NHE6 depletion corrects ApoE4-mediated synaptic impairments and reduces amyloid plaque load

Theresa Pohlkamp1,2,*, Xunde Xian1,2,†*, Connie H Wong1,2,†, Murat S Durakoglugil1,2, Gordon Chandler Werthmann1,2, Takaomi C Saido4, Bret M Evers2, Charles L White III5, Jade Connor1,2, Robert E Hammer6, Joachim Herz1,2,7,8,*

1Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, United States; 2Center for Translational Neurodegeneration Research, Dallas, United States; 3Institute of Cardiovascular Sciences and Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Peking University, Beijing, China; 4Laboratory for Proteolytic Neuroscience, Riken Center for Brain Science, Wako, Japan; 5Pathology, University of Texas Southwestern Medical Center, Dallas, United States; 6Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, United States; 7Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, United States; 8Department of Neurology and Neurotherapeutics, University of Texas Southwestern Medical Center, Dallas, United States

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 EEs 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.

Introduction ApoE is the principal lipid transport protein in the brain. Three different ApoE isoforms are common in humans: ApoE2 (ɛ2), ApoE3 (ɛ3), and ApoE4 (ɛ4). Each ApoE4 allele reduces the age of Alzheimer’s disease (AD) onset by approximately 3–5 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 4–5), which is required for the digestion of endocyted biomolecules (Figure 1A; Casey et al., 2010; Naslavsky and Caplan, 2018). For maturation of the early endosomes (EEs) 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 al., 2002). Dysregulation of endolysosomal trafficking by ApoE4 causes an age-dependent increase in EE number and size (Nuriel et al., 2017).

Based on these observations, we have proposed a model in which destabilization of ApoE4 in the acidic EE environment, combined with a greater propensity for self-association, results in delayed detachment from its receptors (Figure 1B). Subsequent endosomal swelling through K\(^+\), Na\(^+\), and H\(_2\)O influx further impairs cargo delivery, receptor recycling, and ligand re-secretion. Importantly, in neurons, ApoE and its receptor Apoer2 travel together with glutamate receptors through the endosomal recycling pathway (Chen et al., 2010; Xian et al., 2018). Rapid endocytosis and subsequent recycling of synaptic receptors is triggered by the synaptic homeostatic modulator and Apoer2 ligand Reelin (Hiesberger et al., 1999; Trommsdorff et al., 1999). We previously showed that ApoE4, in contrast to ApoE3 and ApoE2, prevents Reelin-mediated glutamate receptor insertion at the synapse, a state we refer to as ApoE4-mediated Reelin resistance (Chen et al., 2010; Durakoglugil et al., 2009; Lane-Donovan and Herz, 2017; Lane-Donovan et al., 2014; Xian et al., 2018). Reduction of endosomal pH and increasing the differential to the ApoE4 IEP abolishes this effect in vitro (Xian et al., 2018).

The pH of EE compartments is controlled by the vATPase-dependent proton pump and proton leakage through NHE6 (Nakamura et al., 2005; Basu et al., 1976; Davis et al., 1987; Ruddenko et al., 2002). NHE6 is encoded by the gene Slc9a6. We showed that EE acidification by pharmaceutical pan-NHE inhibition or selective NHE6/Slc9a6 knockdown in neurons prevents the ApoE4-caused trafficking delay of ApoE and glutamate receptors (Xian et al., 2018). NHE6 deficiency in humans causes neurodevelopmental defects, which result in Christianson syndrome, an X-linked genetic disorder characterized by cognitive dysfunction, autism, ataxia, and epilepsy. However, some Slc9a6 mutant variants causing Christianson syndrome in humans do not significantly alter the ion exchange properties of NHE6 (Ilie et al., 2020; Ilie et al., 2019) suggesting that Christianson syndrome could be caused by loss of NHE6 scaffolding functions and not by loss of endosomal pH regulation. To investigate whether NHE6 depletion can reverse ApoE4 pathology in vivo, we generated a conditional Slc9a6 knockout (KO) mouse line (Slc9a6\(^{flox}\);CAG-Cre\(^{ER}\)) to avoid complications caused by neurodevelopmental defects by temporally and spatially controlling NHE6 ablation. We show that genetic NHE6 ablation attenuates both, the ApoE4-induced Reelin resistance and impaired synaptic plasticity in ApoE4 targeted replacement (Apoe\(^{apo}\)) 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.
Figure 1. ApoE4 induces endolysosomal trafficking delay. (A) pH regulation within the endolysosomal pathway. Upon receptor binding, ApoE is endocytosed along with glutamate receptors (AMPA and NMDA receptors). Cargo that has entered the early endosome (EE) can undergo recycling through a fast direct route without further acidification (fast recycling) or through slower sorting pathways that require further acidification (Casey et al., 2010; Naslavsky and Caplan, 2018). While lipid components are targeted to the lysosome, the majority of receptors, as well as ApoE, remain in the EE.

Figure 1 continued on next page
endosomal compartments at the cellular periphery where they rapidly move back to the surface (Heeren et al., 1999). The increasingly acidic luminal pH is illustrated as a color gradient and depicted on the left. (B) In the presence of ApoE4, early endosomal trafficking and fast recycling are delayed. At the pH of the EE, ApoE4 is near its isoelectric point where solubility is reduced (Wintersteiner and Abramson, 1933), impairing receptor dissociation and resulting in delayed endosomal maturation with a concomitant entrapment of co-endocytosed glutamate receptors. Endosomal pH is regulated by the vesicular ATPase and the counterregulatory action of the proton leakage channel NHE6. NHE6 is an antiporter that exchanges a Na⁺ or K⁺ ion for each proton. As the pH decreases, ligands dissociate from their receptors allowing the EE to mature. If dissociation is delayed, as in case of ApoE4, endosomal trafficking is arrested, leading to progressive acidification as Na⁺, K⁺, and Cl⁻ ions continue to enter the endosome to maintain charge neutrality while also drawing in water molecules due to osmotic pressure. We thus propose a model in which delayed ApoE4-receptor dissociation prevents early endosomal maturation and causes osmotic swelling while the pH continues to decrease until dissociation occurs. (C) Accelerated endosomal acidification by inhibition of the proton leak channel NHE6 resolves ApoE4 accumulation, promotes rapid receptor dissociation, and promotes the vesicle entry into the lysosomal delivery or recycling pathways.

**Results**

### NHE6 is required for postnatal Purkinje cell survival

NHE6 germline KO mice (Slc9a6) and tamoxifen-inducible conditional NHE6 KO mice (Slc9a6<sup>Cre<sup>ERT2</sup></sup>) 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 (cKO) of Slc9a6, tamoxifen was administered to Slc9a6<sup>Cre<sup>ERT2</sup></sup> mice at 2 months (Figure 3A) and experiments were performed at the indicated time points. Slc9a6 KO efficiency in the brains of tamoxifen-injected Slc9a6<sup>Cre<sup>ERT2</sup></sup> mice was 65–82% and varied between brain regions (Figure 3B). To further investigate Cre<sup>ERT2</sup> activity upon tamoxifen injection, we bred the CAG-Cre<sup>ERT2</sup> 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 month after Slc9a6 ablation, Purkinje cell loss was indistinguishable from that seen in the germline KO (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
Figure 2. Generation of Slc9a6\(^{fl}\) and Slc9a6\(^{-}\) mice. (A) Gene targeting strategy. LoxP sites were introduced to flank the first exon (E1) of Slc9a6 (located on the X-chromosome) by gene targeting in embryonic stem cells. The targeting construct contained a long arm of homology (LA, gray) upstream of the first loxP site and the first exon. An loxP/FRT-flanked neomycin resistance cassette was cloned downstream of the first exon, followed by a short arm of homology (SA, gray). The targeted locus is shown below. Targeted stem cells were used to generate chimeric Slc9a6\(^{fl}\) mice. Germline NHE6 knockout mice (NHE6\(^{-/-}\) [female], NHE6\(^{-/-}\) [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 Figure 2 continued on next page
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’s t-test. (**p < 0.01) (F) The percent of vesicles with pH >6.4 is significantly decreased in Slc9a6 fibroblasts. (G) CAG-CreERT2 activity after tamoxifen application in a reporter mouse line expressing tdTomato. CreERT2 recombination activity without (left panel) or with (middle panel) tamoxifen application in the CAG-CreERT2 line bred with Rosa26RasStop-tomato 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 sodium-hydrogen exchanger 6 (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 1 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 three brains in each group were examined. (C–F) NHE6 deficiency leads to cerebellar Purkinje cell loss in germline (Slc9a6-, C) and conditional (Slc9a6fl;CAG-CreERT2, D–F) knockout mice. Slc9a6- includes both female wildtypes (Slc9a6+/+) and male wildtypes (Slc9a6y/+) mice. Slc9a6- includes both female knockouts (Slc9a6-/+) and male knockouts (Slc9a6y/-) mice. In addition, Slc9a6fl mice includes both female Slc9a6fl/fl and male Slc9a6y/fl. The timeline shows that Slc9a6fl;CAG-CreERT2 and control mice were tamoxifen-injected at 2 months and analyzed 1 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 2 months of age, also led to massive Purkinje cells loss when mice were examined 1 year after NHE6 ablation (E, lower panel). (F) Quantification of Purkinje cell loss in the cerebellum of Slc9a6+,CAG-CreERT2 mice. Values are expressed as mean ± SEM from four independent experiments. Statistical analysis was performed using Student’s t-test. *p < 0.05.
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 min in the presence or absence of naturally secreted, receptor-binding competent ApoE (Figure 4). Subsequently, surface biotinylation is performed and cells are harvested for immunoblotting to quantify the amount of Apoer2 and glutamate receptors expressed on the neuronal surface (Chen et al., 2010; Xian et al., 2018).

We have shown previously that in the presence of ApoE4, Apoer2 and glutamate receptors recycle poorly to the neuronal surface. This recycling block could be resolved by endosomal acidification induced by shRNA knockdown of Slc9a6 or by applying the NHE inhibitor EMD87580 (Xian et al.,...
Conditional disruption of NHE6 relieves synaptic Reelin resistance in ApoeAPOE4 mice

Reelin can enhance long-term potentiation (LTP) in hippocampal field recordings of ApoeAPOE3 but not ApoeAPOE4 acute brain slices (Chen et al., 2010). We previously showed that this Reelin resistance in ApoeAPOE4 slices was attenuated by pharmacological NHE inhibition (Xian et al., 2018). To investigate if endogenous loss of NHE6 also restores synaptic plasticity in the presence of ApoE4, we performed hippocampal field recordings on Slc9a6-;CAG-CreERT2 mice bred to ApoAPOE3 or ApoAPOE4 mice. To avoid potentially compounding effects of NHE6 deficiency during embryonic development (Ouyang et al., 2013), NHE6/Slc9a6 gene disruption was induced at 8 weeks by intraperitoneal tamoxifen injection (Lane-Donovan et al., 2015). Electrophysiology was performed 3–4 weeks after NHE6 depletion in 3-month-old mice (Slc9a6-;CAG-CreERT2, Figure 5B, D, F and H). Tamoxifen-injected Slc9a6-;CAG-CreERT2-negative mice expressing human ApoE3 or ApoE4 served as controls (ApoAPOE3 and ApoAPOE4, Figure 5A, C, E and G). For field recordings, hippocampal slices were perfused with Reelin as described (Beffert et al., 2005; Chen et al., 2010; Durakoglougl i et al., 2009; Weeber et al., 2002). Conditional genetic loss of NHE6 resulted in a moderate reduction of the ability of Reelin to enhance LTP in ApoAPOE3 mice (comparing Figure 5A and E to B and F). By contrast, as reported previously (Chen et al., 2010), hippocampal slices from ApoAPOE4 mice were completely resistant to LTP enhancement by Reelin (Figure 5C and G). This resistance was abolished when NHE6 was genetically disrupted in ApoAPOE4 mice: Reelin application enhanced LTP (Figure 5D and H) in ApoAPOE3;Slc9a6-;CAG-CreERT2 to a comparable extent as in the ApoAPOE3;Slc9a6-;CAG-CreERT2 mice (Figure 5B). Synaptic transmission was monitored and input-output curves were generated by plotting the fiber volley amplitude, measured at increasing stimulus intensities, against the fEPSP slope. No significant differences were found (Figure 5I and J).

NHE inhibition or NHE6 knockdown does not alter β-CTF generation in vitro

Cleavage of APP by γ-secretase and the BACE1 generates the short neurotoxic polypeptide Aβ. Cleavage by BACE1 results in a membrane anchored fragment called β-CTF, which is further processed by γ-secretase to yield the soluble Aβ peptide. BACE1 processing of APP occurs in the Golgi complex, on the cell membrane, and after endocytosis in endosomes (Caporaso et al., 1994; Vassar et al., 1999). It has been reported that BACE1 activity increases with lower pH (Hook et al., 2002). In a recent in vitro study in an HEK293 cell line overexpressing APP and BACE1, NHE6 overexpression reportedly led to a reduction of Aβ production and conversely shRNA knockdown of NHE6/Slc9a6 resulted in an increase in Aβ production (Prasad and Rao, 2015). To investigate if NHE6/Slc9a6 deficiency contributes to APP processing by BACE1 in neurons, we used primary neurons derived from AppSwe (Tg2576) mice, an Alzheimer’s disease (AD) mouse model that over-expresses human APP with the ‘Swedish’ mutation (Hsiao et al., 1996). Neurons were treated with the NHE inhibitor EMD87580 or transduced with lentiviral shRNA directed against NHE6/Slc9a6 in the presence or absence of the γ-secretase inhibitor L-685458. β-CTF was detected using the monoclonal antibody 6E10 directed against Aβ residues 1–16 (Figure 6). Inhibition of γ-secretase in ApoE4-treated AppSwe neurons strongly enhanced β-CTF accumulation, however, additional treatment with EMD87580 did not alter the amount of β-CTF in the cell lysates (Figure 6A). NHE6 knockdown using lentiviral shRNA also had no effect on the amount of β-CTF (Figure 6B). We conclude that NHE6 inhibition is unlikely to increase Aβ production under near-physiological conditions.
Figure 5. Effect of conditional sodium-hydrogen exchanger 6 (NHE6) knockout on Reelin-potentiated synaptic plasticity. (A–H) Conditional knockout of NHE6 restores Reelin-enhanced long-term potentiation (LTP) in ApoeAPOE3 mice. Reelin facilitated induction of LTP in ApoeAPOE3 (A, E), but not ApoeAPOE4 (C, G) control (Slc9a6fl) mice. Slc9a6 deficiency in ApoeAPOE3 mice caused a reduction in Reelin-enhanced LTP, such that it is not significantly different from the control LTP (B, F). Importantly, in ApoeAPOE3;Slc9a6fl;CAG-CreERT2 mice Reelin was able to enhance theta burst-induced potentiation (D, H). Hippocampal slices were prepared from 3-month-old double mutant mice with either human ApoeAPOE3 or ApoeAPOE4 crossed with Slc9a6 conditional knockout mice (Slc9a6fl;CAG-CreERT2, 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 min of stable baseline. Representative traces are shown in each panel, before TBS induction (black) and 40 min after TBS (gray). (E–H) Quantitative analysis of normalized fEPSP slopes at time intervals as indicated. (I, J) Input output curves of ApoeAPOE3 (I) and ApoeAPOE4 (J) mice with or without Slc9a6fl;CAG-CreERT2. Slc9a6fl mice includes both female Slc9a6fl/fl and male Slc9a6y/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’s t-test.
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 AppNL-F mice (Saito et al., 2014) to our germline NHE6 KO line (Slc9a6). In these AppNL-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. AppNL-F;Slc9a6- and control AppNL-F littermates were aged to 1 year. Perfusion- fixed brains were harvested and analyzed by hematoxylin and eosin (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 wildtype 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 AppNL-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...
Figure 7. Sodium-hydrogen exchanger 6 (NHE6) deficiency decreases plaque formation in both AppNL-F and AppNL-F,ApoeAPOE4 mice. (A, B) NHE6-deficient AppNL-F and control AppNL-F mice were analyzed for plaque deposition at an age of 12 months. Thioflavin S staining was performed to visualize plaques. Plaques were found more frequently in the control AppNL-F mice (left panel in A), magnifications of the boxed areas are shown in the two middle panels. The plaque load between Slc9a6-/-;AppNL-F;ApoeAPOE4 littermates, the plaque load between Slc9a6-/-;AppNL-F;ApoeAPOE4 littermates was compared and analyzed. (B) In the Slc9a6-/- littermates, the plaque load between Slc9a6-/-;AppNL-F;ApoeAPOE4 and control AppNL-F;ApoeAPOE4 littermates was compared and analyzed. (C, D, E, F, G, H, I) H&E staining was performed to visualize the brain region of the corresponding conditions.
number was reduced, when compared to controls. (C–D) Slc9a6<sup>−/−</sup>;CAG-Cre<sup>Ert2</sup>;App<sup>Nl-F</sup>;Apoe<sup>APOE4</sup> and Slc9a6<sup>+/+</sup>;App<sup>Nl-F</sup>;Apoe<sup>APOE4</sup> mice were analyzed for plaque deposition. NHE6 was ablated at 2 months and brains were analyzed at 13.5–16 months. 4G8-immunolabeling against Aβ was performed to visualize plaques. In App<sup>Nl-F</sup>;Apoe<sup>APOE4</sup> mice conditional Slc9a6 knockout caused a reduction in plaque load compared to the Slc9a6<sup>−/−</sup> control littermates. (C) Magnifications of the boxed areas in C are shown in the middle. (D) Plaque load was analyzed and compared between Slc9a6<sup>−/−</sup>;CAG-Cre<sup>Ert2</sup> and Slc9a6<sup>+/+</sup>;App<sup>Nl-F</sup>;Apoe<sup>APOE4</sup> mice. (F–I) Brain area (F), cortical thickness (G), hippocampal (HC) area (H), and CA1 thickness (I) were analyzed. Student's t-test did not reveal a significant difference. Plaques were differentiated by size or staining density as described in detail in the supplements. Plaque load was analyzed and compared between Slc9a6<sup>−/−</sup>;CAG-Cre<sup>Ert2</sup> and Slc9a6<sup>+/+</sup>;App<sup>Nl-F</sup>;Apoe<sup>APOE4</sup> mice. (F–I) Brain area (F), cortical thickness (G), hippocampal (HC) area (H), and CA1 thickness (I) were analyzed. Student's t-test did not reveal a significant difference. Plaques were differentiated by size or staining density as described in detail in the supplements.

The online version of this article includes the following figure supplement(s) for figure 7:

**Figure supplement 1.** Gross anatomical brain structure in Slc9a6<sup>−/−</sup> mice.

**Figure supplement 2.** Example of Thioflavin S stained plaques for quantification.

**Figure supplement 3.** Sodium-hydrogen exchanger 6 (NHE6) deficiency decreases plaque formation in both App<sup>Nl-F</sup> and App<sup>Nl-F</sup>;Apoe<sup>APOE4</sup> mice.

to their control littermates (Figure 7—figure supplement 3C–E). The −50 % reduction in soluble Aβ was statistically not significant in Slc9a6<sup>−/−</sup> lysates (TBS fraction).

**NHE6/Slc9a6 deficiency reduces plaque load in App<sup>Nl-F</sup>;Apoe<sup>APOE4</sup>**

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<sup>−/−</sup>;CAG-Cre<sup>Ert2</sup>; Apoe<sup>APOE4</sup> with App<sup>Nl-F</sup> mice. At 2 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, G) and 4G8 immunoreactivity (Figure 7C and D). The age-dependent increase in plaque load in Apoe<sup>APOE4</sup>;App<sup>Nl-F</sup> mice was abolished or delayed when Slc9a6 was depleted after 2 months of age (Figure 8). App<sup>Nl-F</sup> mice expressing murine ApoE developed plaques at 12 months (Figure 7A and B), compared to Apoe<sup>APOE4</sup>;App<sup>Nl-F</sup> which showed a similar number of plaques at 15–16 months (Figures 7C–D and 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 2 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<sup>Nl-F</sup>;Apoe<sup>APOE4</sup> cortices**

Xu et al., 2017, reported reduced cortex thickness and hippocampus volume in Slc9a6<sup>−/−</sup> mice, which we were able to reproduce in our germline Slc9a6<sup>−/−</sup> 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<sup>Nl-F</sup>;Apoe<sup>APOE4</sup> model (Figure 7E–I). We measured brain size, cortical thickness, hippocampal area, and CA1 thickness. In contrast to germline Slc9a6<sup>−/−</sup> mice (Figure 7—figure supplement 1), none of the analyzed parameters differed significantly between Slc9a6<sup>−/−</sup>;CAG-Cre<sup>Ert2</sup> and controls (Figure 7E–I), and we specifically did not detect any reduction in brain size compared to Slc9a6<sup>−/−</sup>;App<sup>Nl-F</sup>;Apoe<sup>APOE4</sup> 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.
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 AppNL-F;Slc9a6−/− mice (Figure 9—figure supplement 1A, C, and D) and AppNL-F;ApoeAPOE4;Slc9a6−/− mice were analyzed for plaque deposition. Neuron-friendly exchange 6 (NHE6) was ablated at 2 months and brains were analyzed at 13.5–16 months. 4G8-immunolabeling against Aβ (A, B) and Thioflavin S staining (C–E) were performed to visualize plaques (Figure 7C and Figure 7—figure supplement 3F). Plaque load was analyzed and compared between Slc9a6−/−;CAG-CreERT2 mice and floxed control littermates. Plaques were differentiated by staining intensity (A, B) or size (C–E) as described in the supplements (Figure 7—figure supplement 2). In the time range analyzed, plaque load increased by age in control, but not in Slc9a6−/−;CAG-CreERT2 mice. Plaques were analyzed by a blinded observer. Plaque count (Slc9a6−/−;CAG-CreERT2 n = 8, Slc9a6−/− n = 8 for A) (B), Slc9a6−/−;CAG-CreERT2 n = 12, Slc9a6−/− n = 10 in (C–E) is plotted against age of mice. Slc9a6−/− mice includes both female Slc9a6y/y and male Slc9a6y/y mice. Apoe mice are homozygous for APOE4. AppNL-F mice are homozygous for human NL-F knockin mutation (AppNL-F/NL-F).
Microglia and astrocytes surround plaques in both \textit{slc9a6}−/− and control \textit{AppNL-F} brains. (A–B) Co-labeling of microglia (Iba1, green, A) or astrocytes (GFAP, green, B) with Aβ (6E10, red) in brain slices of \textit{AppNL-F} and \textit{AppNL-F};\textit{Slc9a6}−/− mice. (C) Quantification of plaques in control and \textit{Slc9a6}−/− brains. (D) Bar graph showing the intensity density of Iba1/6E10 as quantitative measure of microglia surrounding plaques. (E) Statistical analysis of 6E10 positive microglia and (F) the intensity of 6E10 signal within microglia. (G) Bar graph showing the intensity density of GFAP/Aβ as quantitative measure of astrocytes surrounding plaques. Data were analyzed by a blinded observer. All data are expressed as mean ± SEM. Data were obtained from \(n=4\) (control) and \(n=5\) (\textit{Slc9a6}) mice (A–G), \(n=33\) (control) and \(n=23\) (\textit{Slc9a6}) plaques were analyzed, in (E) \(n=22\) (control) and \(n=24\) (\textit{Slc9a6}) microscopical pictures were analyzed, in (F) \(n=31\) (control) and \(n=38\) (\textit{Slc9a6}) 6E10 positive (defined as signal intensity above 500) microglia were analyzed, in (G) \(n=33\) (control) and \(n=18\) (\textit{Slc9a6}) plaques were analyzed. Student’s t-test revealed a difference in (C) (**\(p<0.01\)) and did not reveal significant differences in (D–G). \textit{Slc9a6}−/− represents both female wildtypes (\textit{Slc9a6}+/−) and male wildtypes (\textit{Slc9a6}−/−). \textit{Slc9a6} represents both female knockouts (\textit{Slc9a6}−/−) and male knockouts (\textit{Slc9a6}+/−). \textit{AppNL-F} mice are homozygous for human NL-F knockin mutation (\textit{AppNL-F/NL-F}).

The online version of this article includes the following figure supplement(s) for figure 9:

- Figure supplement 1. Sodium-hydrogen exchanger 6 (NHE6) deficiency causes an increase in Iba1 and glial fibrillary acidic protein (GFAP) immunoreactivity in both \textit{AppNL-F} and \textit{AppNL-F};\textit{Apoe}\textit{APOE4} mice.
- Figure supplement 2. Examples of plaques surrounded by microglia and astrocytes.

Microglia and astrocytes surround plaques in both \textit{slc9a6}−/− and control \textit{AppNL-F} mice

Conditional and germline \textit{Slc9a6} deficient \textit{AppNL-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 co-staining 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 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 EEs 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 AD 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 endolysosomal 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 ∼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 (Strømme 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 (Fernández-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).

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 GWAS, 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 (Briehl 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, that is, overexpression in immortalized kidney cells on one hand and primary cortical neuronal cultures on the other. Using a humanized AppNL-F knockin mouse model, we show that NHE6/Slc9a6 deficiency in 1-year-old animals reduces plaque deposition by ~80%. In AppNL-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 AppNL-F animals aged 18 months had a reduction in insoluble Aβ by approximately 71%. NHE6/Slc9a6 depletion in AppNL-F/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<sup>NL-F</sup> 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<sup>APOE4</sup> might be the result of a maximally accelerated early endosomal maturation and cargo transport in the absence of NHE6. When compared to control App<sup>NL-F</sup> mice, Slc9a6-deficient App<sup>NL-F</sup> 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<sup>-</sup> mice, the proportion of microglial structures containing Aβ (6E10 antibody) was comparable between Slc9a6<sup>-</sup> deficient and control App<sup>NL-F</sup> mice, as was the intensity signal for 6E10. This suggests that microglia in the Slc9a6<sup>-</sup> 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<sup>NL-F</sup> mice may involve an increased catabolic rate (Shi et al., 2021), brought about by the accelerated acidification and vesicular trafficking of EEs.

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<sup>NL-F</sup> mouse model used in our study develops plaques at 12 months (Saito et al., 2014) in the presence of murine ApoE. However, the onset of plaque deposition in human Apoe<sup>APOE4</sup> mice was delayed by ~3 months. The effect of germline Slc9a6 deficiency and conditional Slc9a6 deficiency induced at 2 months had a comparable effect on plaque reduction in App<sup>NL-F</sup> and App<sup>NL-F</sup>;Apoe<sup>APOE4</sup> mice.

Slc9a6<sup>-</sup> and Slc9a6<sup>-</sup>;CAG-Cre<sup>ERT2</sup> both show progressive Purkinje cell loss in the cerebellum, indicating that NHE6 requirement is cell-autonomous and not developmentally determined. Slc9a6<sup>-</sup> and Slc9a6<sup>-</sup>;CAG-Cre<sup>ERT2</sup> 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<sup>-</sup> 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<sup>-</sup>;CAG-Cre<sup>ERT2</sup> mice do not present with a reduction in cortical or hippocampal volume (Figure 7), yet have comparable immunoreactivity for markers of glial activation. Our study supports a temporally distinct function of NHE6 in the adult brain where the cerebellum requires NHE6 for development, but not for postnatal neuronal survival, whereas Purkinje cells in the cerebellum do. A similar dual function for NHE6 has been described previously by Xu et al., 2017. Moreover, this further indicates that reduced plaque load is also not an effect of neuronal loss. Therefore, our two mouse models together suggest that the observed increased glial activation is not caused by neuronal cell loss, but rather is likely a direct cell-autonomous effect of NHE6 loss of function. It thus remains to be determined whether endosomal acidification in NHE6-deficient microglia alone is sufficient to induce Aβ degradation and plaque reduction.

NHE6 is ubiquitously expressed in all cells of the body, however, in the CNS it is highly expressed in neurons where abundant synaptic vesicles and neurotransmitter receptors recycle in synapses (Lee et al., 2020; Lee et al., 2021) and to a lesser extent in glial cells (Zhang et al., 2014). Neuronal NHE6 plays a direct role in both synaptic development and plasticity, potentially through BDNF/TrkB (Deane et al., 2013; Ouyang et al., 2013) and other pathways. The impact of NHE6 loss in microglia and astrocytes is still unknown. Global Slc9a6 deficiency causes glial activation in vivo, which could be mediated through either a primary cell-autonomous mechanism or a secondary mechanism induced by damaged neurons. In ApoE4-expressing astrocytes, others have shown that overexpression of
NHE6 increases LRP1 on the surface, which correlated with an increase in Aβ uptake (Prasad and Rao, 2018). Microglia are the primary glial cells that degrade Aβ; thus, it will be imperative to determine how NHE6 levels alter Aβ degradation selectively in astrocytes and microglia, respectively.

Early endosomal pH balance in phagocytic cells plays an important role in viral and bacterial infection response. In phagocytic cells, the deacidification of endosomes using pharmacological inhibitors like chloroquine has been shown to reduce endosomal toll-like receptor (TLR) response (de Bouteiller et al., 2005; Fox, 2019; Kuznik et al., 2011; Wozniacka et al., 2006; Yang et al., 2016), suggesting that NHE6 depletion in microglia might conversely augment TLR response.

It would also be intriguing to test whether Slc9a6 deficiency increases cognitive performance in AD mouse models. In the current study we used AppNL-F and AppNL-F;ApoeAPOE4 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;CAG-CreERT2 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 and 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 EEs 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 worldwide 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

Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Strain, strain background (Mus musculus) | Mouse/Slc9a6<sup>Δ</sup> | This study | | Refer to Materials and methods section for detailed description of mouse model production |
| Strain, strain background (Mus musculus) | Mouse/Slc9a6 | This study | | Refer to Materials and methods section for detailed description of mouse model production |
| Strain, strain background (Mus musculus) | Mouse/Apoe<sup>APOE3</sup> | Sullivan et al., 1997 IMSR_TAC:2,542 | Apo<sup>APOE3</sup> | |
| Strain, strain background (Mus musculus) | Mouse/Apo<sup>APOE4</sup> | Knouff et al., 1999 IMSR_TAC:3,518 | Apo<sup>APOE4</sup> | |
| Strain, strain background (Mus musculus) | Mouse/B6.Cg-Gt(Rosa26Sortm9(CAG-tdTomato))Hze/J | The Jackson Laboratory Madisen et al., 2010 JAX #007909 | ROSA<sup>floxedStop-tmTomato</sup> | |
| Strain, strain background (Mus musculus) | Mouse/CAG-cre/Esr15Amc/J | The Jackson Laboratory Hayashi and McMahon, 2002 JAX #004682 | CAG-Cre<sup>ERT2</sup> | |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Strain, strain background (Mus musculus) | Mouse/B6.129S4-Meox2tm1(cre)Sor/J | The Jackson Laboratory Talquist and Soriano, 2000 | JAX 003755 | Meox-Cre |
| Strain, strain background (Mus musculus) | AppNS-F | Saito et al., 2014 |  | AppNS-F |
| Strain, strain background (Mus musculus) | Tg2576 | Charles River Hsiao et al., 1996 | Charles River Tg2576 | Tg2576, APPSwe |
| Strain, strain background (Rattus norvegicus) | SD rat | Charles River | SC.400 | |
| 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 Materials and methods section for detailed description of MEF production |
| Cell line (Mus musculus) | Slc9a6+ MEFs (Slc9a6 littermate) | This study |  | Refer to Materials and 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 4 G8) (mouse monoclonal) | Covance | SIG-39220, RRID:AB_10175152 | IHC (1:1000) |
| Antibody | Anti-phospho tyrosine (clone 4 G10) (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 | 4,207 S, 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) |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|----------------------------------|-------------|--------------------|-------------|------------------------|
| 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 (50 ×), serum free | Thermo Fisher | 17504044 |
| 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 (1 ×) | Gibco | 14175 |
| Chemical compound, drug          | L- Glutamic acid (glutamate) | Sigma-Aldrich | G1251 |
| Chemical compound, drug          | y-Secretase inhibitor L-685458 | Tocris Bioscience | 2627 |
| Chemical compound, drug          | Penicillin-streptomycin solution, 100 × | Corning | 30–002 CI |
| Chemical compound, drug          | Neurobasal Medium (1 ×) 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 % Paraformaldehyde AQ solution | Fisher Scientific | 15714 S |
| Chemical compound, drug          | PBS (1 ×) | Sigma-Aldrich | D8537 |
| Chemical compound, drug          | Penicillin-streptomycin | Corning | 30–002 CI |
| Chemical compound, drug          | Phosphatase inhibitor cocktail | Thermo Fisher | 78420 |
| 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 |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|--------------------|-------------|------------------------|
| 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 Xian et al., 2018 | TRCN0000068828 | Refer to shNHE6-a |
| Transfected construct (Mus musculus) | psPAX2 | Addgene | 12260 | Plasmid was a gift from Didier Trono |
| Transfected construct (Mus musculus) | pMD2.G | Addgene | 12259 | Plasmid was a gift from Didier Trono |
| Transfected construct (Mus musculus) | pJB-NHE6 targeting vector | This study | N/A | Refer to Materials and methods section for detailed description |
| Recombinant DNA reagent           | pJB1 (plasmid) | Braybrooke et al., 2000 | N/A |                        |
| Recombinant DNA reagent           | pCR4-TOPO (plasmid) | Thermo Fisher | K457502 |                        |
| Recombinant DNA reagent           | pLVCMVfull (plasmid) | Xian et al., 2018 | N/A |                        |
| Recombinant DNA reagent           | pME (plasmid) | Stawicki et al., 2014 | Addgene #73794 | Plasmid was a gift from David Raible |
| Recombinant DNA reagent           | pLVCMV Vamp3pHluorin2 (plasmid) | This study | N/A | Refer to Materials and methods section for detailed description |
| Recombinant DNA reagent           | BAC containing murine NHE6 sequence (bacterial artificial chromosome) | BACPAC Resources Center | RP23 364 F14 |                        |
| 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               | NDPview2 | Hamamatsu Photonics |              |                        |
| Software, algorithm               | Odyssey Imaging System | LI-COR | RRID:SCR_014579 |                        |
| Software, algorithm               | Clustal Omega | EMBL-EBI | RRID:SCR_001591 |                        |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|--------------------|-------------|------------------------|
| Software, algorithm               | Leica TCS SPE | Leica             | RRID:SCR_002140 |                        |
| Sequence-based reagent            | SA forward  | IDT                | GGATCCGTTGT GGTGTTGAGG GAGAGGA |                        |
| Sequence-based reagent            | SA reverse  | Integrated DNA Technology | CTCAGCTCTCAC AATCAGCTCTTT AAATATGCC |                        |
| Sequence-based reagent            | GAP repair US forward | Integrated DNA Technology | AAGCTTGGCAGC GCTTCAATTTCTG TCTTGCTACTG |                        |
| Sequence-based reagent            | GAP repair US reverse | Integrated DNA Technology | AGATCTCAAGAA AGTTAGCTAGA AGTGTC |                        |
| Sequence-based reagent            | GAP repair DS forward | Integrated DNA Technology | AGATCTGTAAGA GAGTTGAGGA AAGAGAG |                        |
| Sequence-based reagent            | GAP repair DS reverse | Integrated DNA Technology | GTGAGCCGGG CCGACACACA CAGATAATAAA CTCAAAGA |                        |
| Sequence-based reagent            | 5' flanking 1st LoxP fragment forward | Integrated DNA Technology | GCATTTCCTCG AGCAAGATGCAAC |                        |
| Sequence-based reagent            | 5' flanking 1st LoxP fragment reverse | Integrated DNA Technology | GATACGAGCA GTTACGACCAA GACCTCAACC TATGTGCTCTATA TGACAAAC |                        |
| Sequence-based reagent            | 3' flanking 1st LoxP fragment forward | Integrated DNA Technology | GTCTGTGGT GGA TCTGCAGAATGA AAAGTTCAATG |                        |
| Sequence-based reagent            | 3' flanking 1st LoxP fragment reverse | Integrated DNA Technology | ATCGATTTCA TAACCAATC CGAGATA |                        |
| Sequence-based reagent            | LoxP Oligo forward | Integrated DNA Technology | GATACGAGCA GTTACGACCAA GACCTCAACC TATGTGCTCTATA TGACAAAC |                        |
| Sequence-based reagent            | LoxP Oligo reverse | Integrated DNA Technology | CATAACTTTCA TAAATCTGCTATG TACATCTTACAC AAGTATGTAC |                        |
| Sequence-based reagent            | Genotyping NHE6-floxed and wt forward | Integrated DNA Technology | GAGGAAGC AAAATGTCA GCTCC |                        |
| Sequence-based reagent            | Genotyping NHE6-floxed and wt reverse | Integrated DNA Technology | CTAATCCCTCTC GAGATGCTCTC |                        |
| Sequence-based reagent            | Genotyping NHE6-KO forward | Integrated DNA Technology | GAGGAAGC AAAATGTCA GCTCC |                        |
| Sequence-based reagent            | Genotyping NHE6-KO reverse | Integrated DNA Technology | CCTCACAAGACT AGAGAAATGTTCC |                        |

*Continued on next page*
### 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.

The mouse lines Rosa-stop-tdTomato B6.Cg-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze/J (Madisen et al., 2010) (JAX #007909) and CAG-CreER<T> B6.Cg-Tg(CAG-cre/Esr1)5Amc/J mice (Hayashi and McMahon, 2002) (JAX #004682) were obtained from The Jackson Laboratories (Bar Harbor, ME). ApoE3 and ApoE4 targeted replacement mice ([**ApoeAPOE3**](https://www.jax.org/searchdb/getRecord?acc=004682) ([**Knouff et al., 1999**](https://doi.org/10.1073/pnas.96.4.1382) ([**Sullivan et al., 1997**](https://doi.org/10.1073/pnas.94.10.5184)) were kind gifts of Dr Nobuyo Maeda. AppSwe ([**Tg2576**](https://www.jax.org/searchdb/getRecord?acc=004682) ([**Hsiao et al., 1996**](https://doi.org/10.1073/pnas.93.12.5351)) was generated by Hsiao et al., 1996. The Meox-Cre B6.129S4- Meox2tm1(cre)Sor/J mice (JAX 003755) were provided by Drs M Tallquist and P Soriano (Tallquist and Soriano, 2000). Conditional NHE6 KO (Slc9a6<sup>−/−</sup>;CAG-Cre<sup>ERT2</sup>) and germline NHE6 KO (Slc9a6<sup>−</sup>/<sup>−</sup>) mice were generated as described below. The human APP knockin line ([**App NL-F**](https://doi.org/10.1073/pnas.1015470107)) ([**Saito et al., 2014**](https://doi.org/10.1073/pnas.1320616111)) has been described earlier. Pregnant female SD (Sprague Dawley) rats were obtained from Charles River (SC:400). Mice were group-housed in a standard 12 hr light/dark cycle and fed ad libitum standard mouse chow (Envigo, Teklad 2016 diet as standard and Teklad 2018 diet for breeding cages).

To generate Slc9a6<sup>−/−</sup> mice, the first exon of NHE6 was flanked with loxP sites ([Figure 2A](https://doi.org/10.7554/eLife.72034)). A loxP site was inserted 2 kb upstream of the first exon of the X-chromosomal NHE6 gene and a Neo-cassette (flanked with loxP and FRT sites) was inserted 1 kb downstream of the first exon. Insertion sites were chosen based on low conservation (mVISTA) between mammalian species (rat, human, mouse). To create the targeting vector for the Slc9a6<sup>−/−</sup> mouse line, pJB1 ([**Braybrooke et al., 2000**](https://doi.org/10.1073/pnas.97.4.1346)) was used as backbone. Murine C57Bl/6 J embryonic stem (ES) cell DNA was used as template to amplify the short arm of homology (SA; 0.88 kb of the first intron starting 1 kb 3' downstream of exon 1), which was inserted between the Neo and HSVTK selection marker genes of pJB1 (BamHI and XhoI sites) to create an intermediate plasmid referred to as pJB1- NHE6SA. To create the intermediate plasmid pNHE6- LA for the long homology arm, a fragment spanning from 13 kb 5' upstream to 1 kb 3' downstream of the first Slc9a6 (NHE6) exon was integrated into pCR4-TOPO (Thermo Fisher) by using a bacterial artificial chromosome (RP23 364F14, Children's Hospital Oakland Research Institute [CHORI]) and the GAP repair technique ([**Lee et al., 2001**](https://doi.org/10.1073/pnas.002940698)) (primers to amplify the upstream (US) and downstream (DS) homology boxes are listed in the Key resources table). To create the intermediate plasmid pNHE6- LA for the long homology arm, a fragment spanning from 13 kb 5' upstream to 1 kb 3' downstream of the first Slc9a6 (NHE6) exon was integrated into pCR4-TOPO (Thermo Fisher) by using a bacterial artificial chromosome (RP23 364F14, Children's Hospital Oakland Research Institute [CHORI]) and the GAP repair technique (Lee et al., 2001) (primers to amplify the upstream (US) and downstream (DS) homology boxes are listed in the Key resources table). In a parallel cloning step, a 2.4 kb Xhol-EcoRV Slc9a6 promoter region fragment spanning from 2.7 to 0.4 kb 5' upstream of the Slc9a6 start-codon was modified with the 1st loxP site to generate pLoxP: three fragments (1) 0.7 kb 5' loxP flanking Slc9a6-promoter 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 Xhol-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/6 J ES cells. Gene targeting-positive C57Bl/6 J ES cells (PCR screen) were injected into albino C57Bl/6 J blastocysts, resulting in chimeric mice. The chimeras were crossed to C57Bl/6 J 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-CreERT2, Slc9a6/fl females were crossed to CAG-CreERT2 mice to obtain Slc9a6/fl;CAG-CreERT2 and Slc9a6/fl;CAG-CreERT2 mice (Slc9a6/fl;CAG-CreERT2 and CAG-CreERT2 negative control littermates (Slc9a6/fl)). Slc9a6/fl;CAG-CreERT2 mice were backcrossed to ApoeAPOE3 or ApoeAPOE4 mice. Breeding pairs were set up in which only one parent was CAG-CreERT2 positive. The following genotypes were used for hippocampal field recordings: (1) ApoeAPOE3;Slc9a6y/fl;CreERT2 (short: ApoeAPOE3;Slc9a6y/fl;CreERT2), (2) ApoeAPOE4;Slc9a6y/fl;CreERT2 (short: ApoeAPOE4;Slc9a6y/fl;CreERT2), (3) ApoeAPOE4;Slc9a6y/fl;CreERT2 and (4) ApoeAPOE4;Slc9a6y/fl;CreERT2 (short: ApoeAPOE4;Slc9a6y/fl;CreERT2). The ApoeAPOE4;Slc9a6y/fl;CAG-CreERT2 line was further crossed with the AppNL line to generate ApoeAPOE4;Slc9a6y/fl;CAG-CreERT2, AppNL and ApoeAPOE4;Slc9a6y/fl;AppNL 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 5 consecutive days. Tamoxifen was dissolved in sunflower oil (Sigma, W530285) and 10 % EtOH.

To generate the germline NHE6 KO line (Slc9a6/fl), heterozygous Slc9a6y/fl females were crossed to Meox-Cre to yield germline mutant Slc9a6y/fl females and Slc9a6y/fl males (Slc9a6/fl). Genotyping: The Slc9a6/fl PCR amplifies 250 bp of the wildtype, 270 bp of the floxed, and no fragment in the KO 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 AppNL (Saito et al., 2014) mice. AppNL;Slc9a6 (AppNL;Slc9a6 or AppNL;Slc9a6) and control (AppNL = AppNL;Slc9a6 or AppNL;Slc9a6) littermates were obtained by crossing Slc9a6/fl;AppNL females with either Slc9a6y/fl;AppNL females or Slc9a6y/fl;AppNL males.

DNA constructs, recombinant proteins, lentivirus production

Lentiviral plasmids with shRNA directed against NHE6/Slc9a6 and the scrambled control have been described in Xian et al., 2018. Plasmids encoding ApoE3 and ApoE4 (Chen et al., 2010) and Reelin described in Xian et al., 2018. Plasmids encoding ApoE3 and ApoE4 (Chen et al., 2010) and the scrambled control have been described before. The lentiviral plasmid encoding the Vamp3-pHluorin2 fusion protein was cloned by inserting mouse Vamp3 cDNA, a linker and pHluorin2 (5' - CATTGTCATCATCATCATCAGTGTGTTCTCCTAAGGTGAAACACGCCGCCTGAGCG CACCGCGGCCCCGGCCAGCATGCCACTACCAAGTTAC-3') into pcDNA3.1 and the fusion protein was then transferred with NheI and EcoRI into pLVCMVfull. Lentiviral plasmids psPAX2 and pMD2.g were a kind gift from Dr D Trono and obtained from Addgene.

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

For lentivirus production HEK 293 T cells were co-transfected with psPAX2, pMD2.g, and the individual shRNA encoding transfer or Vamp3-pHluorin2 constructs. Media was replaced after 12–16 hr. Viral particle containing media was collected after centrifuging cellular debris. The viral particles were 10 x concentrated by ultra-centrifugation and resuspended in Neurobasal medium. To infect neurons on DIV 1/10th of the culture medium was replaced by concentrated virus. After 24 hr, the virus was removed.
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 hr 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 Rosa26foxedStop-tdTomato;CAGCreERT2 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 hr 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 hr at 4 °C. Slices were subsequently washed 4 × 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 hr at room temperature. After washing, slices were mounted with Antifade Mounting medium with DAPI (Vectashield). Images were taken with an Axioplan two 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 % KMnO₄ for 20 min. After washing with water, slices were bleached with 1 % K₂S₂O₅ / C₆H₂O₄ for 2 min, washed in water, and treated with 2 % NaOH/H₂O₂ for 20 min. After washing with water, slices were acidified in 0.25 % CH₃COOH 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, 1:2000), Iba1 (Wako 019–19741, Rabbit, 1:1000), and Aβ (4G8, Covance or 6E10, Biolegend, both mouse, 1:1000). 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. DAB chromagen was used to detect the immunoperoxidase signal (Sinclair et al., 1981) (Vector; anti-mouse and anti-rabbit IgG kits). Fluorescence-labeled slices were counterstained with DAPI (mounting media with DAPI, Vectashield). Standard protocols were utilized for staining of paraffin sections with 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 two 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, SUC9αβ, AppSwe) (E18) as described previously (Chen et al., 2005). Neurons were cultured in poly-D-lysine coated six-well plates (1 million/9 cm²) or on coverslips (30,000 neurons/1.1 cm²) in presence of Neurobasal medium supplemented with B27, glutamine, and penicillin-streptomycin at 37 °C and 5 % CO₂. 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 hr post-infection, vesicular pH was measured on a Zeiss LSM880 Airyscan confocal microscope as described in 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 % CO2 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, six fields of view were imaged for Slc9a6+ and four fields of view for Slc9a6- fibroblast. Between 4 and 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 with cold PBS containing 100 mM glycine. Neurons were lysed in 160 µl/9 cm² lysis buffer (PBS with 0.1 % SDS, 1 % Triton X-100, and protease inhibitors) at 4 °C for 20 min. Cell debris were pelleted at 14,000 rpm for 10 min at 4 °C. The protein concentration was measured using the Bradford Protein Assay (Bio-Rad). One hundred µg of total proteins were incubated with 50 µl of NeutrAvidin agarose at 4 °C for 1 hr. Agarose beads were washed three times using washing buffer (500 mM NaCl; 15 mM Tris-HCl, pH 8.0; 0.5 % Triton X-100), biotinylated surface proteins were eluted from agarose beads by boiling in 2 × SDS sample loading buffer and loaded on an SDS-PAGE gel for Western blot analysis. GST-control and GST-RAP (50 µg/ml) pre-treatment of neurons was performed for 1 hr.

To analyze BACE1 activity, β-CTF was detected by immunoblotting. BACE1 activity was examined after pharmacological NHE inhibition or genetic Slc9a6 knockdown in primary neurons of AppSwe mice. For NHE inhibition DIV10 neurons were treated with 5 µg/ml ApoE4, 3 µM EMD87580 (Merck), and/or 1 µM γ-secretase inhibitor L-685458 (Merck) for 5 hr. For knockdown of NHE6/Slc9a6 DIV7 neurons of AppSwe mice were infected with lentivirus encoding shRNA against NHE6 or a scrambled control shRNA. On DIV13 neurons were treated with γ-secretase inhibitor for 12 hr. Proteins were extracted for Western blot analysis: Cells were washed three times with cold PBS and lysed for 20 min on ice in RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 % Nonidet P-40; phosphatase and proteinase inhibitors). Cell debris were pelleted at 14,000 rpm for 10 min at 4 °C. Protein concentration was measured using the Bradford Protein Assay (Bio-Rad). After adding 4× SDS loading buffer (0.1 M Tris-HCl, pH 6.8, 2 % SDS, 5 % β-mercaptoethanol, 10 % glycerol, and 0.05 % bromophenol blue), samples were denatured at 80 °C for 10 min. Proteins were separated via SDS-PAGE and transferred to a nitrocellulose membrane for Western blotting using different antibodies listed in the Key resources table.

Brain tissue was dissected and prepared for immunoblotting as follows: After removal, brains were placed in ice-cold PBS containing protease inhibitors. Anatomical sectioning was performed under a microscope. The hippocampus was dissected out and transversal slices were further separated into cornu ammonis (CA) 1, CA3, and dentate gyrus. Respective pieces of the same anatomical regions of
one brain were pooled, shock-frozen in liquid nitrogen, and stored at −80 C. Frozen tissue was homogenized in 10 × 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 4× SDS 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 protease inhibitors at 100 mg protein/ml using a glass dounce homogenizer. Crude lysate was centrifuged at 800 × g for 5 min at 4 °C. The supernatant was further centrifuged at 100,000 × g 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 protease inhibitors, centrifuged at 100,000 × g for 30 min at 4 °C and collected as 1 % Triton-soluble lysate. The Triton-pellet was incubated with 5 M guanidine-HCl rotating at RT for 1 hr. Guanidine-soluble lysate (Aβ-insoluble) was collected after centrifugation at 21,000 × g 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 × g for 15 min at 4 °C. Soluble and insoluble Aβ levels were measured in duplicates using a commercial Aβ42 ELISA kit (Thermo Fisher, 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; Slc9a6fl;CAG-CreERT2 mice or Slc9a6fl mice with ApoeAPOE3 or ApoeAPOE4. The brains were quickly removed and placed in ice-cold high sucrose cutting solution (in mM: 110 sucrose, 3 KCl, 1.25 NaH2PO4, 28 NaHCO3, 0.5 CaCl2, 5 glucose, 0.6 ascorbic acid, 7 MgSO4), bubbled with a gas mixture of 95 % O2 and 5 % CO2 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 NaH2PO4, 26 NaHCO3, 10 D-glucose, 2 CaCl2, 1 MgSO4) and 50 % sucrose cutting solution, oxygenated with 95% O2/5% CO2. 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 CB7CBB) 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 four pulses at 100 Hz repeated 10 times with 200 ms intervals; repeated five 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).

**Acknowledgements**

This work was supported by NIH grants R37 HL063762, R01 NS093382, R01 NS108115, and RF1 AG053931 to JH and 1F31 AG067708-01 to CHW as well as funding from the Darrell K Royal Research Fund to MD. While this work was ongoing, JH was further supported by the Bright Focus Foundation (A20135245) and Harrington Discovery Institute; and Circle of Friends Pilot Synergy Grant; and the Blue Field Project to Cure FTD. We are indebted Rebekah Hewitt, Barsha Subba, Huichuan Reyna, Issac Rocha, Tamara Terrones, Emily Boyle, Alisa Gilloon, Travis Wolff, and Eric Hall for their excellent technical assistance. We thank Dr Yuan Yang for creating the NHE6-FLAG plasmid.
and the UTSW Whole Brain Microscopy Facility (WBMF) in the Department of Neurology and Neurotherapeutics for assistance with slide scanning. The WBMF is supported by the Texas Institute for Brain Injury and Repair (TIBIR). John Shelton and the UT Southwestern’s Histopathology Core provided help with paraffin sectioning as well as H&E and Thioflavin S staining. We thank Wolfgang Scholz for providing EMD87580.

### Additional information

#### Competing interests

Xunde Xian, Joachim Herz: Inventor of Patent: https://patents.google.com/patent/US20110136832A1/en. The other authors declare that no competing interests exist.

#### Funding

**Funder**  | **Grant reference number** | **Author**
---|---|---
National Institutes of Health  | HL063762  | Joachim Herz
BrightFocus Foundation  | A20135245  | Joachim Herz
National Institute on Aging  | 1F31AG067708-01  | Connie H Wong
Darrell K Royal Research Fund  |  | Murat S Durakoglugil
Harrington Discovery Institute  |  | Joachim Herz
Circle of Friends Pilot Synergy  |  | Joachim Herz
Blue Field Project to Cure FTD  |  | Joachim Herz
National Institutes of Health  | NS093382  | Joachim Herz
National Institutes of Health  | NS108115  | Joachim Herz
National Institutes of Health  | AG053391  | Joachim Herz
BrightFocus Foundation  | A20163965  | Joachim Herz

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

#### Author contributions

Theresa Pohlkamp, Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing; Xunde Xian, Conceptualization, Investigation, Methodology, Resources, Validation, Visualization; Connie H Wong, Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review and editing; Murat S Durakoglugil, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization; Gordon Chandler Werthmann, Formal analysis, Investigation, Methodology, Validation; Takaomi C Saico, Charles L White, Resources; Bret M Evers, Jade Connor, Investigation; Robert E Hammer, Methodology; Joachim Herz, Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review and editing

#### Author ORCIDs

Theresa Pohlkamp: http://orcid.org/0000-0003-3923-1917
Connie H Wong: http://orcid.org/0000-0002-6452-7966
Murat S Durakoglugil: http://orcid.org/0000-0003-4483-8166
Ethics
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.

Decision letter and Author response
Decision letter https://doi.org/10.7554/eLife.72034.sa1
Author response https://doi.org/10.7554/eLife.72034.sa2

Additional files
Supplementary files
• Transparent reporting form

Data availability
All relevant data are included in the manuscript.

References
Arboleda-Velasquez JF, Lopera F, O’Hare M, Delgado-Tirado S, Marino C, Chmielewska N, Saez-Torres KL, Amarnani D, Schultz AP, Sperling RA, Leyton-Cifuentes D, Chen K, Baena A, Aguillon D, Rios-Romenets S, Giraldo M, Guzmán-Vélez E, Norton DJ, Pardilla-Delgado E, Artola A, et al. 2019. Resistance to autosomal dominant Alzheimer’s disease in an APOE3 Christchurch homozygote: a case report. Nature Medicine 25: 1680–1683. DOI: https://doi.org/10.1038/s41591-019-0611-3, PMID: 31686034
Basu SK, Goldstein JL, Anderson GW, Brown MS. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. PNAS 73: 3178–3182. DOI: https://doi.org/10.1073/pnas.73.9.3178, PMID: 184464
Beffert U, Wreeber EJ, Durudas A, Qiu S, Masiulis I, Sweatt JD, Li W-P, Adelmann G, Frotscher M, Hammer RE, Herz J. 2005. Modulation of synaptic plasticity and memory by Reelin involves differential splicing of the lipoprotein receptor Apoer2. Neuron 47: 567–579. DOI: https://doi.org/10.1016/j.neuron.2005.07.007, PMID: 16102539
Benilova I, Karran E, De Strooper B. 2012. The toxic Aβ oligomer and Alzheimer’s disease: an emperor in need of clothes. Nature Neuroscience 15: 349–357. DOI: https://doi.org/10.1038/nn.3028, PMID: 22286176
Braybrooke JP, Spink KG, Thacker J, Hickson ID. 2000. The RAD51 family member, RAD51L3, is a DNA-stimulated ATPase that forms a complex with XRCC2. The Journal of Biological Chemistry 275: 29100–29106. DOI: https://doi.org/10.1074/jbc.M002075200, PMID: 10871607
Brich J, Shie F-S, Howell BW, Li R, Tus K, Wakeland EK, Jin L-W, Mumbey M, Churchill G, Herz J, Cooper JA. 2003. Genetic modulation of tau phosphorylation in the mouse. The Journal of Neuroscience 23: 187–192. DOI: https://doi.org/10.1523/JNEUROSCI.23-01-00187.2003, PMID: 12514215
Cataldo AM, Peterhoff CM, Troncoso JC, Gomez-Isla T, Hyman BT, Nixon RA. 2000. Endocytic pathway abnormalities precede amyloid beta deposition in sporadic Alzheimer’s disease and Down syndrome: differential effects of APOE genotype and presenilin mutations. The American Journal of Pathology 157: 277–286. DOI: https://doi.org/10.1016/s0002-9440(10)64538-5, PMID: 10880397
Casey JR, Grinstein S, Orlowski J. 2010. Sensors and regulators of intracellular pH. Nature Reviews. Molecular Cell Biology 11: 50–61. DOI: https://doi.org/10.1038/nrm2820, PMID: 19997129
Corder EH, Saunders AM, Risch NJ, Strittmatter WJ, Schmechel DE, Gaskell PC, Rimmell JB, Locke PA, Conneally PM, Schmader KE. 1994. Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. Nature Genetics 7: 180–184. DOI: https://doi.org/10.1038/ng0694-180, PMID: 7920638
Jovic M, Sharma M, Rahajeng J, Caplan S. 2010. The early endosome: a busy sorting station for proteins at the crossroads. *Histology and Histopathology* 25: 99–112. DOI: https://doi.org/10.1080/03008200903341626

Knopman DS, Amieva H, Petersen RC, Chételat G, Holtzman DM, Hyman BT, Nixon RA, Jones DT. 2021. Alzheimer disease. *Nature Reviews. Disease Primers* 7: 33. DOI: https://doi.org/10.1038/s41572-021-00269-y, PMID: 33986301

Kernoff C, Hindsdale ME, Mezdour H, Altenburg MK, Watanabe M, Quarfordt SH, Sullivan PM, Maeda N. 1999. Apo E structure determines VLDL clearance and atherosclerosis risk in mice. *The Journal of Clinical Investigation* 103: 1579–1586. DOI: https://doi.org/10.1172/JCI1672, PMID: 10359567

Kunkle BW, Grenier-Boley B, Sims R, Bis JC, Damotte V, Naj AC, Boland A, Vronskaya M, van der Lee SJ, Amlie-Wolf A, Bellenguez C, Frizatti A, Chouraki V, Martin ER, Sleegers K, Badarinarayan N, Jakobsdottir J, Hamilton-Nelson KL, Moreno-Grau S, Olaso R, et al., 2019. Genetic meta-analysis of diagnosed Alzheimer’s disease identifies new risk loci and implicates Aβ, tau, immunity and lipid processing. *Nature Genetics* 51: 414–430. DOI: https://doi.org/10.1038/s41588-019-0358-2, PMID: 30802047

Kuznik A, Bencina M, Sjagger U, Jeras M, Rozman B, Jerala R. 2011. Mechanism of endosomal TLR inhibition by antimarial drugs and imidazoquinolines. *Journal of Immunology* 186: 4794–4804. DOI: https://doi.org/10.4049/jimmunol.1000702, PMID: 21398612

Lane-Donovan C, Philips GT, Herz J. 2014. More than cholesterol transporters: lipoprotein receptors in CNS function and neurodegeneration. *Neuron* 83: 771–787. DOI: https://doi.org/10.1016/j.neuron.2014.08.005, PMID: 25144875

Lane-Donovan C, Philips GT, Wasser CR, Durakoglugil MS, Masuilis I, Upadhaya A, Pohlkamp T, Coskun C, Kotti T, Steller L, Hammer RE, Frotscher M, Bock HH, Herz J. 2015. Reelin protects against amyloid β toxicity in vivo. *Science Signaling* 8: ra67. DOI: https://doi.org/10.1126/scisignal.aac6674, PMID: 26152694

Lane-Donovan C, Herz J. 2017. ApoE, ApoE Receptors, and the Synapse in Alzheimer’s Disease. *Trends in Endocrinology and Metabolism* 28: 273–284. DOI: https://doi.org/10.1016/j.tem.2016.12.001, PMID: 28057414

Lee EC, Yu D, Martinez de Velasco J, Tessarollo L, Swing DA, Court DL, Jenkins NA, Copeland NG. 2001. A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* 73: 56–65. DOI: https://doi.org/10.1006/geno.2000.6451, PMID: 11352566

Lee U, Choi C, Ryu SH, Park D, Lee SE, Kim K, Kim Y, Chang S. 2020. SCAMP5 plays a critical role in axonal trafficking and synaptic localization of NHE6 to adjust quantal size at glutamatergic synapses. *PNAS* 118: e2011371118. DOI: https://doi.org/10.1073/pnas.2011371118

Lee U, Ryu SH, Chang S. 2021. SCAMP5 mediates activity-dependent enhancement of NHE6 recruitment to synaptic vesicles during synaptic plasticity. *Molecular Brain* 14: 47. DOI: https://doi.org/10.1186/s13041-021-00763-0

Liao F, Zhang TJ, Jiang H, Lefton KB, Robinson GO, Vassar R, Sullivan PM, Holtzman DM. 2015. Murine versus human apolipoprotein E4: differential facilitation of and co-localization in cerebral amyloid angiopathy and amyloid plaques in APP transgenic mouse models. *Acta Neuropathologica Communications* 3: 70. DOI: https://doi.org/10.1186/s40478-015-0250-y, PMID: 26556230

Lucien F, Pelletier PP, Lavoie RR, Lacroix JM, Roy S, Parent JL, Arsenault D, Harper K, Dubois CM. 2017. Hypoxia-induced mobilization of NHE6 to the plasma membrane triggers endosome hyperacidification and chemoresistance. *Nature Communications* 8: 15884. DOI: https://doi.org/10.1038/ncomms15884, PMID: 28635961

Ma L, Ouyang Q, Werthmann GC, Thompson HM, Morrow EM. 2017. Live-cell Microscopy and Fluorescence-based Measurement of Luminal pH in Intracellular Organelles. *Frontiers in Cell and Developmental Biology* 5: 71. DOI: https://doi.org/10.3389/fccl.2017.00071, PMID: 28871281

Madisen L, Zwangman TA, Sunkin SM, Oh SW, Zarivala HA, Gu H, Ng LL, Palmer MD, Hawrylycz MJ, Jones AR, Lein ES, Zeng H. 2010. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nature Neuroscience* 13: 133–140. DOI: https://doi.org/10.1038/nn.2467, PMID: 20023653

Meyer-Luehmann M, Spires-Jones TL, Prada C, Garcia-Alloza M, de Calignon A, Rozkalne A, Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, Lein ES, Zeng H. 2010. A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* 73: 56–65. DOI: https://doi.org/10.1006/geno.2000.6451, PMID: 11352566

Minett T, Clasey J, Matthews FE, Fahrenholt M, Taga M, Brayne C, Ince PG, Nicoll JR, Boche D, MRC CFAS. 2016. Microglial immunophenotype in dementia with Alzheimer’s pathology. *Journal of Neuroinflammation* 13: 143. DOI: https://doi.org/10.1186/s12974-016-0601-z, PMID: 27256292

Morrow JA, Hatters DM, Lu B, Hochtl P, Oberg KA, Rupp B, Weisgraber KH. 2002. Apolipoprotein E4 forms a molten globule. A potential basis for its association with disease. *The Journal of Biological Chemistry* 277: 50380–50385. DOI: https://doi.org/10.1074/jbc.M204898200, PMID: 12393895

Nakamura N, Tanaka S, Teko Y, Mitsui K, Kanazawa H. 2005. Four Na+/H+ exchanger isoforms are distributed to Golgi and post-Golgi compartments and are involved in organelle pH regulation. *The Journal of Biological Chemistry* 280: 1561–1572. DOI: https://doi.org/10.1074/jbc.M410041200, PMID: 15522866

Naslavsky N, Caplan S. 2018. The enigmatic endosome - sorting the ins and outs of endocytic trafficking. *Journal of Cell Science* 131: jcs216499. DOI: https://doi.org/10.1242/jcs.216499, PMID: 29980602

Nixon RA. 2017. Amyloid precursor protein and endosomal-lysosomal dysfunction in Alzheimer’s disease: inseparable partners in a multifactorial disease. *FASEB Journal* 31: 2729–2743. DOI: https://doi.org/10.1096/fj.201700359, PMID: 28663518
Pohlkamp, Xian, et al. eLife 2021;10:e72034. DOI: https://doi.org/10.7554/eLife.72034  32 of 34

Neuroscience

Serrano-Pozo A, Muzikansky A, Gómez-Isla T, Growdon JH, Betensky RA, Frosch MP, Hyman BT. 2013. Reciprocal regulation of signaling and endocytosis: Implications for the evolving cancer cell. The Journal of Clinical Investigation 129: 1293–1304. DOI: https://doi.org/10.1172/JCI90606, PMID: 28862638

Panza F, Solfrezzi V, Torres F, Mastroianni F, Colacchio AM, Basile AM, Capuroso C, D’Introno A, Del Parigi A, Capurso A. 2000. Apolipoprotein E in Southern Italy: protective effect of epsilon 2 allele in early- and late-onset sporadic Alzheimer’s disease. Neurology Letters 292: 79–82. DOI: https://doi.org/10.1016/s0304-3903(00)01447-6, PMID: 10998553

Parhizkar S, Arzberger T, Brendel M, Kleinberger G, Deussing M, Focke C, Nuscher B, Xiong M, Ghasemigharagoz A, Katzmarski N, Krassmann S, Lichtenenthaler SF, Müller SA, Colombo A, Monsarz LS, Tahirovic S, Herms J, Willem M, Pettekin K, Butovsky O, et al. 2019. Loss of TREM2 function increases amyloid seeding but reduces plaque-associated ApoE. Nature Neuroscience 22: 191–204. DOI: https://doi.org/10.1038/s41593-018-0296-9, PMID: 30617257

Prasad H, Fatima, Mouchababi-Constance S, Davidova A, Ferland CE, Orlowski J, Sharif-Naeini R. 2020. Loss of SLC9A6/NHE6 impairs nociception in a mouse model of Chrisonintos syndrome. Pain 161: 2619–2628. DOI: https://doi.org/10.1016/j.pain.2019.07.017, PMID: 32895089

Pohilkamp T, Wasser CR, Herz J. 2017. Functional Roles of the Interaction of APP and Lipoprotein Receptors. Frontiers in Molecular Neuroscience 10: 54. DOI: https://doi.org/10.3389/fnmol.2017.00054, PMID: 28298885

Prasad H, Rao R. 2015. The Na+/H+ exchanger NHE6 modulates endosomal pH to control processing of amyloid precursor protein in a cell culture model of Alzheimer disease. The Journal of Biological Chemistry 290: 5311–5327. DOI: https://doi.org/10.1074/jbc.M114.602219, PMID: 25561733

Prasad H, Rao R. 2018. Amyloid clearance defect in ApoE4 astrocytes is reversed by epigenetic correction of endosomal pH. PNAS 115: E6640–E6649. DOI: https://doi.org/10.1073/pnas.1801612115

Rawat V, Wang S, Sima J, Bar R, Lira O, Gundimeda U, Parekh T, Chan J, Johansson MO, Tang C, Chui HC, Harrington MG, Michaelson DM, Yassine HN. 2019. ApoE4 Alters ABCA1 Membrane Trafficking in Astrocytes. The Journal of Neuroscience 39: 9611–9622. DOI: https://doi.org/10.1523/JNEUROSCI.1400-19.2019, PMID: 31641056

Rosen AD. 1994. Apolipoprotein E affects the rate of Alzheimer disease expression: beta-amyloid burden is a secondary consequence dependent on APOE genotype and duration of disease. Journal of Neurobiology and Experimental Neurology 53: 429–437. DOI: https://doi.org/10.1007/978-94-017-2779-8_9, PMID: 8083686

Rudenko G, Henry L, Henderson K, Ichtenchenko K, Brown MS, Goldstein JL, Deisenhofer J. 2002. Structure of the LDL receptor extracellular domain at endosomal pH. Science 298: 2353–2358. DOI: https://doi.org/10.1126/science.1078124, PMID: 12459547

Saito T, Matsuba Y, Mihiara N, Takano J, Nilsson P, Itohara S, Iwata N, Saida TC. 2014. Single App knock-in mouse models of Alzheimer’s disease. Nature Neuroscience 17: 661–663. DOI: https://doi.org/10.1038/nn.3697, PMID: 24728269

Sando SB, Melquist S, Cannon A, Hutton ML, Sletvold O, Saltvedt I, White LR, Lydersen S, Aasly JO. 2008. APOE epsilon 4 lowers age at onset and is a high risk factor for Alzheimer’s disease; a case control study from central Norway. BMC Neurology 8: 9. DOI: https://doi.org/10.1186/1471-2377-8-9, PMID: 18416843

Sarlis H, Heneka MT. 2017. Microglia in Alzheimer’s disease. The Journal of Clinical Investigation 127: 3240–3249. DOI: https://doi.org/10.1172/JCI90606, PMID: 28862638

Schmid SL. 2017. Reciprocal regulation of signaling and endocytosis: Implications for the evolving cancer cell. The Journal of Cell Biology 216: 2623–2632. DOI: https://doi.org/10.1083/jcb.201705017, PMID: 28674108

Serrano-Pozo A, Muzikansky A, Gómez-Isla T, Growdon JH, Betensky RA, Frosch MP, Hyman BT. 2013. Differential relationships of reactive astrocytes and microglia to fibrillar amyloid deposits in Alzheimer disease.
Neuroscience Research article

 Warnick GR, Mayfield C, Albers JJ, Hazzard WR. 1979. Gel isoelectric focusing method for specific diagnosis of familial hyperlipoproteinemia type 3. *Clinical Chemistry*: 279–284. PMID: 215348.

 Sullivan PM, Simoes-Spassov S, Mayeux R, Petsko GA. 2017. Endosomal Traffic Jams Represent a Pathogenic Hub for Amyloidosis and Type 2 Diabetes. *Science*: 592–602. DOI: https://doi.org/10.1126/science.aan1695, PMID: 28962801

 Stawicki TM, Owens KN, Linbo T, Reinhart KE, Rubel EW, Raible DW. 2014. The zebrafish merovingian mutant reveals a role for pH regulation in hair cell toxicity and function. *Disease Models & Mechanisms*: 34–35. DOI: https://doi.org/10.1242/dmm.016576, PMID: 24973752

 Strømme P, Dobrenis K, Sillitoe RV, Gulinello M, Ali NF, Davidson C, Micsenyi MC, Stephney G, Ellevog L, Sholl DA, Chiao P, Bussière T, Weinreb PH, Williams L, Maier M, Dunstan R, Salloway S, Chen T, Ling Y, Wang M, Jiang H, Parsadanian M, Hartman RE, Bales KR, Paul SM, Holtzman DM. 2005. Deletion of Abca1 is required for normal central nervous system ApoE levels and for lipidation of astrocyte-secreted apoE. *Cellular and Molecular Biology*: 40987–40993. DOI: https://doi.org/10.1074/jbc.M407963200, PMID: 15269217

 Sullivan PM, Mezdour H, Aratani Y, Knouff C, Najib J, Reddick RL, Quarfordt SH, Maeda N. 1997. Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis. *The Journal of Biological Chemistry*: 17972–17980. DOI: https://doi.org/10.1074/jbc.272.29.17972, PMID: 9218423

 Tallquist MD, Soriano P. 2000. Epiblast-restricted Cre expression in MORE mice: a tool to distinguish embryonic vs. extra-embryonic gene function. *Genesis*: 26:113–115. DOI: https://doi.org/10.1002/(SICI)1096-9848(20000226)26:2<113::AID-GENE3>3.0.CO;2-2, PMID: 10686601

 Trommsdorff M, Gotthardt M, Hiesberger T, Shelton J, Stockinger W, Nimip J, Hammer RE, Richardson JA, Herz J. 1999. Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell*: 697–701. DOI: https://doi.org/10.1016/S0092-8674(99)80782-5, PMID: 10380922

 Van Acker ZP, Bretou M, Annaert W. 2019. Endo-lysosomal dysregulations and late-onset Alzheimer’s disease: impact of genetic risk factors. *Molecular Neurodegeneration*: 14:20. DOI: https://doi.org/10.1186/s13024-019-0323-7, PMID: 31159836

 Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendia EA, Denis P, Teplov DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Burgess T, Louis JC, et al. 1999. Beta-secretase cleavage of Alzheimer’s amyloid precursor protein by the transmembrane aspartic protease BACE. *Science*: 735–741. DOI: https://doi.org/10.1126/science.286.5440.735, PMID: 10531052

 Verheijen J, Sleevers K. 2018. Understanding Alzheimer Disease at the Interface between Genetics and Transcriptomics. *Trends in Genetics*: 434–447. DOI: https://doi.org/10.1016/j.tig.2018.02.007, PMID: 29573818

 Wahrle S.E, Jiang H, Parsadanian M, Legleiter J, Han X, Fryer JD, Kowalewski T, Holtzman DM. 2004. ABCA1 is required for normal central nervous system ApoE levels and for lipidation of astrocyte-secreted apoE. *The Journal of Biological Chemistry*: 40987–40993. DOI: https://doi.org/10.1074/jbc.M407963200, PMID: 15269217

 Wahrle S.E, Jiang H, Parsadanian M, Hartman RE, Bales KR, Paul SM, Holtzman DM. 2005. Deletion of Abca1 increases Abeta deposition in the PDAPP transgenic mouse model of Alzheimer disease. *The Journal of Biological Chemistry*: 43236–43242. DOI: https://doi.org/10.1074/jbc.M508780200, PMID: 16207708

 Wahrle SE, Jiang H, Parsadanian M, Kim J, Li A, Knoten A, Jain S, Hirsch-Reinshagen V, Wellington CL, Bales KR, Paul SM, Holtzman DM. 2008. Overexpression of ABCA1 reduces amyloid deposition in the PDAPP mouse model of Alzheimer disease. *The Journal of Clinical Investigation*: 118:671–682. DOI: https://doi.org/10.1172/JCI33622, PMID: 18204999

 Wang M, Jing T, Wang X, Yao D. 2018. Beta-secretase/BACE1 promotes APP endocytosis and processing in the endosomes and on cell membrane. *Neuroscience Letters*: 685:63–67. DOI: https://doi.org/10.1016/j.neulet.2018.08.016, PMID: 30120949

 Warnick GR, Mayfield C, Albers JJ, Hazzard WR. 1979. Gel isoelectric focusing method for specific diagnosis of familial hyperlipoproteinemia type 3. *Clinical Chemistry*: 279–284. PMID: 215348.
Weeber EJ, Beffert U, Jones C, Christian JM, Forster E, Sweat JD, Herz J. 2002. Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity and learning. The Journal of Biological Chemistry 277: 39944–39952. DOI: https://doi.org/10.1074/jbc.M205147200, PMID: 12167620

West HL, Rebeck GW, Hyman BT. 1994. Frequency of the apolipoprotein E epsilon 2 allele is diminished in sporadic Alzheimer disease. Neuroscience Letters 175: 46–48. DOI: https://doi.org/10.1016/0304-3940(94)91074-x, PMID: 7970208

Wintersteiner O, Abramson HA. 1933. The isoelectric point of insulin: Electrical properties of adsorbed and crystalline insulin. The Journal of Biological Chemistry 99: 741–753.

Woźniacka A, Lesiak A, Narbutt J, McCauliffe DP, Sysa-Jedrzejowska A. 2006. Chloroquine treatment influences proinflammatory cytokine levels in systemic lupus erythematosus patients. Lupus 15: 268–275. DOI: https://doi.org/10.1191/0961203306lu2299oa, PMID: 16761500

Xian X, Pohlkamp T, Durakoglugil MS, Wong CH, Beck JK, Lane-Donovan C, Plattner F, Herz J. 2018. Reversal of ApoE4-induced recycling block as a novel prevention approach for Alzheimer’s disease. eLife 7: e40048. DOI: https://doi.org/10.7554/eLife.40048, PMID: 30375977

Xinhan L, Matsushita M, Numaza M, Taguchi A, Mitsui K, Kanazawa H. 2011. Na+/H+ exchanger isoform 6 (NHE6/SLC9A6) is involved in clathrin-dependent endocytosis of transferrin. American Journal of Physiology-Cell Physiology 301: C1431–C1444. DOI: https://doi.org/10.1152/ajpcell.00154.2011

Yang H, Kozicky L, Saferali A, Fung S-Y, Afacan N, Cai B, Falsafi R, Gill E, Liu M, Kollmann TR, Hancock REW, Sly LM, Turvey SE. 2016. Endosomal pH modulation by peptide-gold nanoparticle hybrids enables potent anti-inflammatory activity in phagocytic immune cells. Biomaterials 111: 90–102. DOI: https://doi.org/10.1016/j.biomaterials.2016.09.032, PMID: 27728817

Yanuck SF. 2019. Microglial Phagocytosis of Neurons: Diminishing Neuronal Loss in Traumatic, Infectious, Inflammatory, and Autoimmune CNS Disorders. Frontiers in Psychiatry 10: 712. DOI: https://doi.org/10.3389/fpsyt.2019.00712, PMID: 31632307

Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O’Keeffe S, Phatnani HP, Guarneri P, Caneda C, Rudenis N, Deng S, Liddelow SA, Zhang C, Daneman R, Maniatis T, Barres BA, Wu JQ. 2014. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. The Journal of Neuroscience 34: 11929–11947. DOI: https://doi.org/10.1523/JNEUROSCI.1860-14.2014, PMID: 25186741

Zhong L, Xu Y, Zhuo R, Wang T, Wang K, Huang R, Wang D, Gao Y, Zhu Y, Sheng X, Chen K, Wang N, Zhu L, Can D, Marten Y, Shinozaka M, Liu C-C, Du D, Sun H, Wen L, et al. 2019. Author Correction: Soluble TREM2 ameliorates pathological phenotypes by modulating microglial functions in an Alzheimer’s disease model. Nature Communications 10: 2923. DOI: https://doi.org/10.1038/s41467-019-10950-2, PMID: 31266955

Ziegler-Waldkirch S, Meyer-Luehmann M. 2018. The Role of Glial Cells and Synapse Loss in Mouse Models of Alzheimer’s Disease. Frontiers in Cellular Neuroscience 12: 473. DOI: https://doi.org/10.3389/fncel.2018.00473, PMID: 30618627