*^{fl}) mice, the first exon of NHE6 was flanked with loxP
538   sites (**Figure 2A**). A loxP site was inserted 2 kb upstream of the first exon of the X-chromosomal
539   NHE6 gene and a Neo-cassette (flanked with loxP and FRT sites) was inserted 1 kb
540   downstream of the first exon. Insertion sites were chosen based on low conservation (mVISTA)
541   between mammalian species (rat, human, mouse). To create the targeting vector for the *Slc9a6*^{fl}
542   mouse line, pJB1 (Braybrooke et al., 2000) was used as backbone. Murine C57Bl/6J embryonic
543   stem (ES) cell DNA was used as template to amplify the short arm of homology (SA; 0.88 kb of
544   the first intron starting 1kb 3' downstream of exon 1), which was inserted between the Neo and
545   HSVTK selection marker genes of pJB1 (BamHI and XhoI sites) to create an intermediate
546   plasmid referred to as pJB1-NHE6SA. To create the intermediate plasmid pNHE6-LA for the
547   long homology arm, a fragment spanning from 13kb 5' upstream to 1kb 3' downstream of the
548   first *Slc9a6* (NHE6) exon was integrated into pCR4-TOPO (Thermo Fisher) by using a bacterial
549   artificial chromosome (BAC, RP23 364F14, Children's Hospital Oakland Research Institute
550   (CHORI)) and the GAP repair technique (Lee et al., 2001) (primers to amplify the upstream (US)
551   and downstream (DS) homology boxes are listed in the Key Resources Table). In a parallel
552   cloning step a 2.4 kb XhoI-EcoRV *Slc9a6*-promoter region fragment spanning from 2.7 kb to 0.4
553   kp 5' upstream of the *Slc9a6* start codon was modified with the 1st loxP site to generate pLoxP:
554   three fragments (1) 0.7 kb 5' loxP flanking *Slc9a6*-fragment, (2) 1.7 kb 3' loxP flanking *Slc9a6-*

fragment, and (3) 100 bp loxP oligo (primers/oligos for each fragment are listed in the Key Resources Table) were cloned into pCR4-TOPO. The 2.5 kb loxP-modified XhoI-EcoRV *Slc9a6*-promoter fragment of pLoxP was cloned into pNHE6-LA to create pNHE6-LA-LoxP. To obtain the final targeting construct pJB-NHE6-TV, the NotI-EagI fragment of pNHE6-LA-LoxP containing the long arm (11 kb 5' upstream of the 1st LoxP) and the 1st loxP site was cloned into the NotI-site of pJB1-NHE6SA and checked for orientation (pJB1-NHE6 targeting vector). pJB-NHE6-TV was linearized with NotI and electroporated into C57Bl/6J ES cells. Gene targeting-positive C57Bl/6J ES cells (PCR-screen) were injected into albino C57Bl/6J blastocysts, resulting in chimeric mice. The chimeras were crossed to C57Bl/6J mice, resulting in *Slc9a6*$^{fl/+}$ females. Genotyping: The *Slc9a6*^{fl} PCR amplifies a 250 bp of the wildtype and 270 bp of the floxed allele, primers are listed in the Key Resources Table.

To generate *Slc9a6*^{fl}*;CAG-Cre*^{ERT2}, *Slc9a6*$^{fl/+}$ females were crossed to *CAG-Cre*^{ERT2} mice to obtain *Slc9a6*$^{fl/fl}$*;CAG-Cre*^{ERT2} and *Slc9a6*$^{y/fl}$*;CAG-Cre*^{ERT2} mice (*Slc9a6*^{fl}*;CAG-Cre*^{ERT2}) and *CAG-Cre*^{ERT2} negative control littermates (*Slc9a6*^{fl}). *Slc9a6*^{fl}*;CAG-Cre*^{ERT2} mice were backcrossed to *Apoe*^{APOE3} or *Apoe*^{APOE4} mice. Breeding pairs were set up in which only one parent was *CAG-Cre*^{ERT2} positive. The following genotypes were used for hippocampal field recordings: (1) *Apoe*^{APOE3}*;SLC9a6*$^{y/fl}$ (short:*Apoe*^{APOE3}), (2) *Apoe*^{APOE3}*;Slc9a6*$^{y/fl}$*;Cre*^{ERT2} (short: *Apoe*^{APOE3}; *Slc9a6*^{fl}*;CAG-Cre*^{ERT2}), (3) *Apoe*^{APOE4}*;Slc9a6*$^{y/fl}$ (short: *Apoe*^{APOE4}), and (4) *Apoe*^{APOE4}*;SLc9a6*$^{y/fl}$*;Cre*^{ERT2} (short: *Apoe*^{APOE4} *;Slc9a6*^{fl}*;CAG-Cre*^{ERT2}). The *Apoe*^{APOE4}*;Slc9a6*^{fl}*;CAG-Cre*^{ERT2} line was further crossed with the *App*$^{NL-F}$ line to generate *Apoe*^{APOE4}*;Slc9a6*^{fl}*;CAG-Cre*^{ERT2}*;App*$^{NL-F}$ and *Apoe*^{APOE4}*;Slc9a6*^{fl}*;App*$^{NL-F}$ control littermates. To induce genetic depletion of NHE6, tamoxifen (120 mg/kg) was intraperitoneally injected at 6-8 weeks of age. Injections were applied for five consecutive days. Tamoxifen was dissolved in sunflower oil (Sigma, W530285) and 10% EtOH.

To generate the germline NHE6 knockout line (*Slc9a6*$^{-}$), heterozygous *Slc9a6*$^{fl/+}$ females were crossed to *Meox-Cre* to yield germline mutant *Slc9a6*$^{-/-}$ females and *Slc9a6*$^{y/-}$ males (*Slc9a6*$^{-}$). Genotyping: The *Slc9a6*^{fl} PCR amplifies 250 bp of the wildtype, 270 bp of the floxed, and no fragment in the knockout alleles. Recombination was verified with the NHE6-rec PCR, which amplifies 400 bp if recombination has occurred. PCR-primers are listed in the Key Resources Table. *Slc9a6*$^{-}$ animals were further crossed with *App*$^{NL-F}$ (Saito et al., 2014) mice. *App*$^{NL-F}$*;Slc9a6*$^{-}$ (*App*$^{NL-F/NL-F}$*;Slc9a6*$^{y/-}$ or *App*$^{NL-F/NL-F}$*;Slc9a6*$^{-/-}$) and control (*App*$^{NL-F}$ = *App*$^{NL-F/NL-}$

586 ^{F};Slc9a6$^{y/+}$ or App$^{NL-F/NL-F}$;Slc9a6$^{+/+}$) littermates were obtained by crossing Slc9a6$^{+/-}$;App$^{NL-F/NL-F}$
587 females with either Slc9a6$^{y/+}$;App$^{NL-F/NL-F}$ or Slc9a6$^{y/-}$;App$^{NL-F/NL-F}$ males.

588

DNA Constructs, Recombinant Proteins, Lentivirus Production

590   Lentiviral plasmids with shRNA directed against NHE6/Slc9a6 and the scrambled control
591 have been described in Xian et al. (Xian et al., 2018). Plasmids encoding ApoE3 and ApoE4
592 (Chen et al., 2010), and Reelin (D'Arcangelo et al., 1997) have been described before. The
593 lentiviral plasmid encoding the Vamp3-pHluorin2 fusion protein was cloned by inserting mouse
594 Vamp3 cDNA, a linker and pHluorin2 (pME, Addgene #73794) (Stawicki et al., 2014) into
595 pLVCMVfull (Xian et al., 2018). For Vamp3 the forward primer (5'-
596 TTCAAGCTTCACCATGTCTACAGGTGTGCCTTCGGGGTC-3') contains a Kozak sequence,
597 the reverse primer encodes a KLSNSAVDGTAGPGSIAT-linker (Nakamura et al., 2005) (5'
598 CATTGTCATCATCATCGTGTGGTGTGTCTCTAAGCTGAGCAACAGCGCCGTGGACGGC
599 ACCGCCGGCCCCGGCAGCATCGCCACCAAGCTTAAC-3'). The pHluorin2 primers were
600 forward 5'-CCGGTCCCAAGCTTATGGTGAGCAAGGGCGAGGAGCTGTTC-3' and reverse 5'-
601 GCCCTCTTCTAGAGAATTCACTTGTACAGCTCGTCCATGCCGTG-3'. The fragments were
602 sequentially cloned into pcDNA3.1 and the fusion protein was then transferred with NheI and
603 EcoRI into pLVCMVfull. Lentiviral plasmids psPAX2 and pMD2.g were a kind gift from Dr D.
604 Trono and obtained from Addgene.

605   Recombinant Reelin and ApoE were generated in 293HEK cells. Reelin was purified as
606 described before (Weeber et al., 2002). ApoE-conditioned medium was collected from HEK293
607 cell cultures transiently transfected with pcDNA3.1-ApoE constructs or empty control vector
608 (pcDNA3.1-Zeo). ApoE concentration was measured as described before (Xian et al., 2018).

609   For lentivirus production HEK 293-T cells were co-transfected with psPAX2, pMD2.g, and
610 the individual shRNA encoding transfer or Vamp3-pHluorin2 constructs. Media was replaced
611 after 12-16 hours. Viral particle containing media was collected after centrifuging cellular debris.
612 The viral particles were 10x concentrated by ultra-centrifugation and resuspended in Neurobasal
613 medium. To infect neurons on DIV7 1/10th of the culture medium was replaced by concentrated
614 virus. After 24 hours the virus was removed.

615

Histochemistry

Mice were euthanized with isoflurane and perfused with PBS followed by 4% paraformaldehyde (PFA) in PBS. Brains were removed and post-fixed for 24 hours in 4% PFA. Fresh fixed brains were immobilized in 5% agarose in PBS and 50 µm thick sections were sliced on a vibratome (Leica, Wetzlar, Germany). Vibratome slices of Rosa26$^{floxedStop-tdTomato}$;CAGCre^{ERT2} mice, with or without tamoxifen injection at 8 weeks of age were mounted with Antifade Mounting medium containing DAPI (Vectashield). For immunofluorescence, vibratome slices were labeled for Calbindin after permeabilization with 0.3% Triton X in PBS and blocking for 1 hour in blocking buffer (10% normal goat serum, 3% BSA, and 0.3% Triton X in PBS). The primary antibody mouse anti-Calbindin (Swant CB300) was diluted in blocking buffer (1:1000) and added to the slices for 24-48 h at 4°C. Slices were subsequently washed 4x 15 min with PBS containing 0.3% Triton X. Slices were incubated with anti-mouse IgG coupled to Alexa594 (1:500 in blocking buffer) for 2 hours at room temperature. After washing, slices were mounted with Antifade Mounting medium with DAPI (Vectashield). Images were taken with an Axioplan 2 microscope (Zeiss).

For immunohistochemistry staining, PFA fixed brains were block-sectioned into coronal slabs, paraffin-embedded, and serially sectioned on a rotating microtome (Leica) at 5 µm. Deparaffinized sections were stained with Thioflavin S as described before (Guntern et al., 1992). Briefly, deparaffinized slices were oxidized in 0.25% $KMnO_4$ for 20 min. After washing with water slices were bleached with 1% $K_2S_2O_5$ / $C_2H_2O_4$ for 2 min, washed in water, and treated with 2% $NaOH/H_2O_2$ for 20 min. After washing with water slices were acidified in 0.25% CH_3COOH for 1 min, washed with water and equilibrated in 50% EtOH for 2 min, and stained in Thioflavin S solution for 7 min. Reaction was stopped by washing in 50% EtOH. Slices were dehydrated with 95% EtOH, followed by 100% EtOH and Xylene. Slices were mounted with Cytoseal 60 (Thermo Scientific). Deparaffinized sections were labeled using antibodies raised against GFAP (Abcam AB5804, Rabbit, 1:2,000), Iba1 (Wako 019-19741, Rabbit, 1:1,000), and Aβ (4G8, Covance or 6E10, Biolegend, both mouse, 1:1,000). Briefly, 5 µm sections were deparaffinized, subjected to microwave antigen retrieval (citrate buffer, pH 6.0), permeabilized with 0.3% (vol/vol) Triton X, endogenous peroxidases activity was quenched for

Diaminobenzidine (DAB) staining. Slices were blocked with goat serum (2.5 %) prior to overnight incubation with primary antibodies at 4°C. Primary antibodies were detected by either fluorescent secondary antibodies (goat-anti-mouse Alexa594, goat-anti-rabbit Alexa488) or sequential incubation with biotinylated secondary antisera and streptavidin-peroxidase for DAB staining. Diaminobenzidine chromagen was used to detect the immunoperoxidase signal (Sinclair et al., 1981) (Vector; anti-mouse and anti-rabbit IgG kits). Fluorescence-labeled slices were counter-stained with DAPI (mounting media with DAPI, Vectashield). Standard protocols were utilized for staining of paraffin sections with hematoxylin and eosin (H&E; Leica) (Fischer et al., 2008). Microscopy was performed on a high-throughput microscope (NanoZoomer 2.0-HT, Hamamatsu) for DAB stained tissue or with an Axioplan 2 microscope (Zeiss) for immunofluorescence analysis. The analysis of DAB labeled antibodies was performed after exporting the images with NDP.view2 software with ImageJ. For Thioflavin S staining and Aβ labeling plaques were quantified by categorizing them as small, medium, and large (Thioflavin S) or dense and diffuse (4G8) as depicted in **Figure 7 – Figure Supplement 2**. Co-localization analysis of microglia (Iba1) and astrocytes (GFAP) with plaques (6E10) was performed with ImageJ. A blind observer selected the area of plaques with circles of 20, 40, or 80 μm diameter and analyzed the intensity of 6E10 and Iba1 or GFAP by using the ImageJ plugin RGB_measure. 6E10 positive microglia (Iba1) were quantified by a blind observer by first identifying microglia structures/cells in the green channel (Iba1), and then analyzing the proportion of 6E10 positive structures and signal intensity (red channel).

Primary Cortical Neuronal Cultures

Primary cortical neuronal cultures were prepared from rat (SD, Charles River) or various mouse lines (*wildtype, Slc9a6⁻, AppSwe*) (E18) as described previously (Chen et al., 2005). Neurons were cultured in Poly-D-Lysine coated 6-well plates (1 million / 9 cm^2) or on coverslips (30,000 neurons / 1.1 cm^2) in presence of Neurobasal medium supplemented with B27, glutamine, and Penicillin-Streptomycin at 37°C and 5% CO_2. At indicated days in vitro (DIV) primary neurons were used for experiments.

Mouse Embryonic Fibroblasts

Fibroblasts were isolated from *Slc9a6⁻* and wildtype littermate control embryos (E13.5). After removing the head and the liver, the tissue was trypsinized (0.05% trypsin-EDTA) at 4°C overnight, followed by 30 min at 37°C. Suspended cells were cultivated in DMEM high glucose with 15% FCS, 2 mM L-glutamine, and Pen/Strep. Fibroblasts were serially passaged until proliferation slowed down. Immortalization was achieved by keeping fibroblasts in culture under high confluency until they overcame their growth-crisis.

pH Measurements

Mouse embryonic fibroblasts derived from either *Slc9a6⁻* or wildtype littermate embryos were infected with Vamp3-pHluorin2 lentivirus. 24-48 hours post infection, vesicular pH was measured on a Zeiss LSM880 Airyscan confocal microscope as described in Ma et al. (Ma et al., 2017). For the standard calibration curve, cells were washed and incubated with pH standard curve buffer (125 mM KCl, 25 mM NaCl, 10 µM monensin, 25 mM HEPES for pH > 7.0 or 25 mM MES for pH < 7.0; pH adjusted with NaOH and HCl) and imaged in 5% CO_2 at 37°C. For vesicular pH measurements, cells were washed and imaged with pH standard curve at pH 7 without monensin. Samples were excited at 405 and 488 nm with an emission of 510 nm. For quantification, 6 fields of view were imaged for *Slc9a6⁺* and 4 fields of view for *Slc9a6⁻* fibroblast. Between 4-10 pHluorin-positive vesicles were measured per field of view which resulted in n=32 wildtype vesicle and n=28 *Slc9a6⁻* vesicles. The same settings were used for every image, and images were analyzed using ImageJ Software. The intensity of excitations with 405 and 488 nm was measured, individual vesicles were marked as regions of interest, and the 405/488 ratio was calculated and plotted against pH for the standard curve.

Biochemistry

To analyze receptor recycling cell surface biotinylation was performed. At DIV10-14, primary neurons were pre-treated for 30 min with ApoE-conditioned medium (5 µg/ml) and incubated with Reelin (2 µg/ml) for an additional 30 min (see timeline in **Figure 4A**). After treatment, cells were washed with cold PBS and incubated in PBS containing sulfo-NHS-SS-biotin for 30 min at 4°C. Subsequently excess reagent was quenched by rinsing the neurons

24

704   with cold PBS containing 100 mM glycine. Neurons were lysed in 160 μl/ 9 cm^2 lysis buffer (PBS

705   with 0.1% SDS, 1% Triton X-100, and protease inhibitors) at 4°C for 20 min. Cell debris were

706   pelleted at 14,000 rpm for 10 min at 4°C. The protein concentration was measured using the

707   Bradford Protein Assay (Bio-Rad). One hundred μg of total proteins were incubated with 50 μl of

708   NeutrAvidin agarose at 4°C for 1 hour. Agarose beads were washed three times using washing

709   buffer (500 mM NaCl; 15 mM Tris-HCl, pH 8.0; 0.5% Triton X-100), biotinylated surface proteins

710   were eluted from agarose beads by boiling in 2x SDS sample loading buffer and loaded on an

711   SDS-PAGE gel for Western blot analysis. GST-control and GST-RAP (50 μg/ml) pre-treatment

712   of neurons was performed for 1 hour.

713   To analyze BACE1 activity, βCTF was detected by immune blotting. BACE1 activity was

714   examined after pharmacological NHE inhibition or genetic *Slc9a6* knockdown in primary

715   neurons of *AppSwe* mice. For NHE inhibition DIV10 neurons were treated with 5 μg/ml ApoE4, 3

716   μM EMD87580 (Merck), and/or 1 μM γ-secretase inhibitor L-685458 (Merck) for 5 hours. For

717   knockdown of NHE6/*Slc9a6* DIV7 neurons of *AppSwe* mice were infected with lentivirus

718   encoding shRNA against NHE6 or a scrambled control shRNA. On DIV13 neurons were treated

719   with γ-secretase inhibitor for 12 hours. Proteins were extracted for Western blot analysis: Cells

720   were washed three times with cold PBS, and lysed for 20 min on ice in RIPA buffer (50 mM Tris-

721   HCl, pH 8.0; 150 mM NaCl; 1% Nonidet P-40; phosphatase and proteinase inhibitors). Cell

722   debris were pelleted at 14,000 rpm for 10 min at 4°C. Protein concentration was measured

723   using the Bradford Protein Assay (Bio-Rad). After adding 4xSDS loading buffer (0.1 M Tris-HCl,

724   pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue) samples

725   were denatured at 80°C for 10 min. Proteins were separated via SDS-PAGE and transferred to

726   a nitrocellulose membrane for Western blotting using different antibodies listed in the Key

727   Resources Table.

728   Brain tissue was dissected and prepared for immunoblotting as followed: After removal,

729   brains were placed in ice-cold PBS containing proteinase inhibitors. Anatomical sectioning was

730   performed under a microscope. The hippocampus was dissected out and transversal slices

731   were further separated into Cornu Ammonis (CA) 1, CA3, and dentate gyrus. Respective pieces

732   of the same anatomical regions of one brain were pooled, shock-frozen in liquid nitrogen and

733   stored at -80C. Frozen tissue was homogenized in 10x volume of RIPA buffer and incubated on

ice for 30 min. Cell debris were pelleted for 10 min with 14,000 rpm at 4°C. After adding 4xSDS loading buffer the samples were denatured at 80°C for 10 min and used for immunoblotting.

To measure soluble and insoluble Aβ species, a sequential homogenization procedure was employed. After removal of the brains from PBS perfused mice, cortical tissue was dissected and flash frozen. Frozen cortical tissue was homogenized in TBS supplemented with phosphatase and proteinase inhibitors at 100 mg protein/ml using a glass dounce homogenizer. Crude lysate was centrifuged at 800 xg for 5 min at 4°C. The supernatant was further centrifuged at 100,000 xg for 30 min at 4°C and collected as TBS-soluble lysate (Aβ-soluble)**.** The TBS-pellet was further homogenized in 1% Triton-TBS containing phosphatase and proteinase inhibitors, centrifuged at 100,000 xg for 30 min at 4°C and collected as 1% Triton-soluble lysate. The Triton-pellet was incubated with 5M guanidine-HCl rotating at RT for 1 hour. Guanidine soluble lysate (Aβ-insoluble) was collected after centrifugation at 21,000 xg for 15 min at 4°C. The guanidine-pellet was further solubilized in 1/20th volume with 70% Formic Acid (Aβ-insoluble) and centrifuged at 21,000 xg for 15 min at 4°C. Soluble and insoluble Aβ levels were measured in duplicates using a commercial Aβ42 ELISA kit (ThermoFisher, KHB3441) following the manufacturer's instructions.

Extracellular Field Recordings

Hippocampal slices were prepared from 3-month-old mice (tamoxifen-injected at 8 weeks). Slices of mice were obtained from four different genotypes; *Slc9a6^{fl};CAG-Cre^{ERT2}* mice or *Slc9a6^{fl}* mice with *Apoe^{APOE3}* or *Apoe^{APOE4}*. The brains were quickly removed and placed in ice cold high sucrose cutting solution (in mM: 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 28 NaHCO$_3$, 0.5 CaCl$_2$, 5 glucose, 0.6 Ascorbic acid, 7 MgSO$_4$), bubbled with a gas mixture of 95% O$_2$ and 5% CO$_2$ for oxygenation. 350 µm transverse sections were cut using a vibratome (Leica). Slices were transferred into an incubation chamber containing 50% artificial cerebrospinal fluid (aCSF, in mM: 124 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 26 NaHCO$_3$, 10 D-glucose, 2 CaCl$_2$, 1 MgSO$_4$) and 50% sucrose cutting solution, oxygenated with 95%O$_2$/5%CO$_2$. Slices were transferred into an oxygenated interface chamber and perfused with aCSF with or without Reelin (2 µg/ml). The stimulating electrode was placed on the Schaffer-collateral of the CA1-pyramidal neurons and the recording electrode on the dendrites of the CA3-pyramidal neurons.

Once baseline was stably recorded for 20 min, theta burst was applied and traces collected for an hour. For stimulation concentric bipolar electrodes (FHC, catalog no CBBRC75) were placed into the stratum radiatum. Stimulus intensity was set at 40-60% of maximum response and delivered at 33 mHz through an Isolated Pulse Stimulator (A-M Systems, Model 2100). A custom written program in LabView 7.0 was used for recording and analysis of LTP experiments. A theta burst (TBS; train of 4 pulses at 100 Hz repeated 10 times with 200 ms intervals; repeated 5 times at 10 s intervals) was used as a conditioning stimulus.

Statistical Methods

Data were expressed as the mean ± SEM and evaluated using two-tailed Student's t-test for two groups with one variable tested and equal variances, one-way analysis of variance (ANOVA) with Dunnett's post-hoc or Tukey's post-hoc for multiple groups with only variable tested, or two-way ANOVA with Sidak's post-hoc for plaque quantification (two independent variables of NHE6 genotype and plaque classification). The differences were considered to be significant at $p < 0.05$ (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Software used for data analysis was ImageJ (NIH), LabView7.0 (National Instruments), Odyssey Imaging Systems (Li-Cor), Prism7.0 (GraphPad Software).

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796

797 **AUTHOR CONTRIBUTION**

798 Theresa Pohlkamp designed and performed research, analyzed data, and wrote the
799 paper. Xunde Xian, designed and performed research, analyzed data, reviewed and edited the
800 paper. Connie H Wong, designed and performed research, analyzed data, illustrated figures,
801 reviewed and edited the paper. Murat S Durakoglugil, designed and performed research,
802 analyzed data, reviewed and edited the paper. Takaomi Saido provided the humanized $App^{NL\text{-}F}$
803 mice. Jade Connor, performed research. Bret M Evers, performed research. Charles L White,
804 analyzed data. Robert E Hammer, performed mouse manipulations to create the new $Slc9a6^{fl}$
805 mouse line. Joachim Herz, conceptualization, research design, resources, formal analysis,
806 supervision, funding acquisition, validation, investigation, methodology, writing and editing the
807 paper.
808

FIGURE LEGENDS

810   **Figure 1: ApoE4 Induces Endolysosomal Trafficking Delay.**

811   **(A)** pH regulation within the endolysosomal pathway. Upon receptor binding ApoE is
812   endocytosed along with glutamate receptors (AMPA and NMDA receptors). Cargo that has
813   entered the early endosome (EE) can undergo recycling through a fast direct route without
814   further acidification (fast recycling) or through slower sorting pathways that require further
815   acidification (Casey et al., 2010; Naslavsky and Caplan, 2018). While lipid components are
816   targeted to the lysosome, he majority of receptors, as well as ApoE, remain in endosomal
817   compartments at the cellular periphery where they rapidly move back to the surface (Heeren et
818   al., 1999). The increasingly acidic luminal pH is illustrated as a color gradient and depicted on
819   the left. **(B)** In the presence of ApoE4 early endosomal trafficking and fast recycling are delayed.
820   At the pH of the EE, ApoE4 is near its isoelectric point where solubility is reduced (Wintersteiner
821   and Abramson, 1933), impairing receptor dissociation and resulting in delayed endosomal
822   maturation with a concomitant entrapment of co-endocytosed glutamate receptors. Endosomal
823   pH is regulated by the vesicular ATPase and the counterregulatory action of the proton leakage
824   channel NHE6. NHE6 is an antiporter that exchanges a Na+ or K+ ion for each proton. As the
825   pH decreases, ligands dissociate from their receptors allowing the EE to mature. If dissociation
826   is delayed, as in case of ApoE4, endosomal trafficking is arrested, leading to progressive
827   acidification as Na+, K+ and Cl- ions continue to enter the endosome to maintain charge
828   neutrality while also drawing in water molecules due to osmotic pressure. We thus propose a
829   model in which delayed ApoE4-receptor dissociation prevents early endosomal maturation and
830   causes osmotic swelling while the pH continues to decrease until dissociation occurs. **(C)**
831   Accelerated endosomal acidification by inhibition of the proton leak channel NHE6 resolves
832   ApoE4 accumulation, promotes rapid receptor dissociation and promotes the vesicle entry into
833   the lysosomal delivery or recycling pathways.

834

835   **Figure 2: Generation of *Slc9a6^{fl}* and *Slc9a6^{-}* Mice.**

836   **(A)** Gene targeting strategy. LoxP sites were introduced to flank the first exon (E1) of
837   *Slc9a6* (located on the X-Chromosome) by gene targeting in embryonic stem cells. The
838   targeting construct contained a long arm of homology (LA, grey) upstream of the first loxP site

and the first exon. A loxP/FRT-flanked neomycin resistance cassette was cloned downstream of the first exon, followed by a short arm of homology (SA, grey). The targeted locus is shown below. Targeted stem cells were used to generate chimeric $Slc9a6^{fl}$ mice. Germline NHE6 knockout mice (NHE6$^{-/-}$ (female), NHE6$^{y/-}$ (male); rec indicates recombined allele) were generated by breeding the $Slc9a6^{fl}$ line with Meox-Cre mice. **(B)** Genotyping of wildtype (wt, **+**), floxed (fl), and recombined (rec, -) NHE6 alleles. The PCR amplified regions are indicated in panel A. The wildtype and floxed allele PCR products differ by 50 bp (270 for floxed, 220 for wildtype). **(C)** Western blot showing brain lysates (left) of different NHE6 genotypes after Meox-Cre induced germline recombination. **(D)** Mouse embryonic fibroblasts from $Slc9a6^{-}$ and control littermate were infected with Vamp3-pHluorin2 and excited at 408 and 488 nm with emission measured at 510 nm. **(E)** Vesicular pH measured using a standard curve was significantly decreased in $Slc9a6^{-}$ fibroblasts. Data is expressed as mean ± SEM. Statistical analysis was performed using Student t-test. (**p<0.01) **(F)** The percent of vesicles with pH >6.4 is significantly decreased in $Slc9a6^{-}$ fibroblasts. **(G)** $CAG\text{-}Cre^{ERT2}$ activity after tamoxifen application in a reporter mouse line expressing tdTomato. Cre^{ERT2} recombination activity without (left panel) or with (middle panel) tamoxifen application in the $CAG\text{-}Cre^{ERT2}$-line bred with Rosa26$^{floxStop\text{-}tdTomato}$ line. After tamoxifen induction CreERT2 activity led to a robust tdTomato signal in the hippocampus (middle panel). Pyramidal neurons in the CA1 pyramidal cell layer (PCL) (middle panel) are shown magnified in the right panel.

Figure 3: Long-Term NHE6-Deficiency Induced After Purkinje Cell Maturation Causes Purkinje Cell-loss.

(A) Experimental timeline for B, mice were injected with tamoxifen at 2 months; after one month the brains were analyzed for NHE6 expression (Tam = tamoxifen, Exp. = experiment, mo. = months). **(B)** Western blot showing the efficiency of tamoxifen-induced NHE6 knockout in different brain regions (CA1, CA3, dentate gyrus, cortex, and cerebellum). The knockout efficiency differed between brain regions, it was 80±2% in CA1, 82±5.7% in the CA3, 67±6.8% in the dentate gyrus, 65±11.2% in the cortex, and 74±4.7% in the cerebellum. A total of 3 brains in each group were examined. **(C-F)** NHE6 deficiency leads to cerebellar Purkinje cell loss in germline ($Slc9a6^{-}$, C) and conditional ($Slc9a6^{fl}$;$CAG\text{-}Cre^{ERT2}$, D-F) knockout mice. $Slc9a6^{+}$ includes both female wildtypes ($Slc9a6^{+/+}$) and male wildtypes ($Slc9a6^{y/+}$) mice. $Slc9a6^{-}$ includes

both female knockouts ($Slc9a6^{-/-}$) and male knockouts ($Slc9a6^{y/-}$) mice. In addition, $Slc9a6^{fl}$ mice includes both female $Slc9a6^{fl/fl}$ and male $Slc9a6^{y/fl}$. The timeline shows that $Slc9a6^{fl};CAG-Cre^{ERT2}$ and control mice were tamoxifen-injected at two months and analyzed one year after (D). Calbindin was fluorescently labeled to highlight Purkinje cells in the cerebellum. Massive loss of Purkinje cells was found in $Slc9a6^{-}$ (C, lower panel), compared to wildtype $Slc9a6^{+}$ control (C, upper panel). Long-term loss of NHE6, induced after Purkinje cell maturation at two months of age, also led to massive Purkinje cells-loss when mice were examined one year after NHE6-ablation (E, lower panel). **(F)** Quantification of Purkinje cell-loss in the cerebellum of $Slc9a6^{fl};CAG-Cre^{ERT2}$ mice. Values are expressed as mean ± SEM from 4 independent experiments. Statistical analysis was performed using Student *t*-test. *p<0.05.

Figure 4: NHE6 Deficiency Alleviates ApoE4-Impaired Surface Trafficking Deficits of Apoer2 and Glutamate Receptors.

(A) Timeline for the receptor surface expression assay applied for the experiments shown in B-F. Primary neurons were treated with naturally secreted ApoE3 or ApoE4 and/or Reelin before they underwent surface biotinylation. **(B-F)** Wildtype and $Slc9a6^{-}$ primary neurons were prepared from littermates and used in the receptor surface expression assay described in A. . $Slc9a6^{+}$ includes both female wildtypes ($Slc9a6^{+/+}$) and male wildtypes ($Slc9a6^{y/+}$) mice. $Slc9a6^{-}$ includes both female knockouts ($Slc9a6^{-/-}$) and male knockouts ($Slc9a6^{y/-}$) mice. **(B)** NHE6-deficiency was confirmed via Western blot, β-actin was used as loading control. **(C-F)** ApoE-conditioned media treatment reduces the surface expression of Apoer2 and glutamate receptors in presence of Reelin in primary neurons. Receptor surface levels show a stronger reduction with ApoE4 than ApoE3. NHE6 depletion counteracts the ApoE4-induced reduction of receptor surface expression. Cell surface biotinylation assay was performed for Apoer2 (C), GluN2B (D), GluA1 (E) and GluA2/3 (F). Total levels were analyzed by immunoblotting of whole cell lysates against the same antibodies. β-actin was used as loading control. Quantitative analysis of immunoblot signals is shown in the lower panels (C-F). All data are expressed as mean ± SEM from 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.005. Statistical analysis was performed using one-way ANOVA and Dunnett's post hoc test **(C-F)**.

Figure 5: Effect of Conditional NHE6 Knockout on Reelin-potentiated Synaptic Plasticity.

(A-H) Conditional knockout of NHE6 restores Reelin-enhanced long-term potentiation (LTP) in $Apoe^{APOE4}$ mice. Reelin facilitated induction of LTP in $Apoe^{APOE3}$ **(A, E)**, but not $Apoe^{APOE4}$ **(C, G)** control ($Slc9a6^{fl}$) mice. $Slc9a6$-deficiency in $Apoe^{APOE3}$ mice caused a reduction in Reelin enhanced LTP, such that it is not significantly different from the control LTP **(B, F)**. Importantly, in $Apoe^{APOE4}$;$Slc9a6^{fl}$;CAG-Cre^{ERT2} mice Reelin was able to enhance theta-burst induced potentiation **(D, H)**. Hippocampal slices were prepared from 3 months old double mutant mice with either human $Apoe^{APOE3}$ or $Apoe^{APOE4}$ crossed with $Slc9a6$ conditional knockout mice ($Slc9a6^{fl}$;CAG-Cre^{ERT2}, tamoxifen-injections at 6-8 weeks). Extracellular field recordings were performed in slices treated with or without Reelin. Theta burst stimulation (TBS) was performed after 20 minutes of stable baseline. Representative traces are shown in each panel, before TBS induction (black) and 40 min after TBS (grey). **(E-H)** Quantitative analysis of normalized fEPSP slopes at time intervals as indicated. **(I, J)** Input output curves of $Apoe^{APOE3}$ **(I)** and $Apoe^{APOE4}$**(J)** mice with or without $Slc9a6^{fl}$;CAG-Cre^{ERT2}. $Slc9a6^{fl}$ mice includes both female $Slc9a6^{fl/fl}$ and male $Slc9a6^{y/fl}$ mice. $Apoe$ mice are homozygous for APOE3 or APOE4. All data are expressed as mean ± SEM. N-numbers for each genotype group and treatment are indicated in panels A-D. *p < 0.05. Statistical analysis was performed using Student t-test.

Figure 6: NHE Inhibition or NHE6 Knockdown Does Not Alter BACE1 Activity in Primary Neurons.

(A,B) Pan-NHE inhibition by EMD87580 or lentiviral knockdown of $Slc9a6$ (NHE6) did not alter BACE1 activity in primary neurons of $AppSwe$ mice (Tg2576). **(A)** DIV10 primary neurons were treated with γ-secretase inhibitor L-685458, EMD87580, and/or ApoE4 (as indicated) and harvested for immunoblotting against Aβ-containing C-terminal fragment of APP (βCTF). β-actin was blotted as loading control. Bar graph shows the statistics of n = 3 experiments. **(B)** Primary neurons of $AppSwe$ mice were infected with lentivirus for shRNA expression directed against $Slc9a6$(NHE6) (sh$Slc9a6$) or a scramble control sequence (-) at DIV7. At DIV13 neurons were treated with L-685458 overnight and harvested for immunoblotting against NHE6 and βCTF on DIV14. RAP was blotted as loading control. Bar graph shows the statistics of n = 6 experiments.

930 All data are expressed as mean ± SEM. Statistical analysis was performed using Student *t*-test.

931 n.s. = not significant.

932

933 **Figure 7: NHE6-Deficiency Decreases Plaque Formation in Both *App^{NL-F}* and *App^{NL-}***

934 ***^{F};Apoe^{APOE4}* Mice.**

935 **(A,B)** NHE6-deficient *App^{NL-F}* and control *App^{NL-F}* mice were analyzed for plaque

936 deposition at an age of 12 months. Thioflavin S staining was performed to visualize plaques.

937 Plaques were found more frequently in the control *App^{NL-F}* mice (left panel in **A**), magnifications

938 of the boxed areas are shown in the two middle panels. The plaque load between *Slc9a6^{-}* mice

939 and control littermates (all *App^{NL-F}*) was compared and analyzed. **(B)** In the *Slc9a6^{-}* littermates

940 the plaque number was reduced, when compared to controls. **(C-D)** *Slc9a6^{fl};CAG-*

941 *Cre^{ERT2};App^{NL-F};Apoe^{APOE4}* and *Slc9a6^{fl};App^{NL-F};Apoe^{APOE4}* mice were analyzed for plaque

942 deposition. NHE6 was ablated at two months and brains were analyzed at 13.5-16 months.

943 4G8-immunolabeling against Aβ was performed to visualize plaques. In *App^{NL-F};Apoe^{APOE4}* mice

944 conditional *Slc9a6* knockout caused a reduction in plaque load compared to the *Slc9a6^{fl}* control

945 littermates (**C**). Magnifications of the boxed areas in C are shown in the middle. **(D)** Plaque load

946 was analyzed and compared between *Slc9a6^{fl};CAG-Cre^{ERT2}* mice and floxed control littermates.

947 **I** Hematoxylin and eosin staining (H&E) was performed to investigate for gross anatomic

948 abnormalities in the *Slc9a6^{fl};CAG-Cre^{ERT};App^{NL-F};Apoe^{APOE4}* and *Slc9a6^{fl};App^{NL-F};Apoe^{APOE4}*

949 mice. **(F-I)** Brain area **(F)**, cortical thickness **(G)**, hippocampal (HC) area **(H)**, and CA1 thickness

950 **(I)** were analyzed. Student *t*-test did not reveal a significant difference. Plaques were

951 differentiated by size or staining density as described in detail in the supplements (**Figure 7 –**

952 **Figure Supplement 2**). Labeled plaques were analyzed by a blinded observer. All data are

953 expressed as mean ± SEM. **(B)** *Slc9a6^{-}* n=5, control n=8, **(C)** *Slc9a6^{fl}* n=8, *Slc9a6^{fl};CAG-Cre^{ERT2}*

954 n=8), in **(F-I)** derived from n=5 (*Slc9a6^{fl}*) and n=6 (*Slc9a6^{fl};CAG-Cre^{ERT2}*) animals. *p < 0.05.

955 **p<0.01, ***p<0.005. *Slc9a6^{+}* represents both female wildtypes (*Slc9a6^{+/+}*) and male wildtypes

956 (*Slc9a6^{y/+}*). *Slc9a6^{-}* represents both female knockouts (*Slc9a6^{-/-}*) and male knockouts (*Slc9a6^{y/-}*).

957 In addition, *Slc9a6^{fl}* mice includes both female *Slc9a6^{fl/fl}* and male *Slc9a6^{y/fl}* mice. *Apoe* mice are

958 homozygous for APOE4 (*Apoe^{APOE4}*). *App^{NL-F}* mice are homozygous for human NL-F knockin

959 mutation (*App^{NL-F/NL-F}*). Statistical analysis was performed using two-way ANOVA with Sidak's

960 post-hoc test **(B and D)** and Student *t*-test **(F-I)**.

961

Figure 7 – Figure Supplement 1: Gross Anatomical Brain Structure in *Slc9a6*‾ Mice.

(A) Hematoxylin and eosin staining (H&E) was performed to investigate for gross anatomic abnormalities in the *Slc9a6*‾;*App^{NL-F}* and *App^{NL-F}* mice. Structures representing plaques were found in the *App^{NL-F}* control groups (magnified example is shown in the middle panel). **(B-E)** Brain area **(B)**, cortical thickness **(C)**, hippocampal (HC) area **(D)**, and CA1 thickness **(E)** were analyzed. All data are expressed as mean ± SEM. Student *t*-test did not reveal significant differences, n=3 (control) and n=4 (*Slc9a6*‾). *Slc9a6*^+ represents both female wildtypes (*Slc9a6*^{+/+}) and male wildtypes (*Slc9a6*^{y/+}). *Slc9a6*‾ represents both female knockouts (*Slc9a6*^{-/-}) and male knockouts (*Slc9a6*^{y/-}). *App^{NL-F}* mice are homozygous for human NL-F knockin mutation (*App^{NL-F/NL-F}*).

972

Figure 7 – Figure Supplement 2: Example of Thioflavin S Stained Plaques for Quantification.

Different types of Thioflavin S stained plaques and 4G8-immunoreactive accumulations in *App^{NL-F}* brains are shown. Different sizes of plaques were grouped together for quantification Thioflavin S labeled plaques (**Figure 7 and Figure 7 – Figure Supplement 3**). Plaques bigger in diameter than 20µm with a dense core were defined as big. Medium sized plaques had a diameter between 10-20µm with a dense core. Small plaques were smaller than 10µm and often represented individual cells. 4G8-labeled plaques were differentiated by diffuse or dense appearance as depicted in the examples.

982

Figure 7 – Figure Supplement 3: NHE6-Deficiency Decreases Plaque Formation in Both *App^{NL-F}* and *App^{NL-F}*;*Apoe^{APOE4}* Mice.

(A-B) NHE6-deficient *App^{NL-F}* and control *App^{NL-F}* mice were analyzed for plaque deposition at an age of 12 months. 4G8-immunolabeling against Aβ **(A)** visualized more plaques in the control *App^{NL-F}* mice. The plaque load between *Slc9a6*‾ mice and control littermates (all *App^{NL-F}*) was compared and analyzed **(B).** In the *Slc9a6*‾ littermates the plaque number was reduced, when compared to controls. **(C-E)** Soluble (TBS) and insoluble (GuHCl and 70% FA) Aβ fractions of cortical lysates were analyzed by commercial ELISA. 1.5-year-old *Slc9a6*‾ mice

991 showed less insoluble Aβ than their control littermates (all App^{NL-F}). **(F-G)** $Slc9a6^{fl};CAG$-

992 $Cre^{ERT2};App^{NL-F};Apoe^{APOE4}$ and $Slc9a6^{fl};App^{NL-F};Apoe^{APOE4}$ mice were analyzed for plaque

993 deposition. NHE6 was ablated at two months and brains were analyzed at 13.5-16 months.

994 Thioflavin S staining was performed to visualize plaques. With $App^{NL-F};Apoe^{APOE4}$ background

995 the $Slc9a6^{fl};CAG-Cre^{ERT2}$ mice had a reduced plaque load compared to the $Slc9a6^{fl}$ control mice

996 (left panel in **F**). Magnifications of the boxed areas in left panel are shown in the middle. **(G)**

997 Plaque load was analyzed and compared between $Slc9a6^{fl};CAG-Cre^{ERT2}$ mice and floxed control

998 littermates. Plaques were differentiated by size or staining density as described in detail in the

999 supplements (**Figure 7 – Figure Supplement 2**). Labeled plaques were analyzed by a blinded

1000 observer **(B, G)**. All data (immunohistochemistry: $Slc9a6^{-}$ n=5, control n=4, $Slc9a6^{fl}$ n=10,

1001 $Slc9a6^{fl};CAG-Cre^{ERT2}$ n=12; biochemistry: $Slc9a6^{-}$ n=11, control n=7) are expressed as mean ±

1002 SEM. *$p < 0.05$. **$p<0.01$, ***$p<0.005$. $Slc9a6^{+}$ represents both female wildtypes ($Slc9a6^{+/+}$) and

1003 male wildtypes ($Slc9a6^{y/+}$). $Slc9a6^{-}$ represents both female knockouts ($Slc9a6^{-/-}$) and male

1004 knockouts ($Slc9a6^{y/-}$). In addition, $Slc9a6^{fl}$ mice includes both female $Slc9a6^{fl/fl}$ and male

1005 $Slc9a6^{y/fl}$ mice. $Apoe$ mice are homozygous for APOE4. App^{NL-F} mice are homozygous for

1006 human NL-F knockin mutation ($App^{NL-F/NL-F}$). Statistical analysis was performed using two-way

1007 ANOVA with Sidak's post-hoc test.

1008

1009 **Figure 8. Age Dependent Increase in Plaque Load is abolished in $Slc9a6^{fl};CAG$-**

1010 **$Cre^{ERT2};App^{NL-F};Apoe^{APOE4}$ Mice.**

1011 **(A-E)** $Slc9a6^{fl};CAG-Cre^{ERT2};App^{NL-F};Apoe^{APOE4}$ and $Slc9a6^{fl};App^{NL-F};Apoe^{APOE4}$ mice were

1012 analyzed for plaque deposition. NHE6 was ablated at two months and brains were analyzed at

1013 13.5-16 months. 4G8-immunolabeling against Aβ **(A,B)** and Thioflavin S staining **(C-E)** were

1014 performed to visualize plaques **(Figure 7C and Figure 7 – Figure Supplement 3F).** Plaque

1015 load was analyzed and compared between $Slc9a6^{fl};CAG-Cre^{ERT2}$ mice and floxed control

1016 littermates. Plaques were differentiated by staining intensity **(A, B)** or size **(C-E)** as described in

1017 the supplements (**Figure 7 – Figure Supplement 2**). In the time range analyzed, plaque load

1018 increased by age in control, but not in $Slc9a6^{fl};CAG-Cre^{ERT2}$ mice. Plaques were analyzed by a

1019 blinded observer. Plaque count ($Slc9a6^{fl};CAG-Cre^{ERT2}$ n=8, $Slc9a6^{fl}$ n=8 for **A** and **B**;

1020 $Slc9a6^{fl};CAG-Cre^{ERT2}$ n=12; $Slc9a6^{fl}$ n=10 in **C-E**) is plotted against age of mice. $Slc9a6^{fl}$ mice

1021 includes both female *Slc9a6$^{fl/fl}$* and male *Slc9a6$^{y/fl}$* mice. *Apoe* mice are homozygous for APOE4.

1022 *App^{NL-F}* mice are homozygous for human NL-F knockin mutation (*App$^{NL-F/NL-F}$*).

1023

1024 **Figure 9. Microglia and Astrocytes Surround Plaques in Both *App^{NL-F}* Control and**

1025 ***App^{NL-F}*;*Slc9a6$^-$* Brains**

1026 **(A-B)** Co-labeling of microglia (Iba1, green, **A**) or astrocytes (GFAP, green, **B**) with Aβ

1027 (6E10, red) in brain slices of *App^{NL-F}* and *App^{NL-F}*;*Slc9a6$^-$* mice. **(C)** Quantification of plaques in

1028 control and *Slc9a6$^-$* brain slices. **(D)** Bar graph showing the intensity density of Iba1/6E10 as

1029 quantitative measure of microglia surrounding plaques. **(E)** Statistical analysis of 6E10 positive

1030 microglia and **(F)** the intensity of 6E10 signal within microglia. **(G)** Bar graph showing the

1031 intensity density of GFAP/Aβ as quantitative measure of astrocytes surrounding plaques. Data

1032 were analyzed by a blinded observer. All data are expressed as mean ± SEM. Data were

1033 obtained from n=4 (control) and n=5 (*Slc9a6$^-$*) mice **(A-G)**. **(D)** n=33 (control) and n=23 (*Slc9a6$^-$*)

1034 plaques were analyzed, in **(E)** n=22 (control) and n=24 (*Slc9a6$^-$*) microscopical pictures were

1035 analyzed, in **(F)** n=31 (control) and n=38 (*Slc9a6$^-$*) 6E10 postive (defined as signal intensity

1036 above 500) microglia were analyzed, in **(G)** n=33 (control) and n=18 (*Slc9a6$^-$*) plaques were

1037 analyzed. Student *t*-test revealed a difference in **C** (**p<0.01) and did not reveal significant

1038 differences in **D-G**. *Slc9a6$^+$* represents both female wildtypes (*Slc9a6$^{+/+}$*) and male wildtypes

1039 (*Slc9a6$^{y/+}$*). *Slc9a6$^-$* represents both female knockouts (*Slc9a6$^{-/-}$*) and male knockouts (*Slc9a6$^{y/-}$*).

1040 *App^{NL-F}* mice are homozygous for human NL-F knockin mutation (*App$^{NL-F/NL-F}$*).

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1043 **Figure 9 – Figure Supplement 1: NHE6-Deficiency Causes an Increase in Iba1 and**

1044 **GFAP Immunoreactivity in Both *App^{NL-F}* and *App^{NL-F}*;*Apoe^{APOE4}* mice.**

1045 **(A)** Immunohistochemistry against glial fibrillary acidic protein (GFAP) and ionized calcium-

1046 binding adapter molecule (Iba1) was performed on brain slices obtained from one-year-old

1047 *App^{NL-F}* mice deficient for NHE6 (*App^{NL-F}*;*Slc9a6$^-$*) and control littermates (*App^{NL-F}*). GFAP (upper

1048 panels) and Iba1 (lower panels) immunoreactivity was increased in NHE6-deficient *App^{NL-F}* mice

1049 when compared to NHE6 expressing controls. **(B)** *Slc9a6^{fl}*;*CAG-Cre^{ERT2}*;*App^{NL-F}*;*Apoe^{APOE4}* and

1050 *Slc9a6^{fl}*;*App^{NL-F}*;*Apoe^{APOE4}* mice were analyzed for immunoreactivity GFAP and Iba1. Mice were

1051 injected with tamoxifen at two months and brain slices obtained from 13.5-16 months old mice.

1052 GFAP (upper panels) and Iba1 (lower panels) immunoreactivity was increased in NHE6-

1053 deficient App^{NL-F};$Apoe^{APOE4}$ mice when compared to NHE6 expressing controls. **(C-F)** Intensity

1054 of the staining in various areas was compared for GFAP **(C)** and Iba1 **(D)** in App^{NL-F};$Slc9a6^-$ and

1055 App^{NL-F} mice. Intensity of the staining for GFAP **(E)** and Iba1 **(F)** in $Slc9a6^{fl}$;CAG-Cre^{ERT2};App^{NL-}

1056 ^{F};$Apoe^{APOE4}$ and $Slc9a6^{fl}$;App^{NL-F};$Apoe^{APOE4}$ mice. Analysis was performed by a blinded

1057 observer. 'White matter' comprises corpus callosum, cingulum and external capsule. All data

1058 ($Slc9a6^-$ n=5; control n=4; $Slc9a6^{fl}$;CAG-Cre^{ERT2} n=6; $Slc9a6^{fl}$ n=8) are expressed as mean ±

1059 SEM. *p < 0.05. **p<0.01, ***p<0.005. $Slc9a6^+$ represents both female wildtypes ($Slc9a6^{+/+}$) and

1060 male wildtypes ($Slc9a6^{y/+}$). $Slc9a6^-$ represents both female knockouts ($Slc9a6^{-/-}$) and male

1061 knockouts ($Slc9a6^{y/-}$). In addition, $Slc9a6^{fl}$ mice includes both female $Slc9a6^{fl/fl}$ and male

1062 $Slc9a6^{y/fl}$ mice. For the Cag-Cre^{ERT2}, Cre^+ indicates the absence of Cre ($Slc9a6^{fl}$ alone) and

1063 Cag-Cre^{ERT2} indicates the presence of Cre ($Slc9a6^{fl}$;CAG-Cre^{ERT2}), which are both injected with

1064 Tamoxifen at 2 months of age. $Apoe$ mice are homozygous for APOE4. App^{NL-F} mice are

1065 homozygous for human NL-F knockin mutation ($App^{NL-F/NL-F}$). Statistical analysis was performed

1066 using Student t-test.

1067

1068   **Figure 9 – Figure Supplement 2: Examples of Plaques Surrounded by Microglia**

1069 **and Astrocytes.**

1070   **(A)** Co-labeling of microglia (Iba1, green) and Aβ (6E10, red) in brain slices of App^{NL-F}

1071 and App^{NL-F};$Slc9a6^-$ mice. **(B)** Co-labeling of astrocytes (GFAP, green) and Aβ (6E10, red) in

1072 brain slices of App^{NL-F} and App^{NL-F}; $Slc9a6^-$ mice. $Slc9a6^+$ represents both female wildtypes

1073 ($Slc9a6^{+/+}$) and male wildtypes ($Slc9a6^{y/+}$). $Slc9a6^-$ represents both female knockouts ($Slc9a6^{-/-}$)

1074 and male knockouts ($Slc9a6^{y/-}$). App^{NL-F} mice are homozygous for human NL-F knockin mutation

1075 ($App^{NL-F/NL-F}$).

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1407

1 *Key Resources Table*

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
strain, strain background (*Mus musculus*)	Mouse/*Slc9a6^{fl}*	This study		Refer to Methods section for detailed description of mouse model production.
strain, strain background (*Mus musculus*)	Mouse/*Slc9a6$^-$*	This study		Refer to Methods section for detailed description of mouse model production.
strain, strain background (*Mus musculus*)	Mouse/*Apoe^{APOE3}*	Sullivan et al., 1997	IMSR_TAC:2542	*Apoe^{APOE3}*
strain, strain background (*Mus musculus*)	Mouse/*Apoe^{APOE4}*	Knouff et al., 1999	IMSR_TAC:3518	*Apoe^{APOE4}*
strain, strain background (*Mus musculus*)	Mouse/B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J	The Jackson Laboratory Madisen et al., 2010	JAX #007909	*ROSA$^{floxedStop-tdTomato}$*
strain, strain background (*Mus musculus*)	Mouse/CAG-cre/Esr1)5Amc/J	The Jackson Laboratory Hayashi et al., 2002	JAX #004682	*CAG-Cre^{ERT2}*
strain, strain background (*Mus musculus*)	Mouse/B6.129S4-Meox2tm1(cre)Sor/J	The Jackson Laboratory Tallquist and Soriano 2000	JAX 003755	*Meox-Cre*
strain, strain background (*Mus musculus*)	*App^{NL-F}*	Saito et al., 2014		*App^{NL-F}*
strain, strain background (*Mus musculus*)	Tg2576	Charles River Hsiao et al., 1996	Charles River Tg2576	Tg2576, *APPSwe*
strain, strain background (*Rattus*	SD rat	Charles River	SC:400	

norvegicus)				
cell line (*Homo sapiens*)	HEK293	Thermo Fisher	R70507, RRID:CVCL_0045	
cell line (*Homo sapiens*)	HEK293-T	ATCC	CRL-3216	
cell line (*Mus musculus*)	Neuro-2a	ATCC	CCL-131	
cell line (*Mus musculus*)	NHE6-KO (*Slc9a6*-) mouse embryonic fibroblasts (MEFs)	This study		Refer to Methods section for detailed description of MEF production.
cell line (*Mus musculus*)	*Slc9a6*+ MEFs (*Slc9a6*- littermate)	This study		Refer to Methods section for detailed description of MEF production.
antibody	anti-Aβ (clone 6E10) (Mouse monoclonal)	Covance	SIG-39320 RRID:AB_662798	WB and IHC (1:1000)
antibody	anti-Aβ (clone 4G8) (Mouse monoclonal)	Covance	SIG-39220 RRID:AB_10175152	IHC (1:1000)
antibody	anti-phospho tyrosine (clone 4G10) (Mouse monoclonal)	EMD Millipore	Millipore Cat# 05-321, RRID:AB_309678	WB (1:1000)
antibody	anti-Apoer2 (Rabbit polyclonal)	Herz Lab, #2561, Trommsdorff et al., 1999		WB (1:1000)
antibody	anti-β-Actin (Rabbit polyclonal)	Abcam	Ab8227, RRID:AB_2305186	WB (1:3000)
antibody	Anti-Calbindin D-28k (Mouse monoclonal)	Swant	Swant Cat# 300, RRID:AB_10000347	IHC (1:1000)

antibody	Anti-GFAP (Rabbit polyclonal)	Abcam	Abcam Cat# ab7260, RRID:AB_305808	IHC (1:2000)
antibody	Anti-GluA1 (Rabbit polyclonal)	Abcam	ab31232, RRID:AB_2113447	WB (1:1000)
antibody	Anti-GluA2/3 (Rabbit polyclonal)	EMD Millipore	07-598, RRID:AB_310741	WB (1:1000)
antibody	Anti-GluN2B (Rabbit polyclonal)	Cell Signaling Technology	4207S, RRID:AB_1264223	WB (1:1000)
antibody	Anti-Iba1 (Rabbit polyclonal)	Wako	019-19741, RRID:AB_839504	IHC (1:1000)
antibody	Anti-NHE6 (C-terminus) (Rabbit polyclonal)	Herz Lab, Xian et al., 2018		WB (1:1000)
antibody	Anti-mouse-IgG AF594 (Goat polyclonal)	Thermo Fisher	A-11032, RRID:AB_2534091	IHC (1:500)
antibody	Anti-rabbit-IgG AF488 (Goat polyclonal)	Thermo Fisher	A-11034, RRID:AB_2576217	IHC (1:500)
Commercial assay or kit	Anti-mouse-IgG staining kit	Vector	MP-7602, RRID:AB_2336532	
commercial assay or kit	Anti-rabbit-IgG staining kit	Vector	MP-7601, RRID:AB_2336533	
chemical compound, drug	Antigen retrieval citrate buffer	BioGenex, Cat	HK086-9K	
chemical compound, drug	B-27 Supplement	Thermo Fisher	17504044	

	(50X), serum free			
chemical compound, drug	Cytoseal 60	Thermo Fisher	8310	
chemical compound, drug	DMEM	Sigma-Aldrich	D6046	
chemical compound, drug	FuGENE	Promega	E2311	
chemical compound, drug	HBSS (1X)	Gibco	14175	
chemical compound, drug	L-Glutamic acid (Glutamate)	Sigma-Aldrich	G1251	
chemical compound, drug	γ-secretase inhibitor L-685458	Tocris Bioscience	2627	
chemical compound, drug	Penicillin-Streptomycin Solution, 100X	Corning	30-002-CI	
chemical compound, drug	Neurobasal Medium (1X) Liquid without Phenol Red	Thermo Fisher	12348017	
chemical compound, drug	NeutrAvidin Agarose	Thermo Fisher	29201	
chemical compound, drug	Nonidet P-40 Alternative	EMD Millipore	492016	
chemical compound, drug	32% Paraformaldeh yde AQ solution	Fisher Scientific	15714S	
chemical compound, drug	PBS (1X)	Sigma-Aldrich	D8537	
chemical compound, drug	Penisillin-Streptomycin	Corning	30-002-CI	
chemical compound, drug	Phosphatase Inhibitor	Thermo Fisher	78420	

	Cocktail			
chemical compound, drug	Poly-D-Lysine	Sigma-Aldrich	A-003-M	
chemical compound, drug	Protein A-Sepharose 4B	Thermo Fisher	101042	
chemical compound, drug	Proteinase Inhibitor Cocktail	Sigma-Aldrich	P8340	
chemical compound, drug	Sulfo-NHS-SS-biotin	Pierce	21331	
chemical compound, drug	Triton X-100	Sigma-Aldrich	CAS9002-93-1	
chemical compound, drug	Tween 20	Sigma	P1379	
other	Vectashield with DAPI	Vector Labs	H-1200	(DAPI 1.5 µg/mL)
transfected construct (*Mus musculus*)	pCrl, Reelin expression vector	(D'Arcangelo et al., 1997)	N/A	
transfected construct (*Homo sapiens*)	pcDNA3.1-ApoE3	Chen et al., 2010	N/A	progenitor pcDNA3.1-Zeo
transfected construct (*Homo sapiens*)	pcDNA3.1-ApoE4	Chen et al., 2010	N/A	progenitor pcDNA3.1-Zeo
transfected construct (*Mus musculus*)	pLKO.1 scramble shRNA	Xian et al., 2018	N/A	
transfected construct (*Mus musculus*)	pLKO.1 shNHE6	Open Biosystem	TRCN0000068828	Refers to shNHE6-a in Xian et al., 2018
transfected construct (*Mus musculus*)	psPAX2	Addgene	12260	Plasmid was a gift from Didier Trono
transfected	pMD2.G	Addgene	12259	Plasmid was a gift from Didier

construct (*Mus musculus*)				Trono
transfected construct (*Mus musculus*)	pJB-NHE6 targeting vector	This study	N/A	Refer to Methods section for detailed description.
Recombinant DNA reagent	pJB1 (plasmid)	Braybrooke et al., 2000	N/A	
Recombinant DNA reagent	pCR4-TOPO (plasmid)	Thermofisher	K457502	
Recombinant DNA reagent	pLVCMVfull (plasmid)	Xian et al., 2018	N/A	
Recombinant DNA reagent	pME (plasmid)	Stawicki et al., 2014	Addgene #73794	Plasmd was a gift from David Raible
Recombinant DNA reagent	pLVCMV Vamp3-pHluorin2 (plasmid)	This study	N/A	Refer to Methods section for detailed description.
Recombinant DNA reagent	BAC containing murine NHE6 sequence (Bacterial artificial chromosome)	BACPAC Resources Center	RP23 364F14	
software, algorithm	Adobe Creative Cloud	Adobe	RRID:SCR_010279	
software, algorithm	GraphPad Prism 7.0	GraphPad Software	RRID:SCR_002798	
software, algorithm	Fiji/ImageJ	NIH	RRID:SCR_002285	
software, algorithm	LabView7.0	National Instruments	RRID:SCR_014325	
software, algorithm	NDP.view2	Hamamatsu Photonics		
software, algorithm	Odyssey Imaging System	LI-COR	RRID:SCR_014579	

Type	Name	Source	Identifier	Sequence
software, algorithm	Clustal Omega	EMBL-EBI	RRID:SCR_001591	
software, algorithm	Leica TCS SPE	Leica	RRID:SCR_002140	
Sequence-based reagent	SA forward	IDT		GGATCCGTGTGTGTGTTGGGGGAGGGA
Sequence-based reagent	SA reverese	Integrated DNA Technology		CTCGAGCTCACAATCAGCCCTTTAAATATGCC
Sequence-based reagent	GAP repair US forward	Integrated DNA Technology		AAGCTTGCGGCCGCTTCAATTTCTGTCCTTGCTACTG
Sequence-based reagent	GAP repair US reverse	Integrated DNA Technology		AGATCTCAAGAAAGTTAGCTAGAAGTGTGTC
Sequence-based reagent	GAP repair DS forward	Integrated DNA Technology		AGATCTGTAGAGGATGTGGGAAAGAGAG
Sequence-based reagent	GAP repair DS reverse	Integrated DNA Technology		GTCGACGCGGCCGACACACACAGATAAATAACCTCAAAAG
Sequence-based reagent	5' flanking 1st LoxP fragment forward	Integrated DNA Technology		GCTTCTCTCGAGCAAGAGTCAAC
Sequence-based reagent	5' flanking 1st LoxP fragment reverse	Integrated DNA Technology		GATATCAGCAGGTACCACCAAGATCTCAACCTTATTGTCCTATATGCACAAAC
Sequence-based reagent	3' flanking 1st LoxP fragment forward	Integrated DNA Technology		GTCTTGTTGGTACCTGATGAAATGGACTACCTCCACTTG
Sequence-based reagent	3' flanking 1st LoxP fragment reverse	Integrated DNA Technology		ATCGATCTTCATAACCCATCTGGATA
Sequence-based reagent	LoxP Oligo forward	Integrated DNA Technology		GATCTGCTCAGCATAACTTCGTATAGCATACATTATACGAAGTTATGGTAC
Sequence-based reagent	LoxP Oligo reverse	Integrated DNA Technology		CATAACTTCGTATAATGTATGCTATACGAAGTTATGCTGAGCAGATC
Sequence-based	genotyping	Integrated DNA		GAGGAAGCAAAGTGTCAGC

reagent	NHE6-floxed and wt forward	Technology		TCC
Sequence-based reagent	genotyping NHE6-floxed and wt reverse	Integrated DNA Technology		CTAATCCCCTCGGATGCTGC TC
Sequence-based reagent	genotyping NHE6-KO forward	Integrated DNA Technology		GAGGAAGCAAAGTGTCAGC TCC
Sequence-based reagent	genotyping NHE6-KO reverse	Integrated DNA Technology		CCTCACAAGACTAGAGAAAT GGTTC
Sequence-based reagent	Vamp3 forward	Integrated DNA Technology		TTCAAGCTTCACCATGTCTA CAGGTGTGCCTTCGGGGTC
Sequence-based reagent	Vamp3 reverse	Integrated DNA Technology		CATTGTCATCATCATCATCG TGTGGTGTGTCTCTAAGCTG AGCAACAGCGCCGTGGACG GCACCGCCGGCCCCGGCAG CATCGCCACCAAGCTTAAC
Sequence-based reagent	pHluorin2 forward	Integrated DNA Technology		CCGGTCCCAAGCTTATGGTG AGCAAGGGCGAGGAGCTGT TC
Sequence-based reagent	pHluorin2 reverse	Integrated DNA Technology		GCCCTCTTCTAGAGAATTCA CTTGTACAGCTCGTCCATGC CGTG

2

A

Extracellular pH 7.4

Intracellular pH 7.3

Recycling Endosomes pH 6.5

Early Endosomes pH 6.4

Sorting Endosomes pH 6.2

Late Endosomes pH 5.5

Lysosomes pH < 5

AMPAR   NMDAR   ApoER2

ApoE

NpXY

Fast Recyling

Recycling Endosome

Early Endosome

Sorting Endosome

Late Endosome

Lysosome

B

v-ATPase

H^+

NHE6

Na^+/K^+

H^+

Na^+
K^+
H^+
pH 6.4

Swelling & Recycling block

H^+

Na^+/K^+

H^+

SLOW

Na^+
K^+
H^+

Cl^-
H_2O

pH↓

Cl^-
H_2O

H^+

Na^+/K^+

H^+

Cl^-
H_2O

Na^+
K^+
H^+

pH↓↓

Cl^-
H_2O

C

v-ATPase

H^+

NHE6

Na^+/K^+

H^+

Na^+
K^+
H^+
pH 6.4

FAST

H^+

Na^+
K^+

H^+

pH↓

Recycle to Plasma Membrane

A *Slc9a6* (NHE6) wt locus, X-chr.

220 bp (wt PCR)

E1

homologous recombination

LA

targeting construct

E1   Neo   SA

targeted locus (floxed)

E1   Neo

270 bp (fl PCR)

recombined (rec)

400 bp (rec PCR)

loxP   loxP/FRT

B Meox-Cre

Slc9a6 y/+
Slc9a6 fl/+
Slc9a6 y/fl
Slc9a6 +/+
Slc9a6 y/-
Slc9a6 -/-
Slc9a6 +/-

bp
270
220
400

fl
wt
rec

C E13.5 Brain

Slc9a6 -/-
Slc9a6 +/-
Slc9a6 +/+

kDa
150
100
37

Total NHE6

RAP

D
Excit. 405nm
Excit. 488nm
Merged
5 µm

E

Standard Curve

405/488 Intensity vs pH

Vesicular pH

**

pH

Slc9a6+ (wildtype)
Slc9a6−

F **Vesicular pH Percentage**

pH > 6.4
pH < 6.4

% vesicles

Slc9a6+ (wildtype)
Slc9a6−

G

Rosa26 ^floxStop-tdTomato^; *CAG-Cre^ERT2*

+ Tamoxifen
Rosa26 ^floxStop-tdTomato^; *CAG-Cre^ERT2*

CA1
CA3
MF   DG
PCL

200 µm
50 µm

A

P0   + Tam   Exp

2 mo.   1 mo.

B

kDa	CA1	CA3	Dentate Gyrus	Cortex	Cerebellum	
150						Total NHE6
75						
37						RAP
	− +	− +	− +	− +	− +	CRE
	+ Tam.	+ Tam.	+ Tam.	+ Tam.	+ Tam.	

C

Slc9a6 +

Slc9a6 −

200 µm

D

P0 + Tam   Exp

2 mo.   12 mo.

E

Slc9a6 fl

Slc9a6 fl ; CAG-Cre ERT2

200 µm

F

Relative Cell Count

*

1.5

1.0

0.5

0.0

Slc9a6 fl   *Slc9a6 fl CAG-Cre ERT2*

A ApoE → 30 mins → Reelin → 30 mins → Surface Biotinylation Assay

B

	C+R	E3+R	E4+R	C+R	E3+R	E4+R
kDa — 150, 75, 50

Total NHE6

β-actin

$Slc9a6^+$ $Slc9a6^-$

C Apoer2
$Slc9a6^+$ $Slc9a6^-$
***
Normalized surface/total
100, 50
kDa 150 / 150
Surface / Total
C+R E3+R E4+R C+R E3+R E4+R

D GluN2B
$Slc9a6^+$ $Slc9a6^-$
* ***
150 / 150
C+R E3+R E4+R C+R E3+R E4+R

E GluA1
$Slc9a6^+$ $Slc9a6^-$
* ***
100 / 100
C+R E3+R E4+R C+R E3+R E4+R

F GluA2/3
$Slc9a6^+$ $Slc9a6^-$
* ***
100 / 100
Surface / Total
C+R E3+R E4+R C+R E3+R E4+R

A — *Slc9a6^{fl};Apoe^{APOE3}*

B — *Slc9a6^{fl};CAG-Cre^{ERT2};Apoe^{APOE3}*

C — *Slc9a6^{fl};Apoe^{APOE4}*

D — *Slc9a6^{fl};CAG-Cre^{ERT2};Apoe^{APOE4}*

A. Control (n=12), Reelin (n=13)

B. Control (n=28), Reelin (n=38)

C. Control (n=23), Reelin (n=21)

D. Control (n=29), Reelin (n=49)

0.5 mV
10 msec

E, F, G, H — Normalized LTP; Time (min): 1-60, 1-20, 21-40, 41-60; Control, Reelin

I — *Slc9a6^{fl};Apoe^{APOE3}*, *Slc9a6^{fl};CAG-Cre^{ERT2};Apoe^{APOE3}*

J — *Slc9a6^{fl};Apoe^{APOE4}*, *Slc9a6^{fl};CAG-Cre^{ERT2};Apoe^{APOE4}*

A

	C	E4	E4	E4	E4	
γ-secretase inhibitor	-	-	-	+	+	
EMD87580	-	-	+	-	+	
kDa						
15 —						βCTF
37 —						β-actin

B

shSlc9a6	+	-	+	-	
γ-secretase inhibitor	-	-	+	+	
kDa					NHE6 Dimer
15 —					βCTF
37 —					RAP

A ThioS

App^NL-F

50 μm

Slc9a6^−;App^NL-F

200 μm

B ThioS+

App^{NL-F}
$Slc9a6^-;App^{NL-F}$

Plaque count

Large   Medium   Small

** ***

C 4G8

Slc9a6^fl;App^NL-F; Apoe^APOE4

a
b
c
d

25 μm

Slc9a6^fl;CAG-Cre^ERT2; App^NL-F;Apoe^APOE4

500 μm

D 4G8+

$Slc9a6^{fl};App^{NL-F}; Apoe^{APOE4}$
$Slc9a6^{fl};CAG-Cre^{ERT2}; App^{NL-F};Apoe^{APOE4}$

***

Diffuse   Dense

E

Slc9a6^fl;App^NL-F; Apoe^APOE4

Slc9a6^fl;CAG-Cre^ERT2; App^NL-F;Apoe^APOE4

H&E

1 mm

F Brain Area

Area (mm²)

G Cortical Thickness

Thickness (μm)

H HC Area

Area (μm²)

I CA1 Thickness

Thickness (μm)

$Slc9a6^{fl};App^{NL-F}; Apoe^{APOE4}$
$Slc9a6^{fl};CAG-Cre^{ERT2}; App^{NL-F};Apoe^{APOE4}$

A 4G8+ (Dense)

Plaque load

Age (days)

B 4G8+ (Diffuse)

Plaque load

Age (days)

$Slc9a6^{fl}$;App^{NL-F};$Apoe^{APOE4}$

$Slc9a6^{fl}$;CAG-Cre^{ERT2};App^{NL-F};$Apoe^{APOE4}$

C ThioS+ (Small)

Plaque load

Age (days)

D ThioS+ (Medium)

Plaque count

Age (days)

E ThioS+ (Large)

Plaque load

Age (days)

A

App NL-F

DAPI 6E10 Iba1

*Slc9a6⁻;
App NL-F*

25 μm

B

App NL-F

DAPI 6E10 GFAP

*Slc9a6⁻;
App NL-F*

50 μm

C
6E10+ plaques

Plaque count

15

10

5

0

**

D
**Microglia in
Plaques**

Fluorescence intensity density Iba1/Aβ

15

10

5

0

E
**6E10+
Microglia**

Aβ pos. microglia/total microglia

0.8

0.6

0.4

0.2

0.0

F
**6E10 Reactivity
in Microglia**

6E10 Fluorescence intensity [Log]

100000

10000

1000

100

G
**Astrocytes
in Plaques**

Fluorescence intensity density GFAP/Aβ

8

6

4

2

0

■ *App NL-F*   ■ *Slc9a6⁻;App NL-F*